BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 146–150 (1998)
ARTICLE NO. RC977922

Tyrosine Kinase and MAPK Inhibition of TNF-α- and EGF-Stimulated IEC-6 Cell Growth

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Received November 25, 1997

The role of TNF-α in modulating intestinal crypt cell growth was examined, in comparison with EGF. Both significantly increased IEC-6 cell proliferation. Neither EGF nor TNF-α overcame the inhibitory effect on growth exerted by the tyrosine kinase inhibitor genistein. Immunoblots with phosphotyrosine antibodies showed increased tyrosine phosphorylation of IEC-6 cell proteins in response to EGF and TNF-α stimulation. TNF-α increased ERK1 and ERK2 MAPK phosphorylation. A MAPK assay confirmed the increased activity upon TNF-α stimulation. Selective inhibition of MAPK activation by PD98059 resulted in a dose dependent inhibition of TNF-α or EGF-induced IEC-6 cell growth. These findings suggest a role for TNF-α in the regulation of intestinal epithelial cell growth and that the mitogenic effect of TNF-α requires protein tyrosine phosphorylation and MAPK activation. © 1998 Academic Press

Physiologic intestinal crypt cell growth and differentiation are regulated by various growth factors and hormones, but the mechanisms involved remain largely unclear (1,2). A variety of immune-mediated intestinal inflammatory disorders, including gluten sensitive enteropathy, Crohn's disease and ulcerative colitis, are characterized by abnormal cellular proliferation and differentiation (3). Villous atrophy, crypt hyperplasia, and immature intestinal epithelial cells are common features of these diseases, resulting in diarrhea and malabsorption (1-3). Abnormal levels of cytokines and growth factors have been reported at the sites of inflammation in these pathologic conditions (4). It is therefore postulated that these mediators play a key role in the pathogenesis of the morphologic abnormalities as well as the enterocyte dysfunction observed (5).

IEC-6 cells, a non-transformed rat jejunum crypt cell line, have been widely employed in studies addressing the effect of various growth factors and cytokines on intestinal crypt cell growth and maturation (6,7). Epidermal growth factor (EGF) and transforming growth factor α (TGF-α) have been shown to stimulate IEC-6 cell growth, and to increase the phosphorylation on tyrosine residues of intracellular proteins (8,9,10).

TNF-α, a pleiotropic cytokine secreted primarily by activated monocytes, is a central mediator of diverse inflammatory processes (11). Increased amounts have been described in various inflammatory states, including Crohn's disease, ulcerative colitis and cystic fibrosis (12,13). TNF-α induces cell growth arrest or apoptosis in some cells, yet acts as a growth factor for others (14). The signalling pathways by which TNF exerts its actions remain unclear. Neither of the two TNF receptors contain intrinsic protein kinase activity. A growing number of adaptor proteins have been found to be recruited to the TNF receptors upon stimulation (15-17). These proteins elicit the liberation and/or the formation of second messengers such as ceramide, arachidonic acid metabolites, free radicals and intracellular Ca^{2+}. They also phosphorylate several proteins kinases such as PKA, PKC, MAPK, SAPK and p38 (18). The present study was conducted in order to examine the signal transduction pathways involved in TNF-α mediated IEC-6 growth. We examined the effect of tyrosine kinase inhibition, and the tyrosine phosphorylation levels of proteins in IEC-6 cells following their stimulation with TNF-α, in comparison with EGF. In addition, the role of MAPK in TNF-α induced IEC-6 cell growth was assessed.

MATERIAL AND METHODS

Cell culture and materials. IEC-6 cells (American Type Culture Collection, Rockville, MD) were initially grown at 37° C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM), containing 1% sodium pyruvate and 1% penicillin, supplemented with 0.1 U/ml insulin and 10% decomplemented fetal bovine serum (FBS, Gibco, Grand Island, NY). Cells between passages 16-20 were seeded (3×10^5 cells/well) into 24 well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in DMEM supplemented with 5% FBS. After reaching sub-

0006-291X/98 $25.00

confluence (48 hrs), cells were washed with phosphate buffered saline (PBS) and cultured with FBS-free medium for 24 hours.

The factors used in this study were: hTNF-α (Genzyme, Cambridge, MA) and EGF (Collaborative Research Incorporated, Bedford, MA) at concentrations ranging from 0.1 to 500 ng/ml. The inhibitors used were genistein and PD98059 (Calbiochem, San Diego, CA). At the end of the culture period, cell viability was verified microscopically, using trypan blue exclusion. Antibodies used were: monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, Lake Placid, NY), anti-phosphoMAPK (New England Biolabs, Beverly, MA), mouse anti-MAPK antibody (clone ZO33, Zymed Laboratories, San Francisco, CA), and anti-mouse IgG, conjugated to alkaline phosphatase (Bio-Rad, Richmond, CA).

Analysis of IEC-6 cell proliferation. The effect of the growth modulatory factors, of the inhibitors, and of the two combined were determined by incubating IEC-6 cells and then measuring their thymidine incorporation. Inhibitors were added 1 hour before the factors. After 18 hours of culture, ^{3}[H]-thymidine (1μCi, specific activity 20 Ci/mmol; DuPont, Markham, Ont) was added to each well. Cells were incubated for 2 hours, washed with PBS, fixed with ethanol/acetic acid (3:1) for 15 min, washed twice with PBS and solubilized with NaOH 0.2N-SDS 0.3%. In some experiments, cells were washed with PBS and then harvested with a rubber policeman in phosphate buffer (0.05 M sodium phosphate, 2 M NaCl, 0.04% sodium azide, and 2 mM EDTA, pH 7.4). Cells were sonicated and the incorporation of ^{3}[H]-thymidine in DNA was measured in homogenates (cpm/mg DNA). Results of wells treated with the factors and/or inhibitors were expressed as a percentage of thymidine incorporation relative to those IEC-6 cells maintained in medium alone (controls). Results were comparable with the two methods, with a variation of less than 10%.

Immunoblotting. IEC-6 cells seeded (1.5×10^6) in 25 cm^2 flasks were stimulated with TNF-α or EGF, with or without inhibitors, as described above. Lysates were prepared by washing cells with ice cold HBSS and harvesting into 0.7 ml of ice cold lysis buffer [50 mM TRIS, 150 mM NaCl, 10 mM EDTA, 1% Triton], 2mM sodium orthovanadate, 5mM sodium pyrophosphate and a mixture of protease inhibitors (Complete, Boehringer, Laval, Quebec). Lysates were clarified by a quick spin in an Eppendorf Microfuge and protein determination was performed using a modified micromethod (19).

Western blots were performed as described previously (20). Lysate samples containing equivalent protein content were mixed with 2$\times$ sample buffer (4% (w/v) SDS, 20% (v/w) glycerol, 100 mM Tris, pH 6.8, 0.5% Bromphenol Blue, 200 mM DTT) and boiled for 5 minutes. Proteins were resolved on 8%, as well as 10% polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad), and probed with the appropriate antibody. Western blots were developed as follows: initially, blocking was performed with 5% (w/v) BSA in Tris-buffered saline/Tween -20 (TBST: 20mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween-20), then incubated for 2 hours with the appropriate primary antibody dissolved in TBST (anti-phosphotyrosine and anti-phosphoMAPK at 1:1000 or anti-mitogen activated protein kinase (MAPK) at 1:4000), followed by incubation with the alkaline phosphatase-conjugated anti-mouse IgG (1:3000) for 2 hours. Alternatively, a peroxydase conjugated antibody (1:7000) was added and the bands were read by chemiluminescence.

MAPK assay. MAPK activity was determined in a 25 μg aliquot of cell protein using a kit (Upstate Biotechnology, Lake Placid, NY) which measures incorporation of ^{32}[P]-ATP in myelin basic protein (MBP) in the presence of PKA, PKC and calmodulin kinase inhibitors. Activity was assessed by blotting 25μl of the reaction on phosphocellulose paper and counting in a liquid scintillation counter. Specificity of the measurement was confirmed by Phosphorimager (Molecular Dynamics, Sunnyvale, CA) analysis of radioactivity incorporation in MBP after PAGE.

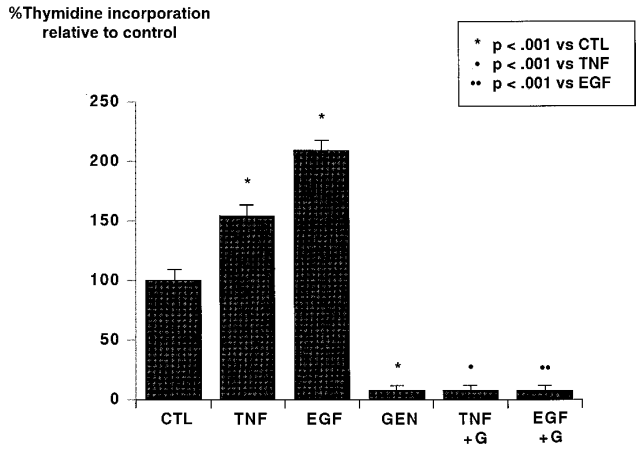

FIG. 1. Effect of EGF, TNF-α, and of the tyrosine kinase inhibitor genistein (GEN) on IEC-6 cell proliferation, measured by ^{3}[H]-thymidine incorporation. IEC-6 cells were treated with 50 ng/ml, 1 ng/ml, and 50 mg/ml of EGF, TNF-α, and genistein respectively, as indicated in the Material and Methods section.

RESULTS

Cytokine modulated IEC-6 cell proliferation. The addition of either EGF or TNF-α to the medium resulted in significantly ($p < 0.001$) increased ^{3}[H]-thymidine uptake by IEC-6 cells (Fig. 1). A significant increase was observed with incremental concentrations of TNF between 0.1 and 500 ng/ml. The dose used in experiments with inhibitors (1-10 ng/ml) represented 60% of the maximal stimulatory effect of TNF-α.

Effect of tyrosine kinase inhibition. The incubation of cells in the presence of genistein alone significantly decreased the spontaneous growth of IEC-6 cells, when compared with control wells ($p < 0.001$), as shown in Fig. 1. Cell viability, as assessed by trypan blue exclusion, was nevertheless uniformly greater than 94% throughout these experiments. Neither EGF nor TNF-α, at the doses used, was able to overcome the inhibitory effect of genistein on IEC-6 cell growth (Fig. 1).

Tyrosine phosphorylation in response to EGF and TNF-α. IEC-6 cells were incubated with 50 ng/mL EGF or 1 to 10 ng/ml TNF-α for 1 to 45 minutes. Immunoblotting revealed that several proteins were rapidly phosphorylated by EGF, with approximate molecular masses of 42, 44, 80, 150, and 175 kDa (Fig. 2A, lane 2). TNF-α induced phosphorylation of proteins with approximate molecular masses of 42, 44, 55, 85, and 110 kDa (Fig. 3A, lane 2). The EGF induced phosphorylation of the proteins corresponding to the 80 and 150 kDa bands was transient, starting at 1 min (not shown). The most prominent and sustained phosphorylation induced by TNF-α was observed for the protein corresponding to the 55 kDa band. Although EGF and TNF-α both induced phosphorylation of proteins corre-

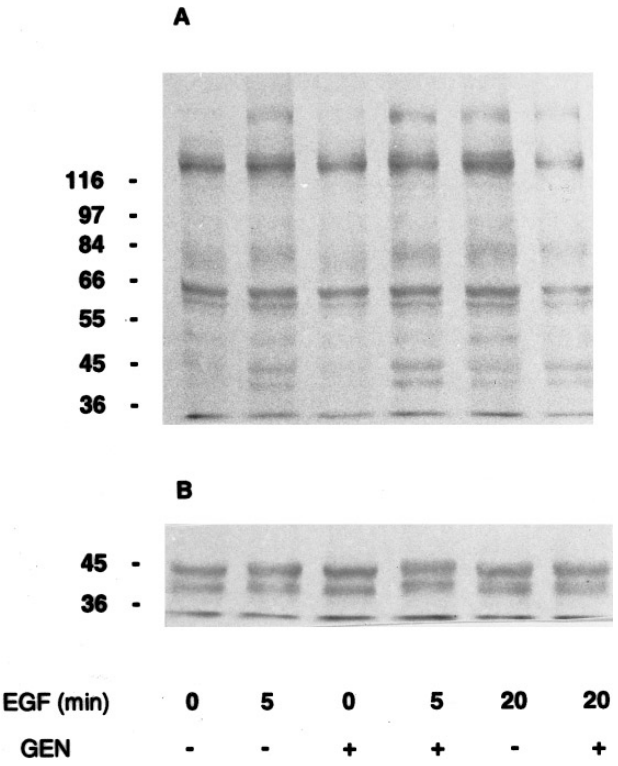

FIG. 2. Effect of EGF and genistein (GEN) on tyrosine phosphorylation in IEC-6 cells. IEC-6 cells were incubated with DMEM alone for 5 or 20 minutes (lanes 1, 4), with 50 ng/ml EGF for 5 or 20 minutes (lanes 2, 5), or with 50 ng/ml genistein and EGF for 5 or 20 minutes (lanes 3, 6). (**A**) Immunoblot illustrating that EGF increased the phosphorylation on tyrosine residues of proteins of approximate molecular weights of 175, 150, 80, 44, and 42 kDa, respectively. Phosphorylation of these proteins was inhibited by genistein. (**B**) Immunoblot performed with anti-MAPK. The two bands in lane 2 of immunoblot B appear to be located at a higher molecular mass than the corresponding bands in lane 1 (control), likely indicating a shift or activation (by phosphorylation) of MAPK in response to EGF.

sponding to the 42 kDa and 44 kDa bands, the phosphorylation induced by EGF was more prominent, and appeared sooner. EGF greatly increased phosphorylation levels of these proteins with peak at 3 to 5 min, afterwhich the phosphorylation decreased progressively but remained above basal levels to 45 min. The phosphorylation levels induced by TNF-α increased only slightly at 5 min, were higher by 10 min and returned to basal levels by 30 min.

When immunoblots were performed using the anti-MAPK antibody, the 42 and 44 kDa isoforms aligned precisely with p42 and p44 (Fig 2B, 3B). These bands were observed at slightly higher molecular masses than those of the control cells, implying their phosphorylation subsequent to EGF or TNF-α stimulation.

Effect of tyrosine phosphorylation inhibition. Preincubating IEC-6 cells with genistein (50 ng/ml for 1 h) caused a decrease in the EGF and TNF-α induced tyrosine phosphorylation of most proteins, especially that of the 42 and the 44 kDa bands (Figs 2A, 3A). Inhibition was incomplete at 50 ng/ml, but higher doses of genistein were cytotoxic.

Effect of MAPK inhibition. Pre-incubation of cells with the selective MAPK kinase (MEK) inhibitor PD98059 decreased spontaneous IEC-6 cell proliferation by 22 and 30% at 20 and 40μM, respectively. PD98059 at 20 μM decreased TNF-α and EGF-induced proliferation by 38 and 41%, respectively. At 40 μM, TNF-α and EGF-induced IEC-6 cell proliferation was decreased by 63 and 67%, respectively (Figure 4). Preincubation of IEC-6 cells with 100 μM PD98059 partially inhibited basal (Figure 5, lane 2) as well as TNF-α -induced (Figure 5, lane 4) MAPK phosphorylation. The MAPK kinase assay showed that radioactivity incorporation in MBP was increased by 24% by TNF-α, while EGF induced MAPK activity by 1.5 to 1.8 fold (Table 1). Treatment with PD98059 reduced the TNF-α induced activity by approximatively 50% (11 vs 24% increase over basal value).

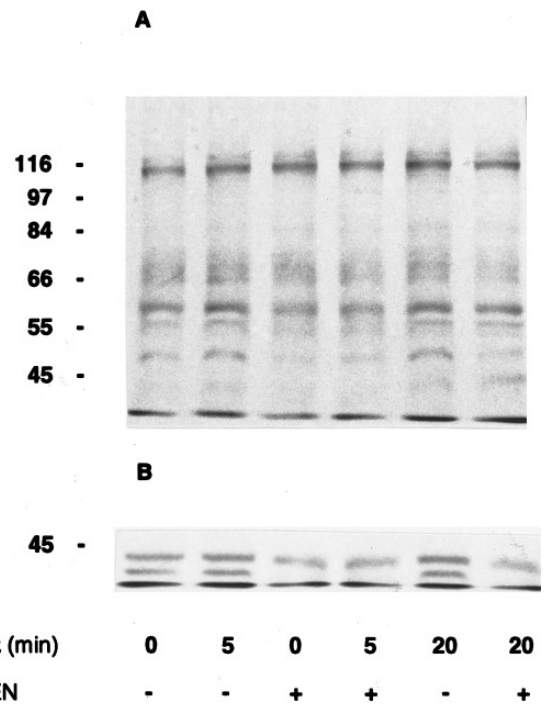

FIG. 3. Effect of TNF-α and genistein (GEN) on tyrosine phosphorylation in IEC-6 cells. IEC-6 cells were incubated with DMEM alone for 5 or 20 minutes (lanes 1, 4), with 1 ng/ml TNF-α for 5 or 20 minutes (lanes 2, 5), or with 50 ng/ml genistein and TNF-α for 5 or 20 minutes (lanes 3, 6). (**A**) Immunoblot illustrates that TNF-α increased the phosphorylation on tyrosine residues of proteins of approximate molecular mass of 110, 85, 55, and 44 kDa, respectively. A fainter band was also noted at 42 kDa. The bands all appear more intense at later time points (lane 5 vs lane 2; 20 vs 5 min stimulation with TNF-α). Preincubation with genistein partially inhibited phosphorylation of these proteins. (**B**) Immunoblot performed with anti-MAPK. A shift upwards in molecular mass is less evident compared to EGF stimulated cells (lanes 2 and 5).

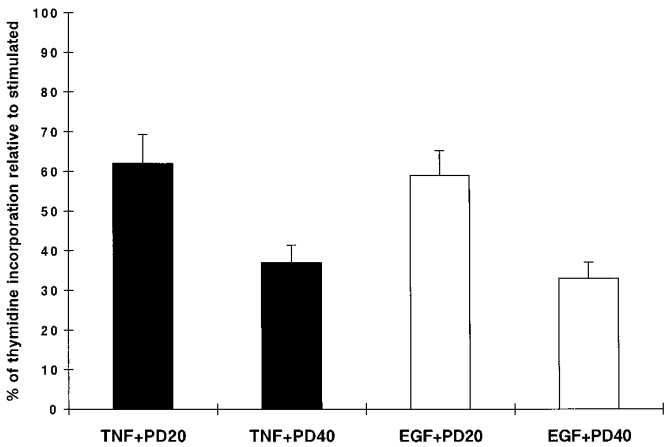

FIG. 4. Incubation of IEC-6 cells with the selective MAPK kinase inhibitor PD98059 decreased TNF and EGF-induced proliferation ($p \leq 0.01$). Results are expressed as percent of cytokine treated cells without an inhibitor.

DISCUSSION

Our results demonstrate that TNF-α increased IEC-6 cell proliferation over a broad range of concentrations. This implies that TNF-α may act as a mitogen for intestinal crypt cells, resulting in the increased epithelial cell turnover encountered in inflammatory states. Genistein abolished spontaneous as well as EGF or TNF-α induced proliferation, suggesting that tyrosine kinase activation is essential to IEC-6 cell growth. TNF-α elicits rapid tyrosine phosphorylation of several proteins (21). That EGF and TNF-α promote the stimulation of tyrosine kinase was shown by the increased phosphorylation on tyrosine of several IEC-6 cell proteins. It is well established that the phosphorylation on tyrosine residues of certain proteins is closely linked to the initiation of cell proliferation (22). Protein tyrosine kinases also are involved in the regulation of intestinal crypt cell growth when stimulated by gastrin (23).

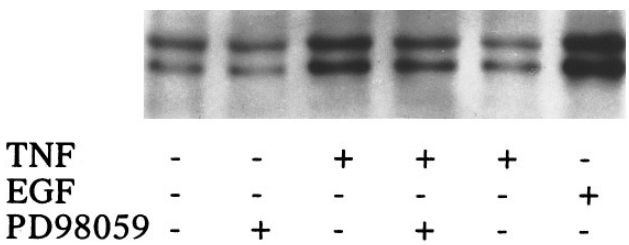

FIG. 5. Effect of TNF, EGF and PD98059 on MAPK tyrosine phosphorylation. Western blotting was performed with a phospho-specific antibody that detects phosphorylated tyrosine 204 of p42 and p44 MAPK. TNF (10 ng/ml, 10 min) increased MAPK phosphorylation (lane 3). At 30 min, MAPK phosphorylation was returned to basal levels (lane 5 vs lane 1). PD98059 decreased basal (lane 2) as well as TNF-induced (lane 4) MAPK phosphorylation. EGF (50 ng/ml, 10 min) elicited a stronger MAPK phosphorylation (lane 6).

TABLE 1

MAPK Activity in Stimulated IEC-6 Cells

TNF (1 ng/ml, 10 min)	124 ± 3%	(n = 4)
TNF + PD98059	111 ± 2%	(n = 3)
EGF (50 ng/ml, 3 min)	182 ± 4%	(n = 3)
EGF (50 ng/ml, 15 min)	151 ± 4%	(n = 3)

TNF treatment increased MAPK activity, which was partially blocked by PD98059 (100 μM, 1 h). Activity is given as % of non stimulated.

Furthermore, altered levels of protein tyrosine kinases have been detected in biopsy specimens obtained from patients with ulcerative colitis (24), an immune-mediated bowel disorder with enhanced crypt cell turnover.

It is of interest that MAPK were phosphorylated in IEC-6 cells in response to both EGF or TNF-α. MAPK are a family of serine-threonine kinases which constitute an important intermediate step in the network of cellular signal transduction pathways, spanning from protein tyrosine kinase receptors to downstream transcriptional events (25). The phosphorylation of MAPK on tyrosine and threonine activates the enzyme, which in turn phosphorylates several substrates involved in the regulation of cell growth, including c-fos, elk1, p90rsk, and others (18). Activation of MAPK by TNF-α was reported for several cell types such as fibroblasts, in which it stimulated proliferation (26). While EGF activation of MAPK in IEC-6 cells has been recently reported (10), we are the first to demonstrate MAPK activation in TNF-α stimulated IEC-6 cells. Activation of two independent signalling pathways have been defined upon binding of TNF to TNF-Rp55. The first one involves neutral sphingomyelinase, ceramide-activated protein kinase and Raf-1 kinase, while the other leads to NF-KB activation through phosphatidylcholine-specific phospholipase C, PKC and acid sphingomyelinase (27). MAPK activation was located in the first pathway in which Raf is believed to activate MAPK through MEK (28). However, MAPK activation by TNF was reported to be independent of raf-1 in some cells (29).

The molecular mechanisms that make a cell resistant or sensitive to TNF-induced apoptosis have not been clarified. It has recently been proposed that TNF stimulates simultaneous pathways: some that convey programmed cell death and others that protect against apoptosis (30). Initial studies pointed to a role of MAPK in cell survival, while SAPK and p38 kinase activation was associated with apoptosis (31,32). However no role for SAPK in apoptosis was found in other studies. (15, 33). Furthermore, MAPK activation is not sufficient to protect against apoptosis in certain cells (34). There is now evidence that TNF-α can activate several MAPK pathways (ERKs, SAPK and p38)(35). One recently identified protective pathway elicited by TNF is NFκB activation (30,36). In addition to the MAPKs ERK 1

and ERK2, we observed that TNF-α-induced transient phosphorylation of SAPK and p38 kinase in IEC-6 cells (data not shown). The role of these kinases in TNF-induced proliferation vs programmed cell death is currently under investigation.

In summary, our results show that TNF-α stimulates IEC-6 intestinal crypt cell proliferation and that this mitogenic effect is in large part mediated by MAPK activation. Establishing that TNF-α stimulates the phosphorylation of tyrosine residues on MAPK and other regulatory proteins will increase our understanding of the signal transduction mechanisms of TNF-α modulation of intestinal epithelial cell growth and function.

ACKNOWLEDGMENTS

This study was supported by a Research Initiative Award from the Canadian Association of Gastroenterology/Astra Pharma MRC-PMAC Program (S.D., I.D.D., E.G.S.) and Research Scholarship Awards from the Fonds de Recherche en Santé du Québec (E.S., E.L.).

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