Effects of glucagon-like peptide-2 on TNF-alpha/actinomycin D-induced intestinal epithelial injury: a scanning electron microscopic and immunohistochemical study

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Abstract

Glucagon-like peptide-2 (GLP-2) is a peptide hormone with intestinotrophic activity, which is released from the intestinal endocrine cells. The aim of this study was to investigate the effects observed by scanning electron microscope of GLP-2 in intestinal epithelial injury induced by tumor necrosis factor-alpha (TNF-α)/actinomycin D (Act D). In addition, it aimed to elucidate whether the action mechanism of GLP-2 might be mediated by gastrointestinal hormones such as cholecystokinin and somatostatin. The intestinal epithelial injury was induced by intraperitoneal administration of 15 µg/kg TNF-α and 800 µg/kg Act D per mouse. Animals were injected subcutaneously 200 µg/kg GLP-2 analogue every 12 hr for 10 consecutive days prior to the administration of TNF-α and Act D. Scanning electron microscopic examination revealed severe epithelial damage in the small intestine of TNF-α/Act D-administered mice. The administration of TNF-α/Act D was significantly increased the number of somatostatin-immunoreactive endocrine cells, but did not affect the number of cholecystokinin-immunoreactive endocrine cells in the intestinal mucosa. On the other hand, GLP-2 analogue pretreatment prevented TNF-α/Act D-induced intestinal epithelial injury by causing a marked decrease in the degenerative changes and the number of somatostatin-immunoreactive endocrine cells, and a significant increase in the number of cholecystokinin-immunoreactive endocrine cells. As a result, the present study indicates that GLP-2 has a protective effect against TNF-α/Act D-induced intestinal epithelial injury. Morever protective effect of GLP-2 might be related to somatostatin and cholecystokinin.

Keywords: Glucagon-like peptide-2; TNF-α/actinomycin D; Intestinal epithelial injury; Cholecystokinin; Somatostatin
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TNF-alfa/aktinomisin D ile oluşturulan ince bağırsak epitel hasarında glukagon-benzeri peptid-2'nin etkileri: taramalı elektron mikroskobik ve immunohistokimyasal bir çalışma

Özet

Bağırsaktaki endokrin hücrelerden salınan glukagon-benzeri peptid-2 (GLP-2) intestinotrofik aktiviteye sahip peptid yapıda bir hormondur. Bu çalışmanın amacı, tümör nekroz faktör-alfa (TNF-α)/aktinomisin D (Act D) ile ince bağırsakta oluşturulan epitel hasarında GLP-2'nin taramalı elektron mikroskobu ile gözlenen etkilerini

incelemekti. Bunun yanı sıra, GLP-2'nin etki mekanizmasının kolesistokinin ve somatostatin gibi gastrointestinal hormonlar aracılı olup olmadığını da aydınlatabilmek amaçlanmıştır. İnce bağırsak epitel hasarı, her fareye intraperitonal olarak 15 µg/kg TNF-α ve 800 µg/kg Act D uygulanması ile oluşturuldu. Hayvanlara TNF-α ve Act D uygulamasından önce 10 gün süreyle her 12 saatte bir subkutanöz olarak 200 µg/kg dozda GLP-2 analoğu enjekte edildi. Taramalı elektron mikroskobu ile yapılan incelemelerde TNF-α/Act D uygulanan farelerin ince bağırsak dokusunda şiddetli epitel hasarı tespit edildi. TNF-α/Act D uygulaması bağırsak mukozasındaki somatostatin immunreaktif endokrin hücre sayısını önemli ölçüde arttırdı, ancak kolesistokinin immunreaktif endokrin hücre sayısını değiştirmedi. Öte yandan, GLP-2 ön uygulaması TNF-α/Act D ile oluşturulan ince bağırsak epitel hasarını dejeneratif değişikliklerde ve somatostatin immunreaktif endokrin hücre sayısında belirgin bir azalmaya, kolesistokinin immunreaktif endokrin hücre sayısında ise belirgin bir artışa neden olarak önledi. Sonuç olarak bu çalışma, GLP-2'nin TNF-α/Act D ile ince bağırsakta oluşturulan epitel hasarına karşı koruyucu etkiye sahip olduğunu göstermiştir. Ayrıca, GLP-2'nin koruyucu etkisi kolesistokinin ve somatostatin ile ilişkilendirilebilir.

Anahtar Kelimeler: Glukagon-benzeri peptid-2, TNF-α/aktinomisin D, İnce bağırsak epitel hasarı, Kolesistokinin, Somatostatin

Introduction

Glucagon-like peptide-2 (GLP-2) is a proglucagon-derived peptide hormone secreted from intestinal endocrine L cells in response to nutritional, hormonal and neural stimulation. GLP-2 promotes nutrient absorption, and decreases gastrointestinal motility, epithelial permeability and gastric acid secretion. GLP-2 also regulates intestinal mucosal growth by stimulation of epithelial cell proliferation and inhibition of apoptosis in the intestinal mucosa (Drucker 2005; Estal and Drucker 2005). The regenerative and protective effects of GLP-2 on epithelial cells provides the opportunity to discover new treatment methods for patients with intestinal diseases. Teduglutide, h[Gly^2]GLP-2, is a dipeptidyl peptidase IV (DPP-IV)-resistant synthetic analogue of human GLP-2 that differs from GLP-2 only by a single amino acid, and is long-acting (Sinclair and Drucker 2005). Teduglutide has been shown to exert their intestinotrophic, regenerative and protective effects in several experimental models of intestinal injury (Boushey et al. 1999; Tavakkolizadeh et al. 2000; L'Heureux and Brubaker 2003; Martin et al. 2004) or human subjects with bowel diseases such as short bowel syndrome (Jeppesen et al. 2005) and Crohn's disease (Buchman et al. 2010).

Yusta et al. (2000) showed that GLP-2 receptor (GLP-2R) localizes specifically in endocrine cells of the gastrointestinal mucosa. Detection of GLP-2R expression in enteroendocrine cells in the response to GLP-2 might suggest that the intestinotrophic effect of this peptide hormone might occur indirectly through other mediators. It is known that many hormones secreted by endocrine cells in the small intestine have paracrine effects specific to tissues and thus play a role in the local control of intestinal functions and ensure communication among different cells of the gastrointestinal tract. The trophic gastrointestinal hormones, such as cholecystokinin (CCK) and somatostatin (SS) are important chemical messengers that regulate the physiological functions of the intestine (Thomas et al. 2003). CCK is a positive enterotrophic factor for the gut, stimulating intestinal motility regulating gastric acid secretion and gastric emptying (Chandra ve Liddle 2007). Somatostatin is a regulatory-inhibitory peptide that inhibits the release of all known gastrointestinal hormones. In addition, somatostatin can inhibit the growth of normal tissues, gastric acid secretion, intestinal absorption and motility (Ferjoux et al. 2000; Thomas et al. 2003).

Tumor necrosis factor-alpha (TNF-α) is a multifunctional cytokine, which activates many signaling molecules, second messengers and transcription factors (Van Horssen et al. 2006).

TNF-α can induce cytotoxicity and cell death in a number of cell types. It has been implicated in the regulation of many inflammatory processes, including experimentally induced intestinal inflammation in animals and also inflammatory bowel diseases and septic shock in humans (Mueller 2002; Chang and Teppermann 2003). Actinomycin D (Act D) inhibits cellular RNA and protein synthesis in consequence of its binding to DNA (Goldberg and Rabinowitz 1962). Blocking the synthesis of protective proteins through a transcriptional inhibitor such as Act D sensitizes many cell types to TNF-α toxicity. TNF-α/Act D combination is commonly used in *in vitro* models in order to cause to cytotoxicity, and stimulate apoptosis (Leist et al. 1994; Jones et al. 2000).

The administration of TNF-α/Act D is the novel experimental model for the intestinal disorders (Arda-Pirincci and Bolkent 2011). Thus the effects of GLP-2 or its analogue on intestinal epithelial injury induced by TNF-α/Act D have not been investigated by electron microscopic scan. Moreover, the actions of GLP-2 on CCK- and SS-immunoreactive endocrine cells in the intestinal mucosa have not yet been studied. The purpose of the present study was to investigate the effects of GLP-2 in the mouse model of intestinal injury induced by TNF-α/Act D by scanning electron microscopy. In addition, we aimed to examine immunohistochemically whether or not the effects of GLP-2 on intestinal epithelial injury might be mediated by gastrointestinal hormones such as CCK and SS.

Materials and Methods

Animals and experimental design

In this study, 8- to 10-wk-old male BALB/c mice (n=26) from DETAE (Experimental Medical Research Institute of Istanbul University) were used. The experiments were reviewed and approved by the Animal Care and Use Committee of Istanbul University (approval number: 45/ 20.09.2005). The animals were fed with Purina Laboratory Rodent Diet 5001 and tap water *ad libitium* but fasted for 16 h prior to the experiments. The animals were randomly divided into six groups. Group I: control animals were injected 0.1% DMSO, and PBS (pH 7.4) intraperitoneally, group II: animals injected intraperitoneally 15 μg/kg TNF-α (Sigma) dissolved in 0.4 ml PBS, group III: mice injected intraperitoneally 800 μg/kg Act D (Sigma) dissolved in 0.4 ml DMSO, group IV: animals receiving Act D, prior to 2 minutes of the administration of TNF-α at the same doses, group V: animals injected subcutaneously 200 μg/kg h[Gly^2]GLP-2 (Bachem) dissolved in 0.25 ml PBS at every 12 hr for 10 consecutive days, group VI: animals given Act D prior to 2 minutes of the administration of TNF-α at 11th day after receiving h[Gly^2]GLP-2 for 10 days. The mice in group V were sacrificed 16.5 hr after the last treatment, and the other animals were sacrificed 4.5 hr after the last treatment by cervical dislocation. Samples from the jejunum were taken from the animals for all analyses.

Scanning electron microscopy (SEM)

Jejunum samples were fixed for 2 hr in a 2% glutaraldehyde, buffered to pH 7.2 with 0.1 M phosphate, and then post fixed for 1 hr in a 1% osmium tetroxide with phosphate buffer. The tissues were dehydrated with graded concentrations of ethyl alcohol, infiltrated with amyl acetate, and dried using by a Bio-Rad critical point dryer. The tissue samples were coated with gold by use of a Bio-Rad SC 502, and were examined under a Jeol 5200 JSM scanning electron microscope.

Immunohistochemisrty

Deparaffinized sections were permeabilized with 0.3% Triton X-100 for 10 min and then were heated in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven. After blocking endogenous peroxidase activity with 3% hydrogen peroxide in methanol, Ultra Vision Detection System (Lab Vision) for streptavidin-biotin-peroxidase technique was employed for immunohistochemical analysis. SS or CCK

immunoreactivity was measured using antisera directed against the 1-14 sequence of SS (Thermo RB-038) or the 26-33 sequence of CCK (CCK-8; Thermo RB-9418) at +4˚C for 24 hours. Each antibody was used at a dilution of 1:50. Control slides were incubated for the same period with phosphate buffer. Peroxidase activity of the tissue was demonstrated by 3-amino-9-ethylcarbazole and then hematoxylin was applied onto the sections.

Quantification of SS or CCK immunoreactive endocrine cells in crypts and villi was performed in all fields of each section by using an Olympus CX41 light microscope at x400 magnification. The mucosal areas including the immunoreactive cells were measured by PC-based image analysis system, using Olympus Analysis 5 Digital Imaging software with an Olympus DP 71 digital camera (35 mm). The number of the SS or CCK positive cells per mm^2 mucosal area was calculated for each section.

Statistics

All data are expressed as mean ± standard error (SE). The results of and SS and CCK immunohistochemistry were analyzed by nonparametric Kruskal-Wallis test and Mann-Whitney U test. Differences were considered statistically significant at $p<0.05$.

Results

Scanning Electron Microscopy

Scanning electron microscope examination of jejunum samples from the control group showed normal intestinal mucus content and mucosal topography. In the group given TNF-α, epithelial rupture with denuded basal lamina on indicated severe epithelial damage and deformation of villar shape in the animals receiving Act D. The administration of TNF-α/Act D resulted in epithelial desquamation, totally exposed basal lamina along with flattened and disrupted villar structures. Scanning electron microscopy of the h[Gly^2]GLP-2 group revealed a normal mucosal topography of small intestinal epithelium with regular villi. The h[Gly^2]GLP-2 and TNF-α /Act D-administered group demonstrates a regular intestinal epithelial topography with slight degeneration (Fig. 1).

Immunohistochemisrty

The photomicrographs and quantitative assessment of SS-immunoreactive cells in the jejunal mucosa is presented in Fig. 2 and 3. Somatostatin-immunoreactive endocrine cells were observed in the epithelium of both crypts and villi. Microscopic observation showed that the number of SS cells increase significantly in the TNF-α/Act D group as compared with the control group ($p<0.01$). However, treatment with h[Gly^2]GLP-2 significantly attenuated the number of SS-immunoreactive cells in the epithelium compared to the group given TNF-α/Act D ($p<0.01$).

The photomicrographs and quantitative assessment of CCK-immunoreactive cells in the jejunal mucosa is shown in Fig. 4 and 5. Cholecystokinin-immunoreactive endocrine cells were observed in the epithelium of both crypts and villi, but they are predominantly located in crypts of Lieberkühn. h[Gly^2]GLP-2 pretreatment of the TNF-α/Act D group markedly stimulated CCK expression in the endocrine cells of the jejunal mucosa when compared with the control or TNF-α/Act D group ($p<0.05$). There was no alteration in the number of CCK-immunoreactive cells of the other groups as compared to the control group.

Discussion

In vivo animal models that are produced experimentally with the use of cytotoxic agents such as radiation, cyclophosphamide or actinomycin D which cause injury in small intestinal tissue, nonsteroidal anti-inflammatory medicines, some cytokines such as TNF-α or bile salt can be used to help us understand the pathogenesis of intestinal diseases and can also be used to gain preclinical data for novel treatments

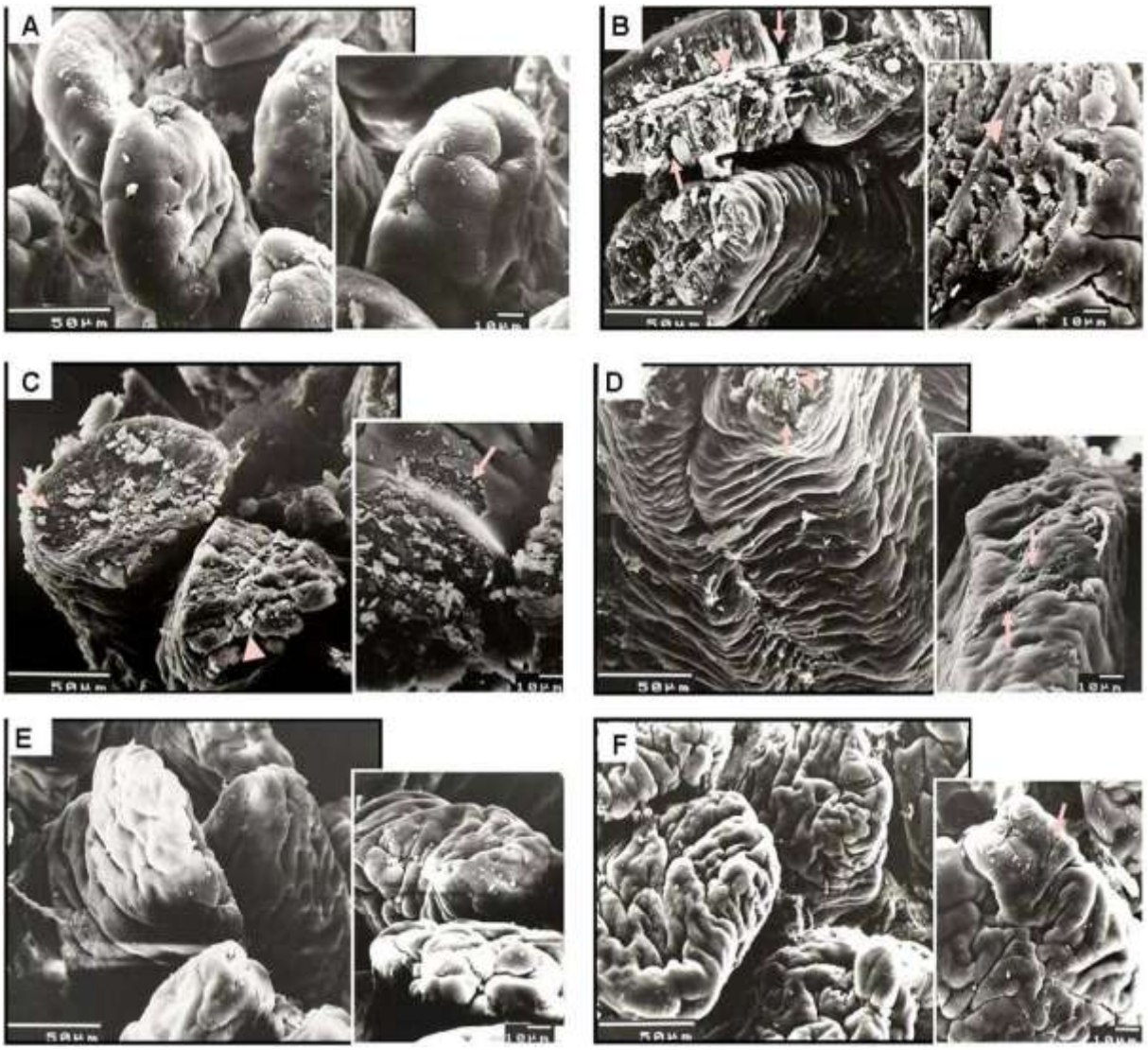

Figure 1. Scanning electron micrographs of all groups. Control **(A)** and GLP-2 **(E)** groups electron micrographs represent normal topography of small intestinal epithelium with regular villar structures. TNF-α **(B)**, Act D **(C)**, TNF-α/Act D **(D)** groups indicate extensive mucosal degeneration with totally exposed basal lamina (▼), severe epithelial damage (➡). GLP-2 treated TNF-α/Act D group **(F)** demonstrates a regular intestinal epithelial topography with slight degeneration (➡).

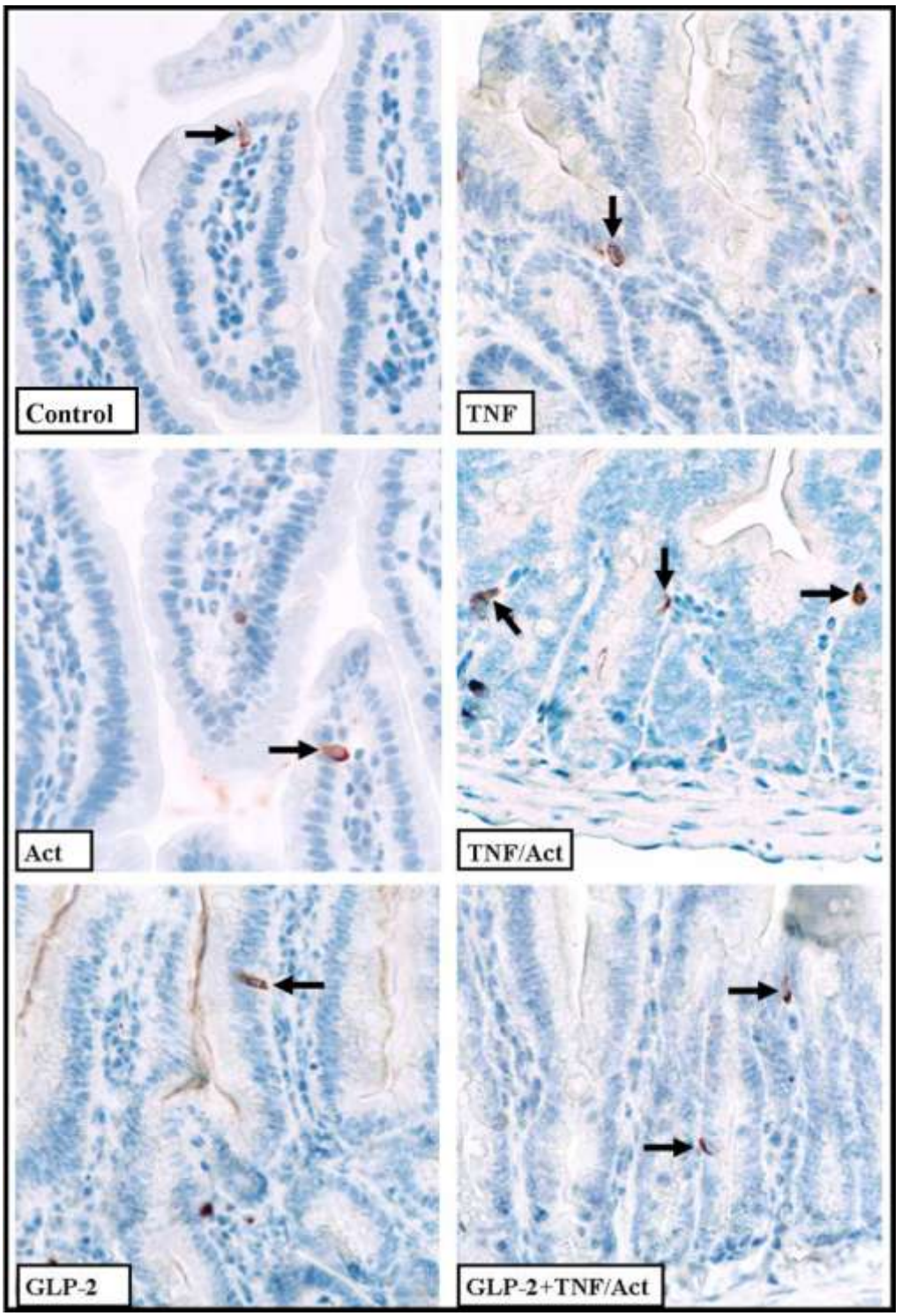

Figure 2. Representative photomicrographs showing the somatostatin immunoreactive cells (➡) in jejunal mucosa of all groups. Original magnification X400.

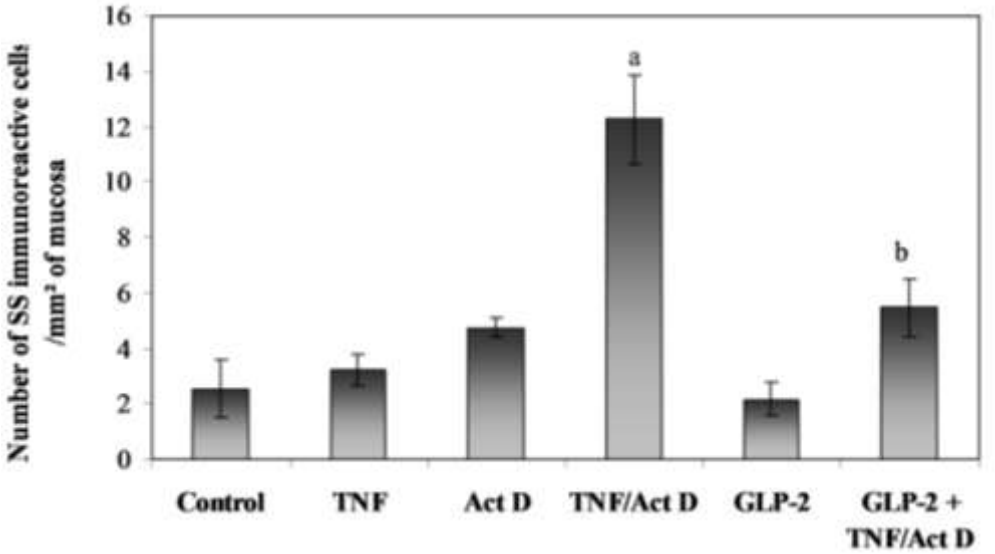

Figure 3. The quantitative assessment of somatostatin immunoreactive cells in jejunal mucosa for all experimental groups. The data were given as mean ± SE for per group. **Control:** 2.54 ± 1.05, **TNF-α:** 3.24 ± 0.57, **Act D:** 4.75 ± 0.37, **TNF-α/Act D:** 12.26 ± 1.61, **GLP-2:** 2.17 ± 0.6, **GLP-2 treated TNF-α/Act D:** 5.45±1.01. [a] ($p<0.01$) versus control group, [b] ($p<0.01$) versus TNF-α/Act D group.

TNF functions as an important mediator of many biological and pathophysiological pathways by activating certain signal molecules, secondary messengers such as phospholipases, kinases and phosphatases, and oxygen radicals, and transcription factors in some cell types (Pastorino et al. 1996). It was shown that TNF-α is able to regulate cell proliferation, differentiation, survival and death in *in vitro* models (Kunstle et al. 1997). The injection of recombinant TNF-α into animals for experimental purposes and into cancer patients for treatment purposes may result in serious organ injury. It was shown that TNF-α administered to mice at 1 μg causes the formation of edema, dilatation and epithelial damage in the small intestine (Remick and Kunkel 1993). This finding is similar to the SEM results of our experiment indicating the formation of epithelial rupture and disruption in the integrity of villus. Actinomycin D is one of the most powerful anti-cancer agents since it has direct effects on nucleic acids. The primary effect of actinomycin D is that it inhibits RNA synthesis by combining with specific regions on DNA (Goldberg and Rabinowitz 1962; Sawicki and Godman 1971). During the experiments that were carried out by Schwartz and Sodergren (1968) to monitor the tumor regression with Act D, it was found that actinomycin doses (400-1200 μg/kg) are tumoricidal for the sensitive tumors but have toxic effect on intestinal crypt cells. 24 hr after 800 μg/kg Act D was administered intraperitoneally to rats, severe injury and crypt loss were observed in duodenum (Schwartz et al. 1963).

In our study, 4.5 hr after administration of Act D at the same dose to mice, severe epithelial damage and deformation of villar shape were all observed in jejunum by scanning electron microscope.

In vitro studies carried out on many cell types showed that inhibition of RNA or protein synthesis increases the sensitivity of cells to TNF-α toxicity as many as 100 times (Leist et al. 1994; 1997). *In vitro* studies carried out by Chang and Teppermann (2001; 2003) on

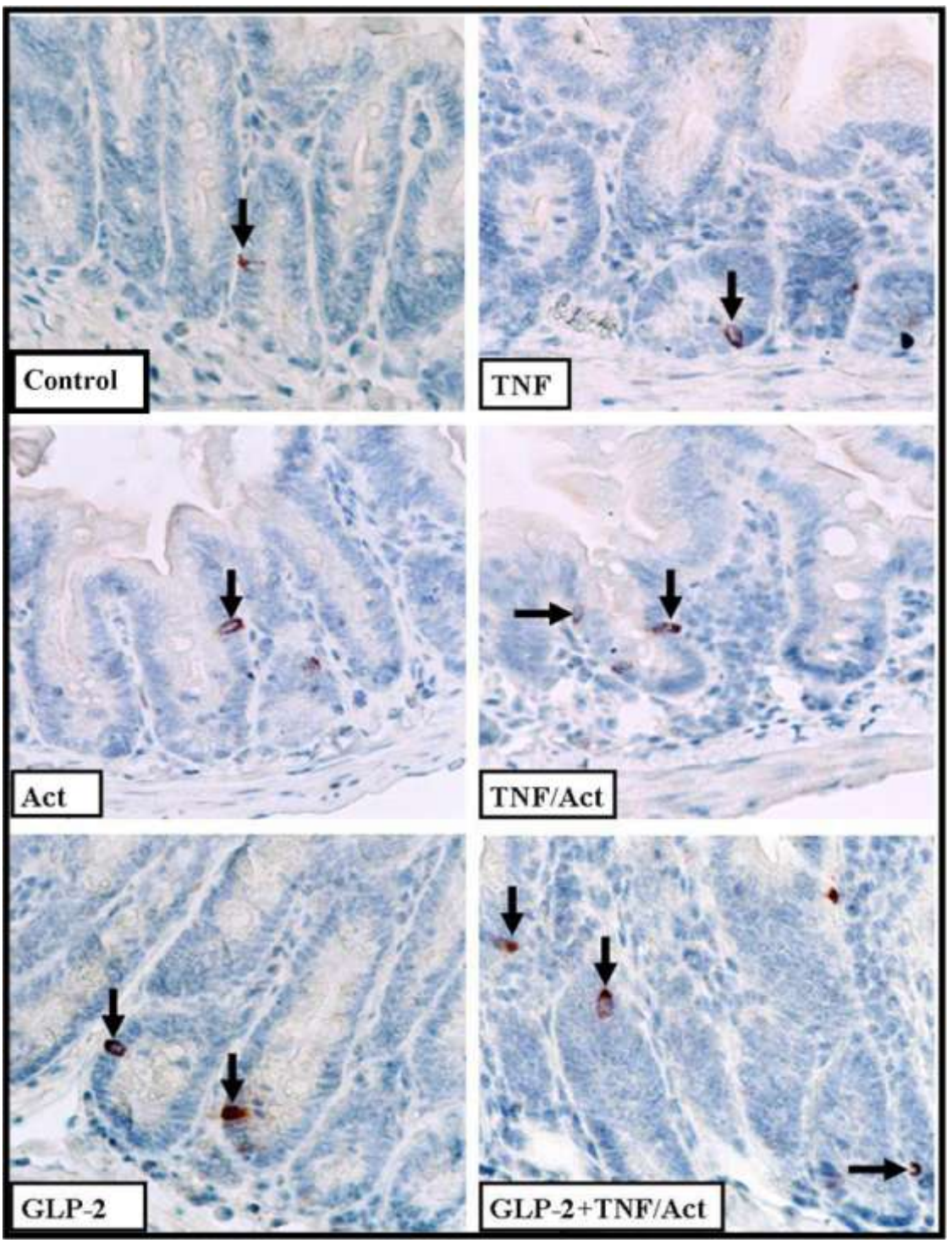

Figure 4. Representative photomicrographs showing the cholecystokinin immunoreactive cells (➡) in jejunal mucosa of all groups. Original magnification X400.

various intestinal epithelial cell lines, it was reported that toxicity produced by TNF-α increased as the result of the addition of Act D to cell culture, and apoptotic cell death was stimulated considerably. Arda-Pirincci and Bolkent (2011) was demonstrated that co-application of TNF-α and Act D to mice was markedly increased apoptotic index both in the villi and crypts of small intestine. In the present study, scanning electron microscopy of the intestinal tissues from the animals receiving TNF-α together with Act D revealed epithelial desquamation, totally exposed basal lamina along with flattened and disrupted villar structures.

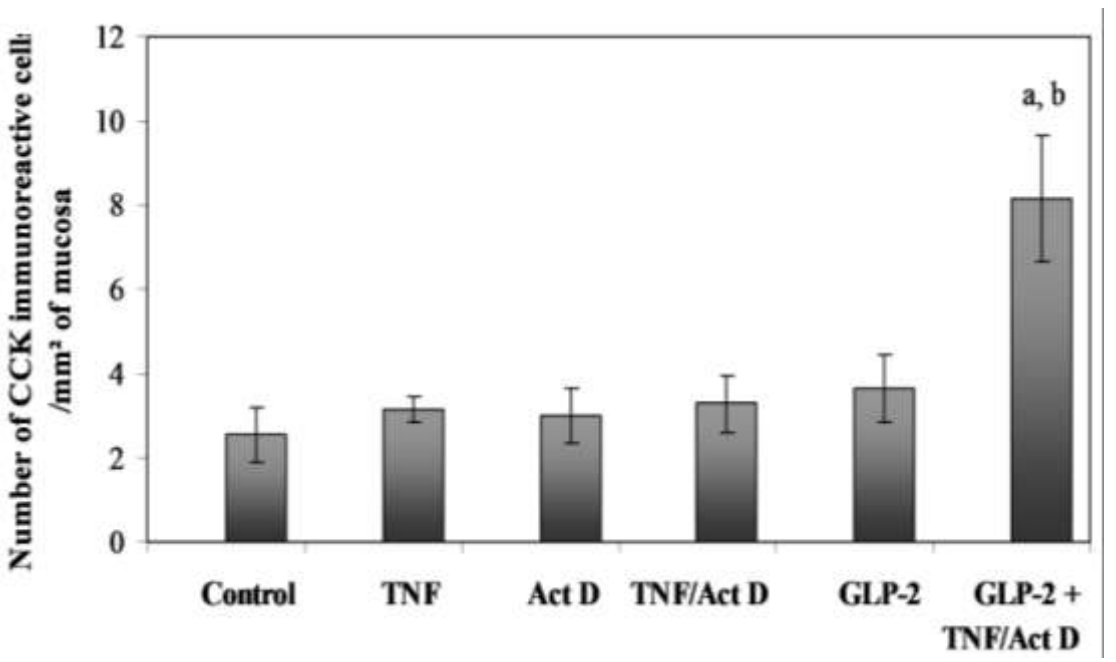

Figure 5. The quantitative assessment of cholecystokinin immunoreactive cells in jejunal mucosa for all experimental groups. The data were given as mean ± SE for per group. **Control:** 2.55 ± 0.63, **TNF-α:** 3.14 ± 0.29, **Act D:** 2.99 ± 0.66, **TNF-α/Act D:** 3.28 ± 0.66, **GLP-2:** 3.64 ± 0.81, **GLP-2 treated TNF-α/Act D:** 8.15 ± 1.49. [a] ($p<0.05$) versus control group, [b] ($p<0.05$) versus TNF-α/Act D group.

The studies in recent years show that the most significant role of GLP-2 is that it elicits trophic effects unique to the small and large intestine by stimulating the proliferation of epithelial cells and ensuring apoptosis and proteolysis inhibition (Drucker et al. 1996; Estall and Drucker 2003; 2005). Animal experiments indicate that the administration of GLP-2 to subjects after chemotherapy increases the rate of intestinal recovery (Tavakkolizadeh et al. 2000) and decreases the death rates correlated with chemotherapy (Boushey et al. 1999). In our study, it is remarkable that subcutaneous injection of h[Gly^2]GLP-2 to mice at the dose of 200 μg/kg for 10 days before the administration of the agents that cause tissue injury maintains normal topography of intestinal epithelium, and prevents degenerative injury in the jejunum due to TNF-α /Act D, thus showing a protective effect.

Somatostatins are known to be inhibitor factors that regulate many physiological functions such as cell proliferation, intestinal motility and inhibition of intestinal nutrition absorption (Benali et al. 2000). Recent studies

have shown that this peptide hormone stimulates apoptosis and plays an active role in the response of immune system to inflammation (De Jonge et al. 2003; Paran and Paran 2003). It is argued that the production of SS and SS receptors are stimulated due to the secretion of proinflammatory cytokines such as TNF-α and IFN-γ in tissue (Panteris and Kouroumalis 2007). On the other hand, in our study no significant change was observed in the number of SS-immunoreactive endocrine cells in the groups that were administered TNF-α only or Act D only compared to the control group while cells showing SS expression due to the combined administration of TNF-α and Act D increased in number considerably. These findings suggest that SS expression increases in the small intestinal epithelium of the mice administered TNF-α/Act D and might stimulate apoptotic cell death. However, pre-administration of h[Gly^2]GLP-2 to the TNF-α /Act D group led to a significant decrease in apoptotic index (Arda-Pirincci and Bolkent 2011) as well as SS expression in the tissue. In the light of these findings, we think that in the TNF-α/Act D-induced intestinal injury there might be a correlation between the anti-apoptotic effect (Arda-Pirincci and Bolkent 2011) and the reduction of SS expression stimulated by h[Gly^2]GLP-2.

It is known that SS hormone inhibits CCK secretion like other peptide hormones secreted from endocrine cells (Green et al. 1989). De Haan et al. (2008) suggest that due to the increase in the CCK receptor activation that inflammation and tissue injury in the intestine decreases. Another group of researchers reported that the administration of GLP-2 to mice in an experimental colitis increases proinflammatory cytokine levels and shows anti-inflammatory effect (Ivory et al. 2008). In our study, it was determined that among all experiment groups, the number of CCK immunoreactive endocrine cells increase considerably only in the h[Gly^2]GLP-2 treated TNF-α/Act D group. This increase in CCK expression after the h[Gly^2]GLP-2 treatment to TNF-α/Act D group in our experimental model suggests that CCK function as an important defense mechanism in the response to inflammation in small intestinal tissue.

This is the first study in the literature that shows that GLP-2 has a protective effect on the topography of intestinal epithelium against TNF-α/Act D-induced intestinal injury. The results of our study indicate that the protective effect of GLP-2 might be due to its ability to affect the number of SS and CCK enteroendocrine cells in intestinal epithelium.

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