Influence of SNN-6010, an elemental diet, on the generation of cytokines, NO, and chemiluminescence from leukocytes and umbilical vein endothelial cells in man

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Aims There is insufficient information on the influence of elemental diet (ED) on leukocytes and endothelial cells although ED has been used increasingly for debilitated and post-operative patients. We here aimed to show the influence of SNN-6010 (Twinline[®]; TL), which contains medium-chain triglycerides, on the generation of cytokines and nitric oxide (NO) and chemiluminescence in human umbilical vein endothelial cells (HUVEC) and leukocytes.

Methods Leukocytes were obtained from 10 healthy volunteers and 10 patients with jaw fractures who underwent TL for 30 days after intermaxillary fixation. Luminol-dependent chemiluminescence and NO generation were measured in polymorphonuclear leukocytes (PMN) and HUVEC and generation of interleukin-1 beta (IL-1 β), tumour necrosis factor-alpha (TNF- α), and prostaglandin E₂ (PGE₂) as well as expression of these cytokines' mRNA were examined in lymphocytes, monocytes, PMN, and HUVEC.

Results TL dose-dependently suppressed generation of IL-1 β , TNF- α , PGE₂, and NO in all kinds of cells from healthy individuals. Lymphocytes from patients who underwent TL feeding for 30 days showed reduced generation of IL-1 β and TNF- α , and the suppressed cytokine generation corresponded with the decreased expression of mRNA of these cytokines. Chemiluminescence by PMN from healthy individuals was suppressed by TL dose-dependently, and PMN from TL-fed patients showed reduced chemiluminescence.

Conclusion These results appear to indicate that TL suppresses the functions of leukocytes and endothelial cells by down-regulating the generation of reactive oxygen intermediates, NO, and cytokines.

Keywords: SNN-6010, cytokines, reactive oxygen intermediates, nitric oxide

Introduction

Elemental diet (ED) has been used increasingly for both post-operative nutritional management and for nutritional treatment of debilitated patients. In addition to improving nutritional status, ED also contributes to maintaining the integrity of cellular immunity [1, 2].

The formation of oxidized lipids, potentially toxic to cells, depends on the composition of the fatty acids in the ED. When the elements in ED are oxidized, they become harmful to the cardio-vascular system, lung, kidney, and many other organs and tissues [3–6]. In addition, if an ED enhances the generation of reactive oxygen intermediates (ROI) from polymorphonuclear leukocytes (PMN), monocytes and macrophages, it may induce tissue damage. Of the ROI, superoxide and hydroxyl radical are the most reactive. Recently, generation of peroxynitrite by reacting superoxide with nitric oxide (NO) has been demonstrated and tissue damage by peroxynitrite has been reported [7–9]. In addition, the marked suppression by ED of NO generation from endothelial cells and other cells may predispose to

tissue ischaemia and hypertension in elderly patients with arteriosclerosis and ischaemic disorders.

The influence of ED on cytokine generation also appears to be important because the immune response is regulated by cytokines [10–14]. We, therefore, examined both *in* and *ex vivo* influences of SNN-6010 (Twinline[®]; TL), an ED containing medium-chain triglycerides, on the generation of ROI, NO and cytokines, and we analyzed the mechanism of action of TL including its effects on endothelial cells.

Methods

Cell preparation

Heparinized peripheral venous blood was obtained from 10 healthy volunteers (eight men and two women, 24 to 46 years old) and 10 patients (eight men and two women, 19 to 56 years old) with mandibular fracture. Blood samples were obtained from the patients before surgical reduction with intermaxillary fixation and on the 8th, 15th and 30th day after the start of administration of TL (Otsuka Pharma. Co., Tokyo, Japan) (Table 1). Administration of TL with a naso-gastric feeding tube was started on the day of operation.

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Table 1 Composition of TL.

Calories (kcal l ⁻¹)	1000	Fe (mmol l ⁻¹)	0.11	Asparagic acid (μmol l ⁻¹)	206.9
Protein (g l ⁻¹)	41.0	Cu (μmol l ⁻¹)	3.6	Glutamic acid (μmol l ⁻¹)	626.8
Fat (g l ⁻¹)	278.0	Zn (μmol l ⁻¹)	145	Glycine (μmol l ⁻¹)	97.1
Vitamin A (I U l ⁻¹)	2070	Mn (μmol l ⁻¹)	29	Proline (μmol l ⁻¹)	270.8
Vitamin D (I U l ⁻¹)	135	Cl (mmol l ⁻¹)	30.2	Serine (μmol l ⁻¹)	208.1
Vitamin E (μmol l ⁻¹)	15.6	Isoleucine (μmol l ⁻¹)	157.5	Caprylic acid (mmol l ⁻¹)	131.4
Vitamin B ₁ (μmol l ⁻¹)	6.0	Leucine (μmol l ⁻¹)	274.8	Capric acid (mmol l ⁻¹)	1.8
Vitamin B ₂ (mmol l ⁻¹)	6.0	Lysine (μmol l ⁻¹)	205.0	Myristic acid (mmol l ⁻¹)	0
Vitamin B ₆ (μmol l ⁻¹)	14.8	Methionine (μmol l ⁻¹)	119.4	Palmitic acid (mmol l ⁻¹)	5.31
Vitamin B ₁₂ (nmol l ⁻¹)	2.4	Cystine (μmol l ⁻¹)	6.7	Stearic acid (mmol l ⁻¹)	3.0
Vitamin C (mmol l ⁻¹)	1.28	Phenylalanine (μmol l ⁻¹)	20.2	Oleic acid (mmol l ⁻¹)	3.6
Pantothenic acid (μmol l ⁻¹)	42.9	Tyrosine (μmol l ⁻¹)	73.8	Linoleic acid (mmol l ⁻¹)	18.9
Biotin (nmol l ⁻¹)	151.5	Threonine (μmol l ⁻¹)	136.0	Linolenic acid (mmol l ⁻¹)	0
Folic acid (nmol l ⁻¹)	567	Tryptophan (μmol l ⁻¹)	27.8	Other fatty acids (mg l ⁻¹)	0
Ca (mmol l ⁻¹)	1.10	Valine (μmol l ⁻¹)	214.3		
P (mmol l ⁻¹)	17.1	Arginine (μmol l ⁻¹)	81.4		
Mg (mmol l ⁻¹)	0.06	Histidine (μmol l ⁻¹)	75.7		
Na (mmol l ⁻¹)	28.8	Alanine (μmol l ⁻¹)	136.3		
K (mmol l ⁻¹)	30.2				

During the period of enteral feeding, nothing else except water was given to the patient. The body weight loss was less than 1.5% of the original weight during the course, and the levels of alimentary parameters such as prealbumin, retinol-binding protein and transferrin showed only slight decrease within the first few days after surgical reduction and soon recovered to the original levels. Lymphocytes and PMN were obtained by centrifugation of heparinized peripheral blood on Ficoll-Paque (Pharmacia Fine Chemical, Piscataway, NJ) gradients at 400 *g* for 30 min. Peripheral blood mononuclear cells (PBMC) were drawn from the top layer, and PMN were collected from just above the red blood cell layer. The obtained cells were resuspended in phosphate buffered saline (PBS) and contaminating erythrocytes were removed by hypotonic treatment. Isolated mononuclear cells and PMN were washed in PBS, and suspended in RPMI1640 (Nissui Pharma. Co., Ltd, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., Gaithersburg, MD) and antibiotics, and in Hanks' balanced salt solution (HBSS), respectively. PBMC were incubated in plastic petri dishes for 2 h, and nonadherent lymphocytes were obtained. More than 95% purity and viability of lymphocytes and PMN were confirmed microscopically by Giemsa staining and trypan blue exclusion test.

Human umbilical vein endothelial cells (HUVEC) were purchased from Morinaga Pharma. Co. (Tokyo, Japan) and cultured in medium containing epithelial growth factor (10 μg ml⁻¹, Morinaga).

Lymphocyte blastogenesis

Peripheral blood lymphocytes (PBL) were obtained from 10 healthy individuals (30 to 40 years old). Lymphocytes (1 × 10⁵ cells) in 0.1 ml of RPMI1640 were seeded into round-bottomed microwells, and 0.1 ml of 1 μg ml⁻¹ phytohemagglutinin-A (PHA, Difco) or 10 μg ml⁻¹ concanavalin A (ConA, Difco) solution was added. Blastogenesis was expressed by incorporated [³H]-thymidine

(New England Nucl, Boston, MA) during the final 6 h of 72 h incubation.

Chemiluminescence

Chemiluminescence was measured with a calcium analyzer (CAF-100, JASCO, Tokyo, Japan). Cells suspended (5 × 10⁵ cells/ml) in HBSS containing 100 μM luminol were incubated for 1 min at 37° C, after which 50 ng ml⁻¹ phorbol myristate acetate (PMA; Sigma, St Louis, Mo.) or 10⁻⁷ M formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma) was added. Activity was expressed as peak chemiluminescence intensity (mV).

Figure 1 Influence of TL on lymphocyte blastogenesis. Human lymphocytes from 10 healthy donors were cultured for 24 h in medium containing TL at the indicated concentrations, and the cells were cultured in medium containing ConA (10 μg ml⁻¹) or PHA (1 μg ml⁻¹) for 72 h. Six h before the end of culture, 1 μCi ml⁻¹ [³H]-thymidine was added, and incorporated isotope was measured. Each bar indicates the mean ± s.d. value from duplicate experiments. *: *P* < 0.05 vs control. □ control (without TL, *n* = 10), ■ TL (0.1%, *n* = 10).

NO (NO_2^-) determination

NO, quantified by accumulation of nitrite (NO_2^-) as a stable end product, was determined by microplate assay [15]. Briefly, 100 μl of the supernatant was incubated with an equal volume of Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined with a microplate reader. Nitrite concentration was calculated from a sodium nitrite standard curve.

Titration of cytokines

Human lymphocytes, monocytes, PMN, and HUVEC were cultured in the presence of the indicated doses of TL for 48 h. Cytokines in the culture supernatants were measured using enzyme amplified sensitivity immunoassay kits for interleukin-1 beta ($\text{IL-1}\beta$), tumour necrosis factor-alpha ($\text{TNF-}\alpha$), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Medgenix Diagnostics, Brussels, Belgium). An enzyme immunoassay kit was used for prostaglandin E_2 (PGE_2) (Amersham, Amersham, UK).

Figure 2 *In vitro* influence of TL on cytokine release from human lymphocytes and monocytes (a) and PMN and HUVEC (b). Lymphocytes, monocytes, PMN, and HUVEC obtained from four healthy individuals were cultured for 48 h in medium containing indicated concentrations of TL ($\square$ 0%, $\square$ 0.01%, $\square$ 0.1%, $\blacksquare$ 1%, $n=4$), and the cytokines released were measured. Each bar indicates the mean $\pm$ s.d. value from triplicate experiments. *: $P<0.05$, †: $P<0.01$, ‡: $P<0.001$, all *vs* control.

Reversed transcription-polymerase chain reaction (RT-PCR)

Total RNA from human peripheral lymphocytes was isolated by standard procedures [16]. The levels of TNF- α - and IL-1 β -mRNA were examined using RT-PCR technique. RT was performed at 42°C for 30 min, and PCR was performed in a Perkin-Elmer thermal cycler Cetus 480 (Veterstetten, Germany). We used a cycle program of 30 s denaturation (94°C), 30 s annealing (60°C), and 90 s amplification (72°C) for 30 cycles with a final 2 min amplification. As a control, cDNA samples were amplified by using β -actin primer. PCR samples electrophoresed on agarose gel were visualized by staining with ethidium bromide. Primers for TNF- α , IL-1 β and iNOS were purchased from Toyobo Inc. (Osaka, Japan). The primer sequences were as follows: TNF- α : sense, 5'GAGTG-ACAAGCCTGTAGCCCATGTTGTAGCA3', antisense, 5'GCAATGATCCCAAAGTAGACCTGCCAGACT3'; IL-1 β : sense, 5'ATGGCAGAAGTACCTAAGCTCGC3', antisense, 5'ACTCAAATTGCATGGTGAAGTCAGTT3'; β -actin: sense, 5'ATCTGGCACCACACCTTCTACAA-TGAGCTGCG3', antisense, 5'CGTCATACTCCTGCT-TGCTGATCCACATCTGC3'.

Statistical analysis

The result were expressed as mean $\pm$ s.d. The mean values were statistically compared using Student's *t*-test. The values presented in the text are also the mean $\pm$ s.d.

Results

Lymphocyte blastogenesis

In vitro TL suppressed [3 H]-thymidine incorporation by human lymphocytes regardless of the stimulators used. Upon stimulation with ConA or PHA, TL-pretreated human lymphocytes incorporated significantly less [3 H]-thymidine than untreated control lymphocytes (Figure 1).

Cytokine generation and cytokine mRNA expression

Cytokines generated from human lymphocytes, monocytes, PMN and HUVEC were suppressed dose-dependently by TL (Figure 2). In lymphocytes and monocytes treated with 0.01% to 1% (v/v) TL for 48 h, the release of TNF- α and IL-1 β was decreased to about half of that in the control cells (Figure 2a). TL dose-dependently suppressed the release of TNF- α and PGE $_2$ from PMN and the release of IL-1 β and GM-CSF from HUVEC (Figure 2b). In addition,

lymphocytes obtained from patients fed TL for 15 and 30 days showed reduced IL-1 β and TNF- α generation compared to those obtained from the patients before TL administration (Table 2). The suppression of cytokine generation was compatible with the suppressed TNF- α - and IL-1 β -mRNA expression in TL-treated lymphocytes (Figure 3). Expression of TNF- α - and IL-1 β -mRNA was strongly suppressed by 0.1% and 1% TL, respectively.

Chemiluminescence

In vitro treatment of human PMN with TL caused suppression of chemiluminescence (Figure 4a). PMA- and FMLP-induced chemiluminescence was decreased by PMN treatment with 0.01% TL, 0.1% TL and 1% TL for 1 h. However, no decrease of chemiluminescence was observed in PMN treated with 0.001% TL (data not shown). PMN obtained from patients who received tube feeding of TL showed time-dependent suppression of chemiluminescence, the values for days 15 and 30 reaching statistical significance (Figure 4b). To identify which components of TL were responsible for the

Figure 3 RT-PCR for cytokine mRNA. Human peripheral lymphocytes were prepared from a healthy individual and treated with 0.1% and 1% TL for 24 h. RNA was then extracted from the cells and blotted with DNA probes for TNF- α and IL-1 β .

Table 2 IL-1 β and TNF- α release by lymphocytes obtained from TL-administered patients.

Time	IL-1 β	TNF- α
Before	131 $\pm$ 20*	615 $\pm$ 43
15 days	102 $\pm$ 24†	568 $\pm$ 34†
30 days	105 $\pm$ 22†	546 $\pm$ 39†

*pg ml $^{-1}$ 10 $^{-6}$ cells, †*P* < 0.05.

Figure 4 *In vitro* (a) and *in vivo* (b) influence of TL on chemiluminescence by human PMN. a) PMN from 10 healthy donors were treated with TL (□ 0%, ▨ 0.01%, ▩ 0.1%, ■ 1%) for 1 h. After being washed, the cells were stimulated with 10^{-7} M FMLP or 50 ng ml^{-1} PMA, and the luminol-dependent chemiluminescence was measured. b) PMN obtained from 10 patients before (□) surgical reduction for mandibular fracture and at 8 (▨), 15 (▩), and 30 (■) days after the operation were stimulated with 10^{-7} M FMLP or 50 ng ml^{-1} PMA. In both a) and b), each bar indicates the mean $\pm$ s.d. of value from duplicate examinations. † $P < 0.001$; * $P < 0.05$.

suppression of ROI generation, we examined the influence on chemiluminescence of the agents included in TL (Figure 5). Fatty acids (0.1% and 1%; v/v) of the same constitution as in TL increased the peak intensity, but ascorbic acid at $12.5 \mu\text{mol l}^{-1}$, the same concentration as in 1% TL, and methionine decreased the peak intensity dose-dependently. However, tryptophan or phenylalanine did not affect the chemiluminescence (data not shown).

NO generation

Suppression of NO generation by TL-treated HUVEC was very slight even in the presence of $1 \mu\text{g ml}^{-1}$ LPS (from *Escherichia coli*, serotype 0127:B81, obtained from Sigma); HUVEC treated with 1% TL for 48 h generated $2.7 \pm 0.2 \mu\text{M NO}_2^-$ and untreated control cells generated $3.5 \pm 0.3 \mu\text{M NO}_2^-$.

Discussion

ROI are closely associated with a variety of biological phenomena such as the killing of microorganisms by phagocytes [17, 18], cell activation via tyrosine phosphorylation of proteins [19], enzyme inactivation [20, 21], and DNA impairment [22]. Suppression of ROI generation allows microbial invasion, especially under distressed condition. Conversely, cell damage may result from excessive ROI and suppressed ROI-scavenging activity.

TL dose-dependently suppressed ROI generation from human PMN. PMA-stimulated chemiluminescence was

significantly decreased by even 0.01% TL. The chemiluminescence-suppressive action of TL was not observed at 8 days after the start of TL administration, although PMN showed reduced chemiluminescence at 15 days and 30 days. In addition, TL at 0.001%, a concentration that appears to be much higher than the intracellular level of TL in TL-administered patients, did not decrease cytokine generation or chemiluminescence (data not shown). These results indicate that the *in vivo* influence of TL on ROI generation is relatively mild. However, the suppressive action of TL may become a risk factor for infection when TL is administered over long periods.

Among the components of TL, fatty acids increased chemiluminescence but ascorbic acid and methionine dose-dependently decreased the peak intensity. In addition, chemiluminescence was decreased markedly by treatment of PMN for 1 h with $12.5 \mu\text{mol ml}^{-1}$ of ascorbic acid, which is the same concentration as in 1% TL. In contrast to ascorbic acid, which scavenges ROI [23], methionine is well known as an inhibitor of myeloperoxidase [24], and indeed, this agent suppressed chemiluminescence from PMN. Therefore, the chemiluminescence-suppressing activity of TL appears to be the net result of the up-regulation by fatty acids and down-regulation by these agents. The suppressive influence of methionine and ascorbic acid is supported by our recent finding that Elental® (Morishita Pharma., Tokyo, Japan), an elemental diet, suppressed ROI generation to a lesser degree than TL, coordinating with the relative concentrations of methionine and ascorbic acid in the two formulas [25].

Figure 5 Influence of agents contained in TL on FMLP-induced chemiluminescence from PMN. PMN from three healthy donors were treated with indicated agents for the indicated times. The cells were washed and stimulated with FMLP, and chemiluminescence was measured as described in Methods. Each bar indicates the mean $\pm$ s.d. value from duplicate experiments. The fatty acid composition in HBSS (% v/v) was the same as that in TL. *: $P < 0.01$, †: $P < 0.001$, vs control.

Cytokines regulate the biological responses of the body, especially immunological reactions [10–14, 26]. *In vitro* treatment of human leukocytes and HUVEC with TL resulted in suppression of TNF- α , IL-1 β , GM-CSF, and PGE₂ generation. These suppressive effects were dependent on the concentration of TL, and a significant decrease of cytokine generation was observed at 0.01% TL, compatible with the suppressed expression of cytokine mRNA. Together with the suppression of chemiluminescence, TL appears to suppress multiple leukocyte functions. The suppressive activity of TL appears to be advantageous in patients in whom the cellular immune activity must be suppressed, for example, in organ and bone marrow-transplant patients and those with autoimmune diseases. Adversely, periodical examination of PMN and PBL activities appears to be requisite in long-term administration of TL to immunologically compromised patients. For the more advantageous usage of TL, it is crucial to recognize these advantages and disadvantages.

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