Elemental Diet Accelerates the Recovery From Oral Mucositis and Dermatitis Induced by 5-Fluorouracil Through the Induction of Fibroblast Growth Factor 2

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Abstract

Mucositis and dermatitis induced by anticancer agents are common complications of anticancer therapies. In this study, we evaluated the efficacy of Elental (Ajinomoto Pharmaceutical Ltd, Tokyo, Japan), an elemental diet with glutamine in the treatment of 5-fluorouracil (5-FU)-induced oral mucositis and dermatitis in vivo and tried to clarify the underlying mechanisms of its action. Oral mucositis and dermatitis was induced through a combination of 5-FU treatment and mild abrasion of the cheek pouch in hamsters and the dorsal skin in nude mice respectively. These animals received saline, dextrin or Elental suspension (18 kcal/100 g) by a gastric tube daily until sacrifice. Elental reduced oral mucositis and dermatitis more effectively than dextrin in the animal model. Moreover, growth facilitating effects of Elental on HaCaT cells were examined in vitro. MTT assay, wound healing assay, and migration assay revealed that Elental could enhance the growth, invasion, and migration ability of HaCaT. ELISA and Western blotting showed upregulated FGF2 in Elental-treated HaCaT. These findings suggest that Elental is effective for the treatment of mucositis and dermatitis, and may accelerate mucosal and skin recovery through FGF2 induction and reepithelization.

Introduction

Oral mucositis and dermatitis are common complications of cancer chemotherapy and radiotherapy. Mucositis causes acute oral pain, and can compromise nutritional intake and oral hygiene in head and neck cancer patients.[1] The detailed mechanism of chemotherapy-induced mucositis is still unclear; however, it might be triggered by multiple factors. Chemotherapeutic agents may damage rapidly dividing immature intestinal crypt cells in the gut, as well as more superficial immature mucosal cells in the oropharynx, oral cavity, and skin.[2-6] In addition, anticancer agent may harm dividing stem cells.[3] It was previously reported that chemotherapy can damage the basal epithelial cell layer directly, which causes the loss of the renewal capacity of the epithelium, with subsequent clonogenic cell death, atrophy, and ulceration. However, recent investigations involving morphologic findings, pro-inflammatory cytokines, platelet aggregation, endothelial and connective tissue injury, and tissue apoptosis have suggested that mucositis is not exclusively an epithelial process but involves all the tissues of the mucosa.[2] Moreover, in the case of gut-related toxicity of chemotherapy and radiotherapy, the phenomenon of bacterial translocation across a malfunctioning gut epithelium may play a role.[2,7,8] Although numerous types of therapy have been introduced for preventing or decreasing chemotherapy-induced mucositis, the efficacy of these treatments remains limited,[9-16] which is also true in case of chemotherapy-induced dermatitis.[6]

Elental (Ajinomoto Pharmaceutical Ltd, Tokyo, Japan), an elemental diet with L-glutamine has been used in Japan for decades as a treatment of malnutrition in patients, which has

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an easily digestible nutrition formula that combines amino acids, carbohydrates, vitamins, minerals and minimal fat.[17,18] Animal studies have shown that supplementation of an elemental diet with glutamine may protect the gut from chemotherapeutic agents and radiation.[3,6] Several authors have reported the benefits of Elental against Crohn's disease,[18-22] and chemotherapy-induced mucositis and stomatitis in cancer patients.[23,24] We have used elemental diet with glutamine (Elental) for improving malnutrition of patients undergoing chemotherapy in these years, and our clinical study revealed the efficacy of Elental for ameliorating chemotherapy-induced oral mucositis and dermatitis in head and neck cancer patients.[25] In this study, we have used animal models to investigate the efficacy of Elental against chemotherapy-induced mucositis and dermatitis in vivo. Moreover, we used Elental in cell cultures to check its growth facilitating effects in vitro and to identify the mechanism of its healing action.

Materials and Methods

Animals

Thirty-six male Syrian hamsters were purchased from Japan SLC, Inc (Hamamatsu, Japan) at 4 weeks age. Fifteen female athymic nude mice with CAnN.Cg-Foxn1nu/CrlCrlj genetic background (CLEA Japan, Inc, Tokyo, Japan) were also purchased at 4 weeks age. They were housed in temperature-controlled rooms and received water and food ad libitum. Surgical procedures and animal treatments were conducted in accordance with the Guidelines for Animal Experimentation of Yamaguchi University.

Induction of Experimental Oral Mucositis and Dermatitis

Oral mucositis was induced in hamsters by 2 intraperitoneal (i.p.) administrations of 5-FU (Wako, Osaka, Japan) on the first and third days of the experiment (60 mg/kg and 60 mg/kg, respectively) and by superficial scratching on the cheek pouch with a metal brush on the second and third day under anesthesia (pentobarbital, 30 mg/kg, i.p.) according to an experimental oral mucositis model.[13-15] In the same way, dermatitis was induced in nude mice by administrations of 5-FU (60 mg/kg, i.p.) twice, on the first and third days of experiment and by superficial scratching on the dorsal skin in nude mice with a metal brush on the second and third days under anesthesia (pentobarbital, 30 mg/kg, i.p.). The metal brush was dragged 3 times in linear fashion across the cheek pouch of hamsters or the dorsum skin of nude mice until erythematous changes were noted.

In Vivo Experimental Groups

Figure 1A shows the experimental design of our in vivo study. We set up the following 3 groups of hamsters and

nude mice, with 12 hamsters or 5 nude mice per group. The 5-FU + abrasion group received saline (1 mL/body/day); the dextrin group received dextrin (18 kcal/100 g body weight/day, Wako); and the Elental group received Elental (18 kcal/100 g body weight/day), which were orally administered daily until sacrifice and 1 hour after the injection of 5-FU on the first and third day of the experiment.

The hamsters were sacrificed on the fifth, sixth, seventh, and eighth days under anesthesia (pentobarbital, 300 mg/kg, i.p.) and then the cheek pouches were removed for the measurement of mucositis area. In case of nude mice, dermatitis area was observed and measured every day. Each lesion was calculated by multiplying the major axis by the minor axis.

Cell Lines and Cell Culture

The immortalized human keratinocyte cell line HaCaT was purchased from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 µg/mL streptomycin/100 U/mL penicillin (Invitrogen) in a humidified atmosphere containing 5% CO_2.

Cell Proliferation Assay

Cells (5×10^3 cells per well) were seeded on 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/Ham's F-12 medium with 10% FBS. Twenty-four hours later, we changed the medium with DMEM/Ham's F-12 with 10% FBS, or D-MEM/Ham's F-12 with 10% FBS plus 5-fluorouracil (final concentration 2 µg/mL). After 24 hours, the cells were treated with different concentrations of Elental (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL), which was dissolved in DMEM/Ham's F-12 medium with 10% FBS or without FBS. After 24 hours, 3-(4, 5-dimethyl-thiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 25 µL/well) was added to the 96-well plate and incubated for 4 hours at 37°C. Next, the culture medium was removed and replaced with dimethyl sulfoxide (100 µL/well) to dissolve the crystals and the absorbance was measured with a spectrophotometer (BioRad Laboratories, Hercules, CA, USA) at 490 nm. Growth inhibitory effects were compared among the groups. All assays were run in triplicate.

Wound Healing Assay

Cells (1.5×10^4 cells per well) were seeded into 24-well plate (Becton Dickinson Labware) and were cultured in DMEM/Ham's F-12 with 10% FBS and 1% penicillin/streptomycin until a monolayer of cells were formed. The cell layer was then gently wounded through the central axis of the plate using a 200 µL pipette tip (yellow tip). After
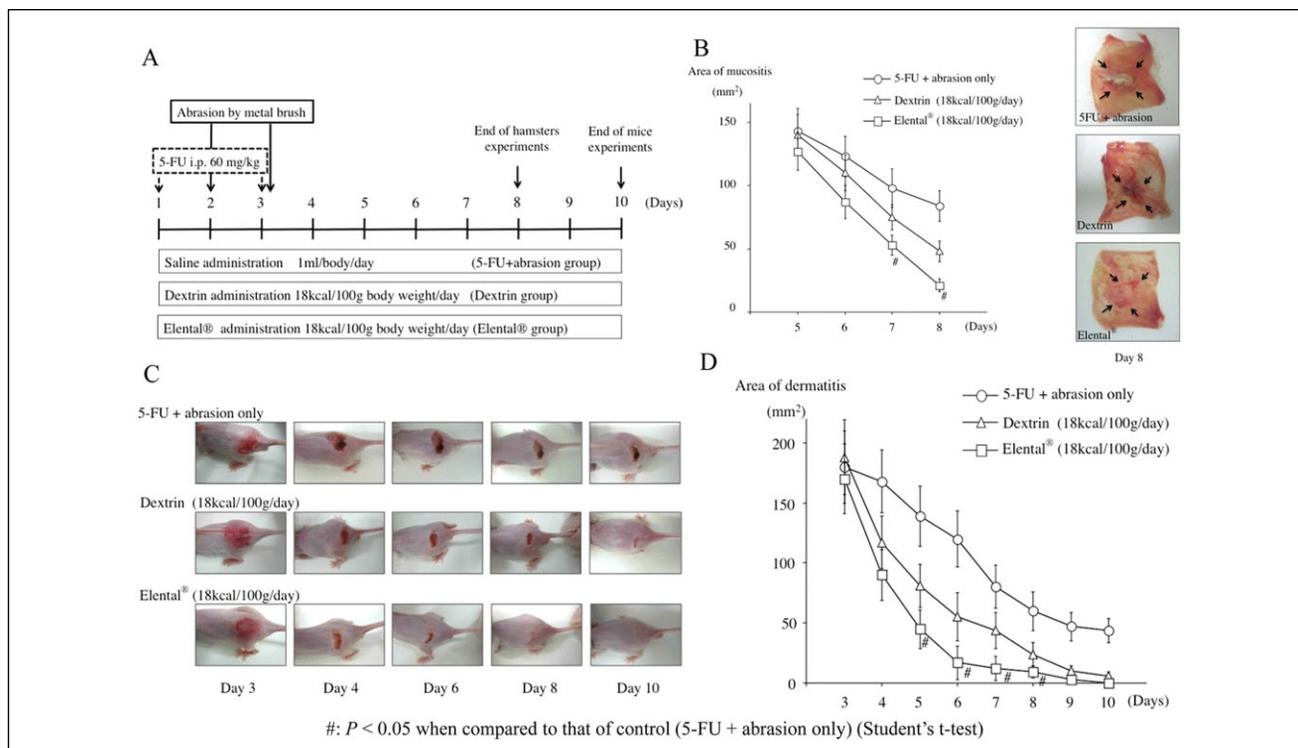
Figure 1. (A) Oral mucositis in hamster cheek pouch and dermatitis on nude mice dorsal skin was induced by the intraperitoneal (i.p.) injection of 5-FU (60 mg/kg, i.p.) on day 1 and day 3 of the experiment followed by mechanical trauma. There were 3 experimental groups; 5-FU + abrasion group (received saline 1 mL/body/day, n = 12), dextrin group (received dextrin 18 kcal/100 g body weight/day, n = 12), and Elental group (received Elental 18 kcal/100 g body weight/day, n = 12) daily until sacrifice. (B) Elental group showed significant healing at day 7 and day 8 of experiment compared to other groups, oral mucositis area is indicated by arrows. Data represent the median values (and range) of macroscopic observation and measurement in at least 3 animals per group. [#]$P < .05$ compared with dextrin-treated animals. Data were analyzed by Student t tests. (C, D) Elental-treated groups showed significant healing effect at days 6 to 8 in dermatitis area of nude mice. Data represent the median values (and range) of macroscopic observation and measurement in 5 animals per group. [#]$P < .05$ compared with dextrin-treated animals. Data were analyzed by Student t tests.

scratching, the cells were treated with different concentrations of Elental (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL) which was dissolved in DMEM/Ham's F-12 medium without FBS. The migration of cells into the wound was observed at 24 hours by microscope (BX-51-33-FLD2, Olympus, PA, USA).

Cell Migration Assay

Cell migration assay was performed using a Boyden chamber according to the manufacturer's instructions (Neuro Probe, Gaithersburg, MD, USA). Briefly, 25 µL DMEM/Ham's F-12 without FBS plus different concentrations of Elental (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL) was added as chemoattractant in the lower chamber. Next, 5×10^3 cells in 50 µL DMEM/Ham's F-12 medium without FBS were seeded on a gelatin-coated polycarbonate membrane in the upper chamber. After the cells were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere, the polycarbonate membrane was washed with phosphate buffered saline, and cells

on the top surface of the polycarbonate membrane were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with hematoxylin solution and counted under a microscope in 5 predetermined fields (200×). All assays were independently repeated at least three times.

Western Blotting

Cells (2.0×10^6 cells in 100 mm dish) were treated with different concentrations of Elental (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL), which was dissolved in DMEM/Ham's F-12 medium without FBS. The cells were lysed with RIPA Buffer (Thermo Fisher Scientific). Whole cell lysates were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels (Thermo Fisher Scientific), and then transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific). After blocking, the membranes were incubated with the anti-fibroblast growth factor 2 (FGF2) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) or
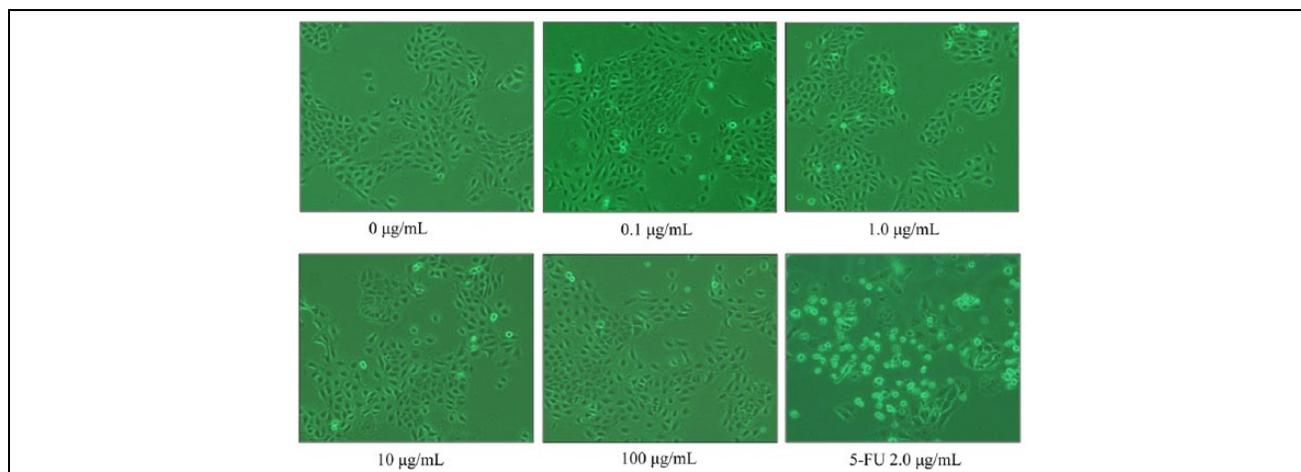
Figure 2. Untreated HaCaT and Elental-treated HaCaT cells had similar cobblestone morphology. Elental did not disturb the growth of HaCaT cells, whereas 5-FU treatment induced apoptosis in HaCaT cells. Floating cells are apoptotic cells.

anti-α-tubulin monoclonal antibody (Santa Cruz Biotech) followed by Novex alkaline-phosphatase conjugated (goat) anti-rabbit or (goat) anti-mouse immunoglobulin G (IgG) secondary antibody (Thermo Fisher Scientific). The antibodies were detected using a chromogenic immunodetection system, WesternBreeze (Thermo Fisher Scientific) according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay for Quantitative Determination of FGF2

FGF2 contained in cultured medium without FBS either from untreated control or from Elental-treated cells was measured by a microtiter-based sandwich enzyme immunoassay system, which is commercially available and specifically estimates the total amount of FGF2. According to the protocol of the enzyme-linked immunosorbent assay (ELISA) kit, cultured medium was subjected to the ELISA using immunoassay kits for FGF2 (R&D Systems, Inc, Minneapolis, MN, USA).

Statistical Analysis

All data were expressed as mean ± SD. The significance of the experiment results was determined by Student t test. The differences were considered statistically significant when $P < .05$.

Results

Effect of Elental on Oral Mucositis of Hamster Cheek pouch

THE combination of 5-FU administration followed by mechanical trauma to the oral mucosal tissue resulted in oral mucositis on the cheek pouch of the hamsters. After the second mechanical irritation (on day 2), all hamster groups

showed severely ulcerated mucosal tissue. Figure 1B shows that the healing rate was faster in animals that had been treated with Elental than in the control group or dextrin-treated group. It was interesting to note that the treatment with Elental reduced oral mucositis more than dextrin of the same caloric value in hamster cheek pouch mucositis model.

Effect of Elental on Dermatitis of Mouse Dorsal Skin

The combination of 5-FU administration followed by mechanical trauma to the dorsum skin tissue resulted in dermatitis on the dorsum of the nude mice. Ulcerated skin tissue was observed in all mice after the second mechanical irritation (on day 2). As shown in Figure 1C and D, the Elental group showed better healing rate than the control or dextrin-treated groups. Similar to our observation in the hamster cheek pouch mucositis model, Elental reduced dermatitis more effectively than dextrin of the same caloric value.

Effect of Elental on Human Keratinocyte Cell Morphology and Proliferation

We could not detect any difference between the morphology of the untreated HaCaT and Elental-treated HaCaT cells. As shown in Figure 2, both cells had the same cobblestone morphology and 5-FU treatment induced apoptosis in HaCaT cells. MTT assay was used to measure the growth rate of the Elental-treated and untreated HaCaT cells. Figure 3 shows that, in the good nutrition condition (10% FBS medium), Elental® (5 µg/mL)-treated HaCaT cells had higher proliferative ability than that of untreated HaCaT cells at 24 hours of culture. Also, in the nutritionally-poor condition (0% FBS medium), the growth rate of Elental (0.5-10 µg/mL)-treated HaCaT cells was higher than that of untreated HaCaT cells at 24 hours of culture. Moreover, Elental (0.5-10 µg/mL)
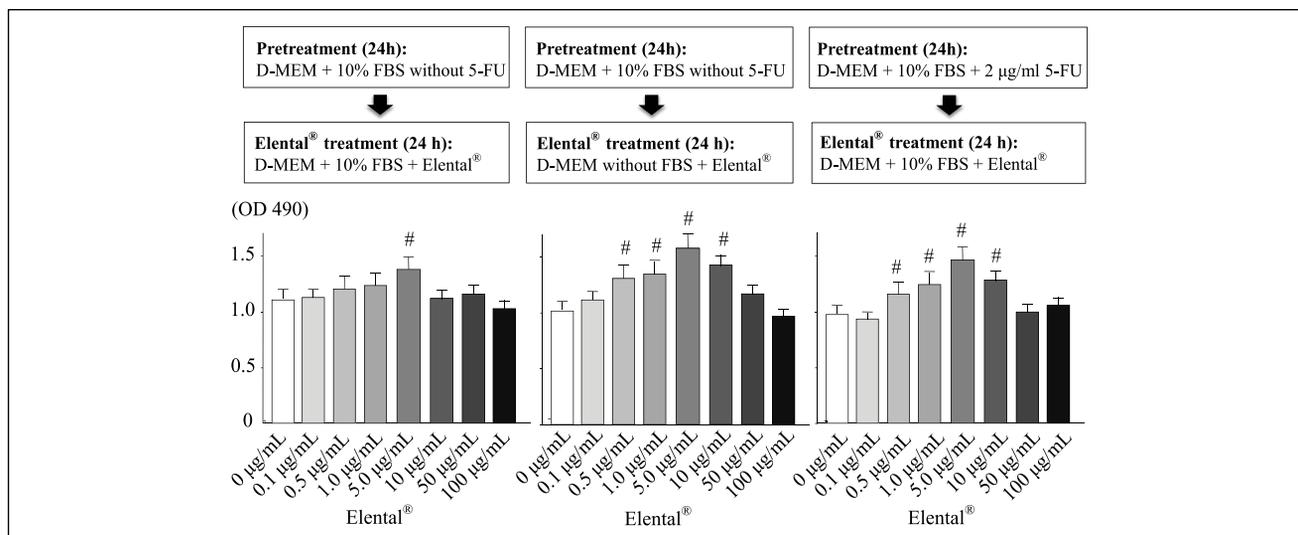
Figure 3. MTT assay was used to measure the growth rate of the Elental-treated HaCaT cells. Elental exerted growth-stimulating effect on cells in poor nutritional condition (Dubecco's modified Eagle medium [DMEM] without fetal bovine serum [FBS]) or on damaged cells (cells pretreated with 5-FU) than on cells in good nutritional condition (DMEM with 10% FBS). #P < .05 when compared with that of control (Student t test).
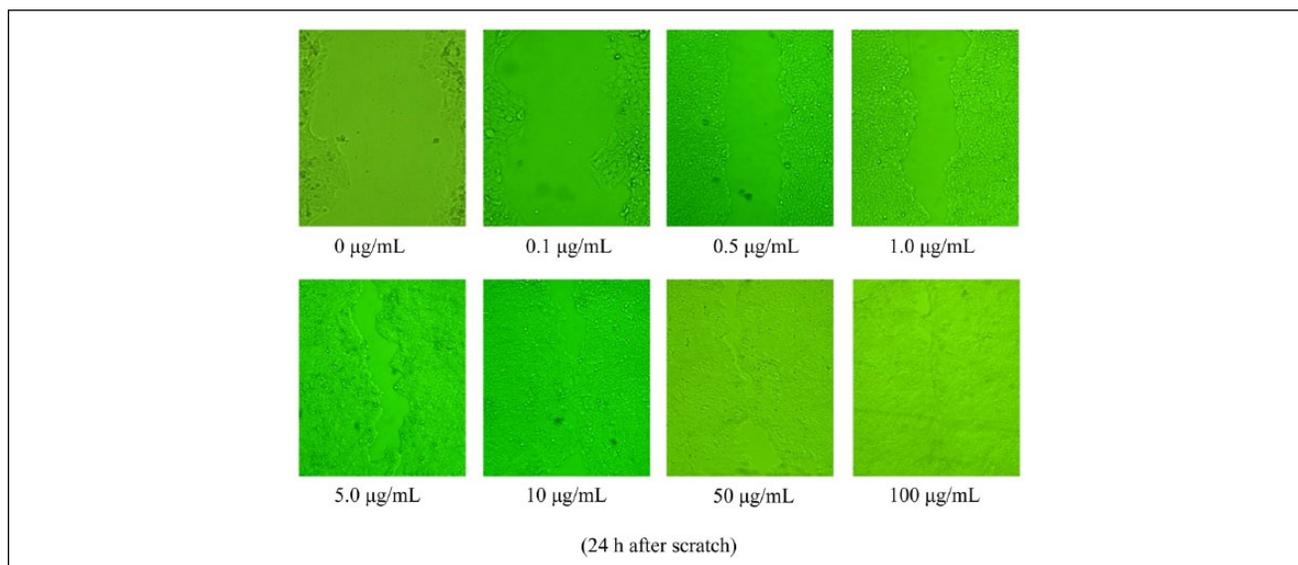

Figure 4. Treatment with Elental promoted the wound healing ability of HaCaT cells does-dependently.

could stimulate the proliferation of 5-FU (2 µg/mL)-pretreated HaCaT cells in substantial condition. Briefly, Elental exerted growth-stimulating effect on all cells, especially on nutritionally-poor cells or damaged cells.

Effect of Elental on Wound Healing Ability

Wound healing assay revealed that Elental-treated HaCaT cells had higher invasive capacity compared with that of untreated HaCaT cells at 24 hours of culture. As shown in Figure 4, Elental® exerted wound healing effects dose-dependently.

However, low concentration of Elental (0.1 µg/mL) could not exert profound effects.

Effect of Elental on Migration Ability

The migration activity of the Elental-treated HaCaT cells was measured with the Boyden chamber. Figure 5 shows that Elental-treated HaCaT cells had significantly higher migration ability than that of untreated HaCaT cells, while 5 µg/mL Elental showed the most noticeable effect on migration than the other concentrations.
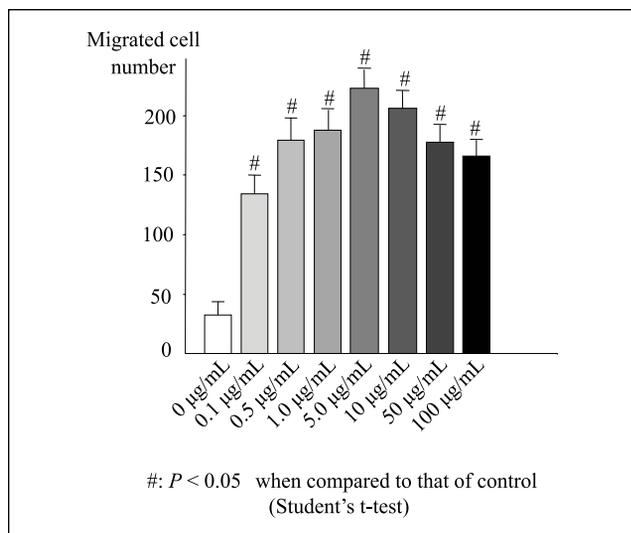
Figure 5. Elental stimulated the migration ability of HaCaT cells. Especially, 5 μg/mL of Elental was most effective in accelerating migration of cells.

Expression of FGF2 in Elental-Treated Cells

To clarify the healing acceleration mechanism of Elental against mucositis and dermatitis, we examined the expression of FGF2 in cells by Western blotting. Figure 6A shows that Elental (0.1-50 μg/mL) enhanced the expression of FGF2 in cells compared with the untreated cells, while 5 μg/mL Elental could strongly increase the expression of FGF2. We measured the amount of FGF2 secreted into the culture medium by ELISA. As shown in Figure 6B, the amount of FGF2 secreted from Elental-treated HaCaT cells was significantly higher than that from untreated HaCaT cells, especially from HaCaT cells treated with 5 to 10 μg/mL Elental.

Discussion

Mucositis, dermatitis, dysphagia, xerostomia, and hematological toxicities are well known as major side effects of chemotherapy, including molecular-targeted agents. Incidence of severe oral mucositis or dermatitis leads to higher unplanned breaks and delays in cancer treatments with radiation or chemotherapy, which is invariably associated with poorer outcome.[26-29] However, effective treatments for radiation- or chemotherapy-induced mucositis and dermatitis have not been established yet.[30-34]

Elental has an easily digestible nutrition formula that combines amino acids, carbohydrates, vitamins, minerals, minimal fat, and L-glutamine, and its safety has been established.[17] It has been approved and covered by public insurance as a prescription treatment indicated for malnutrition in Japan. Elental is inexpensive, costing <US$4.00 per day

and the estimated cost for a 7-week course of Elental is about $112. This elemental diet was reported as useful in the treatment of a number of diseases and in reducing mucosal inflammation in acute Crohn`s disease by lowering the mucosal proinflammatory cytokine production.[18,21,22] Moreover, the effectiveness of Elental in reducing the severity of chemotherapy-induced mucositis and dermatitis was also reported in patients with colorectal cancer and esophageal cancer.[23,24] We previously reported the effectiveness of Elental for the treatment of oral mucositis and dermatitis induced by chemotherapy without any adverse effects related to its clinical use.[25] In this study, we examined the efficacy of Elental against chemotherapy-induced mucositis and dermatitis in vivo and tried to understand the detailed mechanisms of its action in vitro.

Elental had dramatic effects in the recovery of chemotherapy-induced mucositis and dermatitis in our animal models as shown in Figure 1B-D, which was more than our expectations. Interestingly, the treatment with Elental reduced oral mucositis and dermatitis more efficiently than dextrin of the same caloric value in the hamster cheek pouch mucositis model and in the nude mouse dorsum dermatitis model. These findings suggested that Elental might possess actions similar to healing accelerating agents. We therefore investigated the influence of Elental on HaCaT. As Figures 2 and 3 show, Elental did not have any adverse effect on the cells and could exert profound growth-stimulating effect on damaged cells or on cells in nutritionally poor conditions, and mild growth-stimulating effect on cells in good nutritional condition. These data suggest the safety and usefulness of Elental in the treatment of malnutrition. Moreover, the treatment of Elental promoted the wound healing ability and the migration ability of HaCaT cells, as shown in Figures 4 and 5. The above findings imply that Elental might promote the healing of oral stomatitis and dermatitis directly. So, we took particular note of FGF2 because it is reported to play an important role in healing of wounds and skin ulcers.[35] Several studies demonstrated that FGF2 significantly inhibits scar formation after burn injury and can treat wounds that are difficult to cure.[35,36] In fact, the treatment of Elental enhanced the production and secretion of FGF2 as shown in Figure 6A and B. Briefly, Elental may accelerate mucosal and skin recovery through induction of FGF2, and reepithelization.

The human recombinant FGF2 agent, trafermin (Fiblast Spray, Kaken Pharmaceutical, Tokyo, Japan), is thought to be a promising agent for the management of severe oral mucositis and dermatitis with tissue defect.[37] Regardless of its benefit for decreasing oral mucositis and dermatitis, we cannot use the trafermin in cancer patients because trafermin has the potential to promote cancer, as FGF-2 has been found to be involved in cell division, angiogenesis, vascular remodeling, hematopoiesis, and tumor progression.[38,39] Therefore, it is not clear how the FGF2 inducing effect of Elental might affect
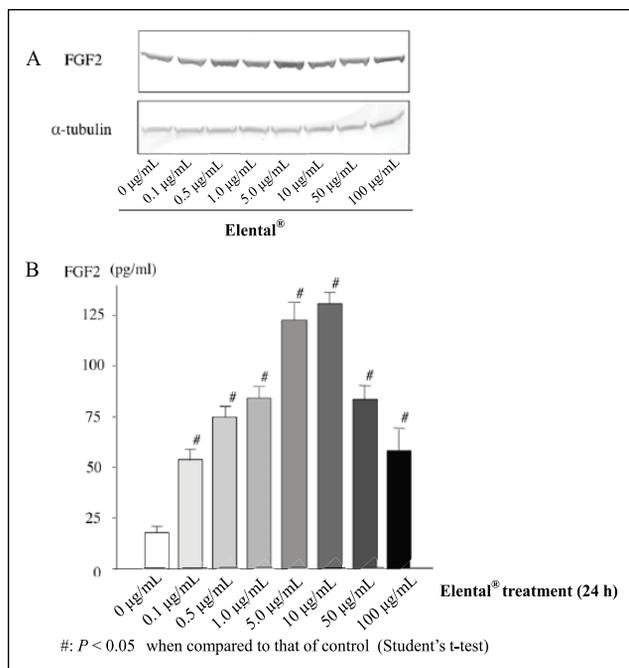
Figure 6. (A) Western blotting revealed that treatment of Elental promoted the production of FGF2 in HaCaT cells, especially 5 μg/mL of Elental increased the expression of FGF2 more profoundly than other concentrations. (B) ELISA was used to measure the amount of FGF2 secreted into the culture medium after Elental treatment. The amount of FGF2 secreted from Elental-treated HaCaT cells was significantly higher than that from untreated HaCaT cells. In particular, a high amount of FGF2 was secreted from HaCaT cells treated with 5 to 10 μg/mL Elental.

the tumors in cancer patients. However, as Elental is not itself a growth factor like trafermin, it might not induce FGF2 as strongly as trafermin. Therefore, we think Elental might be available for the management of severe oral mucositis and dermatitis in cancer patients. There could be other factors that are responsible for the efficacy of Elental against mucositis and dermatitis. Future investigations should aim to identify these unknown factors that would enable us to understand the mechanism of action of Elental more clearly.

Authors' Note

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Declaration of Conflicting Interests

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