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Constitutive Expression of IL-18 and IL-18R in Differentiated IEC-6 Cells: Effect of TNF-α and IFN-γ Treatment

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ABSTRACT

The multifunctional cytokine interleukin-18 (IL-18) is an important mediator in intestinal inflammatory processes. The aim of this study was to evaluate the constitutive expression of IL-18 and its receptors (IL-18Rα and IL-18Rβ) in intestinal epithelial cells (IEC) stimulated by tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). In addition, cellular proliferation and evaluation of brush border enzymes as differentiation markers were studied. Nontransformed rat intestinal epithelial IEC-6 cells were grown on an extracellular matrix (ECM) in medium with or without TNF-α, IFN-γ, or a combination of both. Gene expression of IL-18, its receptors and apoptotic markers was evaluated using real-time PCR. Expression of IL-18Rα protein was demonstrated by flow cytometry and Western blot. Enzymatic activities of brush border enzymes and caspase-1 were determined. The constitutive expression of IL-18, IL-18Rα and IL-18Rβ mRNAs and proteins were detected in IEC-6 cells. The biologically active form of IL-18 was released in response to TNF-α and IFN-γ treatment. Exogenous IL-18 had no effect on cellular proliferation, brush border enzyme activities, and gene expression of apoptotic markers. However, the addition of IL-18 stimulated production and release of the chemokine IL-8. These data suggest that IEC-6 cells may be not only a source of IL-18 but also a target for its action.

INTRODUCTION

INTERLEUKIN-18 (IL-18) is a multifunctional cytokine with structural similarities to the IL-1 cytokine family (Okamura and others 1995b; Nakanishi and others 2001). Initially, IL-18 was described as an interferon-γ (IFN-γ)-inducing factor in mice infected with *Propionibacterium acnes* (Okamura and others 1995a). Later, it was found that IL-18 is detectable in different cell types, including macrophages, keratinocytes (Stoll and others 1997), osteoblasts (Udagawa and others 1997), cardiomyocytes (Chandrasekar and others 2003), rat testis (Strand and others 2005), and epithelial cells (Takeuchi and others 1997). IL-18 is produced as a precursor molecule, which is cleaved by caspase-1 to the mature, bioactive protein (Gu and others 1997) capable of activating the IL-18 receptor complex (Torigoe and others 1997). Although the biologic functions of IL-18 are still not fully understood, IL-18 is considered to be

an important factor in the regulation of inflammatory processes (Reuter and Pizarro 2004).

Intestinal epithelial cells (IECs) line the mucosal surface of the intestine and, in addition to their digestive and absorptive functions, form a barrier to prevent the entry of microbes and antigens into the underlying tissue (Goodrich and McGee 1998). IECs can constitutively, or upon stimulation, produce a number of cytokines, chemokines, and growth factors that play an important role in mucosal immune response (Stadnyk 2002). IECs possess receptors that respond to extrinsic stimuli, such as intestinal infection and cytokines produced by activated immune cells. Specific cytokines, such as IFN-γ and tumor necrosis factor-α (TNF-α), alone (Kaiser and Polk 1997; Chavez and others 1999) or in synergy (Fish and others 1999), may alter enterocyte activity, proliferation, differentiation, and intestinal barrier function (Snoeck and others 2005). Epithelial cell lines cultured with cytokines have been used successfully

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as an *in vitro* model for studying inflammatory processes (Vallee and others 2004).

Proinflammatory cytokines have been implicated in the pathogenesis of inflammatory bowel disease due to their overproduction in inflamed mucosa (Breese and others 1994; Sartor 1994; Niessner and Volk 1995). In the presence of proinflammatory IFN-γ, precursor T cells are polarized toward a Th1 phenotype. Activation of Th1 cells induces an inflammatory cascade that is associated with the production of TNF-α by macrophages and lymphocytes. Many lines of evidence suggest that IL-18 (Pages and others 2000) plays a central role in inflammatory diseases by acting on Th1 cell differentiation and altering the cytokine profile. In patients with Crohn's disease (Pizarro and others 1999), chronic lesions exhibit increased IL-18 protein levels accompanied by overproduction of IL-18-induced cytokines, such as IFN-γ, IL-1β, IL-8, and TNF-α. The presence of these cytokines promotes neutrophil infiltration, leading to tissue injury. Similarly, in experimental models of intestinal injury (Siegmund and others 2001; Halpern and others 2002), overproduction of IL-18 in the inflamed mucosa is correlated with progression of tissue damage. Interestingly, IL-18 is produced not only by various immunocompetent cells but also by the IECs in both normal and inflamed mucosa (Takeuchi and others 1997; Halpern and others 2002; Kalina and others 2002; Okazawa and others 2004). However, the physiologic role of epithelial cell-derived IL-18 remains unclear (Okazawa and others 2004).

In this study, the nontransformed rat IEC derived from rat small intestine (Quaroni and others 1979) was used. To study expression of IL-18 and its receptors (IL-18Rα and IL-18Rβ), we used the nontransformed rat IEC line IEC-6 cultivated on an artificial extracellular matrix (ECM) (He and others 1993), producing differentiated villuslike cells that are more susceptible to apoptosis and cell injury. Such cells were used to elucidate the mechanisms of cytokine action on differentiated cells. The aim of this study was to evaluate the constitutive expression of IL-18, IL-18Rα, and IL-18Rβ mRNAs, IL-18Rα, and IL-18 proteins in IEC-6 cells modulated with TNF-α and IFN-γ. In addition, cellular proliferation and differentiation, assessed by levels of brush border enzymes, were evaluated.

MATERIALS AND METHODS

Cell line, cytokines, and reagents

IEC-6 cells were purchased from American Type Culture Collection (ATCC, CRL-1592, Manassas, VA). Recombinant rat TNF-α was from Serotec (Kidlington, UK), and recombinant rat IFN-γ, recombinant rat IL-18, and goat antimouse IL-18Rα were from R&D Systems, Inc. (Minneapolis, MN). Rat IL-18 ELISA kit was purchased from Biosource International (Nivelles, Belgium). Caspase-1 Colorimetric Assay Kit was provided by BioVision Inc. (THP Medical Products, Vienna, Austria). EMC Gel, CHAPS, pepstatin A, five nucleotide monophosphates (AMP, CMP, IMP, GMP, and UMP), DMEM medium, insulin and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were purchased from Sigma (St. Louis, MO). Glycyl-L-proline-4-nitroanilide was from Sigma-Aldrich (Diesenhofen, Germany). Zwittergent 3–14 was obtained from Calbiochem (La Jolla, CA). Gentamicin was from Krka (Ljubljana, Slovenia). Rabbit antigoat IgG conjugated with FITC and rabbit anti-goat IgG conjugated with alkaline phosphatase were from Vector Laboratories (Burlingame, CA), and 5-L-glutamic acid-4-nitroanilide, p-nitrophenylphosphate, and glycylglycine were from Fluka (Buchs, Switzerland).

IEC-6 cultures

Normal rat small intestinal IEC-6 cells (15–27 passages) were used. The stock culture was grown in DMEM at 37°C, 95% air and 5% CO_2. The medium contained 40 μg/mL gentamicin, 2 mM glutamine, 50 mL/L fetal bovine serum (FBS), and 10 mg/L insulin. For experiments, the cells were plated at a density of $1.6 \times 10^6/cm^2$ onto plastic plates (60 mm in diameter) coated with Matrigel (Engelbreth-Holm-Swarm sarcoma basement membrane). The cultivation medium contained a mixture of five nucleotide monophosphates (10 mg/L each), added on the second day after seeding (He and others 1993). At day 5, the culture medium was replaced with fresh medium containing the nucleotide mixture and either TNF-α, IFN-γ, or a combination of both. In some experiments, IL-18 was added. On day 8, the culture medium was removed and stored at -75°C. The cells were harvested by gentle trypsinization with 0.25% trypsin-0.3% EDTA. Cells were washed with phosphate-buffered saline (PBS) and directly used for flow cytometry or pelleted at 10,000g and homogenized in (1) 0.6 mL lysis buffer RLT for RNA isolation, stored at -75°C, (2) 50 mM KCl for enzyme assays, centrifuged at 100,000g for 45 min, and the pellet solubilized in 0.5% Zwittergent 3–14 in 50 mM KCl at 4°C for 2 h and centrifuged at 100,000g for 45 min, the supernatant stored at -75°C, or (3) 0.6 mL lysis buffer for caspase-1 assay, centrifuged at 100,000g for 45 min, and the supernatant stored at -75°C.

RNA preparation

Total RNA was isolated from IEC-6 cells using the RNeasy Mini Kit (Qiagen, Santa Clara, CA) as described in the manufacturer's protocol and our previous studies (Halpern and others 2003; Clark and others 2005). All samples were incubated with RNase-free DNase (20 U per reaction) for 10 min at 37°C to eliminate DNA contamination. RNA concentration was quantified by ultraviolet spectrophotometry at 260 nm, and the purity was determined by the A_{260}/A_{280} ratio (SPECTRAmax PLUS, Molecular Devices, Sunnydale, CA). The integrity of RNA was verified by electrophoresis on a 1.2% agarose gel containing formaldehyde (2.2 M) and ethidium bromide in $1 \times$ MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, and 1 mM EDTA, pH 8.0) (Halpern and others 2003; Clark and others 2005).

RT and real-time PCR

RT real-time PCR assays were performed to quantify rat IL-18, IL-18Rα, IL-18Rβ, Bcl-2, and Bax steady-state mRNA levels (Halpern and others 2002, 2003; Clark and others 2005). cDNA was synthesized from 0.5 μg DNase-treated total RNA. Target (Bcl-2, Bax, IL-18Rα, and IL-18Rβ) primers and probes were designed using Primer Express Software™ (Applied Biosystems, Foster, CA) (Table 1). Target probes were labeled

TABLE 1. SEQUENCES OF PCR PRIMERS AND PROBES

Gene	Sense primer	Antisense primer	Probe sequence	GenBank
Bcl-2	aggattgtggccttctttgagtt	gccggttcaggtactcagtcat	ccctggtggacaacatcgctct	N016993.1
Bax	ttgctgatggcaacttcaactg	ctttagtgcacagggccttgag	ccgcgtggttgccctcttctactttg	NM017059.1
IL-18Rα	ctcaatgttttatataattgtaccgtggc	ccttggatgtcaggtgtttcttttc	aacgaagaagccacagacac	XM237088.4
IL-18Rβ	ctttctctgtctctcagaggcc	gaggcttaacttccaagacagtct	acacaacaagctgtatcctg	AJ550893.1

with fluorescent reporter dye FAM (Pham and others 2003). Predeveloped TaqMan® primer and probe were used for detection of IL-18 (Applied Biosystems). Reporter dye emission was detected by an automated sequence detector combined with ABI Prism 7700 Sequence Detection System® software (Applied Biosystems). Real-time PCR quantification was then performed using TaqMan 18S controls. Relative quantification of PCR products was based on value differences between the target and 18S control using the comparative C_T method (Menon and others 2001). Cycle parameters were 55°C for 5 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 55°C for 60 sec. All samples were run in triplicate for each PCR reaction.

Flow cytometry

Cultured IEC-6 cells were washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatin (PBS-GEL) and were surface stained for flow cytometry as described previously (Sinkora and others 2005, 2007). Briefly, cells were incubated with goat antimouse IL-18Rα primary polyclonal antibody for 30 min and subsequently washed twice in PBS-GEL. This antibody was shown to cross-react with rat IL-18R (Chandrasekar and others 2003). In control staining, primary antibody was replaced by PBS-GEL alone, by normal heat-inactivated goat serum, or by goat antimouse IgG polyclonal antibody. Rabbit antigoat IgG secondary antibody conjugated with FITC was then added to the cell pellets, and after 30 min, cells were washed three times in PBS-GEL and measured on a FACS Calibur cytometer (BDIS, Mountain View, CA). Damaged and dead cells were excluded from analysis using propidium iodide fluorescence.

Cytokine protein measurements

IL-8 and monocyte chemotactic protein 1 (MCP-1) were measured using a Luminex-based bead array method using the LINCO*plex* simultaneous multianalyte detection system for rats (Linco Research, Inc., St. Charles, MO) following the manufacturer's instructions. The cytokines were simultaneously measured in cultivation medium from each sample. The concentration of IL-18 protein in the cultivation medium was determined using the enzyme-linked immunosorbent assay (ELISA) kit for rat IL-18 according to the manufacturer's instructions (Biosource International).

Caspase-1 assay

Pelleted IEC-6 cells were homogenized in lysis buffer containing 25 mM Na HEPES, 2 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM PMSF, and 1 μM pepstatin A, pH 7.2. The lysate was centrifuged at 4°C at 100,000g. The su-

pernatant was immediately frozen and stored at −75°C until use. The assay was performed using the caspase-1 colorimetric assay kit according to the manufacturer's instructions (BioVision Inc.). Caspase-1 activity was expressed in nanomoles/μg protein. Protein measurement was with Folin phenol reagent.

Western blot

Cells were extracted with lysis buffer containing 2 mM EDTA, 50 mM TRIS-HCl, pH 7.5, 100 mM NaCl, and 1% Nonidet 40. After centrifugation at 15,000g, supernatants were used for further analysis. Cell lysates were diluted in 10 mM KCl to obtain a final concentration of 2 mg/mL protein. The same volume of each lysate (20 μL/well) was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred onto nitrocellulose membranes. IL-18 Rα was detected after incubation of membranes with goat antimouse IL-18R antibodies (1:500 final dilution), followed by incubation with rabbit antigoat IgG conjugated with alkaline phosphatase (1:500 final dilution). Immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Quantification of IL-18Rα expression was performed using dot-blot analysis. Densitometry was performed to compare protein expression between groups with Bio-Rad QuantityOne software (Richmond, VA).

Determination of IEC-6 brush border enzymes

Lactase of the lactase-phlorizinhydrolase complex (β-galactosidase, glycosylceramidase; EC 3.2.1.23/62/108), sucrase (EC 3.2.1.48) of the sucrase-isomaltase complex (sucrose α-glucosidase, oligo-1,6-glucosidase; EC 3.2.1.48/10), and glucoamylase of the maltase-glucoamylase complex (α-glucosidase, glucan 1,4-α-glucosidase; EC 3.2.1.20/3) activities were determined with 50 mM lactose, 50 mM sucrose, and 12 g/L starch as substrates, respectively (Kolinska and Kraml 1972; Kraml and others 1972). The liberated glucose was measured with the Tris-glucose oxidase-peroxidase reagent (Dahlqvist 1964). Alkaline phosphatase (ALP) (EC 3.1.3.1), activity was determined in brush border membrane vesicles (BBMV) with 0.1 M p-nitrophen-ylphosphate as substrate in Tris-HCl buffer, pH 8.5. The liberated p-nitrophenol was measured at A_{405} using the Test Kit (Lachema, Brno, Czech Republic). The activity of γ-glutamyltranspeptidase (GGT; EC 2.3.2.2) was determined with 7 mM 5-L-glutamic acid-4-nitroanilide as donor substrate, 0.1 mol/L glycylglycine, pH 8.2, as acceptor substrate, and 0.1 M NaCl as activator. Released 4-nitroaniline was measured at A_{405} (Thompson and Meister 1976). The activity of dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) was determined with 1.4 mM glycyl-L-proline-4-nitroanilide in 66 mM Tris-HCl buffer, pH 8.0. The reaction was stopped with 1 M

TABLE 2. EFFECT OF TNF-α AND IFN-γ ON IEC-6 CELL COUNTS[a]

| Treatment no. | Control | TNF-α | | IFN-γ | | IFN-γ + TNF-α |
		10 ng/mL	20 ng/mL	100 ng/mL	200 ng/mL	100 ng/mL + 20 ng/mL
1	5.13 ± 2.21	4.53 ± 0.19*				
2	5.95 ± 0.11		5.88 ± 0.21			
3	5.17 ± 0.14			3.95 ± 0.11***		
4	4.97 ± 0.30				3.02 ± 0.40*	
5	5.68 ± 0.05					3.32 ± 0.05***

[a]Values are means ± SEM, represent cell counts × 10^{-6}/60-mm plastic plate. IEC-6 (1.3×10^6/cm^2) passages 15–27 were plated onto the Matrigel-coated plastic plates in DMEM-based medium with nucleotide mixture. Addition of cytokines on cultivation day 5; cells collected on day 8.

*$p < 0.05$; ***$p < 0.001$ vs. control.

sodium acetate buffer, pH 4.2, and the released 4-nitroaniline was measured at A$_{405}$ (Nagatsu and others 1976). Enzyme activities were expressed as nkat/mg protein, 1 nanokatal being the amount of the enzyme that converts 1 nanomole of substrate per second under the given conditions. Protein was estimated with Folin phenol reagent.

Statistics

Statistical analyses between groups were performed using analysis of variance (ANOVA) followed by Fisher protected least significant difference. All statistical analyses were determined using the statistical program StatView (Abacus Concepts, Berkeley, CA). All numerical data are expressed as mean ± SEM. Statistical analysis of brush border enzymes was assessed by nonparametric ANOVA Kruskal-Wallis test, followed by pairwise group comparison using the Mann-Whitney U-test. The data are expressed as mean ± SD.

RESULTS

TNF-α + IFN-γ induce changes in IEC-6 cell number

Table 2 shows there was no change in cell number when monolayers plated on Matrigel were cultivated with either 10 or 20 ng/mL TNF-α. Proliferation of IEC-6 cells was markedly inhibited by IFN-γ at concentrations of 100 and 200 ng/mL. When added together, TNF-α (20 ng/mL) intensified the effect of IFN-γ (100 ng/mL).

TNF-α in combination with IFN-γ significantly inhibits expression of differentiation marker enzymes

Table 3 shows that TNF-α (20 ng/mL) did not affect the expression of any brush border enzyme studied. In contrast, IFN-γ (100 ng/mL) significantly inhibited expression of the main membrane differentiation markers lactase, sucrase, and glucoamylase. TNF-α in combination with IFN-γ markedly inhibited the expression of both glycosidases and peptidases and, to the greatest extent, alkaline phosphatase.

Detection of IL-18 mRNA by real-time RT-PCR

IL-18 mRNA was detectable in nontransformed rat IEC-6 cells plated on a basement membrane matrix and cultured in DMEM-based medium supplemented with insulin and five nucleotide monophosphates (Fig. 1). No significant changes in IL-18 mRNA expression were found after addition of TNF-α (20 ng/mL) or IFN-γ (60 ng/mL) to the cultivation medium on day 5 until harvest. Stimulation of IEC-6 cells with a combination of both TNF-α and IFN-γ (20 ng/mL and 60 ng/mL, respectively) caused a significant decrease in IL-18 mRNA compared with control.

TABLE 3. EFFECT OF TNF-α (20 NG/ML) AND IFN-γ (100 NG/ML) ON ENZYME ACTIVITIES OF MEMBRANE FRACTION OF IEC-6[a]

Enzyme	Control	IFN-γ	TNF-α nkat/mg protein ($\times 10^3$)	IFN-γ + TNF-α
Lactase	106.4 ± 6.4	78.0 ± 12.0**	107.6 ± 15.7	31.0 ± 9.7**
Sucrase	27.1 ± 1.3	16.3 ± 5.9*	23.8 ± 11.5	6.9 ± 5.3**
Glucoamylae	85.5 ± 4.3	63.3 ± 18.8*	91.3 ± 23.8	21.7 ± 6.7**
DPP IV[b]	440 ± 25	413 ± 53	440 ± 31	307 ± 43**
GGT	225 ± 17	217 ± 32	227 ± 21	129 ± 76**
Alkaline phosphatase	96.9 ± 6.9	86.8 ± 9.4	97.4 ± 11.9	8.4 ± 1.2**

[a]Values presented as means ± SD. IEC-6 (1.3×10^6/cm^2) passages 18–25 grown on the Matrigel matrix in DMEM-based medium with nucleotide mixture. Addition of cytokines on cultivation day 5; cells collected on day 8.

[b]DPP IV, dipeptidyl peptidase IV; GGT, γ-glutamyltranspeptidase.

*$p < 0.05$; ***$p < 0.01$ for cytokine-treated cells vs. control.

Effects of TNF-α and IFN-γ on secretion of IL-18 protein from IEC-6 cells

IEC-6 cells secrete IL-18 protein into the cultivation medium without any stimulation. Cells grown in cultivation medium supplemented with either TNF-α or IFN-γ alone or a combination of both significantly increased the secretion of IL-18 protein (Fig. 2). Because of a variation in the IL-18 concentration in supernatants from individual subcultures, combined results from all studies are expressed as mean relative IL-18 levels. The range of the actual IL-18 concentrations expressed in pg/million cells was: control (1–10), IFN-γ (3–26), TNF-α (8–18), and IFN + TNF (19–36).

Detection of IL-18Rα and IL-18Rβ mRNAs by real-time RT-PCR

Stimulation of IEC-6 cells with IFN-γ caused increased expression of IL-18Rα, whereas TNF-α or the combination of both did not have any stimulatory effect on IL-18Rα mRNA level (Fig. 3A). In contrast, gene expression of IL-18Rβ was not affected by IFN-γ or TNF-α alone; however, the combination of both cytokines caused synergistic stimulation of IL-18Rβ expression (Fig. 3B).

Constitutive expression of IL-18Rα protein and its detection on surface of cells

Because an antibody against rat IL-18Rα is not available, we used antimouse IL-18Rα that was shown to cross-react with rat IL-18Rα (Chandrasekar and others 2003). Constitutive expression of IL-18Rα protein was shown in IEC-6 cells after 8 days in culture. IEC-6 lysate resolved on SDS-PAGE and probed with antimouse IL-18Rα revealed three domains of IL-18Rα on Western blot (Fig. 4A). This may indicate IL-18Rα polymorphisms (Azam and others 2003). In Figure 4A, lane 1 without and lane 2 with 20 ng/mL TNF-α added for the last 24 h
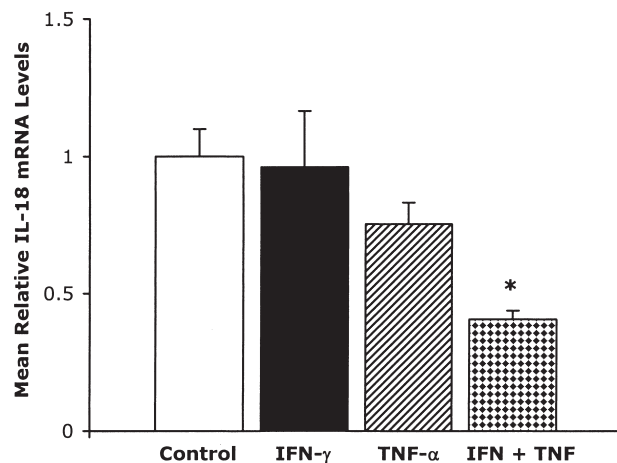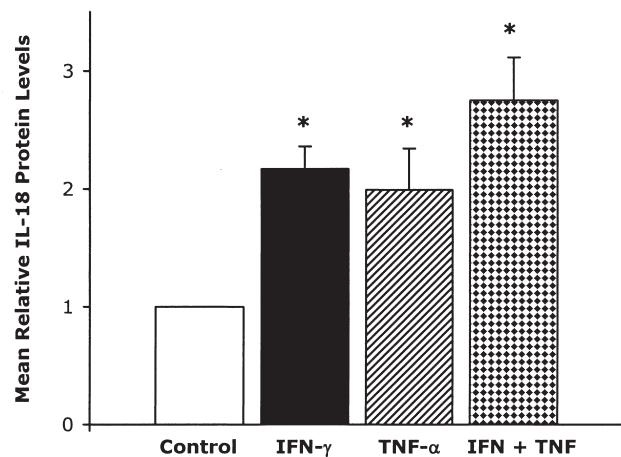

FIG. 2. Secretion of IL-18 protein. TNF-α (20 ng/mL), IFN-γ (60 ng/mL), and TNF-α + IFN-γ (20 ng/mL + 60 ng/mL) added on day 5 of cultivation induced IL-18 protein secretion from IEC-6 cells after 3 days in culture. IL-18 protein levels in culture supernatants were evaluated by ELISA. The mean protein levels from stimulated groups were determined relative to the control group from each experiment. $*p < 0.05$ vs. control.

into the cultivation medium represent the first subculture. There was no visible effect of TNF-α on IL-18Rα expression. In the second subculture (Fig. 4A, lane 3, control), 20 ng/mL TNF-α (lane 4) added on day 5 slightly stimulated IL-18Rα expression on day 8 of IEC-6 cultivation. Lane 1 and lane 3 represent control samples of two independent cell subcultures after 8-day cultivation; they differ remarkably, with much lower expression of IL-18Rα in the first subculture than in the second subculture.

Flow cytometry (Fig. 4B, line A) shows that IL-18Rα, corresponding to the first subculture in Figure 4A, lane 1, was expressed on the surface of about 5%–10% of IEC-6 cells (Fig. 4B, line A). Control staining (Fig. 4B, lines B, C, and D) proved that there is no nonspecific binding of either primary or secondary antibodies. The low percentage of IEC-6 cells that expressed IL-18Rα on the surface may indicate that the cells were not synchronized in the same phase of the cell cycle. As revealed on dot-blot of sequentially diluted cell samples, TNF-α or a combination of TNF-α and IFN-γ significantly increased the expression of IL-18Rα compared with IFN-γ (Fig. 4C) ($*p < 0.05$ vs. IFN-γ).

Caspase-1 activity in IEC-6 cells

Caspase-1 is an intracellular cysteine protease that converts the inactive IL-18 precursor to the mature cytokine secreted into the extracellular space (Melnikov and others 2001). Control IEC-6 cells produced a constitutive level of active caspase-1 (50 ± 2 pmol/μg protein), which was upregulated by IFN-γ (65 ± 6 pmol/μg protein, $p < 0.05$). TNF-α induced a slight but insignificant effect on caspase-1 expression (55 ± 7 pmol/μg protein) compared with the control. These results suggest that IEC-6 cells are capable of secreting mature bioactive IL-18.

FIG. 1. IL-18 mRNA levels were detected after stimulation with TNF-α (20 ng/mL), IFN-γ (60 ng/mL), and TNF-α + IFN-γ 20 ng/mL + 60 ng/mL) for 3 days using real-time RT-PCR. The mean mRNA levels from stimulated groups were determined relative to the control group, which was given a value of 1.0. $*p < 0.05$ vs. control.
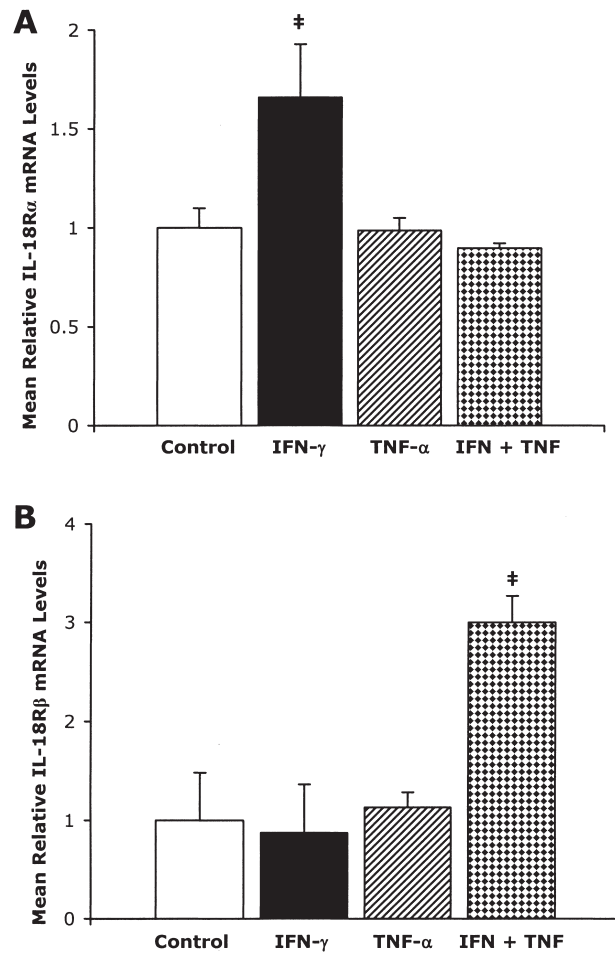
A

B

FIG. 3. Gene expression of IL-18Rα (**A**) and IL-18Rβ (**B**). Messenger RNA levels were detected after stimulation with TNF-α (20 ng/mL), IFN-γ (60 ng/mL), and TNF-α + IFN-γ (20 ng/mL + 60 ng/mL) for 3 days using real-time RT-PCR. The mean mRNA levels from stimulated groups were determined relative to the control group. ‡$p < 0.05$ vs. control.

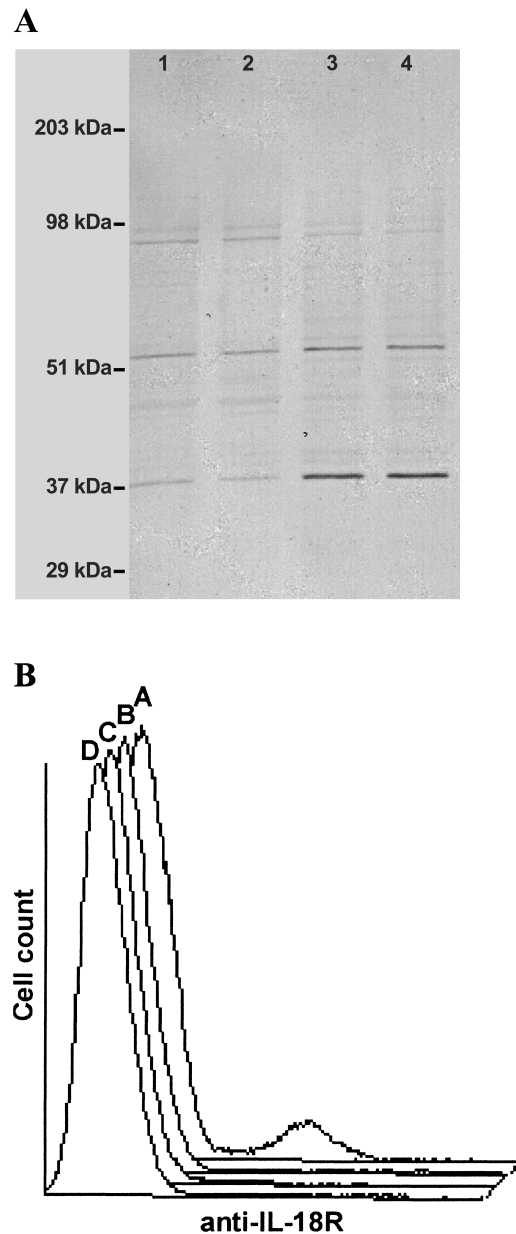**FIG. 4.** Constitutive expression of IL-18 Rα. (**A**) Western blot analysis of IL-18Rα expression in lysates of IEC-6 cells after 8-day cultivation. Experiment I: lane 1, control; lane 2, TNF-α (20 ng/mL) for last 24 h in cultivation medium. Experiment II: lane 3, control; lane 4, TNF-α (20 ng/mL) 72 h in cultivation medium. (**B**) Flow cytometry detection of IL-18Rα on the surface of about 5%–10% rat IEC-6 cells (A), negative control staining with no primary antibody (B), using normal goat serum (C), or using irrelevant goat polyclonal antibody (D) does not show any reactivity with IEC-6 cells. The results are representative of the subculture of low IL-18Rα expression (Fig. 4A, Experiment I, lane 1). (**C**) Quantification of dot-blot analysis of IL-18Rα expression in lysates of IEC-6 cells after addition of TNF-α (20 ng/mL), IFN-γ (60 ng/mL), and TNF-α + IFN-γ (20 ng/mL + 60 ng/mL) in cultivation medium. **$p < 0.05$ vs. IFN-γ.

Detection of mRNA levels of proapoptotic and antiapoptotic markers in IEC-6 cells

Apoptosis is an important regulatory mechanism of homeostasis in the intestinal epithelium (Clark and others 2005), and TNF-α is a potent inducer of cell death (Halpern and others 2006). Gene expression of proapoptotic Bax and antiapoptotic Bcl-2 was evaluated using RT real-time PCR (Clark and others 2005; Halpern and others 2006). Proapoptotic Bax mRNA levels were significantly increased in TNF-α and IFN-γ + TNF-α-treated IEC-6 cells (Fig. 5A). Treatment with IFN-γ or IFN-γ + TNF-α caused a statistically significant decrease of Bcl-2 mRNA levels compared with TNF-α treatment (Fig. 5B).

Appearance of chemokine IL-8 and MCP-1 in cultivation medium following stimulation with IL-18

IEC-6 monolayers were stimulated with rat IL-18 on days 5–8 of cultivation. IL-8 is a marker of cellular activation and
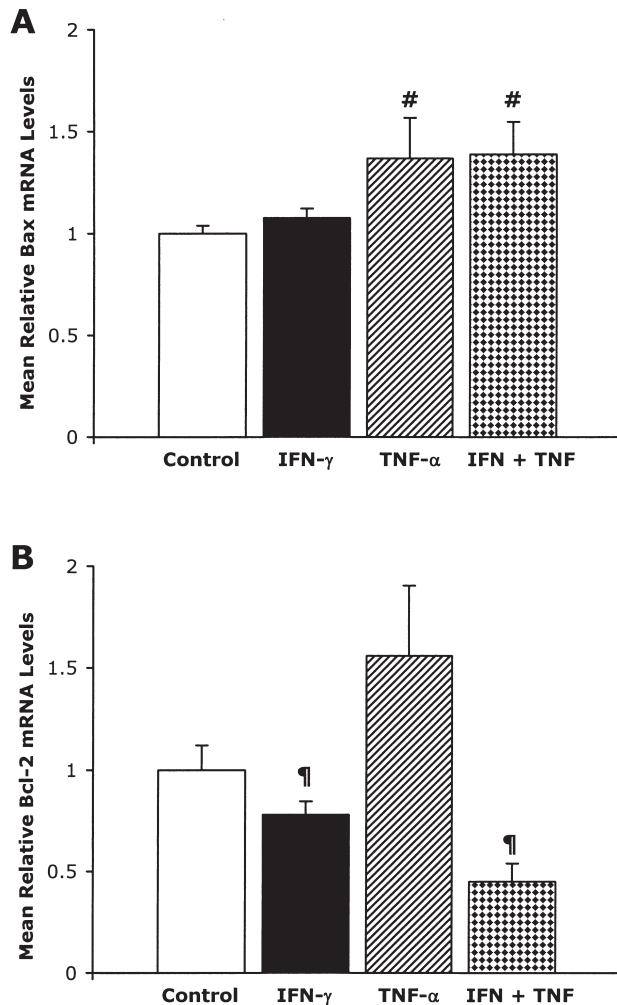
TABLE 4. EFFECT OF EXOGENOUS IL-18 ON IEC-6 CELL COUNTS, APOPTOSIS MARKERS BAX AND BCL-2 MRNAS, SECRETION OF CHEMOKINES (IL-8 AND MCP-1), SUCRASE, AND ALKALINE PHOSPHATASE ACTIVITIES[a]

	Control	IL-18
Cell counts ($\times 10^{-6}$)	5.49 ± 0.34	5.79 ± 0.44
Relative mRNA Bax	1.00 ± 0.09	0.82 ± 0.04
Relative mRNA Bcl-2	1.00 ± 0.19	0.84 ± 0.06
IL-8 (ng/mL)	9.43 ± 2.63	49.14 ± 13.05*
MCP-1 (ng/mL)	1.59 ± 0.16	2.17 ± 0.19
Sucrase	27.1 ± 1.3	27.5 ± 1.2
ALP	96.9 ± 6.9	100.6 ± 14.8

[a]Values are means $\pm$ SEM. IEC-6 grown on Matrigel matrix in DMEM-based medium with nucleotide mixture. Addition of IL-18 (10 ng/mL) on day 5, cells collected on day 8. Assays were performed as indicated in Materials and Methods. Sucrase and alkaline phosphatase activities are expressed in nkat/mg protein ($\times 10^3$).
*$p < 0.01$.

was detected in the cultivation medium at the end of the experiment. Table 4 shows significant induction of IL-8 production and a slight increase in MCP-1 by externally administered IL-18. Other parameters given in Table 4 were not influenced by IL-18. We did not find any effect of TNF-α and IFN-γ on IL-8 protein appearance (data not shown). There were no statistically significant differences in Bax and Bcl-2 expression in the IL-18-treated groups (Table 4).

DISCUSSION

The inflammatory processes in the intestine represent a complex interplay of immune and epithelial cell interactions. In recent years, IL-18 has been shown to be an important mediator in intestinal inflammatory disorders. In the present study, we have shown for the first time that the normal rat intestinal epithelial cell line IEC-6 constitutively expresses IL-18 and heterodimeric IL-18 receptors. In addition, exogenous IL-18 stimulates the production and secretion of IL-8 in IEC-6 cells.

Increased gene expression and protein production of the proinflammatory cytokine IL-18 have been shown in a rat model of necrotizing enterocolitis (NEC) (Halpern and others 2002). In this model, the production of IL-18 is localized in the ileal epithelium, and overproduction of IL-18 correlates with villus damage (Halpern and others 2002). This suggests that both intraepithelial (IEL) and IEC may play an important role during intestinal injury and that their interactions may be regulated by IL-18. In an *in vivo* system, IL-18 may initiate a cascade of inflammatory cytokines by cells of the immune system that express IL-18 receptors (Puren and others 1998). These cells include macrophages, natural killer (NK) cells, dendritic cells, and most T cells (Eaton and others 2003). T cells isolated from inflamed tissue in Crohn's disease respond to IL-18 by increasing production of IFN-γ (Maerten and others 2004).

To characterize the capability of small IECs to produce and use IL-18 in the absence of immune cells, we evaluated IL-18 and IL-18Rα (ligand-binding chain, IL-1Rrp) and associating

FIG. 5. Effect of IFN-γ (60 ng/mL), TNF-α (20 ng/mL), and TNF-α + IFN-γ (20 ng/mL + 60 ng/mL) on relative levels of proapoptotic marker Bax mRNA (**A**) and antiapoptotic marker Bcl-2 mRNA (**B**). The mean mRNA levels from stimulated groups were determined relative to number 1.0 of the control group. #$p < 0.01$ vs. control; ¶$p < 0.01$ vs. TNF-α.

IL-18Rβ (signal-transducing chain/AcPL) gene expression in the differentiated villuslike IEC-6 cell line. It is characterized by the appearance of the differentiation markers sucrase, glucoamylase, and ALP, in accordance with the data of others (He and others 1993; Wood and others 2003). Having evaluated the effects of the lymphocyte-producing cytokines TNF-α and IFN-γ, we asked the question whether TNF-α and IFN-γ have a direct effect on epithelial cell functions and whether they act synergistically. Our data reveal the possibility of both types of action. Further, the results show that costimulation with IFN-γ rendered the IEC-6 cells more susceptible to TNF-α. According to Ruemmele and others (1999) and Fish and others (1999), this may be explained as the enhanced proapoptotic response of TNF-α by IFN-γ via stimulation of TNF-α receptor expression. Under such conditions, we observed not only the reduction of cell number to 70% but a much higher reduction in enzyme activity levels, especially of ALP, which was reduced to 10%. Inhibition of ALP may indicate that the liver isoform of ALP, known to develop in differentiated IEC-6 cells (Wood and others 2003) and characterizing the state of differentiation, is most sensitive to the synergistic effect of TNF-α and IFN-γ-mediated alterations of epithelial cell function. Depression of sucrase activity by IFN-γ in IEC-6 cells could be related to a selective downregulation of sucrase gene expression in villus enterocytes of acutely inflamed Crohn's ileum (Ziambaras and others 1996).

Another example for a synergistic effect of TNF-α and IFN-γ is gene expression of antiapoptotic mitochondrial protein Bcl-2 that stabilizes the mitochondrial inner transmembrane potential and prevents opening of transition pores. In the present study, IFN-γ decreased gene expression of Bcl-2, whereas TNF-α increased Bcl-2 expression. Similarly, TNF-α accelerated IFN-γ-dependent changes in transepithelial resistance of a colonic cell line (Fish and others 1999). However, IFN-γ does not affect the transcript level of the proapoptotic marker Bax, promoting mitochondrial permeability. Interestingly, TNF-α stimulated Bax gene expression without synergy with IFN-γ. The final effect may represent dysregulation of the balance between proapoptotic and antiapoptotic Bcl-2 family members.

This study is the first to describe, in the absence of immune cells, constitutive expression of IL-18 and its receptors in the rat villus enterocyte-like IEC-6 line. Further, we showed that IEC-6 cells constitutively produce both IL-18 protein and ligand-binding IL-18Rα protein. Investigation of culture supernatants indicated detectable secretion of IL-18 into the culture medium, which was stimulated by the proinflammatory cytokines IFN-γ and TNF-α that may partially mimic the presence of a specific T cell. Although neither IFN-γ nor TNF-α alone is involved in gene activation of IL-18, the combined effect of both cytokines leads to decreased IL-18 mRNA. Both cytokines did stimulate IL-18 secretion, but this phenomenon seems to be regulated by posttranslational mechanisms, such as extracellular export. This notion is supported by studies on dendritic cells (Gardella and others 1999) showing that IL-18 precursor protein lacks a signal peptide and its secretion does not occur along the classic endoplasmic reticulum-Golgi pathway.

IL-18 secretion from the intracellular compartment involves proteolytic processing and translocation of cytosolic molecules into secretory lysosomes (Semino and others 2005). In addition, it was reported that secretion of IL-18 requires processing by proteolytic enzymes, particularly by caspase-1 (Fantuzzi and others 1999). As caspase-1 activity is required for cleavage of the precursor form of IL-18 into the mature biologically active IL-18 (Melnikov and others 2001), we evaluated capase-1 enzymatic activity in this system. Our results show the presence of the active form of caspase-1 in IEC-6 cells. Consequently, release of IL-18 protein into the cultivation medium was detected. Thus, IEC-6 cells are capable of expressing and synthesizing the mature IL-18 protein. These data suggest that the first level of regulation of IL-18 precursor protein by other cytokines in IEC-6 cells is also at the nonclassic secretion step. Indeed, upregulation of IL-18 secretion by IFN-γ correlated with increased expression of the caspase-1 activity by this cytokine. The ability of IFN-γ to induce caspase-1 activity in IEC-6 cells correlated with its ability to cause stimulated expression of the caspase-1 gene in the U937 leukemia cell line (Tamura and others 1996). However, we did not observe any effect of TNF-α on caspase-1 activity and still found stimulated secretion of IL-18 from IEC-6 cells. Possibly there exists another, caspase-1-independent system of IL-18 release from the cell. Indeed, in macrophages, Fas/Fas ligand (FasL) was suggested (Tsutsui and others 1999) to operate in this way in caspase-1-deficient mice. Fas and FasL are transmembrane proteins and members of the TNF receptor family. They are also expressed constitutively in some nonimmune cells (Choi and others 1999), and TNF-α and IFN-γ promote their expression. We hypothesize that this system might be involved in mediating IL-18 release from IEC-6 cells. As the combined addition of TNF-α and IFN-γ does not significantly exceed the secretion of IL-18 protein stimulated by either TNF-α or IFN-γ alone, we expect independent effects of these cytokines.

It is known from previous studies that mature IL-18 exerts its effects on binding to two receptor chains, IL-18Rα and IL-18Rβ, essential for signal transduction (Kim and others 2001). Results from our studies indicate constitutive gene expression of both components of IL-18R, in contrast to some other cell types, such as native COS-1 cells (Kim and others 2001) and rat islet β-cells (Hong and others 2000) that lack IL-18Rβ. Our results show different regulation of IL-18Rα and IL-18Rβ by IFN-γ and TNF-α. Expression of IL-18Rα mRNA is upregulated by IFN-γ, but supplementation with TNF-α neutralizes this stimulatory effect. On the other hand, neither cytokine alone affects IL-18Rβ mRNA expression, but additive stimulation by TNF-α and IFN-γ occurs. However, only negligible effects of TNF-α and IFN-γ on the expression of IL-18Rα protein were seen. The discrepancy between IL-18Rα gene and protein levels after stimulation with IFN-γ suggests differences in cytokine regulation of IL-18Rα expression at the transcriptional or translational level in IEC-6 cells.

The presence of functional IL-18Rβ in nonimmunocompetent cells is required for IL-12-independent production of IL-8 (Kim and others 2001). Because of the lack of available IL-18Rβ antibodies, IL-18Rβ protein was not studied here, although gene expression of IL-18Rβ in IEC-6 cells was detected. Thus, the production and release of IL-8 by IEC-6 after stimulation with IL-18 suggest that these cells may be not only a source of IL-18 but also a target for IL-18 actions. Interestingly, the addition of exogenous IL-18 (10 ng/mL) to IEC-6 culture exerts no effect on cellular proliferation, brush border enzyme

activities, and gene expression of the proapoptotic and anti-apoptotic markers Bax and Bcl-2.

In conclusion, these data show that IECs might be a target for proinflammatory cytokines released by IEC themselves, such as IL-18, as well as by proinflammatory cytokines released by other mucosal cells, such as TNF-α and IFN-γ. The expression of IL-18 and IL-18R mRNAs by normal differentiated IEC points to the involvement of IL-18 in normal physiologic processes.

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