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BOMBESIN ENHANCES TGF- β GROWTH INHIBITORY EFFECT THROUGH APOPTOSIS INDUCTION IN INTESTINAL EPITHELIAL CELLS

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Abstract

Mammalian intestinal epithelium undergoes continuous cell turn over, with cell proliferation in the crypts and apoptosis in the villus. Both transforming growth factor (TGF)- β and gastrin-releasing peptide (GRP) are involved in the regulation of intestinal epithelial cells for division, differentiation, adhesion, migration and death. Previously, we have shown that TGF- β and bombesin (BBS) synergistically induce cyclooxygenase-2 (COX-2) expression and subsequent prostaglandin E₂ (PGE₂) production through p38^{MAPK} in rat intestinal epithelial cell line stably transfected with GRPR (RIE/GRPR), suggesting the interaction between TGF- β signaling pathway and GRP-R. The current study examined the biological responses of RIE/GRPR cells to TGF- β and BBS. Treatment with TGF- β 1 (40 pM) and BBS (100 nM) together synergistically inhibited RIE/GRPR growth and induced apoptosis. Pretreatment with SB203580 (10 μ M), a specific inhibitor of p38^{MAPK}, partially blocked the synergistic effect of TGF- β and BBS on apoptosis. In conclusion, BBS enhanced TGF- β growth inhibitory effect through apoptosis induction, which is at least partially mediated by p38^{MAPK}.

Keywords

p38^{MAPK}; Gastrin-releasing peptide receptor; Cell cycle

INTRODUCTION

The intestinal epithelium is one of the most rapidly proliferating tissues in the body. It forms a continuous two-dimensional sheet where new cells are added in the crypts and subsequently removed by apoptosis upon reaching the villus tips three to eight days later [1, 2]. Renewal of the intestinal epithelium involves a complex, dynamic cellular process that is directionally oriented along the crypt-villus axis. Once intestinal epithelial cells migrate to the villus, they undergo cell cycle arrest, differentiation, and eventually apoptosis [3, 4]. In

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normal tissue, renewal of the intestinal epithelium is controlled by signaling pathways that regulate cell division, survival, motility and differentiation. Alteration of these signaling pathways leads to gastrointestinal diseases [5].

Transforming growth factor (TGF)- β is involved in the regulation of epithelial cells on division, differentiation, adhesion, migration and death [6–8]. TGF- β inhibits proliferation of crypt cells in the small intestine and colon *in vivo* [9], and inhibits proliferation of intestinal epithelial cells *in vitro* [10–12]. TGF- β stimulates the migration of intestinal epithelial cells [13, 14] and induces their differentiation [15]. TGF- β also plays an important role in mediating apoptosis in intestinal epithelial cells [16, 17]. In normal epithelial cells and early tumors, TGF- β acts as a tumor suppressor; however, during tumor progression, TGF- β assumes the role as a tumor promoter. Most colon cancers are resistant to the antiproliferative and apoptotic effects of TGF- β , due to loss or mutations of components in the TGF- β signaling pathway, notably TGF- β type I and type II receptors [18] and Smad4 [19, 20].

Bombesin (BBS), a tetradecapeptide, was originally isolated from the skin of the frog *Bombina orientalis* [21]. Later on the mammalian homologue of BBS was identified and named gastrin-releasing peptide (GRP) [22]. In normal tissue, GRP is present in the brain and central nervous system, and the nerve fibres throughout the gastrointestinal tract and pancreas, thymus, prostate, and urethra. The expression of GRPR has also been shown in various fetal or adult tissues, such as the cells lining the gastric antrum [23]. The GRP-like peptides play many physiological roles in addition to the stimulation of gastric acid secretion. They stimulate the release of a variety of hormones including gastrin, somatostatin, CCK, and exocrine secretion from the pancreas, promote smooth muscle contraction [23] and wound repair [24]. GRP/GRPR has been found to mediate the itch sensation in the spinal cord [25] and contribute to intestinal villus growth [26]. Bombesin is a potent gastroprotective agent to prevent gastric injury via the release of endogenous gastrin [27]. It also reduces endotoxemia, intestinal oxidative stress, and apoptosis in experimental obstructive jaundice [28].

We have recently reported that TGF- β and BBS synergistically induced cyclooxygenase-2 (COX-2) expression and subsequently prostaglandin E₂ (PGE₂) secretion in RIE/GRPR cell line expressing GRPR [29]. These effects are mediated in part by p38^{MAPK} and suggest that the TGF- β signaling pathway interacts with that of the GRPR. However, whether this interaction leads to functional consequences is unknown. To address these questions we used RIE/GRPR cells in this study as a model to examine the effects of TGF- β and BBS on cell growth, apoptosis, and cell cycle progression, and the role of p38^{MAPK} in mediating these effects.

MATERIALS AND METHODS

Reagents

BBS (Bachem Inc, Torrance, CA) was dissolved in ethyl alcohol and diluted in PBS. TGF- β 1 (R&D systems Inc, Minneapolis, MN) was diluted in vehicle (0.1% BSA, 4 mM HCl). SB203580 (Calbiochem, La Jolla, CA) was diluted in dimethyl sulfoxide.

Cell lines

Rat intestinal epithelial cell lines stably expressing GRPR (RIE/GRPR) [29] were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 5% dialyzed fetal bovine serum (dFBS, Invitrogen Co., Carlsbad, CA) and G418 (Mediatech Inc., Herndon, VA) at 400 μ g/ml.

Growth study

RIE/GRPR cells were seeded at 5×10^4 cells/well in 12-well plates and cultured for 24 h. On next day, cells were treated with TGF- β (40 pM), BBS (100 nM) alone or together for up to 7 days. Cells were trypsinized and counted daily as described before [30].

Apoptosis assay

DNA fragmentation was quantified by cell death detection ELISA assay (Roche Molecular Biochemicals, Indianapolis, CA) according to manufacturer's instruction. The number of cells in each condition was adjusted as 5×10^5 cells/ml in lysis buffer provided by the manufacturer. The absorbance ($A_{405-490 \text{ nm}}$), an indication of DNA fragmentation, of each sample was measured using a 96-well plate reader (Molecular Devices Co., Sunnyvale, CA) [17]. Apoptotic cells were stained with Annexin V-FITC and propidium iodide using BD ApoAlert™ Annexin V-FITC Apoptosis kit (BD Bioscience, CA). Cells were analyzed by flow cytometry and cells stained only with Annexin V were identified as apoptotic cells as previously described [30].

Cell cycle analysis

The cells were seeded at 1.5×10^5 in 100-mm dishes and incubated at 37°C for 3 days, followed by serum starvation for 2 days. The cells were replated with the dilution of 1/5 and incubated at 37°C for 90 min, and then treated with BBS or TGF- β (40 pM) up to 24 h. The cells were pulsed with 5-bromo-2' deoxyuridine (BrdU) for the last 45 min and harvested for flow cytometric analysis using BrdU flow kit (BD Biosciences, San Jose, CA) according to the manufacturer's instruction [30].

Western blotting analysis

Cultured cells were lysed in 1X cell lysis buffer (Cell Signaling Technology, Inc., MA). Protein concentrations of cell lysates were quantified using a protein assay dye (Bio-Rad Laboratories, CA). Western blotting was performed as previously described [17]. Antibodies of p-p38^{MAPK} (phospho-p38^{MAPK}), p38^{MAPK} α , and α -tubulin were from Cell Signaling Technology, Inc., Upstate Biotechnology, and Santa Cruz Biotechnology, Inc. respectively. Horseradish peroxidase (HRP) conjugated goat anti-rabbit and anti-mouse antibodies were from Bio-Rad Laboratories.

Kinase activity assay

RIE/GRPR cells were pretreated with or without SB203580 (10 μ M) for 30 min followed by treatment with or without TGF- β (40 pM) for 60 min. Cell lysates were analyzed for p38^{MAPK} activity using a recombinant MAPK-activated protein (MAPKAP) kinase-2 as a p38^{MAPK} substrate (Upstate Biotechnology, Lake Placid, NY) [31].

Statistical Analysis

Data were expressed as means $\pm$ SEM. Differences between groups were analyzed by ANOVA with Tukey-Kramer multiple comparisons test, and $p < 0.05$ is considered significant. Jin's formula, $Q = Ea + b / (Ea + Eb - Ea \times Eb)$, was used to evaluate the combined effects of the drugs on cell growth inhibition [32, 33]. Q is the combination index; Ea+b represents the cell growth inhibition rate of the combined drugs; Ea and Eb represent the cell growth inhibition rate of individual drug. Q value < 0.85 , from 0.85 to 1.15, and > 1.15 represents antagonistic, additive, and synergistic effect respectively.

RESULTS

TGF- β and BBS synergistically inhibit growth of RIE/GRPR cells

A seven-day growth study was conducted to determine whether TGF- β and BBS cooperate to regulate cell growth in RIE/GRPR. Treatment with either TGF- β or BBS alone significantly inhibited cell growth. The combined treatment with both peptides resulted in greater growth suppression than treatment with either peptide alone from day 1 to day 5 (Fig. 1). Most notably, the combined treatment of BBS and TGF- β decreased cell number by 72.6% on day 3, which was significantly lower than either BBS (44.9%) or TGF- β (32.8%) treatment alone. According to Jin's formula [32], the Q values were above 1.15 from day 3 to day 6, suggesting TGF- β and BBS synergistically inhibit RIE/GRPR cell growth.

TGF- β and BBS synergistically induce apoptosis in RIE/GRPR cells

Previously, we have shown that TGF- β inhibits growth of intestinal epithelial cells by cell cycle arrest [11, 34] and apoptosis induction [17, 35]. We determined whether the synergistic inhibition of cell growth by TGF- β and BBS is due to increased apoptosis or cell cycle arrest. RIE/GRPR cells were treated with TGF- β and BBS and apoptosis was quantified using a DNA fragmentation ELISA. TGF- β treatment alone induced DNA fragmentation (2.6-fold) compared to the control group (Fig. 2A). However, BBS treatment alone did not induce DNA fragmentation. Treatment with BBS and TGF- β together increased DNA fragmentation by about 6.0-fold compared to the control group (Fig. 2A). Using Annexin V-FITC staining, we found that either TGF- β or BBS alone induced apoptosis; the combined treatment of TGF- β and BBS induced greater apoptosis than either alone (Fig. 2B). Apparently Annexin V-FITC staining was more sensitive to detect an earlier event of apoptosis induced by BBS alone than DNA fragmentation ELISA. Nevertheless, both assays demonstrated the synergistic induction of apoptosis by the combined treatment of TGF- β and BBS.

Cell cycle progression was determined in RIE/GRPR cells by growing cells to confluence followed by 48 h serum starvation to induce mitotic quiescence. Cells were then released into the cell cycle by replating in serum-containing media and analyzed for S phase and G0/G1 phase by BrdU incorporation (Fig. 3). The percentages of G0/G1 phase for the control, TGF- β alone, BBS alone, and combination of TGF- β and BBS were 46.4%, 64.0%, 66.9%, and 74.6% respectively (Fig. 3). The percentages of S phase for the control, TGF- β alone, BBS alone, and combination of TGF- β and BBS were 45.0%, 27.7%, 16.5%, and 9.2% respectively (Fig. 3). According to Jin's formula [32], the Q value is 1.03, suggesting that the cell cycle arrest induced by combined treatment of TGF- β and BBS was an additive effect.

It is well known that the cell count reflects the balance between cell proliferation and cell death [36]. In Fig. 2, we demonstrated that the apoptotic changes induced by TGF- β and BBS were from 24 h time point, which accounted for the decreased cell number in the combined treatment groups. The apoptotic changes continued throughout the time course until day 7 when the cells were confluent. Therefore, the most significant changes in cell number observed from days 2 to 5 are a reflection of the cumulative effects of cell lost due to apoptosis. Taken together, our results of apoptosis and cell cycle arrest suggest that TGF- β and BBS cooperatively induced apoptosis in intestinal epithelial cells, resulting in decreased cell number.

p38^{MAPK} partially mediates BBS- and TGF- β -induced apoptosis

Our previous study demonstrated that p38^{MAPK} is involved in the cross-talk of BBS- and TGF- β -induced COX-2 expression in RIE/GRPR cells [29]. To ascertain whether p38^{MAPK}

signaling pathway mediates the regulation of cellular function by BBS and TGF- β , we used SB203580, a specific p38^{MAPK} inhibitor to study if p38^{MAPK} is required in apoptosis induction by TGF- β and BBS in RIE/GRPR cells. We found that TGF- β induced phosphorylation of p38^{MAPK}, whereas pretreatment of SB203580 blocked TGF- β -induced phosphorylation of p38^{MAPK} by western blotting (Fig. 4A). Correspondingly, using a recombinant MAPKAP kinase-2 as a p38^{MAPK} substrate, we measured p38^{MAPK} enzyme activity in vitro. In RIE/GRPR cells, treatment with TGF- β stimulated p38^{MAPK} activity by 1.4 fold. Pretreatment with SB203580 blocked TGF- β -induced p38^{MAPK} activity and decreased the basal level of p38^{MAPK} activity as well (Fig. 4B). Furthermore, pretreatment with SB203580 decreased apoptosis induction by 42.4% in the combination of TGF- β and BBS groups (Fig. 5), suggesting that BBS enhanced TGF- β -induced apoptosis partially through p38^{MAPK}.

DISCUSSION

Growth factors and gastric peptides interact to regulate gut epithelial cell growth, differentiation, and death to maintain gut homeostasis. To investigate the biological responses to TGF- β and BBS in rat intestinal epithelial cells stably-transfected to overexpress GRP receptor (RIE/GRPR), we demonstrated that TGF- β and BBS work synergistically to inhibit RIE/GRPR cell growth and induce apoptosis, and their effects on apoptosis were partially blocked by a p38^{MAPK} inhibitor.

TGF- β controls cellular processes by signaling through cell surface receptor complexes composed of TGF- β type I and type II receptors. Both receptors are transmembrane serine/threonine kinases. TGF- β binds to type II receptor, which subsequently activates the type I receptor by phosphorylation. Once activated, type I receptor phosphorylates Smad2 and Smad3, allowing them bind to Smad4 to form heteromeric complexes. The complexes then translocate into the nucleus to regulate transcription of target genes. The TGF- β signaling pathway interacts with a variety of other intracellular signaling mechanisms [37]. For example, MAPK, NF- κ B or PI3K/AKT pathways can either be activated by TGF- β , or can modulate the outcome of TGF- β induced Smad signaling [38, 39]. Cross-talk between TGF- β and other ligands, as well as the interaction of the Smad cascade with other intracellular signaling pathways add further complexity to these networks.

The development of colorectal cancers from normal epithelium is a multi-step process requiring inactivation of a series of tumor suppressor genes [40, 41] and the activation of specific oncogenes [42]. Normal human gut epithelium does not express endogenous GRPR or other BBS receptor subtypes [43]. However, a subset of human colorectal cancers expresses GRPR and its ligand GRP [43–45]. Activation of the GRPR by bombesin stimulates growth of colorectal cancer cells [46–49]. In this study we examined the cellular effects of activation of GRPR during colorectal carcinogenesis using RIE-1 cells stably transfected with GRPR [50]. RIE-1 cell line was selected for several reasons: (1) it is derived from rat small intestine and possesses the normal rat diploid number of chromosomes; it is a nontumorigenic and does not form colonies in soft agarose [51]; (2) unlike most human colonic cancer cell lines, it possesses a functional TGF- β signaling axis and responds to TGF- β -induced cell cycle arrest [11, 34] and apoptosis induction [17, 35]; (3) like the normal human colonic epithelium, it does not express endogenous GRPR or other BBS receptor subtypes [29]; (4) it has been used by many investigators to study the activation of oncogenes during colorectal carcinogenesis [30, 52, 53]. We found that RIE/GRPR cells did not develop transforming phenotypes such as colony formation in soft agarose (data not shown). Surprisingly, BBS inhibited cell growth and enhanced TGF- β 's growth inhibitory effects. In contrast, our previous study demonstrated that BBS enhanced TGF- β -induced COX-2 expression [29] that is associated with the tumor promoting effects

of TGF- β [54]. There is substantial evidence to support the contention that TGF- β is a potent growth inhibitor with tumor-suppressing activity. As well, it has tumor promoting effects and contributes to tumor cell aggressiveness due to genetic loss of TGF- β signaling components or because of downstream perturbation of the signaling pathway [54, 55]. Taken together our studies demonstrate that GRPR enhances both the tumor suppressor function and tumor promoting effects of TGF- β , however, overexpression of GRPR alone is insufficient for colorectal carcinogenesis. Therefore, we speculate that activation of GRPR by bombesin promotes cell growth when the TGF- β tumor suppressor function is inactivated late in colorectal carcinogenesis. This novel hypothesis is currently being tested in our laboratory.

In conclusion, we have demonstrated in this study that BBS enhanced TGF- β -induced growth inhibition through induction of apoptosis, suggesting the interaction between TGF- β and GRPR signaling pathways responsible for functional consequences. Furthermore, p38^{MAPK} partially mediated this interaction. Our study provides further evidence that growth factors and gastric peptides interact to regulate gut function, and sheds light on better understanding the complicated network in gut physiological and pathological development.

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Figure 1. TGF- β and BBS inhibited cell growth in RIE/GRPR cells

The cells were seeded in 12-well plates for 24 h, then treated with TGF- β 1 (40 pM), BBS (100 nM), or TGF- β 1 (40 pM) and BBS (100 nM) together. Cell numbers were obtained from triplicate wells and expressed as mean $\pm$ SEM. * p < 0.05 compared with the group of vehicle control. † p < 0.05 compared with either TGF- β 1 or BBS alone.

Figure 2. TGF-β and BBS synergistically induced apoptosis in RIE/GRPR cells

A. The cells were seeded in 12-well plates for 24 h, then treated with TGF-β1 (40 pM), BBS (100 nM), or TGF-β1 (40 pM) and BBS (100 nM) together for 24 h. **A.** DNA fragmentation was quantified by a cell death detection assay. **B.** Cells were harvested and stained with Annexin V-FITC and propidium iodide. Apoptotic cells with Annexin V-FITC only were expressed as mean±SEM. * $p < 0.05$ compared with the group of vehicle control, † $p < 0.05$ compared with the group with treatment of TGF-β alone.

Figure 3. TGF-β and BBS induced cell cycle arrest in RIE/GRPR cells

The cells were seeded for 24 h, then starved for 48 h. The cells were treated with TGF-β1 (40 pM), BBS (100 nM), or TGF-β1 (40 pM) and BBS (100 nM) together for 24 h. The cells were pulsed with BrdU during the last 45 min and harvested for flow cytometric analysis. The cells in G0/G1 phase and S phase from duplicate samples were expressed as mean $\pm$ SEM, * $p < 0.05$ compared with the group of vehicle control.

Figure 4. SB203580 blocked TGF- β -induced p38^{MAPK} activity

RIE/GRPR cells were pretreated with or without SB203580 (10 μ M) for 30 min followed by treatment with or without TGF- β (40 pM) for 60 min. **A.** Whole cell lysates were extracted and subjected to Western blot. p-p38^{MAPK} (phospho-p38^{MAPK}) and p38^{MAPK} _{α} proteins were measured using the specific antibodies, α -tubulin as a loading control. **B.** Cell lysates were assayed for p38^{MAPK} activity using a recombinant MAPK-activated protein (MAPKAP) kinase-2 as a p38^{MAPK} substrate. Results from duplicate wells were expressed as mean $\pm$ SEM. * p < 0.05 compared with the group of vehicle control.

Figure 5. SB203580 attenuated TGF-β-induced apoptosis

The cells were seeded in 12-well plates for 24 h, then treated for 24 h with TGF-β1 (40 pM), BBS (100 nM), or TGF-β (40 pM) and BBS (100 nM) together, with or without pretreatment of SB203580 (10 μM) for 30 min. DNA fragmentation was quantified by a cell death detection assay. Results from triplicate wells are expressed as mean±SEM. * $p < 0.05$ compared with the group of vehicle control, † $p < 0.05$ compared with TGF-β alone, # $p < 0.05$ compared with the group with both TGF-β and BBS and without SB203580 pretreatment.