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Long-Term Exposure of the HT-29 Human Intestinal Epithelial Cell Line to TNF Causes Sustained Up-Regulation of the Polymeric Ig Receptor and Proinflammatory Genes through Transcriptional and Posttranscriptional Mechanisms

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Transport of IgA Abs across intestinal epithelial cells into gut secretions is mediated by the polymeric Ig receptor (pIgR). The cytokine TNF plays a central role in initiating and amplifying inflammatory reactions, and is implicated in the pathogenesis of inflammatory bowel diseases. Acute exposure of intestinal epithelial cell lines to TNF has been shown to up-regulate transcription of genes encoding pIgR and a number of proinflammatory factors, but the effects of chronic exposure to TNF have not been studied. We found that exposure of HT-29 human colon carcinoma cells to TNF for up to 20 days reduced the rate of cell proliferation, but did not cause gross morphological changes. Expression of mRNA encoding pIgR and several proinflammatory genes increased acutely, and then diminished but remained elevated above control levels throughout the experiment. Changes in gene expression were paralleled by increased expression of the transcription factors IFN regulatory factor-1 and the RelB subunit of NF-κB. HT-29 cells activated the endogenous TNF gene in response to TNF treatment, but the level of TNF production was insufficient to maintain pIgR and proinflammatory gene expression after withdrawal of exogenous TNF. Chronic exposure to TNF caused a marked increase in pIgR mRNA stability and a small but significant decrease in TNF mRNA stability, but no change in the half-lives of IL-8, c-Myc, and GAPDH. In summary, we observed different effects of acute vs chronic exposure to TNF on gene expression, and found evidence for transcriptional and posttranscriptional regulation of expression of the pIgR.


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3 Abbreviations used in this paper: pIgR, polymeric Ig receptor; IRF, IFN regulatory factor; CT, threshold cycle.

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published studies in HT-29 cells have focused on the acute response, mainly examining changes in gene expression during the first 72 h following TNF stimulation. In the present study, we found that HT-29 cells tolerated a moderate dose of TNF for up to 3 wk with no obvious morphological changes, although the rate of cellular proliferation declined gradually. Sustained expression of plgR and inflammatory mediators required continuous exposure to exogenous TNF. Our studies also provided novel evidence that TNF regulates steady-state levels of plgR mRNA by transcriptional and posttranscriptional mechanisms.

Materials and Methods

Cell culture

The HT-29v20 subclone of the human colon adenocarcinoma cell line (21) was maintained in DMEM-Ham’s F-12 (1:1) medium supplemented with 5% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml fungizone). All cell culture reagents were from BioWhittaker or Invitrogen Life Technologies. HT-29 cells were plated at an initial concentration of 30–40% and subcultured by trypsinization twice weekly to maintain continuous cellular proliferation. Cells were replated at the same cell density at each passage. Where indicated, cells were treated with human recombinant TNF (R&D Systems) at a final concentration of 10 ng/ml. Culture media were replenished every other day for the experiments described in Figs. 1–3, and daily for the experiments described in Fig. 4. To measure rates of mRNA decay, HT-29 cells were treated with TNF for 5 days, washed to remove residual TNF, and given fresh culture medium containing actinomycin D (8 µM) (Sigma-Aldrich) for 1, 2, 4, or 6 h.

RNA isolation, cDNA synthesis, and real-time RT-PCR

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen), according to the manufacturer’s protocol. Reverse transcriptase reactions to generate cDNA templates were performed using the TaqMan Gold RT-PCR kit from Applied Biosystems, according to the manufacturer’s protocol. Specific mRNA levels were quantified by real-time RT-PCR, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers and fluorescent probes specific for human plgR, TNF, IL-8, IRF-1, and GAPDH were designed using the Primer Express software (Applied Biosystems) (Table I). Predesigned primers and probes for the NF-kB RelA and RelB subunits were purchased from Applied Biosystems. The TaqMan PCR Core Reagent kit was used according to the manufacturer’s protocol. Amplification of the cDNA was measured at every cycle by fluorescence resonance energy transfer (ΔRn). The threshold cycle (Ct), which is inversely proportional to the level of a given mRNA transcript, is defined as the cycle at which the ΔRn exceeds the threshold of fluorescence detection. GAPDH mRNA levels were used as an internal control because this mRNA was constitutively present in the cells and was not affected by the experimental treatments. mRNA levels were normalized to GAPDH mRNA according to the following formula: (2^{-\Delta Ct_{GAPDH}}) × 100%.

Statistical analyses

Statistical differences among treatment groups were determined by ANOVA and Fisher’s protected least significant difference test. For calculation of rates of mRNA decay, regression curves were constructed with time of actinomycin D treatment as the independent variable and mRNA level (relative to the level before addition of actinomycin D) as the dependent variable. mRNA half-lives were calculated from the slopes of the regression curves. StatView software (SAS Institute) was used for all statistical analyses.

Results

A model of chronic exposure of human intestinal epithelial cells to TNF

We previously reported that the optimal dose of rTNF for acute up-regulation of plgR expression in HT-29 cells was 10 ng/ml (19), consistent with the results from other studies of various genes regulated by TNF in this cell line (17, 18, 28–38). To determine the effects of chronic exposure to TNF, HT-29 cells were treated for varying times with 10 ng/ml recombinant human TNF. The initiation of TNF treatments was staggered such that all cultures were harvested at the end of 24 days in culture. To maintain continuous cellular proliferation, cells were subcultured twice weekly. Treatment with TNF for up to 10 days did not affect the rate of cell proliferation (Table II). The number of cell divisions declined gradually with increasing times of TNF exposure for up to 20 days. No obvious morphological changes were observed for up to 3 wk of TNF exposure (Fig. 1, A and B). By 24 days, however, treatment signs of cell necrosis were apparent, including loss of integrity of the nuclear and cell membranes, and accumulation of karyorrhectic debris (Fig. 1C). Further necrotic changes and decreased cell numbers were observed when HT-29 cells were treated with TNF for longer times (data not shown). These results demonstrate that HT-29 cells can be maintained in the presence of 10 ng/ml TNF for ~3 wk without major changes in cell structure and proliferative capacity.

Effects of long-term exposure to TNF on expression of plgR and proinflammatory genes

HT-29 cells were cultured for 21 days with varying times of TNF treatment, and steady-state mRNA levels were determined by real-time RT-PCR for plgR and several proinflammatory genes (Fig. 2). mRNA levels for these target genes were normalized to the mRNA level for the housekeeping gene GAPDH. To determine whether TNF treatment altered GAPDH expression, we performed real-time PCR on cDNA reverse transcribed from 20 ng of total cellular RNA from HT-29 cells treated for varying times with TNF, as described in Materials and Methods. The average C_T for GAPDH across all experimental groups was 17.29 ± 0.055 (mean ± SEM; n = 50). ANOVA demonstrated that the C_T for GAPDH did not vary significantly with TNF treatment (p =
By contrast, expression of plgR and proinflammatory genes increased significantly in response to TNF treatment (Fig. 2). Expression of plgR mRNA increased significantly at 24 h and remained elevated throughout the experiment, although considerable fluctuations were observed over time. Endogenous TNF and IL-8 mRNA increased dramatically in the first 24 h, and then declined gradually, reaching a plateau at 1 wk that was maintained for the subsequent 2 wk of TNF treatment. Significant but less pronounced increases in IL-1β and RANTES mRNA were also observed (data not shown).

It has previously been reported that enhanced transcription of the human PIGR gene during acute exposure to TNF (1–2 days) requires the association of NF-κB, and to a lesser extent IRF-1, with cognate elements in exon 1 and intron 1 (23) (illustrated in Fig. 3A). These investigators recently demonstrated that de novo synthesis of the RelB subunit of NF-κB was correlated with enhanced PIGR transcription (24). To determine whether chronic exposure to TNF alters expression of these transcription factors, RNA samples from the experiment described in Fig. 2 were analyzed for mRNA encoding NF-κB/RelA and RelB, and IRF-1 (Fig. 3B). RelA mRNA levels rose modestly for 3 days, and then remained relatively constant for the remainder of the experiment. By contrast, RelB mRNA levels rose dramatically at 24 h of TNF treatment and remained significantly elevated. As a consequence, the ratio of RelB/RelA mRNA increased from 0.2 to 2.9 at 24 h, and fluctuated from 1.3 to 1.9 at longer times of TNF treatment. IRF-1 mRNA levels also peaked at 24 h, and then declined gradually and plateaued in a pattern similar to that of TNF and IL-8 mRNA.

**Endogenous production of TNF is not maintained following withdrawal of exogenous TNF**

The dramatic increase in endogenous TNF mRNA levels caused by exposure of HT-29 cells to exogenous TNF (Fig. 2) raised the question of whether endogenous production of TNF would be sufficient to stimulate an autologous feedback loop following withdrawal of exogenous TNF. To address this question, HT-29 cells were treated with TNF for 2 days, washed to remove residual TNF (both exogenous and endogenous), and then cultured for 7 additional days in the absence of exogenous TNF (Fig. 4). Endogenous TNF expression increased significantly in response to exogenous TNF treatment, comparable to the results described in Fig. 2. However, within 24 h of removal of TNF from the culture medium, endogenous TNF mRNA had declined to a baseline level that was not significantly different from untreated HT-29 cells. Concurrent with the loss of TNF expression, plgR and IL-8 mRNA levels dropped rapidly after withdrawal of exogenous TNF. By contrast, HT-29 cells treated continuously with TNF for 9 days maintained high levels of TNF, plgR, and IL-8 mRNA. High ratios of RelB/RelA were associated with the presence of an exogenous source of TNF (Fig. 4, lower panel). Levels of RelB mRNA dropped precipitously upon withdrawal of TNF, and within 24 h, the ratio of RelB/RelA had declined to that of untreated HT-29 cells. IRF-1 mRNA levels also increased with TNF addition and decreased to basal levels 24 h after TNF withdrawal (data not shown). These results suggest that exposure of HT-29 cells to exogenous TNF did not significantly affect IRF-1 expression.
not stimulate high enough endogenous levels of TNF production so that, upon removal of TNF from the medium, new release of TNF was rapid enough to accumulate to a threshold level sufficient to stimulate continued synthesis of TNF itself and other target genes of the TNF-signaling pathway.

Effects of TNF on mRNA stability

We found that the levels of expression of the transcription factors NF-κB and IRF-1, following long-term exposure to TNF, were correlated with TNF-induced increases in pIgR mRNA (compare Figs. 2 and 3), consistent with a mechanism involving increased transcription of the PIGR gene. However, it remains possible that posttranscriptional mechanisms may also influence steady-state levels of pIgR mRNA. To determine whether TNF treatment alters the mRNA stability of pIgR and other target genes, rates of mRNA decay were measured in HT-29 cells that had been cultured for 5 days in the presence or absence of TNF (Fig. 5 and Table III). This time was chosen to represent the point at which steady-state levels of mRNA reached a plateau during chronic treatment with TNF (Figs. 2 and 3). The half-life of each message was determined by measuring the rate of decrease in mRNA levels following treatment with 8 μM actinomycin D to block de novo mRNA synthesis. Actinomycin D concentrations of 4–8 μM have been previously used in HT-29 cells to evaluate mRNA stability of several gene products (39–41).

Rates of mRNA decay were measured over 6 h of treatment with actinomycin D.
The cytokine TNF is a key regulator of intestinal immunity, including roles in organogenesis of peripheral lymphoid structures, activation of innate antiviral and antibacterial responses, and transmission of signals to initiate adaptive immune responses (11). We postulate that up-regulation of plgR-mediated IgA transport by TNF contributes both to immune defense and to the regulation of inflammatory responses, for example by neutralizing LPS in the intestinal lumen (42) and within epithelial cells (7). By contrast, chronic exposure of the intestinal epithelium to TNF has been implicated in the pathogenesis of inflammatory bowel diseases (12, 26, 27), and therapeutic agents that neutralize TNF activity have proven effective in the treatment of these conditions (43). TNF has also been shown to play an instrumental role in the development of colitis in several mouse models of inflammatory bowel disease (27). Analyses of intestinal biopsies from patients with Crohn’s disease and ulcerative colitis have demonstrated that expression of TNF is elevated in inflamed relative to uninvolved mucosa (44, 45). One study of seven patients with ulcerative colitis revealed that expression of plgR was normal in mildly inflamed but reduced in severely inflamed mucosa, and inversely related to the degree of cellular dysplasia (46).

To model the chronic inflammatory state in the intestine induced by overexpression of TNF, we studied the effects of long-term treatment of HT-29 cells with recombinant human TNF. We believe that our experimental model represents the first stage of chronic inflammation, before significant damage has occurred to the epithelium. We found that HT-29 cells could be cultured in the presence of 10 ng/ml TNF for up to 3 wk, while still preserving cellular integrity and proliferation (Table II and Fig. 1). To our knowledge, this is the first report of an intestinal epithelial cell culture model suitable for the investigation of changes in gene expression caused by long-term exposure to TNF.

We found that TNF caused a dramatic increase in expression of plgR mRNA levels, which fluctuated but remained high throughout the experiment (Fig. 2). By contrast, expression of IL-8 and TNF peaked early, and then decreased and plateaued at a level significantly elevated over control cells. This pattern suggests that cellular signaling pathways may act to down-regulate proinflammatory gene expression after the acute response to TNF. We found that the endogenous production of TNF could not be maintained following removal of TNF from the culture medium (Fig. 4). Given the short half-life of TNF mRNA (Fig. 5), it appears that de novo secretion of TNF protein was insufficiently rapid to rise to a threshold that would trigger signaling through TNF receptors and maintain transcription of the endogenous TNF gene. In vivo, macrophages and activated T cells are likely to provide an exogenous source of TNF, suggesting that complex immune dysregulation and not simply epithelial overproduction of TNF is required for maintenance of a chronic inflammatory state.

Activation of the transcription factor NF-κB is key to initiation of intestinal inflammatory responses by TNF (47). In mammals, five NF-κB/Rel genes (NFKB1, NFKB2, RELA, RELB, and c-REL) generate seven proteins: NF-κB1 (p105 and its proteolytic product p50), NF-κB2 (p100 and its proteolytic product p52), p65 (RELA), RelB, and c-Rel (48). These factors form multiple homo- and heterodimers that recognize consensus NF-κB sequences in target genes. The classical NF-κB pathway is activated by a variety of inflammatory signals, including TNF, leading to the degradation of the inhibitory protein IκB and nuclear translocation of RelA/p50 dimers (14). Activation of the classical NF-κB pathway results in expression of multiple inflammatory and innate immune genes such as chemokines (e.g., IL-8), cytokines (e.g., TNF), enzymes, and adhesion molecules. The alternative NF-κB pathway is activated through cell surface receptors such as lymphotixin-β receptor, B cell-activating factor belonging to the TNF family/B lymphocyte stimulator, and CD40L, and results in nuclear translocation of RelB/p52 dimers and activation of genes involved in lymphoid organogenesis (14). Targeted disruption of the nfkB2 and relB genes in mice was shown to result in impaired development of Peyser’s patches, demonstrating the importance of this signaling pathway for intestinal immunity (49). Using reporter gene analyses, Schjerven et al. (23) demonstrated that TNF-induced transcription of the PIGR gene was mediated by a novel NF-κB/Rel site in intron 1. Surprisingly, these investigators found that transcriptional activation of the PIGR gene in HT-29 cells required de novo synthesis of RelB and was not stimulated by overexpression of RelA (24). We report here that chronic stimulation of HT-29 cells with TNF leads to continuous up-regulation of RelB mRNA, to levels exceeding those of RelA mRNA (Fig. 3). Our results, along with those of Schjerven et al. (23, 24), suggest that in intestinal epithelial cells TNF may activate proinflammatory gene transcription through the classical NF-κB pathway, while activating transcription of the PIGR gene through the alternative NF-κB pathway.

TNF also induces the synthesis of the transcription factor IRF-1, which binds to an element in exon 1 of the PIGR gene and coordinates with NF-κB to stimulate transcription (20, 21, 23, 24). IRF-1 has been implicated in transcriptional activation of many...
proinflammatory genes, and in down-regulation of cellular proliferation (50). We found that basal expression of IRF-1 was low in HT-29 cells, peaked rapidly in the acute phase of TNF stimulation, and then declined but remained elevated over baseline with chronic TNF exposure (Fig. 3). This pattern of IRF-1 expression was almost identical with the pattern of expression of the proinflammatory genes TNF and IL-8 (compare Figs. 2 and 3), suggesting a role for IRF-1 in maintenance of the inflammatory response as well as the coordinate regulation of PIGR gene transcription by RelB. Sustained expression of IRF-1 may have also contributed to the gradual decrease in cellular proliferation (Table II) and ultimate necrosis of the HT-29 cells after 24 days of TNF exposure (Fig. 1).

In addition to the well-established effects of TNF on gene transcription, this cytokine has been shown to activate signaling pathways that selectively influence mRNA stability. In HT-29 cells, acute exposure to TNF (10 ng/ml) has been shown to decrease the stability of mRNA encoding the cystic fibrosis transmembrane conductance regulator (51) while increasing the stability of mRNA for decay-accelerating factor (32) and IL-8 (52). We found that chronic exposure of HT-29 cells to the same concentration of TNF caused a significant increase in PIGR mRNA stability (Fig. 5 and Table III), indicating that posttranscriptional mechanisms contribute to the sustained expression of this important anti-inflammatory gene product. Our findings represent the first report of TNF-mediated regulation of PIGR mRNA stability, opening a new field of investigation into mechanisms by which PIGR expression is controlled. By contrast, chronic exposure to TNF caused a small-but-significant decrease in TNF mRNA stability, perhaps representing a feedback mechanism to limit the proinflammatory cycle. In contrast to previous reports that acute exposure of HT-29 cells to TNF increased the half-life of IL-8 mRNA (52), we observed no change in IL-8 mRNA stability following 5 days of TNF exposure.

In summary, our results suggest that intestinal epithelial cells adapt to chronic exposure to TNF by modifying programs of gene expression through transcriptional and posttranscriptional mechanisms. The acute phase of the TNF response is characterized by high expression of gene products that contribute to innate defense and promote recruitment of elements of the adaptive immune response. Up-regulation of PIGR expression is an important feature of this acute response, by increasing epithelial transport of protective IgA Abs. In the continuing presence of TNF, which in vivo could also be produced by activated macrophages and T cells recruited to the intestinal mucosa, the program of TNF-induced gene expression may begin to shift from proinflammatory to anti-inflammatory. Continued high expression of PIGR would be important in this phase to limit the inflammatory response to bacterial and viral products, for example via epithelial transport of IgA anti-LPS Abs (7), and through the anti-inflammatory effector functions of the secretory component of PIGR (8). It is important to note that gene products traditionally associated with acute inflammatory responses may also play roles in limiting inflammation and promoting tissue repair. For example, IL-8 has been shown to stimulate the migration of human colonic epithelial cells, necessary for repair of mucosal damage (53). Production of TNF in the intestine in response to TLR signaling has been shown to be associated with protection against chemically induced colitis (54). Sustained production of TNF and IL-8 may represent a normal part of the epithelial repair response. The availability of an intestinal epithelial cell culture model should facilitate further study of the signaling pathways through which chronic exposure to TNF regulates expression of pro- and anti-inflammatory genes.

Disclosures
The authors have no financial conflict of interest.

References
REGULATION OF THE plgR BY TFN


