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Original Research

Growth hormone upregulates intestinal trefoil factor expression in the ileum of rats after γ-radiation

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Abstract

Growth hormone (GH) and intestinal trefoil factor (ITF) have been involved in intestinal protection and repair. This study investigates the effects of GH administration on ITF expression and histological changes associated with tissue injury in an intestinal rat model of radiation. Adult male rats were divided into four groups: control, GH, radiation and radiation + GH (GHyRAD). Ileum samples were obtained at 2 or 72 h after radiation and processed to determine ITF levels (mRNA and protein) by quantitative polymerase chain reaction, Western blot and immunohistochemistry. In addition, goblet ITF-positive cells were identified by immunohistochemistry at 72 h. Our results showed an upregulation of mRNA and protein production of ITF in ileum samples after GH and radiation + GH compared with control and irradiated samples. Irradiation alone affected ITF protein expression. However, irradiation after GH pretreatment produced the highest ITF mRNA and protein levels at both the tested time points. ITF-producing goblet cells were identified in intestinal villi (apical location). GH treatment increased the number of ITF-producing goblet cells, and radiation after GH treatment displayed further increase in the number of ITF-positive goblet cells. GH upregulates ITF in normal intestinal tissue. This upregulation is higher when radiation is given after GH treatment. Nevertheless, the mechanism by which GH regulates ITF expression remains unclear and is still under investigation. These results could open up new avenues in the therapeutic reparative and protective effects of GH during radiotherapy and chemotherapy.

Keywords: growth hormone, intestinal trefoil factor, radiation, tissue repair, goblet cells, quantitative PCR

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Introduction

In recent years, there has been considerable interest in the use of various growth factors to reduce the severity of mucositis induced by cancer therapy; some agents having currently reached human clinical trials.[1] Murine models have been particularly useful to study the effects of growth factors on radiation and chemotherapeutic drug-induced intestinal mucositis.

The intestinal epithelium is constantly renewed from stem cells located near the bottom of small intestinal and colonic crypts.[2,3] After injury induced by γ-radiation, some cells near the bottom of the intestinal crypts are lost by apoptosis.[4] The number of crypts that survive and regenerate following a cytotoxic insult correlates with the severity of the noxious symptoms of the treatments in animal models. Recent evidence suggests that certain growth factors and cytokines can modify cellular radiosensitivity and some have been shown to improve crypt regeneration in murine intestinal epithelium following γ-radiation. Therefore, it has been postulated that growth factors are involved in the process of crypt regeneration and, consequently, tissue repair after a cytotoxic insult such as radiation, in different ways. These factors include basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-I, keratinocyte growth factor and vascular endothelial growth factor (VEGF); b-FGFs and the others all protect the intestine from radiation injury. Furthermore, administration of IL-11 and transforming growth factor (TGF-b) before radiation therapy seems to protect and promote intestinal crypt survival after treatment.[3–9]

Growth hormone (GH), an anabolic hormone secreted by the anterior pituitary gland, stimulates nitrogen and mineral retention.[5] In rats, GH promotes the growth of different tissues by increasing the number of cells in this tissue.[10] The existence of GH receptors in intestinal epithelial cells suggests a direct effect of this hormone on the

gastrointestinal tract proliferative function.[11–13] Interestingly, over the past few years our group, among others, has shown that GH has potent proliferative effects and a protective action against radiotherapy and chemotherapy-induced injury in the gut.[14,15]

The trefoil factor family (TFF) is composed of small peptides that are characterized by one or more three-looped structural motifs. They are mainly secreted in association with mucins into the gut lumen and are concentrated within the mucus layer where they promote epithelial cell migration.[16,17] Among the different trefoil factors, intestinal trefoil factor (ITF, also known as TFF-3) is typically expressed in goblet cells of the small and large intestine.[18–20] ITF is an integral component of the surface of mucous epithelium and is secreted with the mucin MUC2 by intestinal goblet cells. Previous studies have shown that ITF plays an important role in mucosa formation and repair. Although the action of ITF is not fully understood, damage to intestinal mucosa results in upregulation of ITF gene expression and protein production, and the intestinal damage produced after radiotherapy and chemotherapy is also regulated by ITF.[21] Interestingly, recent studies suggest a role for GH in ITF regulation,[22] suggesting a potential role for GH in tissue repair and protection via ITF.

In this study, we attempt to further our understanding of the role of both GH and ITF in intestinal mucosal injury by examining ITF expression in a rat model of γ-radiation-induced mucositis under the action of exogenous GH.

Materials and methods

Animals

Sixty-four ($N = 8$/group) BDIX rats weighing 150–240 g (Janvier, LeGenest, France) were used in this study. Animals were fed with a high-protein diet (50 kcal/rat/d; Impact, Novartis, Basel, Switzerland) that was previously found to potentiate the protective effects of GH on the intestine following radiation.[23] The expected reduction in food intake following radiation was not observed. The study was approved by the Research Committee of our institution and met European Union criteria for protection of animals used for experimental and other scientific purposes (86/609/EU).

Treatments

Groups of rats were treated as follows: (1) the control group received a subcutaneous saline injection on three consecutive days; (2) the GH group received human recombinant growth hormone (Lilly Labs, Madrid, Spain) 1 mg/kg/d subcutaneously injected on three consecutive days; (3) the RAD group received one γ-radiation dose (1000 cGy) according to the protocol described previously;[23] and (4)[4] the GHyRAD group received irradiation as above plus GH for three consecutive days before radiation. In all groups, animals were weighed at the beginning and at the end of experiments.

Tissue sampling

At 2 or 72 h after radiation treatment, the animals were lightly anesthetized with isoflurane and killed by cervical dislocation. Samples from the ileum were taken 1 cm from the ileocecal valve and placed in 10% neutral buffered formalin for 24 h. Fixed tissues were embedded in paraffin blocks, from which 5-μm serial sections were cut and stained with hematoxylin–eosin.

Quantification of ITF mRNA expression: realtime polymerase chain reaction

The expression levels of ITF were detected by realtime polymerase chain reaction (PCR) (LightCycler, Roche Diagnostics, Indianapolis, IN, USA). cDNA from 1 μg of total RNA was used for PCR. Realtime PCR was performed with the Fast-Start DNA master SYBR Green system (Roche). The sequences of the primers were as follows: S26-f, 5′-ATGCGTGCCCAAGGACAAGG-3′; S26-r, 5′-GGCAGCACCCGCAGGTCTAA-3′; ITF-f, 5′-CTTGCTGTCCTCCAGCTCT-3′; ITF-r, 5′-CCGGTTGTTGCACTCCTT-3′.

All results were normalized with respect to the expression of S26, a ribosomal RNA.

The cDNA copy number for each gene of interest was determined using a seven-point standard curve. Standard curves were run with each set of samples. Correlation coefficients ($r2$) for standard curves were typically ≥ 0.98. The precision of target S26 from the same cDNA was specific and the levels were the same. To confirm that each primer pair correctly amplified the sequence of interest, initial PCR products were run on agarose gels, stained with 0.5 μg/mL ethidium bromide, and viewed by UV transillumination for the presence of a single product of the predicted size. To confirm the specificity of the reaction product during each run, the melting profile of each sample was analyzed using the LightCycler. The melting profile was determined by holding the reaction at 80°C for 10 s and then heating slowly to 95°C with a linear rate of 0.1°C/s while the fluorescence emitted was measured. Melting-curve analysis demonstrated that each of the primer pairs described amplified a single product. The rare samples that produced a significant second peak in the melting profile were not used in analysis.

ITF Western blotting

To demonstrate potential changes in ITF expression, Western blotting was performed on total protein samples isolated from ileum tissues using an RNA–DNA–protein separation reagent (Progen Industries, Brisbane, Australia). First, quantification of the protein concentration was performed using the Bradford reagent (Sigma, St Louis, MO, USA) with bovine serum albumin (BSA) as a standard. Equal amounts of protein (10 μg) from each sample, and 2 μg of a broad range-biotinylated protein molecular weight marker (Bio-Rad, Hercules, CA, USA) were treated with a reducing sample buffer, separated on a 12.5% sodium dodecyl-polyacrylamide minigel, and electroblotted onto 0.2-μm nitrocellulose membranes (Amersham,

Barcelona, Spain) (100 V, 500 mA, 4°C, 40 min). Membranes were blocked for two hours in 5% bovine serum albumin and then incubated overnight with a rabbit ITF polyclonal IgG antibody (kindly provided by Dr A Giraud, Melbourne, Australia) at a concentration of 200 μg/mL and a dilution of 1:600. After primary antibody incubation, membranes were washed for 5 × 10 min in tris (hydroxymethyl) aminomethane-buffered saline (TBS), with a third 10-min washing in TBS with 0.05% Tween-20, and then incubated for one hour at 4°C with a 1:1000 dilution of peroxidase-conjugated swine anti-rabbit biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents (Dako, Barcelona, Spain). Membranes were then washed for 5 × 10 min in TBS, followed by a 60-s wash in enhanced chemiluminiscence reagents (Amersham). A high-performance luminescence detection film (Amersham) was exposed to membranes in a film cassette for between 10 s and 2 min and developed.

ITF immunostaining

To identify any potential site-specific changes of ITF protein in the ileum samples in the different study groups, immunohistochemical detection of ITF was performed on paraffin sections of the ileum. After non-specific binding sites were blocked with 5% pig serum, sections were incubated overnight at 4°C with the above rabbit anti-ITF serum at 1:1000 dilution in Tris-buffered saline (TBS, pH 7.4) containing 1% BSA. After sections were washed, staining was visualized with swine anti-rabbit biotinylated IgG and avidin/biotinylated horseradish peroxidase reagents (Dako, Barcelona, Spain) and 3.3′-diaminobecidine substrate (Sigma). ITF specificity was confirmed by the omission of rabbit anti-ITF serum and by including a normal rabbit serum (negative control).

Statistical analysis

Results of quantitative PCR (q-PCR) (ITF gene expression) and Western blot (ITF protein levels) were compared with analysis of variance (ANOVA) between the different groups at two time intervals, 2 or 72 h. The immunohistochemistry data (number of ITF-positive cells and number of goblet cells) were also compared at 72 h.

Results

mRNA expression and protein levels of ITF in ileum mucosa

To determine whether the changes in ITF were apparent at mRNA level in intestinal ileum mucosa, quantitative real-time (qRT)-PCR analysis was employed to measure the changes in ITF gene transcript in the four different previously described groups at two time intervals (2 and 72 h). Figures 1a and b illustrate the results as the ratio of the expression of ITF mRNA to the expression of S26 gene, the latter being employed as a 'housekeeping gene'. At two hours time, we found an increase in the expression of mRNA ITF in the GH and GHyRAD groups compared with the control and RAD groups, with statistical significances of $P < 0.05$ and $P < 0.009$, respectively (Figure 1a).

At 72 h, only the GHyRAD group showed the upregulation of ITF mRNA expression but not the other groups: control, RAD and GH ($P < 0.01$) (see Figure 1b).

Analysis of protein expression by Western blot demonstrated a similar pattern of ITF expression to that obtained by q-PCR analysis of the RNA level. At two hours we observed an increase in ITF protein expression levels in the group treated with GH compared with the controls ($P < 0.05$) and a decrease compared with the GHyRAD group ($P < 0.05$). The group treated with radiation alone showed a decrease in expression compared with the other three groups: controls, GH and GHyRAD with respective significant values of $P < 0.05$, $P < 0.01$ and $P < 0.001$. At 72 h, we observed an increase in protein expression levels in the group treated with GH compared with the control and irradiated groups ($P < 0.05$ in both comparisons). Interestingly, the group irradiated and treated with GH (GHyRAD) showed higher ITF protein expression levels than the GH group ($P < 0.004$) (see Figure 2b). A representative Western

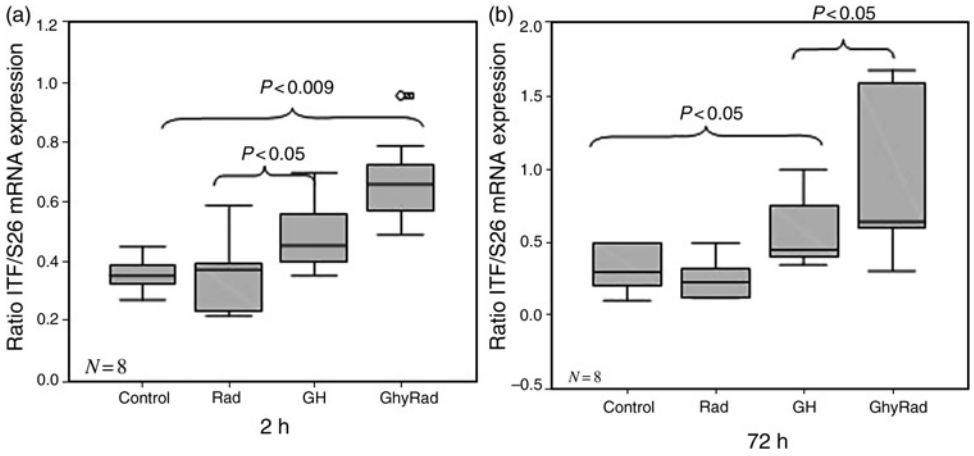

Figure 1 Quantification of ITF in ileum mucosa samples ($n = 8$ per group), as determined by q-PCR at 2 h (a) and 72 h (b). ITF mRNA expression increased markedly in both GH and GHyRAD groups compared with the control and irradiated groups at 2 and 72 h (a and b, respectively). Boxes enclose medians and interquartile ranges. ITF, intestinal trefoil factor; q-PCR, quantitative PCR; GH, growth hormone; GHyRAD, radiation + GH

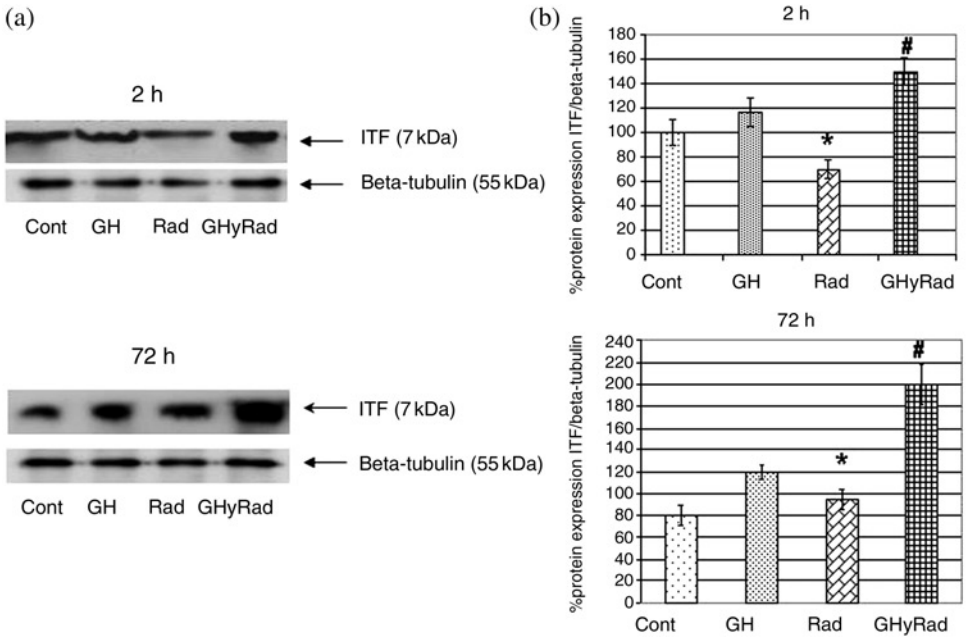

Figure 2 Quantification of ITF in ileum mucosa samples ($n = 8$ per group), as determined by Western blot at 2 and 72 h. (a) A representative Western blot picture of ITF and β-tubulin in ileum for four groups at 2 h (up) and 72 h (down). (b) Densitometric analysis of Western blots showing the levels of ITF protein normalized to β-tubulin expression in the four groups. Western blots were performed three times on two separate pooled samples from four rats. Error bars represent the standard deviations. *Significant at $P < 0.05$ compared with control and $^{\#}P < 0.05$ compared with GH group. ITF, intestinal trefoil factor; GH, growth hormone

blot of ITF and β-tubuline in the ileum of the four groups at the two time points is shown in Figure 2a.

Immunohistochemical findings

Apical goblet ITF immunohistochemistry in the intestinal ileum

Immunohistochemical studies were performed on serial sections of intestinal ileal biopsies. Significant immunostaining of the number ITF-positive goblet cells was detectable in all GH-treated groups at 72 h. Unifactorial ANOVA analysis for positive ITF immunostaining were statistical significant ($P < 0.001$) with a value of $F_{7,31} = 46.06$. When we compared individually each group with the other, the Mann–Whitney analysis showed more ITF-positive apical goblet cells in the GH (Figure 3 and arrows in Figure 4c) group than in the control group (Figure 3 and arrows in Figure 4a) or the RAD (Figure 3 and arrows in Figure 4b) group where few or non-goblet cells were ITF detected at 72 h with a statistical value of $P < 0.001$ in both comparisons. Interestingly, the GHyRAD showed a higher number of goblet cells which were immunoreactive for ITF than the control and RAD sample groups ($P < 0.001$) (see Figure 3 and arrows in Figure 4a, 4b and 4d). There were no significant differences between the control and RAD groups in the number of goblet cells positively immunoreactive for ITF in the apical location of the intestinal ileum villi.

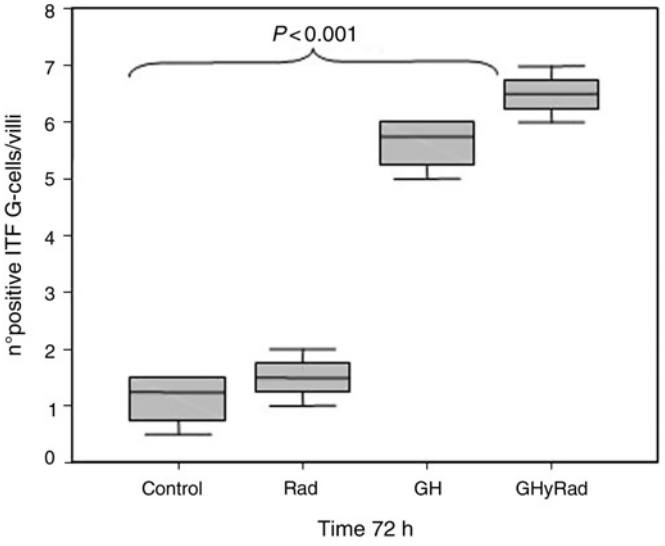

Figure 3 Number of total ITF immunoreactive goblet cells in ileum villi. The number of goblet cells in four groups at 72 h are shown. Boxes enclose medians and interquartile ranges. ITF, intestinal trefoil factor

Discussion

Despite human trials over recent years, using different growth factors to reduce the severity of the mucositis caused by chronic inflammatory diseases of the gastrointestinal tract or cancer therapy,[3,24,25] there is still no significantly effective treatment or preventive therapy against the effects of either chemotherapy or radiotherapy as yet. Several approaches have been suggested, including dietary modulation and administration of growth factors.[15,23,26–28] We hypothesized that GH, a trophic factor that has potent proliferative effects in the intestine and acts as a protective molecule against radiotherapy and chemotherapy injury,[14,15,28] regulates ITF, an intestinal peptide involved

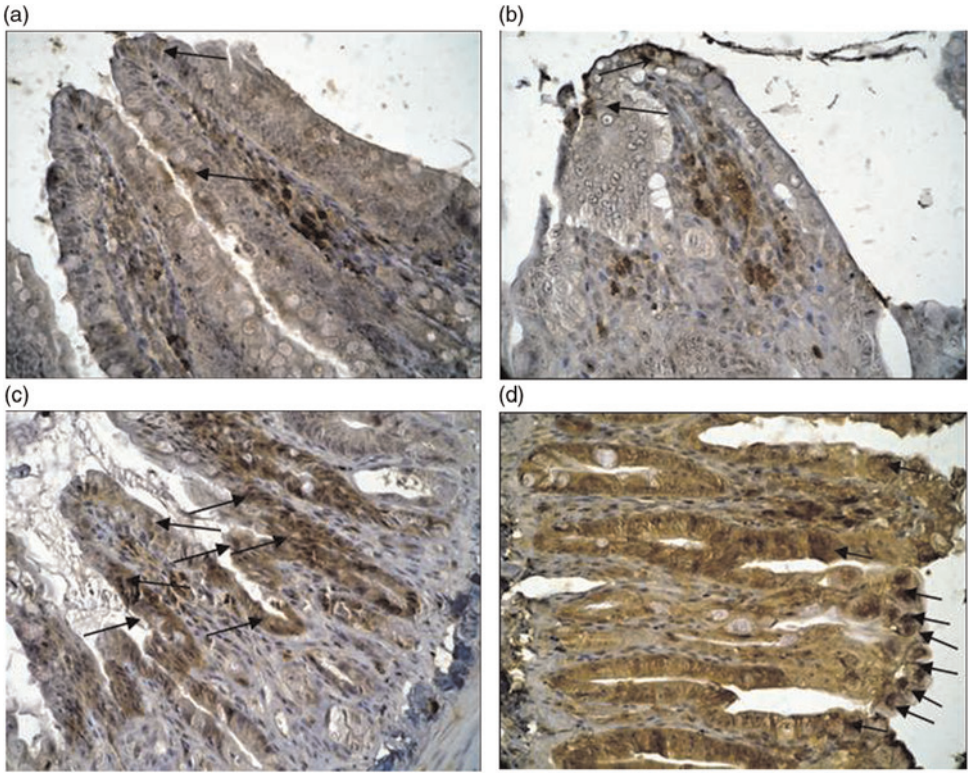

Figure 4 Immunohistochemical localization of ITF in ileum villi at 72 h. Representative immunohistochemical staining with anti-ITF in (a) control, (b) irradiated, (c) GH and (d) GhyRad. Arrows indicate ITF-positive goblet cells in apical location. Photomicrographs in original magnification ×20. ITF, intestinal trefoil factor; GH, growth hormone; GHyRAD, radiation + GH

in tissue protection and repair, in the ileum. We analyzed the effects of GH on ITF expression at the gene and protein level in a rat model of radiation.

In murines the process of mucosal damage after cytotoxic insult such as radiation or chemotherapy is characterized by an initial decrease in crypt cell proliferation that precedes crypt loss, villus shortening and atrophy and goblet cell depletion followed by crypt cell hyperproliferation. In the repair process, the upregulated proliferation of crypt epithelial cells precedes crypt and villus regeneration, repopulation by mucous cells and normalization of mucosal structure. Both of these processes are regulated by ITF.[21,29] Previous studies by our group and others have demonstrated that the small-intestine manifestations of toxicity caused by DNA damage induced by radiotherapy in rats with an acute and transient process of mucosal damage and regeneration can be influenced by the action of GH.[14,28] Our data showed ITF mRNA and protein upregulation in irradiated animals treated with GH compared to those treated with GH without radiation. Interestingly, GH alone seems to increase ITF levels in comparison with control and irradiated rats. ITF levels in ileum mucosal tissue were highest in the group treated with GH and irradiated (GHyRAD) at both analysis times (2 and 72 h). We observed a clear action of GH on ITF expression, indicating that ITF is involved in protective/reparative mechanisms against radiation through upregulation in the villi in the GHyRAD group.

To establish this, we analyzed ITF expression in villi using immunohistochemistry. ITF seems to function locally by augmenting epithelial restitution and resealing epithelial breaches by migration of neighboring cells rather than by proliferation.[30,31] Although it does not generally enhance proliferation of intestinal epithelial cell lines, it has been shown to protect the mucosa against ethanol and indomethacin damage[31] and to increase the rate of cell migration *in vitro* whether administered alone in cooperation with mucus glycoproteins.[17,32,33] Goblet cells are abundant constituents of the surface epithelium within the small and large intestine. *In vitro* and *in vivo* studies have demonstrated that ITF plays an important role in mucosal homeostasis of the intestinal mucosa.[31] The role of ITF in protecting the epithelium from injury and promoting repair is facilitated by its selective expression in goblet cells where it is secreted mostly at the apical surface of the villi.[32,33] Furthermore, trefoil gene expression is rapidly induced by experimentally induced injury.[34] Mucin glycoproteins, the other major products of goblet cells, enhance these effects through synergistic interaction with ITF.[20] Interestingly, ITF immunostaining was increased in the apical villous area, where goblet cells express ITF, in the GHyRAD group, and this would suggest a role for ITF upregulation in the migration produced by GH after radiation. This type of protective/reparative mechanism produced by GH in response to radiation could be involved in the recruitment of ITF-positive migratory cells by the apical villi. Moreover, previous work from our group in the same experimental model as this study showed that GH reduced radiation-induced intestinal injury by improving proliferation and reducing apoptosis and p53 expression;[35] this is

accompanied by GH-mediated increased expression of ITF by the apical goblet cells in the villi after radiation at 72 h. This would implicate cell-dependent and cell migration-mediated mechanisms in the protective and/or reparative processes against radiation in the ileum.

The greater ITF expression in the GHyRAD group than in the radiation group agrees with previously published literature reporting that cytokines, growth factors (such as GH) and peptides (such as ITF) may reduce radiotherapy- and chemotherapy-induced intestinal damage by stimulating cell proliferation, although some of these proteins also have systemic actions.[1,6–9,21,36] Interestingly, recent data show that the exposure of neonatal rat islets or insulinoma cells to GH, a known beta-cell growth factor, resulted in markedly increased ITF mRNA and protein levels.[22] Further studies are needed, and are currently being carried out in our laboratory, to identify the mechanism by which GH regulates ITF and to examine the sensitivity of tumor cells to GH as suggested by others.[37] However, the present results show a clear upregulation of ITF at different levels (gene, protein and tissue) in the groups treated with GH that is particularly marked after radiation. Possible therapeutic objectives include prevention of the mucositis found in inflammatory bowel diseases and effects of radiotherapy (our model) and chemotherapy which result in substantial morbidity.

Author contributions: The work presented here was carried out in collaboration between all authors. SJP and EM defined the research theme. SJP, CL, JJM and EM designed the methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and SJP and EM wrote the paper. GG, ME, VC and IC co-designed the quantitative PCR, Western blot and immunohistochemistry experiments, and co-worked on associated data collection and their interpretation. All authors have contributed to, seen and approved the manuscript. CL and JJM have contributed equally to this manuscript.

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