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Tumor necrosis factor-alpha (TNFα) regulates the epithelial barrier in the human intestinal cell line HT-29/B6

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SUMMARY

Cytokines are supposed to be mediators in diarrhoeal diseases. The aim of this study is to characterize the effect of tumor necrosis factor-alpha (TNFα) on epithelial barrier function in the colonic epithelial cell line HT-29/B6. Active ion transport and barrier function were measured as short-circuit current and transepithelial electrical resistance (R^t), respectively. In parallel, freeze-fracture electron microscopy (EM) of tight junctions (TJ) and immunofluorescence microscopy of the zonula occludens protein-1 (ZO-1) were performed. Serosal addition of TNFα (100 ng/ml) decreased R^t by 81%. This effect was dose-dependent and could be mimicked by antibodies against the p55 form of the TNF receptor. Cytotoxic effects were excluded by a negative lactate dehydrogenase (LDH) assay. Immunofluorescence localization with anti-ZO-1 antibodies revealed no evidence for disruption of the monolayer after TNFα treatment. In freeze-fracture EM, TJ complexity was decreased by TNFα, as indicated by a decrease in the number of strands from 4.7 to 3.4. The tyrosine kinase blocker genistein and the protein kinase A inhibitor H-8 reduced the effect of TNFα. A combination of TNFα with interferon-γ acted synergistically on the epithelial barrier. In conclusion, TNFα impairs epithelial barrier function by altering structure and function of the tight junction, which could be of pathogenic relevance in intestinal inflammation.

Key words: Barrier, Colonic cell line HT-29/B6, Cytokine, Freeze-fracture EM, Interferon-γ, Tight junction, Tumor necrosis factor-α, Tyrosine kinase, Zonula occludens protein-1

INTRODUCTION

The pathogenic mechanisms of diarrhoea in inflammatory bowel disease (IBD) and HIV enteropathy are still unknown. Both diseases show an altered cytokine production, e.g. of tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) (Lähdevirta et al., 1988; Vyarkarnam et al., 1991; Kotler et al., 1993; Sinicco et al., 1993; Braegger and MacDonald, 1994).

So far, it has been shown that TNFα and IL-1 stimulate an indomethacin-sensitive chloride (Cl^-) secretion in the intestine of various species (Chang et al., 1990; Chiossone et al., 1990; Kandil et al., 1994; Schmitz et al., 1996). Therefore, the induction of prostaglandins by cytokines may contribute to diarrhoea in intestinal inflammation. However, the cytokine effects on intestinal ion transport were only of short duration in these in vitro studies. This, and the fact, that in IBD the inflamed colonic mucosa presents decreased and not increased rheogenic transport do not support the hypothesis of a secretory diarrhoeal mechanism driven by electrogenic Cl^- secretion (Sandle et al., 1990). On the other hand, in IBD tissue conductance of inflamed colonic mucosa is increased and epithelial resistance – as can be measured by alternating current impedance analysis – is almost abolished (Sandle et al., 1990; Schulzke et al., 1995). This finding may point to another mechanism being more important in the pathogenesis of diarrhoea in IBD, namely the alteration of the intestinal epithelial barrier function.

Several studies have described cytokine effects on epithelial and endothelial barrier function. IFNγ markedly decreased transepithelial resistance (R^t) in T_{84} cells after 48 hours, while all other cytokines tested (TNFα, IL-1, IL-2) were inactive (Madara and Stafford, 1989). In endothelial cells TNFα and IL-1 enhanced vascular permeability (Brett et al., 1989; Goldblum and Sun, 1990; Campbell et al., 1992; Burke-Gaffney and Keenan, 1993). Also in the porcine kidney epithelial cell line LLC-PK_1 TNFα decreased R^t for about 1 hour, followed by an overshoot recovery (Mullin et al., 1992), and in HT-29cl.19A cells TNFα caused a small decrease in R^t after 48 hours (Heyman et al., 1994; Rodriguez et al., 1995). In the latter cell line, co-incubation of the cells with IFNγ caused a reduction in tight junctional strand number (Rodriguez et al., 1995). This synergistic cytokine effect may

be due to IFNγ-dependent up-regulation of TNF receptors, but could also be caused by metabolic IFNγ effects enhancing TNFα susceptibility, as identified in a study by Aggarwal and Eessalu (1987).

In our study TNFα strongly impaired the barrier function of HT-29/B6 cells. The time course of this effect resembles the pronounced and continuous loss of barrier function seen in active IBD (Schulzke et al., 1995). Therefore, the HT-29/B6 cell line represents an ideal model epithelium for investigating cytokine dependent tight junction regulation and the mediation pathways involved.

MATERIALS AND METHODS

Cell culture

HT-29/B6 cells are a subclone of the human colorectal cancer cell line HT-29. They grow as highly differentiated polarized epithelial monolayers with properties of Cl^-- and mucus-secreting cells (Kreusel et al., 1991). The cells were routinely cultured in 25 cm^2 culture flasks (Nunc). The culture medium (RPMI 1640, Biochrom KG, Berlin, Germany) contained 2% stabilized L-glutamine and was enriched with 10% fetal calf serum (FCS). Culture was performed at 37°C in a 95% air, 5% CO_2 atmosphere. Cells were seeded on Millicell PCF filters (Millipore, effective membrane area 0.6 cm^2) at an average concentration of $7 \cdot 10^5$ cells/cm^2. Three inserts were placed together into one conventional culture dish (OD 60 mm). Confluence of the polarized monolayers was reached after 7 days. Experiments were performed on day 11 or 12, giving transepithelial resistances (R^t) of 250-400 $\Omega \cdot cm^2$. The apical compartment was routinely filled with 500 μl culture medium, and the basolateral compartment contained 10 ml.

Monitoring of transepithelial resistance

Transepithelial resistance (R^t) of the monolayers was measured by a modification of the method described by Kreusel et al. (1991). Briefly, electrical measurements were performed in the culture dishes by two fixed pairs of electrodes (STX-2, World Precision Instruments, USA) connected with an impedance meter (D. Sorgenfrei, Inst. Klinische Physiologie). R^t was calculated from the voltage deflections caused by an external ±10 μA, 21 Hz rectangular current. Depth of immersion and position of the filters was standardized mechanically. The temperature was maintained at 37°C during the measurements by a temperature-controlled warming plate. Resistance values were corrected for the resistance of the empty filter and of the bathing solution. The setup was placed under a plastic hood, and the electrodes were regularly disinfected with 70% ethanol. This proved to be sufficient to allow repetitive measurements in individual dishes over several days without infection.

Flux measurements

For flux experiments filters were incubated with TNFα for 4.5 hours. Then, the complete inserts were mounted into modified Ussing chambers (Kreusel et al., 1991), which were driven by a 6-channel computer-controlled voltage clamp device (CVC 6, Fiebig, Berlin, Germany). The bathing solution was RPMI 1640 and contained TNFα (for the TNFα-incubated cells). The solution was gassed by 95% O_2 and 5% CO_2. The temperature was maintained at 37°C using water-jacketed reservoirs. The pH was 7.4 in all experiments. Measurements were performed under short-circuit conditions. Short-circuit current (I_{SC}), open-circuit transepithelial voltage, and transepithelial resistance were recorded every 10 minutes. The resistance of the bathing solution was determined before each experiment and subtracted from the raw data. Flux studies from serosal-to-mucosal (s-to-m) were performed with $^{22}Na^+$ and $^3H^+$-mannitol as described previously (Schulzke et al., 1987, 1992). After equilibration, samples for flux measurements were taken at regular intervals of 30 minutes.

Cytotoxicity assay

As a monitor of cell deterioration, the lactate dehydrogenase (LDH) release from the cells was measured (Madara and Stafford, 1989; Heyman et al., 1994). Briefly, the post-experimental LDH content in the supernatant of controls and of TNFα-treated cells was determined. After detergent extraction with 2% Triton X-100 for 20 minutes, the total LDH content of the residual cells was measured. Then, the percentage of LDH released into the supernatant could be calculated.

Drugs

Recombinant human (rh) TNFα (10^7 units/mg) and selective agonistic polyclonal anti-TNFα receptor antibodies, either to the p55 or to the p75 TNF receptor, were provided by Schering (Berlin, Germany). The latter were produced by the immunization of rabbits against the human soluble p55 and p75 TNF receptor types, respectively (Barnes et al., 1996). Cycloheximide, genistein and polymyxin B were obtained from Sigma (Deisenhofen, Germany), and rh-IFNγ was from PromoCell (Heidelberg, Germany). Chelerythrine chloride, staurosporine and H-8 were from Biotrend Chemikalien (Köln, Germany). Polyclonal antibodies against zonula occludens protein-1 (ZO-1) were purchased from Zymed (San Francisco, USA) and the subsequent immunofluorescence localization was performed with Texas Red antibodies from Dianova (Hamburg, Germany). Tracer flux measurements were performed with $^{22}Na^+$ and ^3H-mannitol (Du Pont de Nemours, Wilmington, USA). If not stated otherwise, drugs were added to the serosal side.

Immunohistochemistry

Immunofluorescence localization of ZO-1 was performed to test the integrity of the tight junctional domain after TNFα exposure (we thank Prof. Dr Landmann, Basel/Switzerland for advice). Briefly, monolayers were fixed in paraformaldehyde (4%) at room temperature. Then, cells were permeabilized by incubation with 0.25% Triton X-100. To reduce unspecific binding sites, cells were incubated with sodium borohydride (0.5%), skimmed milk powder (1%) and goat serum (1:20). Then, the cells were incubated with anti ZO-1 antibodies (1:200) for 60 minutes followed by extensive washing. Texas Red-immunofluorescence antibodies were added for 20 minutes. After washing fluorescence microscopy was performed.

By means of video micrographs obtained by light microscopy the length of the tight junction network was determined in control and after TNFα treatment, in order to detect changes in the tight junctional domain and in cell size after TNFα treatment.

The occurrence of apoptosis was tested by the cleavage of poly(ADP-ribose) polymerase (PARP) (Kaufmann et al., 1993) as well as by DNA laddering. For PARP assay, cells were rinsed in PBS and lysed with a solution containing 62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue and β-mercaptoethanol. Then the solution was sonicated for 15 seconds and incubated at 65°C for 15 minutes. For electrophoresis, probes were applied to SDS-gels containing 10% polyacrylamide and subsequently transferred to nitrocellulose (PVDF Transfer Membrane, NEN™ Life Science Prod., Boston, MA, USA). For immunodetection a mouse monoclonal anti-PARP-antibody (dilution 1:1000 for 2 hours; Oncogene Research Products, Cambridge, MA, USA) was utilized and bands were visualized by enhanced chemiluminescence.

For detection of nucleosomal DNA fragmentation, genomic DNA was prepared according to the manufacturer's description of the Apoptotic DNA-Ladder Kit (Boehringer Mannheim, Germany).

Freeze-fracture electron microscopy

Freeze-fracture analysis was carried out as described earlier (Bentzel et al., 1980; Schulzke et al., 1992). HT-29/B6 monolayers were

initially fixed at room temperature with phosphate-buffered 2% glutaraldehyde by simultaneously exposing the mucosal and serosal surfaces to the fixative. This was done while the tissue was still mounted in the in vitro setup, in order to guarantee an identical degree of stretch as in the electrophysiological experiments. Monolayers were then frozen in Freon 22 and liquid nitrogen (–100°C) and fractured with a double replica device (Denton CV-502). Freeze-fractures were shadowed with platinum and carbon and examined in a Phillips 200 electron microscope. Morphometric analysis was performed using coded prints of freeze-fracture electron micrographs (×60,000 magnification) on all tight junction regions in which both an apical and a contra-apical strand of the meshwork could be clearly demarcated. Vertical grid lines in 1 cm intervals (equivalent to 167 nm) perpendicular to the most apical strand served to determine the number of horizontally oriented strands in the meshwork of the tight junction (= strand number). The distance between the most apical and the most contra-apical strand along each vertical grid line was defined as the depth of the tight junction (depth). Total depth and depth of the main compact meshwork of tight junctions did not differ in both groups, since aberrant strands were not observed either in control or after TNFα exposure.

In the control group, 280 grid lines from 22 different tight junction regions and in the TNFα group 320 grid lines from 22 tight junctions were analyzed. In addition to the mean tight junctional strand count, the distribution of tight junction strand numbers within each group was also evaluated.

Statistical analysis

All values are given as means ± s.e.m. The unpaired two-tailed *t*-test was used to determine the significance of differences. $P<0.05$ was considered significant.

RESULTS

Effect of TNFα on transepithelial resistance

Incubation of HT-29/B6 cells with TNFα (100 ng/ml) decreased R^t from 376±26 to 73±8 $\Omega \cdot cm^2$ after 8 hours, corresponding to 19±2% of the initial resistance ($P<0.001$, $n=12$; Fig. 1A). Thereafter, R^t transitorily increased to 28±3%, but stabilized at 18±1% after 24 hours ($P<0.001$, versus control). In a subgroup the time of R^t monitoring after TNFα addition was extended to 72 hours. R^t remained diminished at 24±4% and 23±4% of the initial resistance after 48 and 72 hours, respectively ($P<0.001$, $n=3$). Thereafter, R^t increased again in the presence of TNFα, without reaching starting levels after 10 days of incubation (R^t=56±4% of initial R^t). This effect was only observed if TNFα was added to the basolateral side, while apical addition was ineffective (Fig. 1A). TNFα action was dose-dependent with a minimum effect at 5 ng/ml (Fig. 1B).

Cytotoxicity of TNFα

After 24 hours the LDH activity in the supernatant was 120±11 units/l in controls and 128±12 units/l in TNFα-treated HT-29/B6 cells (n.s., $n=6$). The post-experimental LDH content of the cells after treatment with Triton X-100 was also not different in both groups (2947±226 and 3028±340 units/l, respectively, n.s.). Thus, the percentage of LDH released into the supernatants was equal in controls and TNFα-treated cells (4.0±0.3% versus 4.2±0.3%, n.s.), which indicates that the TNFα-induced decrease in R^t was not due to cytokine-induced cell deterioration.

Specificity of the TNFα effect

First, if boiled at 100°C for 30 minutes, TNFα (100 ng/ml) was without effect on R^t (R^t=97±2% after 24 hours, $n=6$). Secondly, co-incubation with the endotoxin inhibitor polymyxin B (20 μg/ml) did not prevent TNFα action (R^t=18±4% after 24 hours, $n=9$), while polymyxin B alone had no effect on R^t (R^t=93±6% after 24 hours, $n=9$). These results indicate that R^t decrease was due to TNFα addition and not to endotoxin contamination of the TNFα preparation.

In addition, the TNF receptor subtype involved in this TNFα effect was determined by experiments with specific polyclonal receptor antibodies against the p75 and the p55 TNF receptors (anti-p75 TNFR-Ab and anti-p55 TNFR-Ab, respectively), which can exhibit agonistic effects on the TNF receptor (Engelmann et al., 1990; Barnes et al., 1996). In a dilution of 1:10 the anti-p75 TNFR-Ab failed to affect R^t (R^t=97±2% after 24 hours), while antibodies against the p55 TNF receptor decreased R^t to 78±1% after 24 hours ($P<0.001$, $n=6$). IFNγ (10 units/ml) did not influence R^t if added alone, but increased the effect of the anti-p55 TNFR-Ab, thereby diminishing R^t to 16±1% after 24 hours ($P<0.001$, $n=6$). In contrast, the anti-p75 TNFR-Ab was ineffective also in combination with IFNγ (R^t=102±1% after 24 hours).

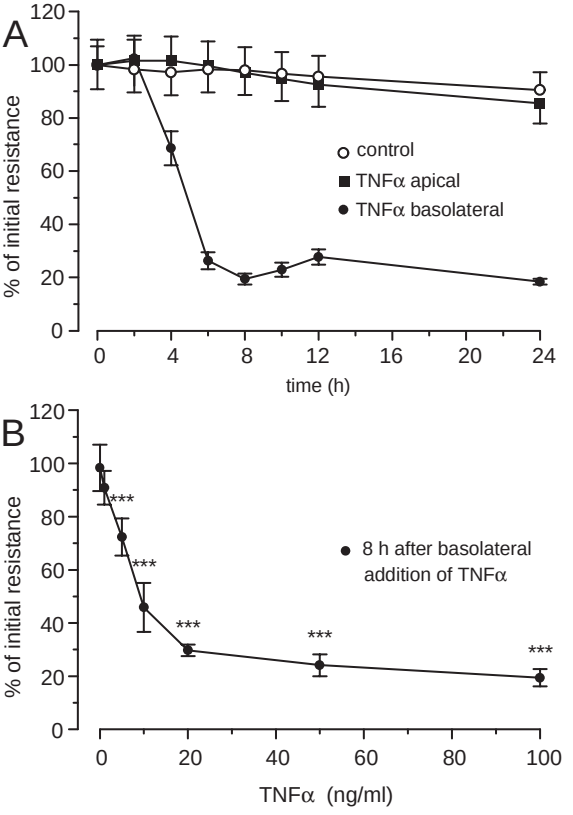

Fig. 1. TNFα action on transepithelial resistance (R^t) of HT-29/B6 colon cells. Data are expressed as the percentage of the initial resistance (=376±26 cm^2 in TNFα-treated cells). (A) Time course. TNFα (100 ng/ml) was added to the basolateral or to the apical side, respectively. Values are means ± s.e.m. of 12 filters. (B) Dose-response curve. Values are means ± s.e.m. (n=9-12; ***$P<0.001$).

Table 1. Effect of TNFα on Na^+ and mannitol s-to-m flux rates, transepithelial resistance and short-circuit current of HT-29/B6 cells

	J^{sm}_{Na} (μmol/hour/cm²)	$J^{sm}_{mannitol}$ (μmol/hour/cm²)	R^t (Ω·cm²)	I_{SC} (μmol/hour/cm²)
Control (*n*=6)	1.6±0.1	0.1±0.01	359±9	0.1±0.01
TNFα (*n*=6)	10.2±1.0	0.4±0.03	53±4	0.1±0.01
P	<0.001	<0.001	<0.001	n.s.

Data are means±s.e.m. of 6 filters under control conditions and after treatment with TNFα (100 ng/ml serosal addition).
Values represent the period of maximum R^t decrease in the TNFα group.
J^{sm}_{Na}, Na^+ s-to-m flux; $J^{sm}_{mannitol}$, mannitol s-to-m flux; R^t, transepithelial resistance; I_{SC}, short-circuit current.
n.s., not significantly different from controls.

Effect of TNFα on I_{SC} and fluxes of Na^+ and mannitol

TNFα-treated cells (100 ng/ml) were pre-incubated in culture dishes for 4.5 hours and then transferred into Ussing chambers (Table 1). The initial I_{SC} of control cells and of the TNFα-treatment group was 0.1±0.01 μmol/hour/cm², respectively, and was constant throughout the whole experiment in both groups. The initial R^t of controls was 334±10 Ω·cm² and slightly decreased to 312±6 Ω·cm² after mounting into the Ussing chamber. In contrast, R^t of TNFα-treated cells was already diminished in the pre-incubation period from 350±17 to 161±13 Ω·cm² (P<0.001). Minimal R^t values of 53±4 Ω·cm² were reached after 7 hours (P<0.001), while R^t of controls increased to 359±9 Ω·cm² (P<0.01). Since I_{SC} was not influenced by TNFα, the decrease in R^t is not due to activation of transporters involved in rheogenic ion transport.

The time course of R^t was paralleled by an increase of Na^+ and mannitol s-to-m fluxes in the TNFα group, while both flux rates were constant in controls at a much lower level (Table 1). Na^+ and mannitol moved across the epithelium in a linear relationship to each other (r^2=0.97, Fig. 2), suggesting that they share the same pathway through the epithelium. The transport ratio of Na^+ and mannitol, which is dependent on their diffusion coefficients as well as on their concentrations (Dawson, 1977), is characterized by the slope of 31.1 (Fig. 2).

Immunohistochemistry on TNFα-treated HT-29/B6 monolayers

Immunofluorescence localization of ZO-1 demonstrated an intact tight junctional network even after TNFα treatment (Fig. 3), although the intensity of the immunofluorescence signal always seemed less intensive than in controls. When measured

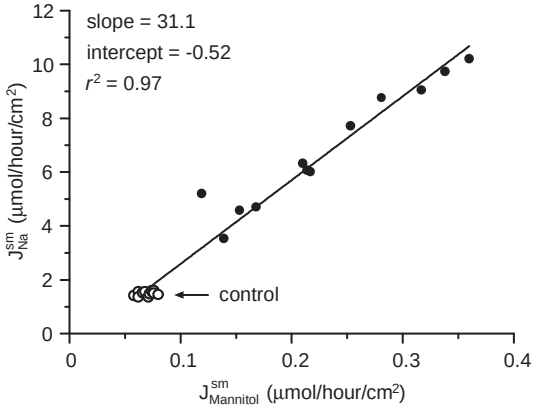

Fig. 2. Correlation of Na^+ and mannitol s-to-m flux under control conditions and at different time points during exposure to 100 ng/ml TNFα.

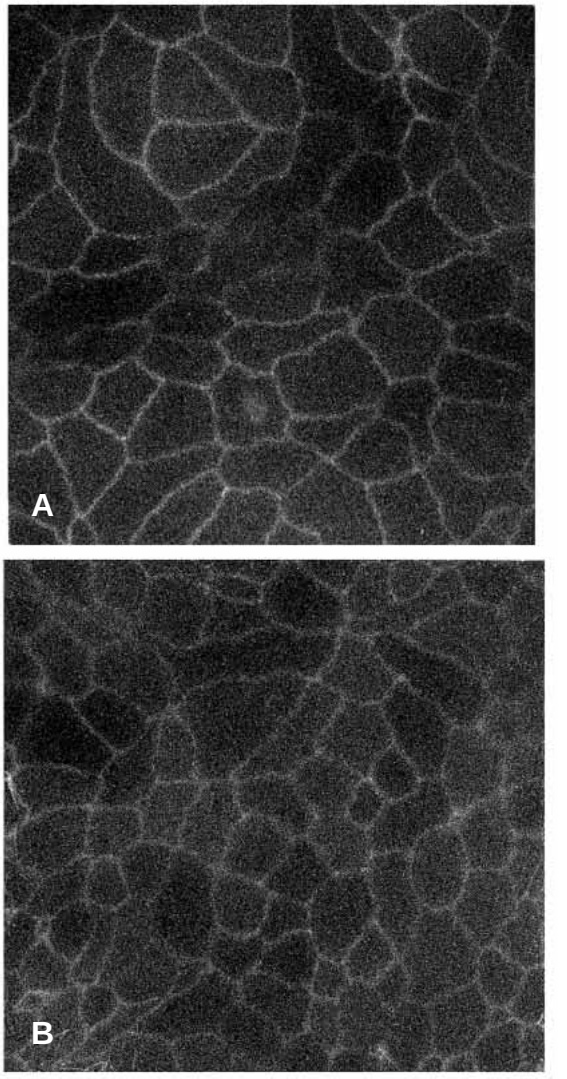

Fig. 3. Immunofluorescence localization of ZO-1 in HT-29/B6 epithelial cells under control conditions (A) and after exposure to TNFα (100 ng/ml) for 24 hours (×630 magnification). Some regions of the monolayer are slightly out of focus, as a result of which the net is not sharp at any region in the picture. However, focusing the respective regions clearly revealed an intact monolayer at all sites.

in a segment of area 3025 μm^2, the length of the junctional network was 833±17 μm (n=6) in controls and 830±18 μm (n=6, n.s.) in TNFα-treated cells. Thus cell size was not affected after treatment with 100 ng/ml TNFα for 24 hours.

Role of apoptosis in TNFα action on HT-29/B6 cells

Since it is known that TNFα induces apoptosis in various cell lines, we determined the extent of PARP proteolytic cleavage, which is observed as a consequence of ICE-protease activation in apoptosis (Kaufmann et al., 1993). In non-irradiated HL-60 cells PARP was nearly exclusively detected in the uncleaved form (116 kDa), whereas UV-irradiated HL-60 cells (as positive controls) displayed predominantly the 85 kDa band of cleaved PARP, which is indicative of apoptosis (Fig. 4A). HT-29/B6 cell monolayers showed some cleavage of PARP even under control conditions. After a 24 hour treatment with TNFα cleavage of PARP was slightly more perceptible than in controls. Similarly, IFNγ induced PARP cleavage that even exceeded the TNFα effect. However, in contrast to TNFα, IFNγ had only a minor effect on R^t (Table 3). Also, neither TNFα- nor IFNγ-treated cells displayed DNA fragmentation (Fig. 4B). Thus, the extent of apoptosis did not correlate with the extent of R^t decrease. This was corroborated by conventional light microscopy, which did not show any difference in gross morphology of TNFα- and IFNγ-treated HT-29/B6 cells compared to control cells after 72 hours (Fig. 5A-C).

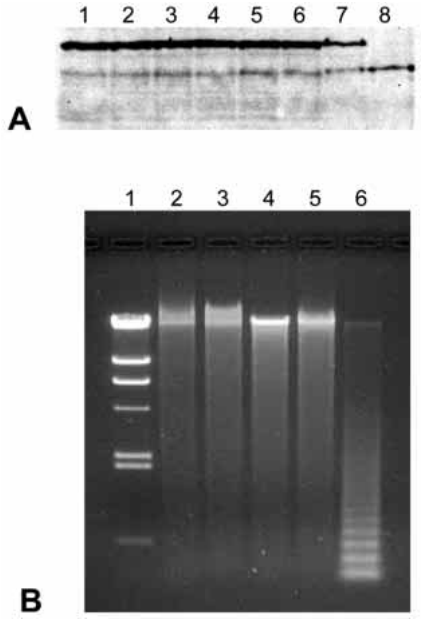

Fig. 4. (A) Cleavage of PARP as a marker for apoptosis of HT-29/B6 cells under control conditions (lanes 1, 2), after treatment with 100 ng/ml TNFα for 24 hours (lanes 3, 4), and after treatment with 1000 units/ml IFNγ after 24 hours (lanes 5, 6). The cleavage of PARP in HL-60 cells (lane 7) after UV irradiation (lane 8) served as a positive control. The upper band shows uncleaved PARP, the lower band cleaved PARP, which indicates apoptosis. (B) Analysis of genomic DNA for the detection of apoptosis in HT-29/B6 cells under control conditions (2), after treatment with TNFα (3), and after treatment with 1000 units/ml IFNγ (4) or 100 units/ml IFNγ (5). Lane 1 shows the marker (λ-DNA/*Hin*DIII fragments) and lane 5 shows the apoptotic laddering of U937 cells treated with camptothecin (10 μg/ml) as a positive control.

Table 2. Tight junction morphometry

	Strands	TJ depth (nm)	n
Control	4.7±0.2	265±17	22
TNFα	3.3±0.2**	200±14*	22

Number of horizontally oriented strands (strands) and depth of tight junction (TJ depth) in control and after 24 hours TNFα treatment.
Values are means±s.e.m.
**P<0.001, *P<0.01.
n, number of tight junctions analyzed.

Freeze-fracture electron microscopy on TNFα-treated HT-29/B6 monolayers

Typical freeze-fracture electron micrographs of control and TNFα-treated cells are shown in Fig. 6. The results of the statistical analysis are demonstrated in Fig. 7 and Table 2. Incubation of HT-29/B6 cells with TNFα (100 ng/ml) decreased the mean number of horizontally oriented strands from 4.7±0.2 to 3.3±0.2 (P<0.001, Table 2) and the depth of tight junctions from 265±17 to 200±14 nm (P<0.01, Table 2). In contrast to control cells, TNFα-treated cells showed a significant nember of tight junctional regions with only 1 or 2 strands as indicated by the distribution of the tight junctional strand counts in Fig. 7A. However, the Gaussian distribution of the strand counts in both groups, the regular shift towards lower strand counts in the TNFα group, and the very low and comparable s.e.m. values for strand count in both groups (control 4.3%, TNF 5.6%), point to an uniform effect of TNFα on the cell monolayer. This is further supported by the fact that the reduction in strand count in the TNFα group cannot be explained by incorporation of a small percentage of tight junctions with very low strand count (Fig. 7B). Taken together, these findings strongly suggest that the TNFα-induced alterations in strand count are homogeneously distributed in HT-29/B6 cells, indicating an orderly interference with the assembly/disassembly process of junction formation.

Variation of incubation time of TNFα

The TNFα-containing basolateral medium was exchanged for TNFα-free solution after incubation periods of 2, 4 and 6 hours, respectively, and R^t was subsequently measured 8 and 24 hours after starting the experiment. No TNFα action was noted, if it was removed after 2 hours (R^t=104±6% after 8 hours, n=4, n.s.). If the cells were incubated for 4 hours with TNFα, R^t was only slightly decreased to 64±9% after 8 hours (n=6, P<0.05), while a 6 hour incubation period sufficed to obtain the full R^t response of 20±3% after 8 hours (n=6, P<0.001). Exchanging the bathing solution after 6 hours provoked a recovery of R^t to 54±1% of the initial resistance after 24 hours (P<0.001), to 80±5% after 48 hours and to 89±3% after 72 hours (data not shown).

Inhibitors of TNFα action

As shown in Fig. 8, the tyrosine kinase inhibitor genistein (185 μM = 50 μg/ml) prevented the TNFα-induced decrease of R^t after 8 hours, while genistein alone had no significant effect on R^t. The protein kinase A inhibitor H-8 (50 μM) was also tested, and on its own also had no significant effect on R^t. H-8 almost

completely inhibited the TNFα effect on R^t after 8 hours, however. In contrast, the protein kinase C (PKC) inhibitors staurosporine (10 nM) and chelerythrine chloride (10 μM) were ineffective in inhibiting TNFα action, suggesting that the PKC pathway is not involved in TNFα action on R^t.

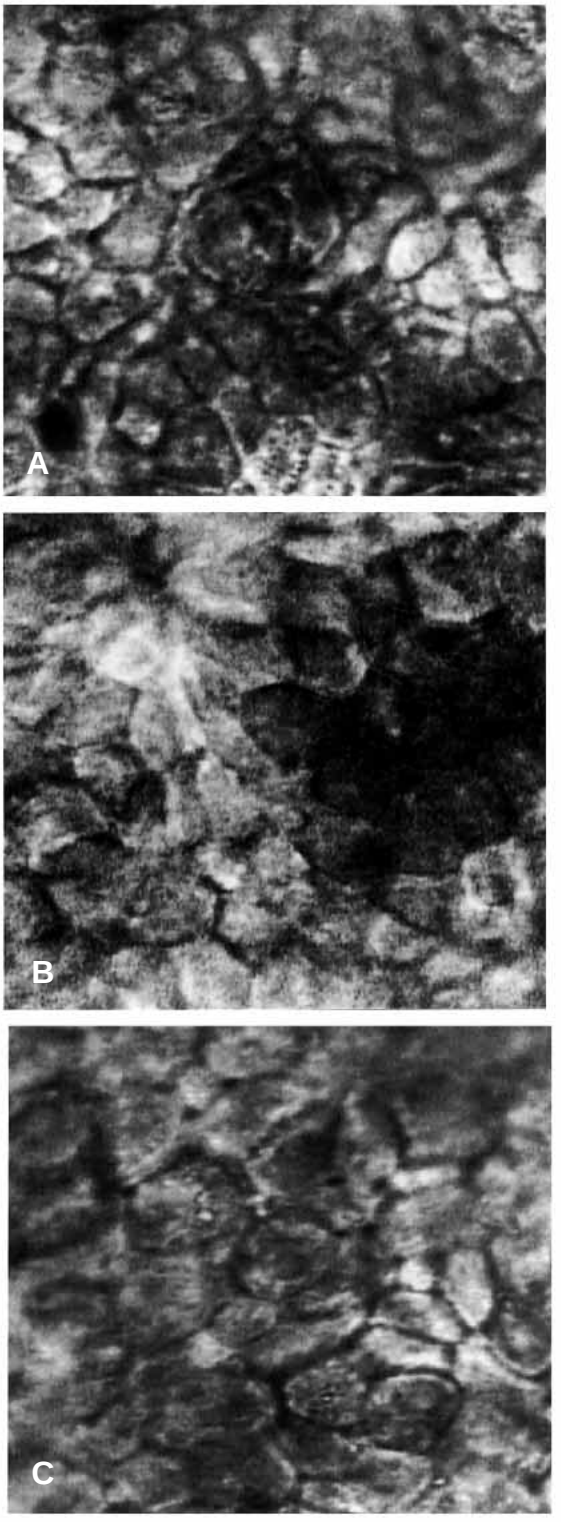

Fig. 5. Representative light micrographs of HT-29/B6 monolayers. (A) Control conditions, (B) after incubation with TNFα for 24 hours and (C) after incubation with IFNγ for 72 hours. ×1000.

Inhibition of protein synthesis and TNFα action

These data are shown in Fig. 9. Cycloheximide (100 μg/ml) alone had no effect on R^t of HT-29/B6 within 12 hours. When cycloheximide was present, the TNFα effect was delayed by about 2 hours, but was not prevented. Furthermore, in the presence of cycloheximide TNFα decreased R^t to 12±2% after 12 hours, whereas R^t showed a partial recovery to 28±3% 12 hours after adding TNFα when cycloheximide was absent (P<0.001). After 24 hours, cycloheximide combined with TNFα led to cell detachment with visible discontinuities of the epithelial layer in light microscopy (R^t=0±3%), whereas there was no significant effect of cycloheximide alone on R^t (R^t=78±8%, P=0.36).

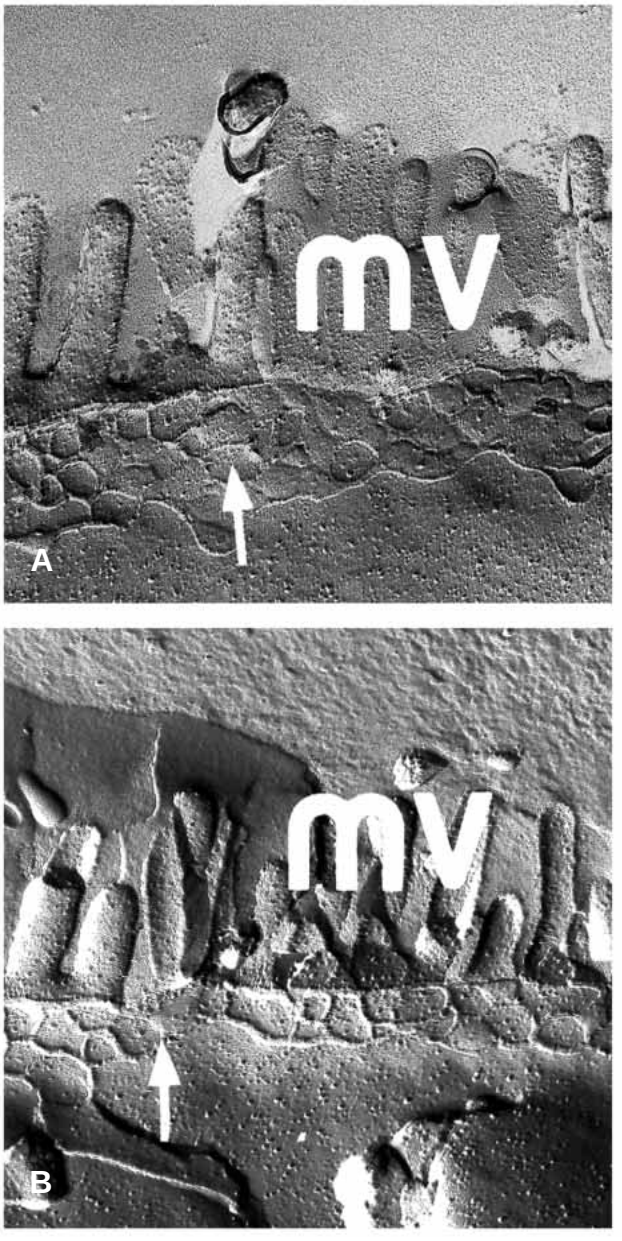

Fig. 6. (A) Freeze-fracture electron micrographs from the junctional region of HT-29/B6 control cells and (B) after incubation with 100 ng/ml TNFα for 24 hours. mv, microvillus; arrow, tight junction (×60,000).

Table 3. Effect of IFNγ and/or TNFα on R^t of HT-29/B6 cells

	Serosal concentration (units/ml)	% of initial resistance		
		After 24 hours	After 72 hours	*n*
Control		98±5	88±4	12
IFNγ	1	98±1^{ns}	87±4^{ns}	6
IFNγ	10	86±5^{ns}	54±6**	6
IFNγ	100	83±7^{ns}	31±4**	15
IFNγ	1000	79±5*	17±1**	15
TNFα	5 ng/ml	69±9*	41±7*	6
TNFα+IFNγ	10	23±4**·‡	11±1**·‡	6

IFNγ was used at a serosal concentration of 1, 10 100 and 1000 units/ml, TNFα was used at 5 ng/ml.

Values are means±s.e.m.

Significance is given versus control. ns, not significant, *P<0.01, **P<0.001.

‡Tested against TNFα alone.

Effect of IFNγ on R^t and TNFα action

Only the high concentration of 1000 units/ml IFNγ had a slight effect on R^t within 24 hours (Table 3), decreasing R^t to 79±5%. If the experiments were prolonged to 72 hours, the effect of 1000 units/ml IFNγ in HT-29/B6 cells was pronounced (R^t=17±1%) and quite similar to the response observed in T_{84} cells (Madara and Stafford, 1989).

If small amounts of TNFα were used, the effect of TNFα on R^t was considerably intensified by IFNγ (10 units/ml). At 5 ng/ml TNFα reduced R^t to 69±9% after 24 hours, but when combined with IFNγ, R^t decreased to 23±4% (P<0.001) and LDH release was not different from controls (4.4±0.2 versus 4.0±0.3 units/ml in controls, n=6, n.s.). This effect continued up to 72 hours. Thus, in the presence of IFNγ, TNFα induced pronounced effects on R^t at much lower concentrations.

DISCUSSION

In inflammatory bowel disease (IBD), colonic ion transport is not characterized by increased active ion secretion, but rather by reduced active absorption and increased mucosal leakiness as the cause for the diarrhoea (Sandle et al., 1990; Schulzke et al., 1995). The pathogenic mechanisms of this barrier defect in IBD are still unclear. In HIV infection diarrhoea is mostly related to secondary infections with enteropathogens, but in 15-50% of cases diarrhoea occurs without an opportunistic infection (Riecken et al., 1990). Also, there is experimental evidence for an epithelial barrier defect from in vivo permeability studies in HIV-infected patients (Ott et al., 1991). Since both IBD and HIV-infected patients exhibited altered cytokine patterns within the intestinal wall and/or in the serum (Lähdevirta et al., 1988; Vyarkarnam et al., 1991; Kotler et al., 1993; Sinicco et al., 1993; Braegger and MacDonald, 1994), we investigated the ability of TNFα to regulate the intestinal barrier in this study. Since cytokine effects may occur even after 48 hours (Madara and Stafford, 1989; Heyman et al., 1994), the highly differentiated colonic epithelial cell line HT-29/B6 was used for our study, which easily allows measurements to be taken for several days. A further advantage of a cell line model is that effects are directly at the epithelial cell level and humoral or neuronal influences can be excluded.

Effect of TNFα on R^t

TNFα strongly impairs the epithelial barrier function of HT-29/B6 cells, beginning after 8 hours and exhibiting its long-lasting effects in a dose-dependent manner. The effect was observed only if TNFα was added to the basolateral side. This suggests that TNFα action relied on TNF receptors expressed on the basolateral cell membrane.

The amount and time course of the TNFα effect in our study is quite different from those found by previous investigators. In the T_{84} cell line model no effect of TNFα was observed (Madara and Stafford, 1989), which may be due to the low TNFα concentration applied. In the porcine kidney epithelial cell line LLC-PK_1 the R^t decrease induced by 50 ng/ml of TNFα lasted only for 1 hour, followed by an overshoot recovery of R^t (Mullin et al., 1992). In the intestinal epithelial

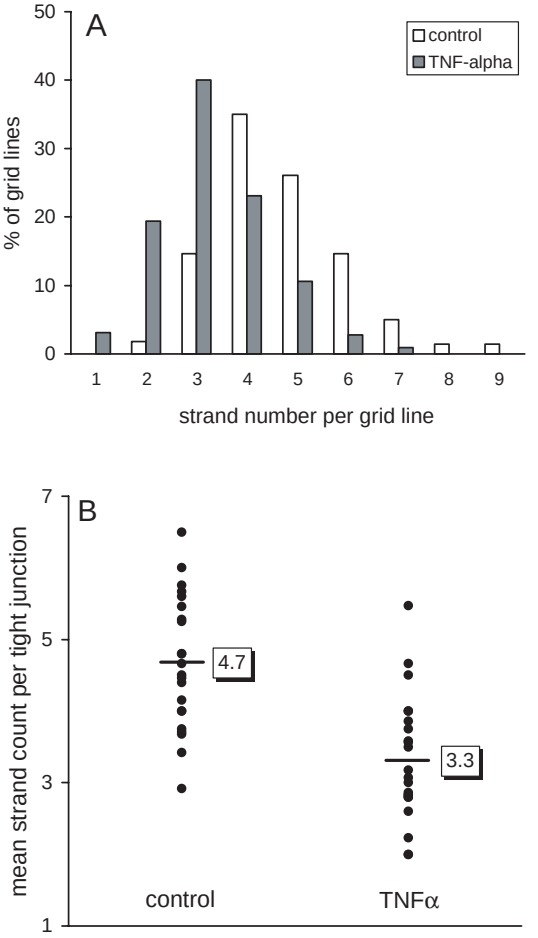

Fig. 7. (A) Distribution of the number of horizontally oriented strands along the tight junction of HT-29/B6 cells under control conditions and after incubation with 100 ng/ml TNFα for 24 hours. Values represent the percentage of grid lines with the respective strand count (related to the total number of grid lines analyzed). For this analysis 280 grid lines of control cells and 320 grid lines of TNFα-treated cells were evaluated (cf. Materials and methods). (B) Mean strand count per tight junction region (n=22) of controls and TNFα-treated cells.

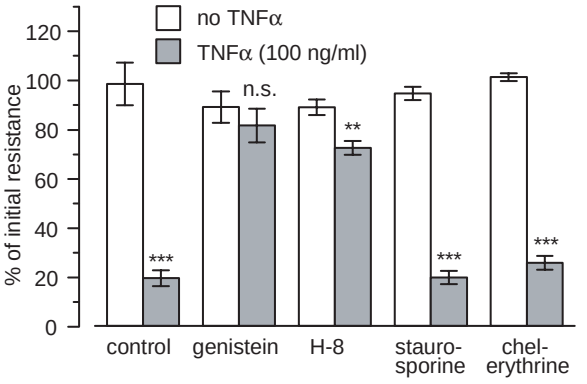

Fig. 8. Inhibitors of TNFα action. Genistein acts as a tyrosine kinase inhibitor (n=11), H-8 is a protein kinase A inhibitor (n=13), and staurosporine and chelerythrine chloride are protein kinase C inhibitors (n=6 each). The inhibitors were given 30 minutes prior to the addition of TNFα. Values are means ± s.e.m. 8 hours after the addition of TNFα (significances are given versus control: **P<0.01, ***P<0.001).

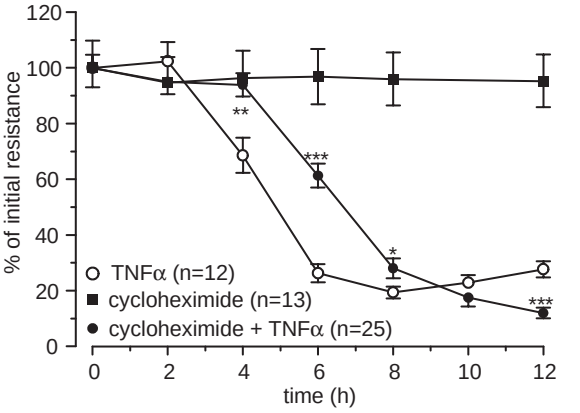

Fig. 9. Influence of the protein synthesis inhibitor cycloheximide (100 μg/ml) on TNFα action. Values represent means ± s.e.m., significance is given versus TNFα effect in the absence of cycloheximide (*P<0.05, **P<0.01, ***P<0.001).

cell line HT-29cl.19A even the high concentration of 100 ng/ml of TNFα had only a very small effect on R^t after 48 hours (Heyman et al., 1994). In a further study by this group, the combination of TNFα with a small dose of IFNγ led to a significant reduction of tight junctional complexity (Rodriguez et al., 1995), which could be due to upregulation of TNFα receptors, as argued by the authors. However, it should be mentioned that IFNγ may also have other, e.g. metabolic, effects in this cell line that could enhance TNFα susceptibility. Aggarwal and coworkers have described such a phenomenon. Induction of TNF receptors by IFNγ was not the major mechanism of synergism, since not only IFNγ but also other interferons acted synergistically with TNFα, yet only IFNγ induced TNF receptors (Aggarwal and Eessalu, 1987).

It is important to note that TNFα without other cytokines affected the barrier function of our HT-29/B6 cell line model. Taking this in account, together with the prolonged time course and the pronounced intensity of the effect, the HT-29/B6 cell line seems to be the most suitable model so far for studying cytokine-dependent regulation of the intestinal barrier.

Cytotoxicity and specificity of TNFα action

In contrast to the results of Heyman and coworkers with cytokine-enriched supernatants obtained from stimulated peripheral blood mononuclear cells of children with cow's milk allergy (Heyman et al., 1994), LDH release was not increased by TNFα in our study, indicating that (1) the TNFα-induced R^t decrease in our study was not due to cell death and (2) the increased LDH release found by Heyman et al. may be due to another mediator, or to the combined action of different cytokines.

The influence of the endotoxin inhibitor polymyxin B, as well as of heat inactivation of TNFα, were tested as standard procedures to discriminate between specific cytokine effects and endotoxin contamination (Sato et al., 1986). The results clearly point against endotoxin contamination.

Receptor subtypes involved in TNFα action

Polyclonal antibodies against the TNF receptor subtypes p55 or p75 can exert TNFα-like effects, most probably by cross-linking of the receptors, which led to the concept that this cross-linking is essential for transduction of the TNFα signal into the cell (Engelmann et al., 1990). It has been demonstrated that only anti-p55 receptor antibodies mimicked the TNFα effect on production of the proinflammatory chemokine, RANTES (**r**egulated upon **a**ctivation, **n**ormally **T** **e**xpressed, and presumably **s**ecreted) in the CH235 astrocytoma cell line (Barnes et al., 1996). In our present study, barrier effects of TNFα were also observed exclusively with the anti-p55 receptor antibodies.

Effect of TNFα on Na^+ and mannitol fluxes

Since the TNFα-induced decrease in R^t was not due to cytotoxicity nor to the activation of rheogenic transporters, the TNFα-induced decrease in R^t should be based on an increase in paracellular permeability. To test this hypothesis, $^{22}Na^+$ and ^{3}H-mannitol (s-to-m) fluxes were measured. TNFα increased Na^+ and mannitol fluxes in a linear relationship (r^2=0.97), indicating that they share the same pathway across the epithelium. The slope of 31.1 approximates quite well the theoretical value of 27, which was calculated by the equation of Dawson (1977) and based on the respective diffusion coefficients in aqueous solution for the solute concentrations used in our study. Thus, Na^+ and mannitol cross the epithelium by simple diffusion through an aqueous path, e.g. through the tight junction and lateral intercellular space.

Morphological studies on HT-29/B6 monolayers

Immunofluorescence localization of ZO-1 indicated that TNFα does not act by disrupting (parts of) the monolayer, a result that is in accordance with the negative LDH assay. Also, TNFα-treated cells did not appear different from control cells when examined by conventional light microscopy, although the fluorescence signal was less intensive in TNFα-treated cells. Although immunohistochemistry does not allow quantification, it seems reasonable to speculate that TNFα may have caused disassembly of tight junction-associated proteins.

Subsequently, apoptosis was tested by measurements of (1) proteolytic cleavage of PARP and (2) nucleosomal DNA

fragmentation as a possible cause of the TNFα-dependent R^t decrease. As the main result, induction of apoptosis by TNFα was only very weak (in the case of DNA laddering it was below the resolution of the method). Furthermore, both assays indicated that the extent of apoptosis in both the TNFα and the IFNγ-treated cell monolayers was similar. Since INFγ only slightly affected R^t, the effect of TNFα has to be mainly attributed to tight junction regulation. However, it cannot be excluded that the induction of apoptosis contributes partly to the observed R^t decrease. For example, Peralta Soler et al. (1996) showed an increase in the rate of apoptosis in LLC-PK_1 renal epithelial cells after TNFα treatment that was accompanied by extensive tissue remodeling and a transient decrease in R^t, although the variation in R^t did not correlate with the number of apoptotic cells.

Furthermore, freeze-fracture analysis of TNFα-treated HT-29/B6 cells revealed a homogeneously distributed decrease in strand number, which is direct evidence for the regulatory effect of TNFα on the tight junction. In this context it is important to note that the relationship between tight junction strand number and ionic conductance is not linear but exponential, as pointed out by Claude (1978). This may explain why a reduction of 1.3 strands caused the dramatic decrease of R^t. Furthermore, not only the mean strand number was altered by TNF, but a significant percentage of the tight junctional area exhibited only 1 or 2 strands, indicating that barrier function is seriously affected by TNFα.

Variation of incubation time of TNFα

Wash-out experiments revealed that TNFα has to be present for at least 4 hours in order to affect R^t and for 6 hours to yield the full R^t response. The long interval between application of TNFα and the decrease in R^t may reflect a complex intracellular mediation pathway. If TNFα was removed after TNFα action had peaked, R^t partially recovered within 24 hours, and changed to almost normal R^t values after 72 hours. Thus, TNFα needs to be continuously present to maintain reduced R^t levels.

Inhibitors of TNFα action

In HT-29/B6 cells, the tyrosine kinase inhibitor genistein completely blocked the TNFα effect on R^t after 8 hours, indicating that a tyrosine kinase is involved in the intracellular signal transduction cascade. That tyrosine kinases are important for the regulation of barrier function is also known from other studies. Tyrosine phosphorylation increased tight junction permeability in MDCK cells (Staddon et al., 1995) and Mullin and coworkers found an inhibitory effect of genistein on the TNFα effect in LLC-PK_1 cells (Mullin et al., 1992). However, in contrast to LLC-PK_1 cells, the protein kinase A (PKA) inhibitor H-8 was also able to inhibit the TNFα-induced decrease in R^t in our HT-29/B6 cell model, suggesting that in addition to tyrosine kinases PKA plays a crucial role as well. This points to an important difference in signal transduction between LLC-PK_1 and HT-29/B6 cells, which could explain the prolonged time course and the more intensive effect.

Inhibition of protein kinase C (PKC) with staurosporine or chelerythrine chloride had no effect on TNFα action. The same result was obtained in other studies (Mullin et al., 1992; Marano et al., 1995), suggesting that TNFα effects on tight junction permeability are independent of the PKC pathway.

Inhibition of protein synthesis and TNFα action

First, cycloheximide alone had no effect on R^t of HT-29/B6 cells within 24 hours, which might indicate that maintenance of barrier function (tight junction integrity) is independent of protein synthesis or at least does not require very much protein synthesis within this limited observation period.

Secondly, cycloheximide delayed the TNFα effect by about 2 hours, a result which could be interpreted in terms of protein synthesis to be required for intracellular signal transduction. That the TNFα effect in LLC-PK_1 cells did not show such a delay (Mullin et al., 1992) points again – as the different H-8 effect mentioned above – to differences in the intracellular signal transduction between HT-29/B6 and LLC-PK_1 cells.

Thirdly, R^t did not stabilize 8 hours after addition of TNFα in the cycloheximide-pretreated group and even showed epithelial discontinuities after 24 hours. A possible explanation could be that stabilization of R^t and integrity of the epithelium after addition of TNFα is due to a new steady state of tight junction degradation and assembly on a reduced level, and for this tight junction formation and/or assembly protein synthesis is required.

Effect of IFNγ on R^t of HT-29/B6 cells

As already observed by Madara and Stafford (1989) on T_{84} cells, IFNγ strongly decreased R^t of HT-29/B6 cells after 72 hours. When used in combination, IFNγ acted synergistically with TNFα. Whether or not this effect is due to up-regulation of TNF receptors by IFNγ is not clear. Aggarwal and Eessalu (1987) found that metabolic IFNγ effects can imitate cytokine synergism. Independent of the underlying mechanisms, the presence of IFNγ can enhance the TNFα effect on barrier function, which may have pathophysiological implications as it can explain significant TNFα effects at very low cytokine concentrations.

Possible role of cytokines in intestinal inflammation

A significant barrier dysfunction in IBD has been documented in several studies (Sandle et al., 1990; Schulzke et al., 1995). Whether TNFα and/or other cytokines contribute to such a barrier dysfunction needs further clarification. According to our present study, cytokines are prominent candidates in this respect. Consistent with this idea, single infusions of anti-TNFα antibodies have been reported to lead to clinical and endoscopic remission for several weeks in patients with therapy-refractory Crohn's disease (van Dullemen et al., 1995).

In conclusion, our study presents a novel cell model (HT-29/B6) for studying cytokine effects on intestinal barrier function, particularly that of the colon. With respect to time course and intensity of cytokine effects, this model epithelium resembles the severe and continuous barrier defects seen in IBD much more than any other intestinal cell line. TNFα seriously impairs the epithelial barrier function, and this is even further enhanced by IFNγ. Since active ion secretion has not been found to be activated in the colon of patients with IBD and conductance is severely affected (Sandle et al., 1990; Schulzke et al., 1995), a cytokine-induced barrier defect could be of pathogenic relevance in IBD, firstly by contributing to inflammation due to the invasion of antigenic or noxious agents, and secondly by leading to a loss of ions and water into the intestinal lumen (leak flux mechanism of diarrhoea; Fasano et al., 1988).

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