

Elemental diet inhibits pro-inflammatory cytokine production in keratinocytes through the suppression of NF- κ B activation

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Abstract. An elemental diet (ED) has been reported to reduce oral mucositis and dermatitis induced by chemotherapy. However, its molecular mechanism of action as an anti-inflammatory agent is still unknown. The aim of the present study was to clarify whether ED confers its anti-inflammatory action via reduction of pro-inflammatory cytokine production in keratinocytes *in vivo* and *in vitro*. We evaluated the efficacy of ED in the treatment of 5-fluorouracil (5-FU)-induced dermatitis of nude mice, and examined the expression of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 using immunohistochemistry. Moreover, we assessed the expression and production of these pro-inflammatory cytokines by western blotting and ELISA assays, respectively, in immortalized human keratinocyte cell line, HaCaT. Furthermore, we investigated the effect of ED on a major inflammation-related factor, nuclear transcription factor- κ B (NF- κ B), since it controls many genes involved in the inflammation pathway. Our results indicated that ED reduced the expression of TNF- α , IL-1 β and IL-6. It also inhibited the nuclear transition of p65 NF- κ B, which is known to regulate inflammatory cytokine expression in keratinocytes suffering from 5-FU-induced dermatitis. In addition, ED reduced the production of TNF- α , IL-1 β and IL-6 in HaCaT cells. Moreover, ED attenuated 5-FU-induced transcriptional activation of NF- κ B. These findings revealed that ED suppresses the expression of pro-inflammatory cytokines by suppressing NF- κ B in keratinocytes, suggesting the potential usefulness of ED in the treatment of various inflammatory diseases of the dermal region.

Introduction

Dermatitis and oral mucositis are the frequent side-effects of chemotherapy and radiotherapy treatment in cancer patients including head and neck cancer. Dermatitis appears as the onset of erythema, swelling, an acneiform rash, severe pruritus, xerosis cutis, blisters and ulceration in cancer patients, which can lead to chronic inflammation, necrosis, fibrosis, hair and nail alterations, stomatitis and lymphedema (1-3). Mucositis causes acute oral pain and difficulty with swallowing, which can result in reduced nutrient intake, significant malnutrition and weight loss, as well as poor oral hygiene in head and neck cancer patients (1,4). Dermatitis as well as mucositis can cause interruption, sometimes even termination of cancer treatments, which adversely affects the prognosis and eventually reduces the survival rates of the patients (1,5,6). There could be multiple factors involved in triggering dermatitis and mucositis, although the detailed mechanism of chemotherapy and/or radiotherapy induced dermatitis and mucositis in cancer patients is still unclear.

Chemotherapeutic agents including 5-fluorouracil (5-FU) may harm rapidly dividing immature keratinocytes, as well as dividing stem cells (7-9). Moreover, the basal cell layer of epithelium can be directly damaged leading to the loss of the renewal capacity of the epithelium, which may result in ulceration. It has been reported that the secretions of pro-inflammatory cytokines by epidermal keratinocytes play a key role in various types of inflammations in the skin (10). Among these pro-inflammatory cytokines, TNF- α has been implicated in the promotion of inflammatory reactions via the activation of cytokines IL-6 and IL-1 β (10,11). Nuclear transcription factor- κ B (NF- κ B) is the most important transcriptional regulator of inflammatory pathways, and it directly controls the cellular expression of these pro-inflammatory cytokines (10,12). Activation of NF- κ B induces the transcription of many inflammation-related genes including the 'classic cachectic cytokines' TNF- α , IL-6 and IL-1 β (10,11,13). Therefore, inhibiting or limiting the production of these pro-inflammatory cytokines may help in the treatment of inflammation and dermatitis in cancer patients. Although various types of therapies have been introduced for the prevention and treatment of chemotherapy-induced mucositis and dermatitis, the efficacy of these treatments remains limited (8,15-21).

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Elental® (EA Pharma Co., Ltd., Tokyo, Japan), an elemental diet (ED) with L-glutamine which has an easily digestible nutrition formula that combines amino acids, carbohydrates, vitamins, minerals and with minimal fat content has been used in Japan as a treatment for malnourished patients (22,23). Elental® has been reported to be useful in the treatment of Crohn's disease (24-27), as well as in the management of chemotherapy-induced mucositis in cancer patients (28,29). We have been using Elental® for the treatment of malnutrition in patients undergoing chemotherapy and/or radiotherapy in our hospital since 2011, and our clinical study revealed the efficacy of Elental® in ameliorating chemotherapy-induced oral mucositis and dermatitis in head and neck cancer patients (30). Recently, we reported that Elental® may accelerate the recovery from 5-FU induced oral mucositis and dermatitis through the induction of fibroblast growth factor (31). However, the detailed mechanism of its action against inflammations is still unclear.

The aim of this study was to clarify the efficacy of ED (Elental®) in reducing pro-inflammatory cytokine production in keratinocytes *in vivo* and *in vitro*. Additionally, we investigated the effect of Elental® on the inhibition of NF- κ B activation.

Materials and methods

Animals. Ten 4-week-old female athymic nude mice with a CAnN.Cg-Foxn1^{nu}/CrJ genetic background were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed in a temperature-controlled room with a 12 h light/dark cycle, under sterile conditions in a pathogen-free environment, and received water and food *ad libitum*. All procedures concerning animal handling and treatment were conducted in accordance with the Guidelines for Animal Experimentation of Yamaguchi University.

Induction of experimental dermatitis. Dermatitis was induced in all nude mice by 2 intraperitoneal (i.p.) administrations of 60 mg/kg 5-FU (Wako, Osaka, Japan) on the first and third day of the experiment accompanied by superficial scratching on the dorsal skin with a metal brush on the second and third day of the experiment under anesthesia (pentobarbital sodium, 30 mg/kg, i.p.; Somunopentyl®; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) (Fig. 1). The metal brush was dragged 3 times in a linear fashion across the dorsum skin of nude mice until erythematous changes of the skin were noted.

In vivo experimental groups. Fig. 1 shows the experimental design of our *in vivo* study. We set up the following two groups of nude mice (5 mice/group) with dermatitis induced by 5-FU + abrasion: The 5-FU + abrasion group was the untreated control in this experiment, and they received saline (1 ml/body/day) only, whereas the Elental® group received Elental® (18 kcal/100 g body weight/day), which was orally administered daily until the wounded area was totally healed at the end of the experiment. The healing process of the dermatitis of each mouse was examined every day and the affected area was assessed. Each lesion was calculated by multiplying the major axis by the minor axis. We purchased Elental® from EA Pharma Co., Ltd. Tissue samples of the wounded area were collected after the mice were sacrificed on the 12th day of the experiment.

Immunohistochemical staining. Tissue samples obtained from inflammatory lesions were examined by immunohistochemical analyses using the universal EnVision™ kit (Dako, Glostrup, Denmark). Paraffin-embedded 4 μ m-thick tissue sections were immersed in xylene, and then in graded alcohols (100-70%). Endogenous peroxidase activity was quenched with a 0.3% hydrogen peroxide/methanol mixture for 20 min. Then, the sections were rinsed in PBS and incubated with 2% blocking serum for 30 min, followed by incubation with the anti-TNF- α -rabbit polyclonal antibody (1:200; no. ab6671; Abcam, Cambridge, UK), the anti-IL-1 β rabbit polyclonal antibody (1:200; no. ab9787; Abcam), the anti-IL-6 mouse monoclonal antibody (1:50; no. ab9324; Abcam), the anti-NF- κ B p65 rabbit polyclonal antibody (1:200; no. sc-109; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or the anti- α -tubulin mouse monoclonal antibody (1:200; no. sc-5286; Santa Cruz Biotechnology, Inc) for 8 h at 4°C. After rinsing the tissue sections in phosphate-buffered saline (PBS) for 10 min, the antibody was detected using the universal EnVision™ kit according to the manufacturer's instructions. Tissues were finally rinsed in tap water, and then counterstained with hematoxylin for 1-2 min. The tissue sections were subsequently dehydrated in graded ethanol, cleared in Histo-Clear® (National Diagnostics, Atlanta, GA, USA), and mounted with glass coverslips using DPX. At least 1,000 cells were counted under a microscope in several random fields of each section. The number of positive cells was divided by the total number of counted cells and each labeling index was expressed as a percentage.

Cell lines and cell culture. Immortalized human keratinocyte cell line, HaCaT was purchased from RIKEN BioResource Center Cell Bank (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM)/Ham's F-12 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μ g/ml streptomycin/100 U/ml penicillin (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell culture with Elental® for western blotting and enzyme-linked immunosorbent assay (ELISA). HaCaT cells (2x10⁶ cells) were cultured in 100 mm plates (BD Biosciences, Franklin Lakes, NJ, USA) with D-MEM/Ham's F-12 medium containing 10% FBS for 48 h. Then, the cells were cultured in D-MEM/Ham's F-12 medium but without FBS and 5-FU (2 μ g/ml) for 24 h to induce cellular injury and apoptosis. Subsequently, the cells were cultured with Elental® (0, 0.1, 0.5, 1, 5, 10, 50 and 100 μ g/ml) dissolved in D-MEM/Ham's F-12 medium without FBS. After 12 h, the cell medium was collected for ELISA, and the cells were collected by scraping for western blotting. We used this culture method for the preparation of samples for western blotting and ELISA.

Western blotting. HaCaT cells were cultured as aforementioned, and then cells were lysed with RIPA Buffer (Thermo Fisher Scientific, Inc.) to extract cell proteins. These whole cell lysates were used as samples for the detection of TNF- α , IL-1 β , IL-6 and α -tubulin expression. For the detection of the expression of nuclear and cytoplasmic NF- κ B (p65), we extracted and

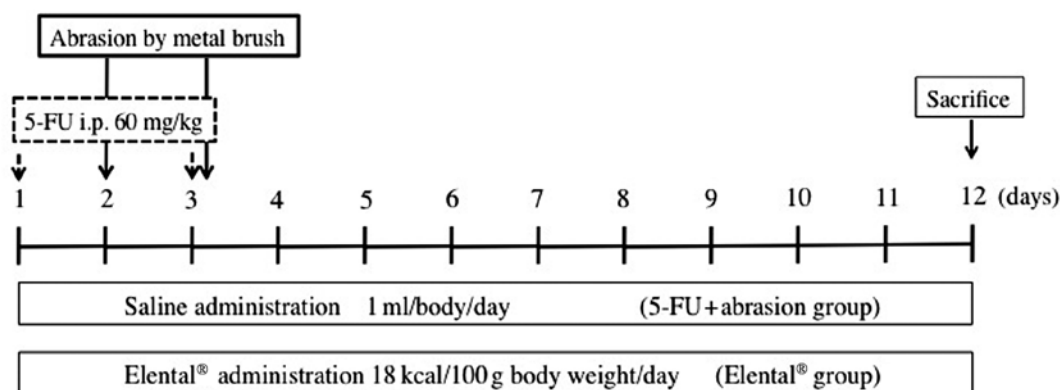


Figure 1. Preparation of experimental dermatitis and Elental® treatment protocol. Dermatitis on nude mice dorsal skin was induced by the intraperitoneal injection of 5-FU (60 mg/kg, i.p.) on day 1 and 3 of the experiment followed by mechanical trauma on days 2 and 3. There were two experimental groups: The 5-FU + abrasion group (received saline only, 1 ml/body/day; n=5) and the Elental® group received Elental® (18 kcal/100 g body weight/day; n=5) daily until sacrifice.

separated cytoplasmic and nuclear protein fractions from cells using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Protein samples containing 50 µg of protein were subjected to electrophoresis on NuPAGE® Novex® Bis-Tris precast gels (Thermo Fisher Scientific, Inc.), and then transferred to a polyvinylidene difluoride (PVDF) membrane using iBlot™ PVDF Transfer Stack and iBlot™ Dry Blotting System (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After blocking the membrane with a blocking solution prepared from WesternBreeze™ Blocker/Diluent Part A and B (Thermo Fisher Scientific, Inc.), the membranes were incubated with the anti-TNF-α rabbit polyclonal antibody (1:500; no. ab6671; Abcam), anti-IL-1β rabbit polyclonal antibody (1:500; no. ab9787; Abcam), anti-IL-6 mouse monoclonal antibody (1:250; no. ab9324; Abcam), anti-NF-κB p65 rabbit polyclonal antibody (1:500; no. sc-109; Santa Cruz Biotechnology, Inc.), or anti-α-tubulin monoclonal antibody (1:500; no. sc-5286; Santa Cruz Biotechnology, Inc.) overnight; followed by Novex® alkaline-phosphatase conjugated goat anti-rabbit (cat. no. WP2007; Thermo Fisher Scientific, Inc.) or (goat) anti-mouse immunoglobulin G (IgG) secondary antibody (cat. no. WP20006; Thermo Fisher Scientific, Inc.). The antibodies were detected using a Novex™ AP Chromogenic Substrate (BCIP/NBT) (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

ELISA for quantitative determination of TNF-α, IL-1β or IL-6. HaCaT cells were cultured as aforementioned, and then we assessed TNF-α, IL-1β and IL-6 proteins that were released into cultured medium from the Elental®-treated or untreated control cells. These proteins were assessed using a microtiter-based sandwich enzyme immunoassay system. We used commercially available ELISA kits (Abcam) according to the manufacturer's protocol and estimated the total amount of TNF-α, IL-1β and IL-6 in the culture medium. Each sample was examined in triplicate.

Immunocytochemical staining. Cells (2.0×10^5 cells/well) were cultured on cover glasses in 6-well plates (BD Biosciences)

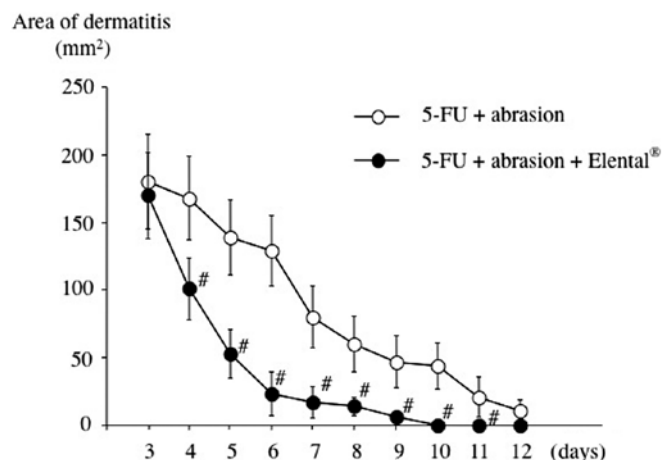


Figure 2. Effect of Elental® on mouse dorsal skin dermatitis. The Elental® group exhibited a significant healing effect on days 4-11 in the dermatitis-affected areas of nude mice compared to the 5-FU + abrasion group, and its affected area healed almost completely on days 9-10 in the Elental® group. Data represent the median values (and range) of macroscopic observations and measurements in 5 animals/group. #P<0.05 compared to the 5-FU + abrasion-treated animals. Data were analyzed by Student's t-tests.

with 10% FBS D-MEM/Ham's F-12 for 48 h. Then, the cells were cultured in D-MEM/Ham's F-12 medium but without FBS and 5-FU (2 µg/ml). After 24 h, the cells were cultured with Elental® (0 and 100 µg/ml) and dissolved in D-MEM/Ham's F-12 medium without FBS. Twelve hours later, the cells were washed with PBS, fixed with 4% paraformaldehyde and incubated for 60 min at 37 °C with anti-NF-κB p65 rabbit polyclonal antibody (1:250; no. sc-109; Santa Cruz Biotechnology, Inc.). After applying the Envision+ System HRP (Dako) for 60 min at room temperature, immunostaining was visualized with diaminobenzidine. The sections were lightly counterstained with hematoxylin.

Statistical analysis. All data are expressed as the mean ± SD. The significance of the experimental results was determined by Student's t-test or Mann-Whitney U test. The differences were considered statistically significant when P<0.05.

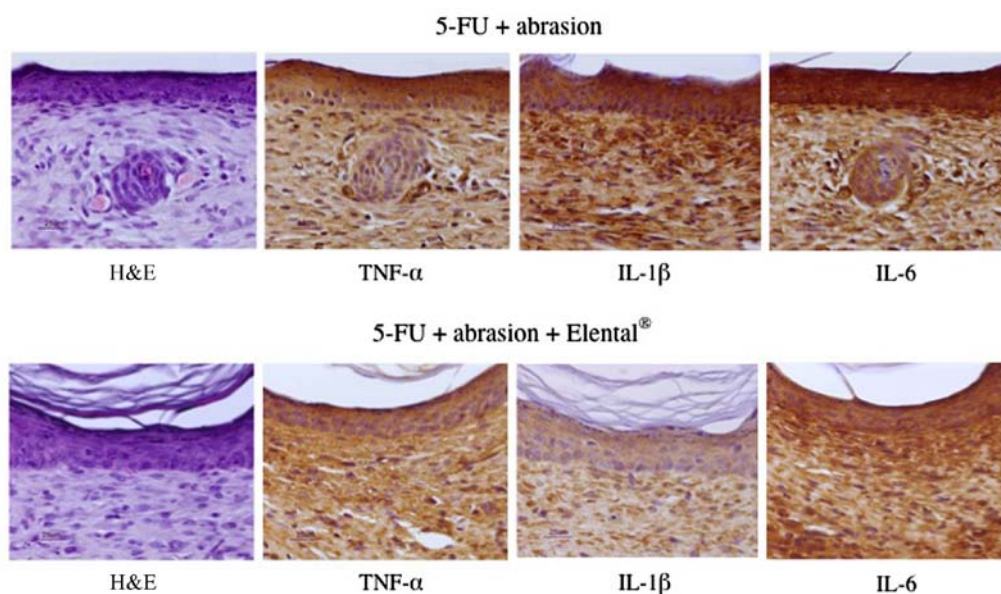


Figure 3. Effect of Elental® on the expression of TNF- α , IL-1 β and IL-6 in mouse dorsal skin dermatitis. Wounded nude mice dorsal skin was collected after sacrificing the mice on the 12th day of the experiment, and the expression of TNF- α , IL-1 β and IL-6 was investigated by immunohistochemistry. High expression of TNF- α , IL-1 β and IL-6 was observed in the 5-FU + abrasion-induced dermatitis regions. Elental® administration reduced the expression of TNF- α , IL-1 β and IL-6 in the dermatitis-affected regions.

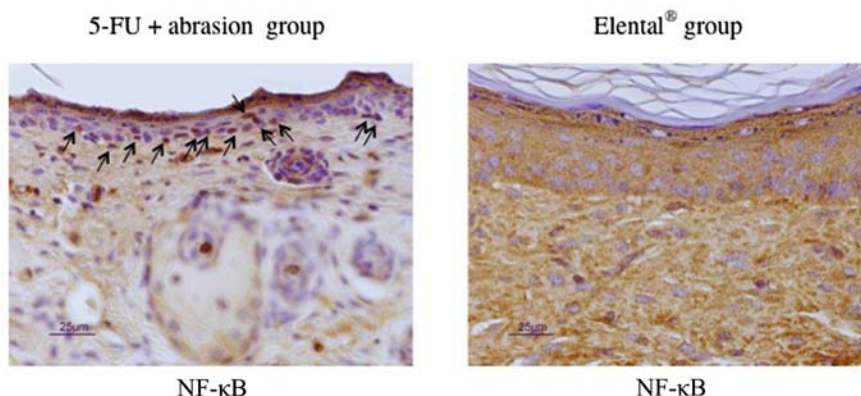


Figure 4. Effect of Elental® on the expression of p65 in mouse dorsal skin dermatitis. After sacrifice, the expression of p65 on nude mice dorsal skin was investigated by immunohistochemistry. The expression of p65 was detected in the nucleus of the 5-FU + abrasion group (arrows); however, it was mainly detected in the cytoplasm of the Elental® group.

Results

Effect of Elental® on mouse dorsal skin dermatitis. To induce dermatitis on the dorsal skin of mice, 5-FU administration and mechanical trauma were used. Ulcerated skin tissue was observed after the second mechanical irritation (on day 3). As shown in Fig. 2, the Elental® group exhibited a better healing rate than the untreated control (5-FU + abrasion group). The affected dorsal area was completely healed on days 9-10 in the Elental® group, however it was healed on day 12 in the case of the 5-FU + abrasion group.

Effect of Elental® on the expression of TNF- α , IL-1 β and IL-6 in mouse dorsal skin dermatitis. We then focused on pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6 as the dermatitis-induced factors in this study. Reduced expression of TNF- α , IL-1 β and IL-6 was detected in the dorsal skin

tissue of the Elental® group compared to the 5-FU + abrasion group (Fig. 3). Particularly, the expression of IL-1 β expression was greatly reduced in the Elental® group compared to the untreated control. This data indicated that Elental® may reduce the expression of TNF- α , IL-1 β and IL-6 in areas affected with dermatitis.

Effect of Elental® on the expression of p65 in mouse dorsal skin dermatitis. Next, we tried to examine the expression of transcription factor NF- κ B in the dermatitis area of mouse dorsal skin by immunohistochemical staining, as NF- κ B plays an important role in the regulation of TNF- α , IL-1 β and IL-6. The expression of p65 was mainly evident in the nucleus of the 5-FU + abrasion group, but was mostly detected in the cytoplasm of the Elental® group (Fig. 4). From this data we can assume that, Elental® may attenuate 5-FU plus abrasion-induced transcriptional activation of NF- κ B.

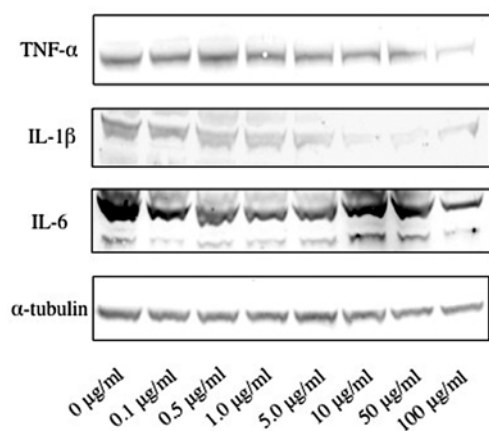


Figure 5. Expression of TNF- α , IL-1 β and IL-6 in Elental[®]-treated cells. The expression of TNF- α , IL-1 β and IL-6 in whole cell lysates was examined by western blotting. Elental[®] suppressed the expression of TNF- α , IL-1 β and IL-6 in cells compared to the untreated cells.

Expression of TNF- α , IL-1 β and IL-6 in Elental[®]-treated cells.

To clarify the mechanism involved in the healing accelerating effect of Elental[®] for dermatitis, we examined the expression of TNF- α , IL-1 β and IL-6 in cells by western blotting. Fig. 5 revealed that Elental[®] (1.0-100 μ g/ml) suppressed the expression of TNF- α in cells compared to the untreated cells, and 100 μ g/ml Elental[®] exhibited the best result. Moreover, Elental[®] (0.1-100 μ g/ml) dose dependently suppressed the expression of IL-1 β , and moderately suppressed IL-6 expression in treated cells compared to the untreated cells. IL-1 β expression was lowest in the 10 and 50 μ g/ml Elental[®] concentrations, however, these doses slightly increased the expression of IL-6. Additionally, IL-6 expression was the lowest in the 100 μ g/ml Elental[®]-treated cells compared to the untreated cells (Fig. 5).

We also assessed the amount of TNF- α , IL-1 β and IL-6 secreted into the culture medium by ELISA. As shown in Fig. 6, the amount of TNF- α , IL-1 β and IL-6 secreted from Elental[®]-treated HaCaT cells was significantly lower than that from untreated HaCaT cells. The lowest expression of TNF- α

was detected with the 100 μ g/ml Elental[®] treatment. In addition, all Elental[®] concentrations decreased the expression of IL-1 β almost at the same rate except for the 5.0 μ g/ml Elental[®] treatment. Moreover, IL-6 expression was lowest with the 50 μ g/ml Elental[®] treatment.

Effect of Elental[®] on the expression of p65 in cultured HaCaT cells. We examined the expression of p65 NF- κ B in cells by western blotting and immunocytochemistry. Some Elental[®] concentrations (5.0, 50 and 100 μ g/ml) increased p65 expression in the cytoplasm; however, 1.0-100 μ g/ml Elental[®] decreased p65 expression in nucleus of HaCaT cells (Fig. 7A). In addition, the expression of p65 was detected in both the nucleus and cytoplasm of 5-FU-treated HaCaT cells. However, p65 expression was mainly detected in the cytoplasm of Elental[®]-treated HaCaT cells (Fig. 7B). This data supports our hypothesis that Elental[®] may have an attenuating effect on 5-FU-induced transcriptional activation of NF- κ B.

Discussion

The major adverse effects of chemotherapy and/or radiotherapy including mucositis, dermatitis, dysphagia, xerostomia and hematological toxicities often hamper cancer treatment, as it can compromise the quality of life of patients (2,5,6). These lead to a higher rate of unplanned breaks and delays in cancer treatments, which always result in a poorer outcome (1,13,32). However, effective treatments for chemotherapy-induced mucositis and dermatitis have not been established, yet (33-37).

Elental[®] is a good source of nitrogen and amino acids but low in fat. It has an easily digestible nutrition formula that rarely requires a fully functional digestive system (22). This ED is inexpensive, safe and has been approved and covered by public insurance as a prescription treatment for malnutrition in Japan. Elental[®] has been reported to be effective in reducing the severity of chemotherapy-induced mucositis and dermatitis in colorectal cancer and esophageal cancer patients, as well as in acute Crohn's disease (19-22). Elental[®] contains

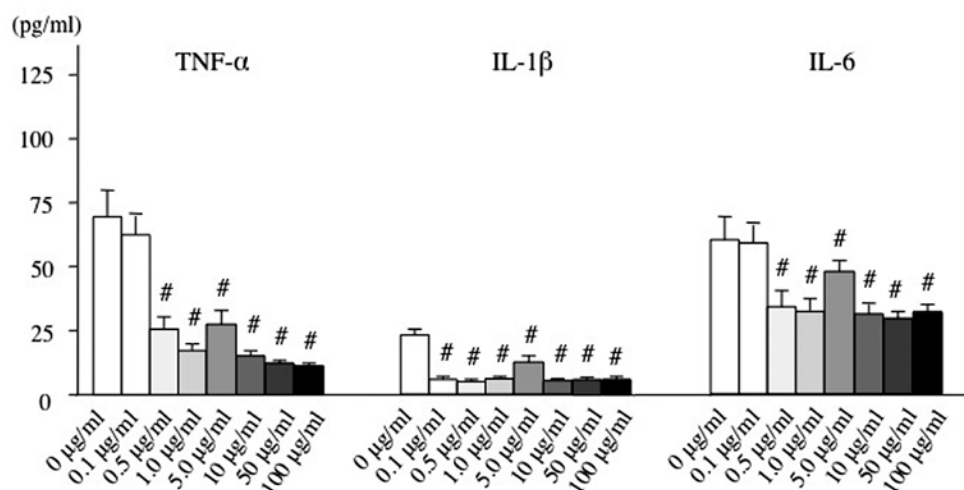


Figure 6. Expression of TNF- α , IL-1 β and IL-6 in Elental[®]-treated cells. The amount of TNF- α , IL-1 β and IL-6 secreted into the culture medium was examined by ELISA. The amount of TNF- α , IL-1 β and IL-6 secreted from the Elental[®]-treated HaCaT cells was significantly lower than that from the untreated HaCaT cells. #P<0.05 compared to the untreated HaCaT cells (Elental[®] 0 μ /ml). Data were analyzed by Mann-Whitney U test.

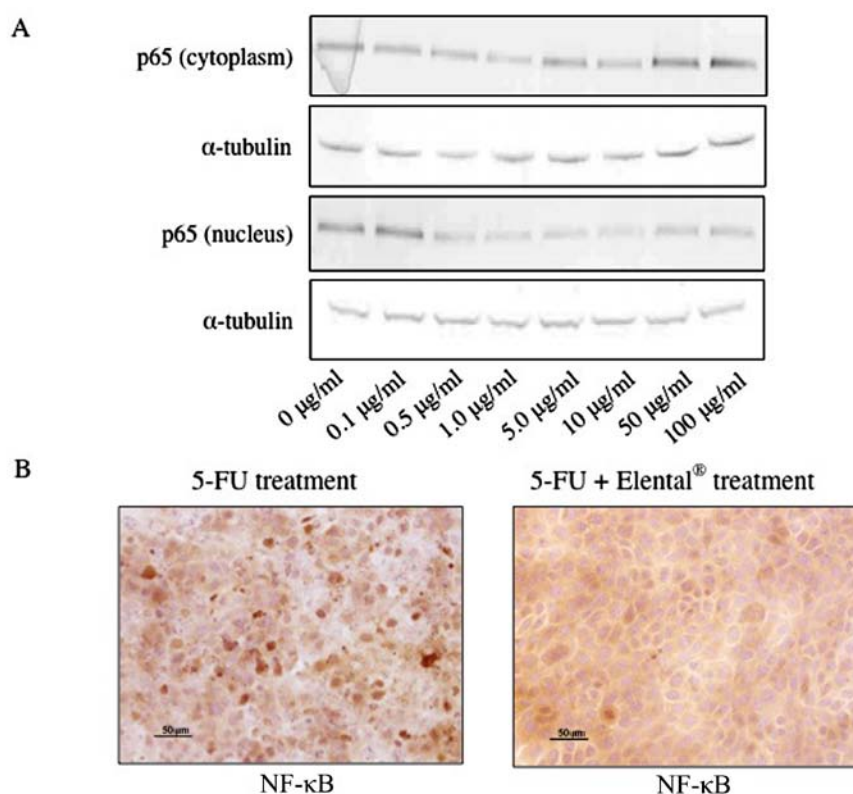


Figure 7. Effect of Elental® on the expression of p65 in cultured HaCaT cells. (A) The expression of p65 in the nuclear and cytoplasmic protein fractions was examined by western blotting. Elental® treatment increased p65 expression in the cytoplasm in most cases, and decreased it in the nucleus of HaCaT cells. (B) The expression of p65 in cultured cells was examined by immunocytochemistry. The expression of p65 was detected in both the nucleus and cytoplasm of the 5-FU-treated HaCaT cells, while p65 expression was mainly detected in the cytoplasm of the Elental®-treated HaCaT cells.

L-glutamine (2.4 g/100 g), which helps in the treatment of cellular injuries, chemotherapy-induced cell toxicities and mucositis (22,32-37). Our previous clinical study revealed the effectiveness of Elental® for the treatment of chemotherapy-induced oral mucositis and dermatitis without causing any adverse effects (30). In the present study, we examined the efficacy of Elental® in reducing pro-inflammatory cytokine production in keratinocytes, and tried to elucidate the detailed mechanisms of its action.

Elental® promoted the healing of chemotherapy-induced dermatitis in our animal models as shown in Fig. 2. Yamamoto *et al* reported that Elental® reduced mucosal inflammation in acute Crohn's disease by lowering the mucosal proinflammatory cytokine production (23). Therefore, we focused on pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 as the dermatitis-inducing factors in this study. In addition, we examined NF- κ B p65 which plays an important role as a transcription factor in regulating many of the pro-inflammatory cytokine genes including TNF- α , IL-1 β and IL-6 (13).

In our animal model of dermatitis, we observed that Elental® reduced the expression of TNF- α , IL-1 β and IL-6, and inhibited the nuclear transition of p65 NF- κ B in the keratinocytes of 5-FU-induced dermatitis regions (Figs. 3 and 4). Next, examined whether Elental® reduced the production of pro-inflammatory cytokines *in vitro*. Our data revealed that Elental® had the desired effects on reducing pro-inflammatory cytokine expression in HaCaT cells damaged by 5-FU-pretreatment. Briefly, Elental® decreased the expression

of TNF- α , IL-1 β and IL-6 in HaCaT cells (Fig. 5), and reduced the production of TNF- α , IL-1 β and IL-6 released in the cultured medium of these cells (Fig. 6). Moreover, Elental® may have functioned as an inhibitor of NF- κ B p65 nuclear transition in 5-FU-pretreated HaCaT cells (Fig. 7).

Our findings revealed that Elental® possibly attenuated 5-FU-induced transcriptional activation of NF- κ B, thereby reducing the expression of pro-inflammatory cytokines. Several authors have reported that other agents, such as caffeic acid, saikosaponin A and palmitic acid, can reduce inflammation by inhibiting NF- κ B, TNF- α , IL-1 β and IL-6 (10,11,38), and possibly Elental® works in the same way. However, Elental® could be more beneficial for cancer patients than those agents, since it is an ED and has other nutritional benefits, but no side-effects. Moreover, Elental® has been used clinically for several years in Japan, therefore, it is already available for the treatment of cancer patients suffering from chemotherapy-induced dermatitis and mucositis.

NF- κ B is considered to be a good target for cancer treatment as its activation directly affects cancer cell proliferation, angiogenesis, metastasis, inflammation and apoptosis (12,39), hence down-regulation of NF- κ B activity by Elental® could be overall useful for cancer patients (40). It is also probable that Elental® has regulatory effects on other oncotargets besides NF- κ B, therefore, extensive research and clinical trials are necessary to identify its additional beneficial effects. We suggest that, ED including Elental® may have great potential for wide clinical application.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

KH was involved in the study design, data analysis, and writing of the manuscript. TF carried out all the experiments, collected and evaluated data; also assisted in manuscript writing and revision. YM and KM revised and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures concerning animal handling and treatment were conducted in accordance with the Guidelines for Animal Experimentation of Yamaguchi University.

Consent for publication

Not applicable.

Competing interests

The authors have declared that they have no competing interest.

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