Detection of Chronic Inflammatory States in Cancer Patients and Screening for Antioxidant Activity of Plant Extracts

February 2013

Yoko SUZUKI

Detection of Chronic Inflammatory States in Cancer Patients and Screening for Antioxidant Activity of Plant Extracts

癌患者における慢性炎症状態の検出および

植物抽出成分による抗酸化活性スクリーニング

February 2013

Waseda University

**Graduate School of
Advanced Science and Engineering**
AND

Tokyo University of Agriculture and Technology

**Graduate School of
Bio-Applications and Systems Engineering**

Cooperative Major in Advanced Health Science

Research on Applied Immunology

Yoko SUZUKI

鈴木　洋子

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LIST OF ABBREVIATIONS

ROS   reactive oxygen species

LmCL   luminol-dependent chemiluminescence

PS   performance status

CRP   C-reactive protein

IL-6   interleukin-6

IL-8   interleukin-8

TNF   tumor necrosis factor

G/L   granulocyte/lymphocyte

MPO   myeloperoxidase

HOCl   hypochlorous acid

S-TGP   scaffold-thermoreversible gelation polymer

HBSS   Hanks' Balanced Salt Solution

WBC   white blood cell

CL   chemiluminescence

COX   cyclooxygenase

EGCG   epigallocatechin-3-gallate

HPLC   high performance liquid chromatography

EC epicatechin

ECG epicatechin gallate

EGC epigallocatechin

GCG gallocatechin-3- gallate

PREFACE

Many carcinomas arise from sites of chronic infection, irritation and inflammation. Besides, patients with end-stage carcinoma show inflammatory state represented by increased number of neutrophils. It is well known that most of the reactive oxygen speacies are produced by neutrophils. Up to now, the pathophysiological role of reactive oxygen species (ROS) produced by neutrophils to that inflammatory state has not yet been defined. Since neutrophils functions are difficult to evaluate due to their short-lived characteristics, with a circulating half-life of 6-8 hours.

Suzuki laboratory has examined an easy method for oxygen radical production and migration capability of neutrophils by use of thermoreversible hydrogel without isolating neutrophils from whole blood. An aqueous solution of thermoreversible gelation polymer (TGP) or scaffolded-TGP (S-TGP) is solidified by raising its temperature more than 20°C. Peripheral blood samples were mixed with luminol and set on the gel at 37°C. During the incubation time, neutrophils migrated into the S-TGP and emitted chemiluminescence but not in the TGP. The cells in the gel were more than 90% of neutrophils. These results suggested that S-TGP can be applied to clinical tests for estimating neutrophil activity.

Preliminary experiment was done with samples from young healthy people, and the luminol-dependent chemiluminescence peak value of the healthy person was approximately 1000 and 3000. There was individual difference by age, sex, health condition, but the peak value of the luminol-dependent chemiluminescence almost appeared 45 minutes later and tended to converge in approximately 90 minutes (Figure 1).
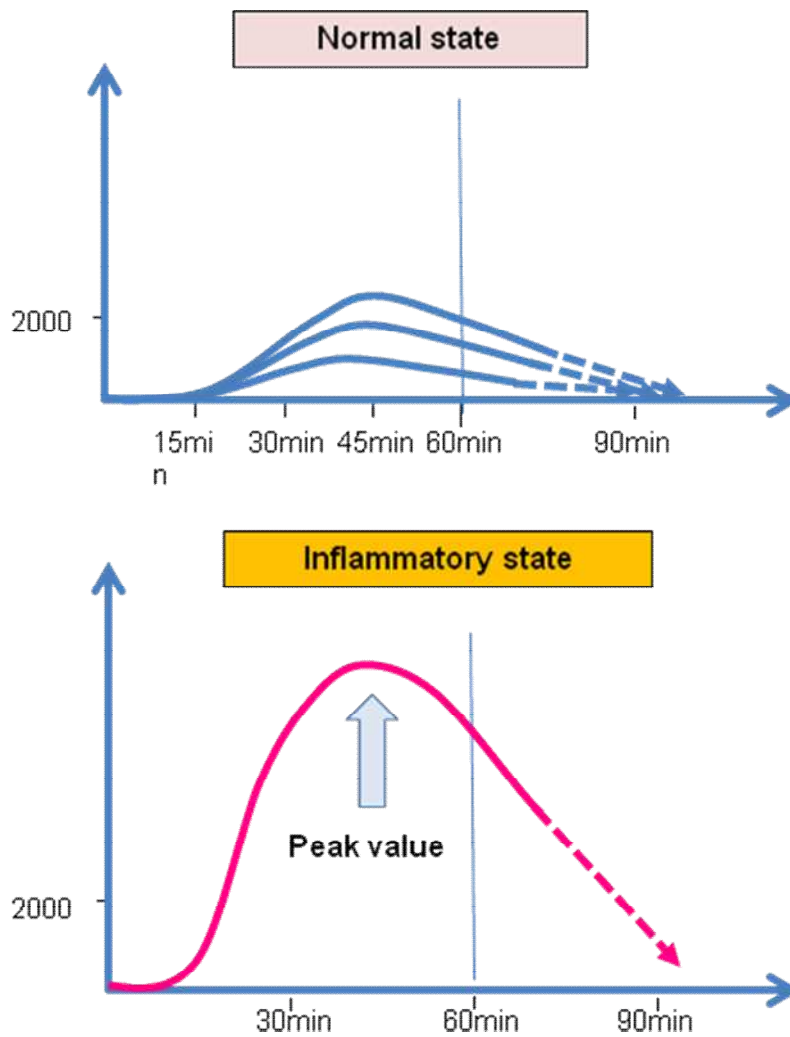
Figure 1. *Luminol-dependent chemiluminescence typical curve (normal)*

Then, we had collaborated for the examination to clinical application with Dr. Yuko Sato and Dr. Jun-ichi Akiyama in National Center for Global Health and Medicine from 2007 to 2009.

Since concise time for the assay was preferred for clinical test, we decided to perform it in 60 minutes for measurement time. However, blood samples from patients with cancer, a large intestine polyp, or the ulcerative colitis did not to show the luminol-dependent emission of light peak within 60 minutes (Figure 2a-b).
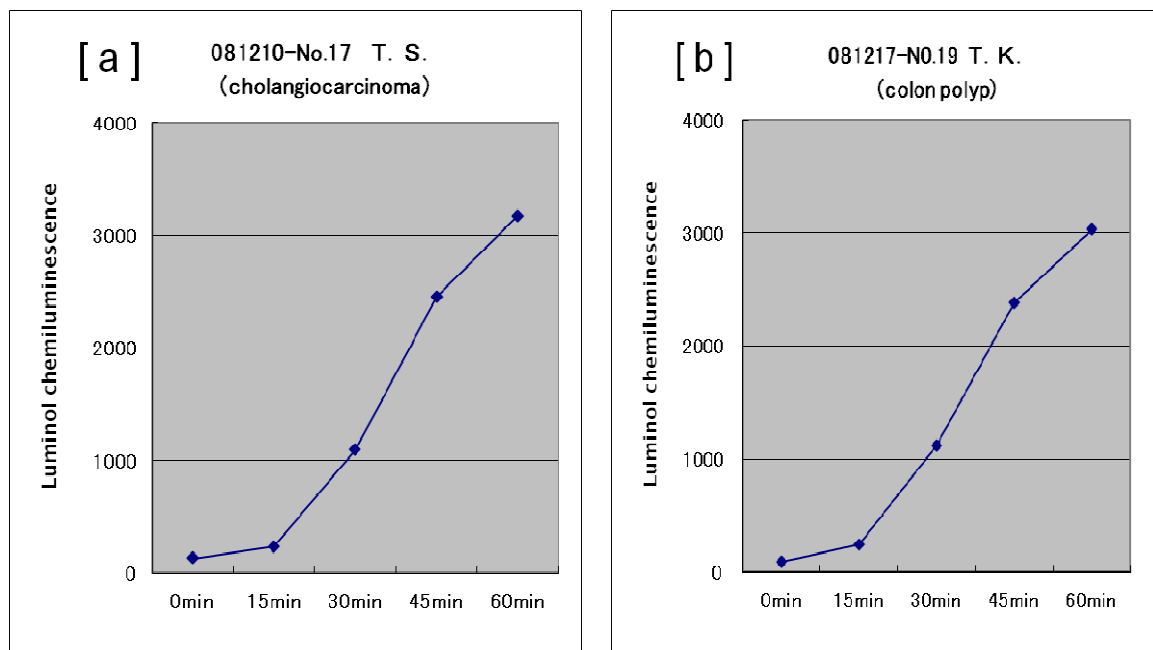
Figure 2a-b. *Measurement for 60 minutes (patient). 2a: cholangiocarcinoma, white blood-cell count 5100 /µl, neutrophils 78.8% (4000 /µl). 2b: colon polyp, white blood-cell count 4900 /µl, neutrophils 53.0% (2600 /µl).*

The author doubted whether we might finish the measurement without the peak value being provided. And she decided to extend measurement time for 90 minutes. However it was measured for 90 minutes, we were not able to get the peak value (Fig. 3).
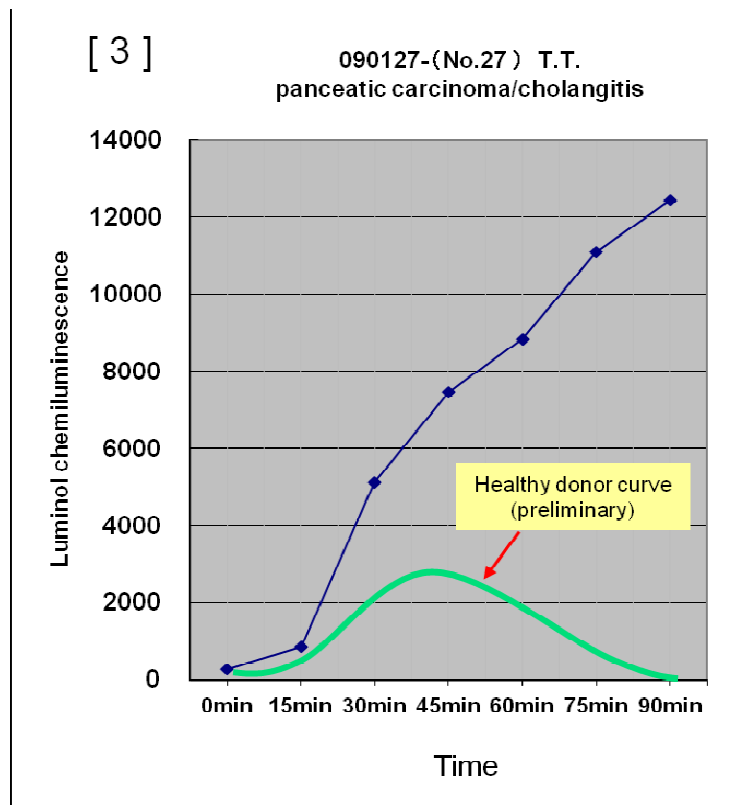
Figure 3. *Measurement for 90 minutes (patient):*
Pancreatic carcinoma / cholangitis, white blood-cell count 9000 /μl,
neutrophils 89.3% (8000 /μl).

Therefore the author extended the measurement until we got the peak value (Figure 4-5).
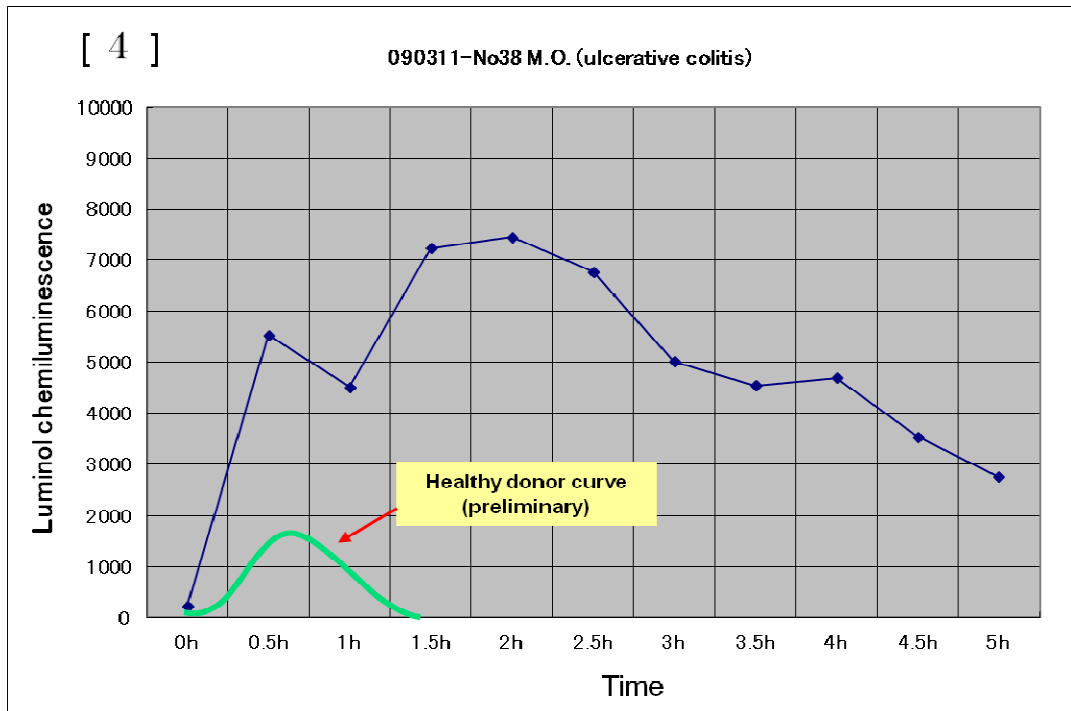
Figure 4. *Measurement time for 5 hours: Ulcerative colitis, white blood-cell count 11500 /μl, neutrophils 89.4% (10200 /μl).*
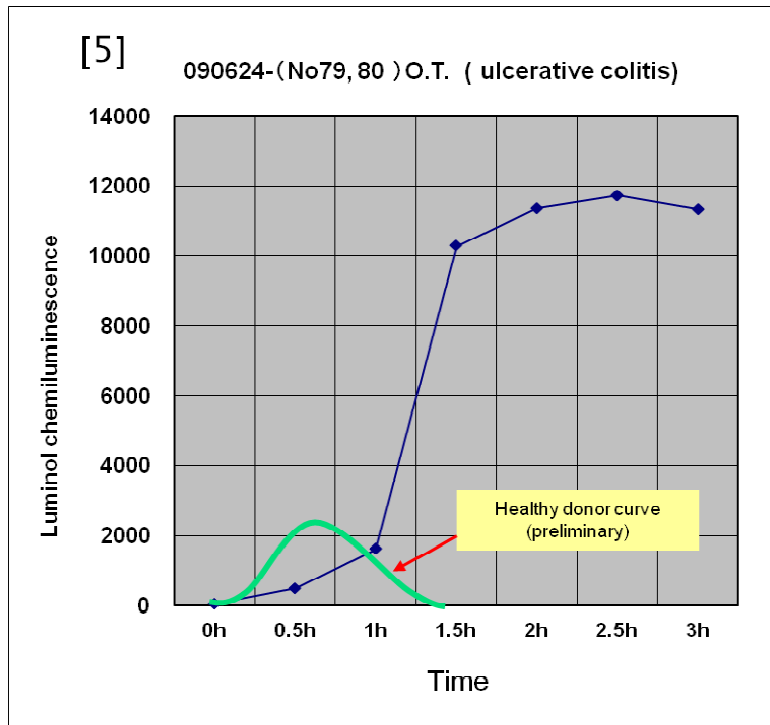
Figure 5. *Measurement time for 3hours: Ulcerative colitis, white blood-cell count 11300 /μl, neutrophils 88.8% (10000 /μl).*

The author obtained an opportunity to participate in collaborative investigation with Tokyo Women's Medical University and Waseda University, and measured the reactive oxygen species produced by neutrophils from patients with cancer at TWIns from June, 2010 to March, 2011.

This study aimed to determine the pathophysiological role of reactive oxygen species produced by neutrophils from patients with end-stage advanced cancer. At the same time, we investigated the effect of green tea extract on reactive oxygen species produced by neutrophils from patients with end-stage advanced cancer.

CHAPTER I

Determination of Chronic Inflammatory States in Cancer Patients

Using Assay of Reactive Oxygen Species Production by Neutrophils

I-1 Introduction

The functional relationship between inflammation and cancer is generally known. Many carcinomas arise from sites of chronic infection, irritation and inflammation. In addition, recent reports have expanded the concept that inflammation is a critical component of tumor progression. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration (1).

The existence of the systemic inflammatory condition can be confirmed in cancer patients. C-reactive protein (CRP) is a nonspecific but sensitive marker of inflammation (2). Erlinger et al. reported that plasma CRP concentrations were higher among all colorectal cancer cases compared to controls (3). Furthermore, up-regulation of CRP was associated with distant metastasis in patients with colon cancer (4). Moreover, increased CRP was associated with shorter survival in patients with several types of cancer (5). Recent research found that these changes were mediated by cytokines such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor (TNF) (6).

The granulocyte/lymphocyte (G/L) ratio appears to be a simple and clinically

relevant parameter for the assessment of perioperative inflammatory stress in patients with cancer (7). Moreover, preoperative evaluations with the G/L ratio may be important prognostic indicators, and its correlation may be a good indicator of the degree of effectiveness in activating anticancer immunity in patients with gastric cancer (8).

Neutrophils as the major type of leukocytes play important roles in host defense against all classes of infectious agents but, paradoxically, they are also involved in the pathology of various inflammatory conditions. Neutrophils are remarkably short-lived, with a circulating half-life of 6-8 hours and hence are produced at a rate of $5 \times 10^{10} - 10 \times 10^{10}$ cells/day. Tight regulation of these cells is vital because they have significant histotoxic capacity and are widely implicated in tissue injury (9). The release of cytotoxic molecules such as reactive oxygen species (ROS) into the extracellular environment can damage healthy tissues (10). ROS and cellular oxidative stress have long been associated with carcinogenesis (11).

The measurement of neutrophil chemiluminescence has been pioneered by Allen *et al.* (12, 13). Neutrophil respiratory burst activity can be measured in the presence of a light emitting reporter molecule (lumiphor) by the production of NADPH oxidase-dependent oxidants such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO)-dependent hypochlorous acid (HOCl)

production using luminal as the lumiphor (14).

Although a chronic inflammatory state is associated with disease progression in patients with cancer, the pathophysiological importance of ROS produced by neutrophils has not been defined. To address this question, the aim of the present work was to investigate the production of ROS from neutrophils in patients with cancer by assessment of luminol-dependent chemiluminescence (LmCL). The LmCL assay has been used to measure myeloperoxidase (MPO)-mediated formation of HOCl (15).

I-2 Patients and Methods

Patients and healthy donor.

The entry criteria were as follows: 16-79 years of age; the presence of inoperable, chemoresistant and radioresistant cancer; estimated survival of more than 3 months; performance status 0-3 (Table I); no severe organ function impairment and the written informed consent of the patient. At least 4 weeks prior to sampling, the patients were free from antitumor treatments, such as surgery, chemotherapy and radiation. Control samples were also obtained from a healthy donor (39-year-old male). The protocol was approved by the Ethical Committee at Tokyo Women's Medical University (approval number: 1692) and Waseda University (approval number: 2010-221).

Synthesis of peptide-bound temperature-responsive polymer (G-TRP).

Twenty-four grams of collagen peptide (SCP-5100; Nitta Gelatin Co., Osaka, Japan) were dissolved in 96 g of distilled water at 37°C, followed by reaction with 3.26 g of *N*-acryloylsuccinimide (Kokusan Kagaku, Tokyo, Japan) for 4 days at 37°C to obtain polymerizable collagen peptide. *N*-Isopropylacrylamide (108.5 g; Kojin, Tokyo, Japan) and *n*-butylmethacrylate (4.26 g; Wako Chemical, Osaka, Japan) were

dissolved in 600 ml of ethanol and then 123 g of the above aqueous solution of polymerizable collagen peptide was added. Under nitrogen atmosphere, 1 ml of *N,N,N',N'-* tetramethylethylenediamine was added to the mixed solution (Wako Chemical) and 10 ml of 10wt% ammonium persulfate (Wako Chemical) aqueous solution reacted for 5 hours at 4˚C, maintaining the nitrogen atmosphere. After the reaction, 30 l of cold (4˚C) distilled water were added and the mixture concentrated to 3 l using an ultrafiltration membrane (molecular weight cut off of 100,000) at 4˚C. This dilution and concentration process was repeated 5 times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105 g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel.

Under a clean-air laminar hood workbench, 0.5 g of G-TRP and 0.5 g of thermoreversible gelation polymer (Mebiol Gel; Mebiol Inc, Kanagawa, Japan) was dissolved in 16.7 ml of HBSS (Hanks' Balanced Salt Solution, calcium chloride, magnesium chloride) at 4˚C overnight, yielding a viscous transparent scaffold-thermoreversible gelation polymer (S-TGP) gel of uniform liquid without any bubbles for use in the experiments (16).

Mebiol Gel is a pure synthesized biocompatible copolymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks, characterized by its temperature-dependent dynamic viscoelastic properties and used as a biocompatible scaffold for three-dimensional culture without any toxicity (17).

S-TGP gel is a peptide-bound thermoreversible gel formed by mixing Mebiol Gel and G-TRP.

Luminol-dependent chemiluminescence (LmCL) assay.

Peripheral blood samples were obtained from patients and the healthy donor using Na-heparin glass tubes (Terumo Venoject II, Terumo Co, Tokyo, Japan). An aqueous solution of S-TGP gel was solidified by raising its temperature. Accordingly, 50 µl S-TGP gel was dispensed into microtubes (2 ml), and spread carefully at 4°C, and set on block incubators at 37°C.

Subsequently, the blood samples were mixed with 2.5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. The luminol-blood samples 150µl were set on the S-TGP gel tubes at 37°C. The production of ROS from neutrophils were detected as the values of LmCL using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan), without vortex in kinetic mode at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 hours (Figure 6).

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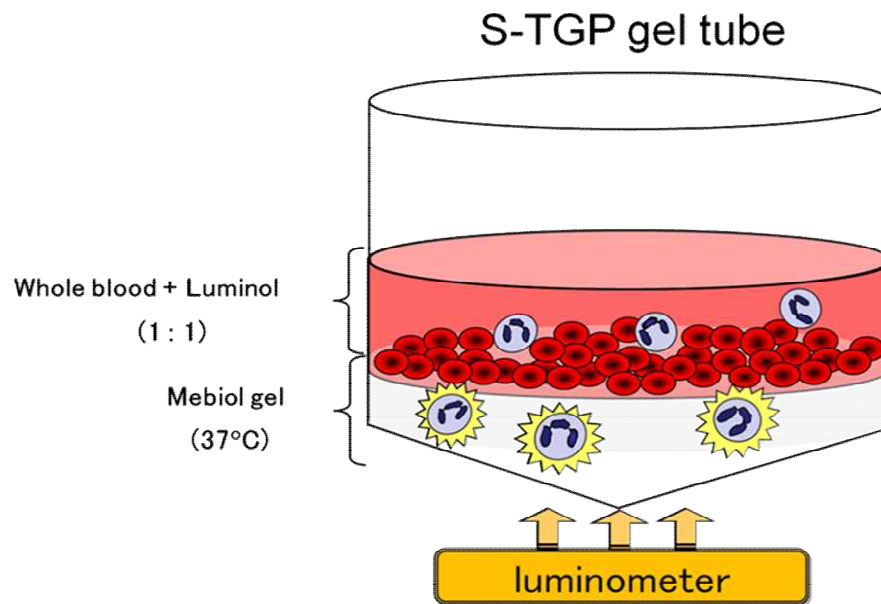
Figure 6. *Peripheral blood samples mixed with 2.5 mM luminol were transferred to microtubes filled with Mebiol Gel to measure the production of reactive oxygen species (ROS). In this assay system, only ROS produced by neutrophils were detected by luminometer.*

Neutrophils migrate from the blood into the S-TGP gel in the tube at 37°C, and LmCL can be detected through the transmissive gel, thereby there is no need to separate neutrophils from blood to determine ROS production, reducing any delay in sample processing that is associated with conventional methods (15). After LmCL was measured at 2.5 h, luminol-blood samples in the tubes were removed and the tubes with 50 μl S-TGP, in which neutrophils migrated, were washed three times with PBS warmed at 37°C. Tubes with gel were then cooled on ice, and 50 μl reagent A and 50 μl reagent B (Chemometec A/S, Allerød, Denmark) were added

and mixed well. This process effectively makes the cell membrane permeable to the DNA staining dye, and is effective in the dispersion of cell aggregates. The samples were aspirated into a NucleoCassett and the cell number was counted by the NucleoCounter (Chemometec A/S) (Figure 7).
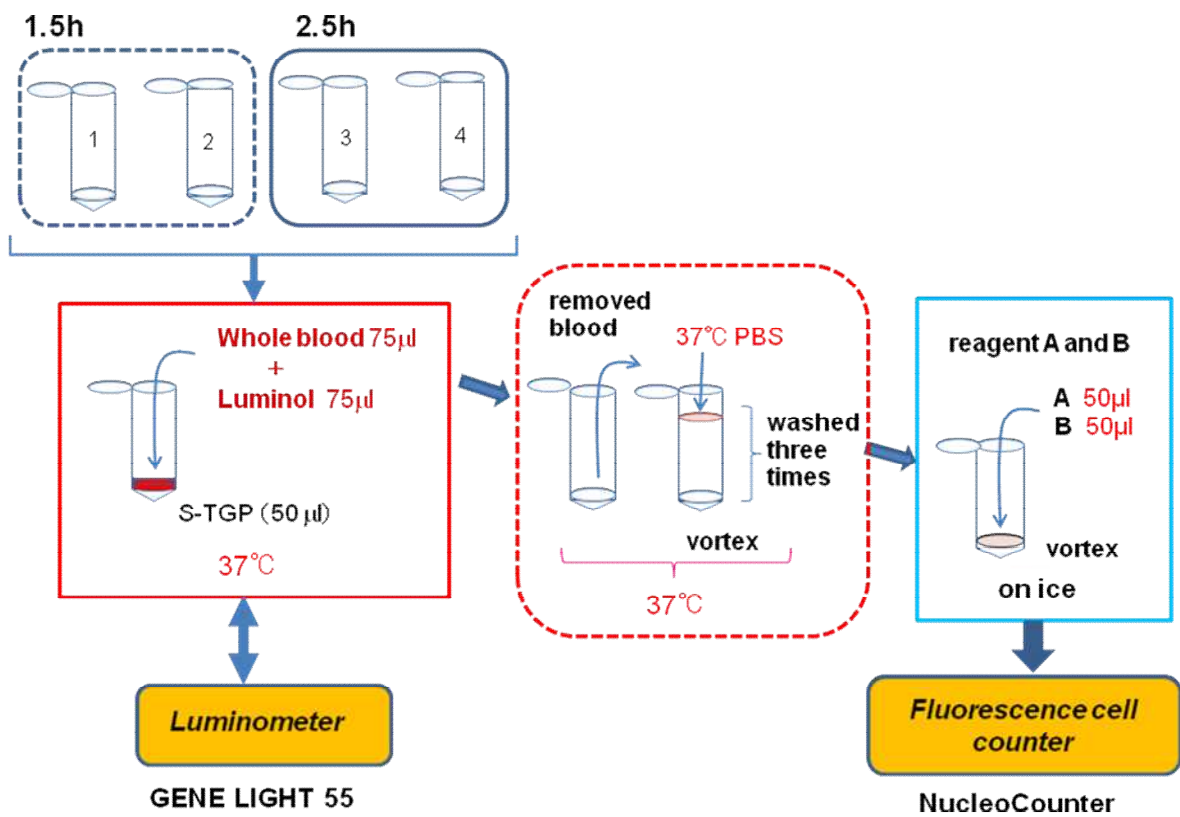

Figure 7. *Procedure of the measurement.*

Statistical analysis.

Differences between test groups were analyzed using the Mann–Whitney, U-test and Kruskal Wallis test. Calculations were performed using the statistical softwarepackage StatVeiw 5.0 (Abacus Concepts, Berkeley, CA, USA).

I-3 Results

Patients' characteristics.

Twenty-one patients were enrolled in this study. The mean age of the 21 enrolled patients was 56.8 years (range 35-76 years). Gender, diagnosis and performance status of patients are shown in Table I.

Table I. Patients' characteristics

No.	Age	Gender	Diagnosis	PS*
1	39	F	cervical cancer	0
2	54	F	epithelioid sarcoma	1
3	67	F	pancreatic cancer	1
4	55	F	colorectal cancer	2
5	45	F	pancreatic cancer	2
6	65	M	pancreatic cancer	1
7	61	F	colorectal cancer	2
8	63	F	colorectal cancer	2
9	61	F	lung cancer	1
10	71	M	lung cancer	2
11	54	F	duodenum papilla cancer	0
12	52	F	ovarian cancer	0
13	35	M	pancreatic cancer	0
14	47	M	gastric cancer	3
15	53	M	colorectal cancer	2
16	74	M	colorectal cancer	2
17	63	F	pancreatic cancer	3
18	46	M	lung cancer	0
19	76	M	gastric cancer	2
20	50	F	ovarian cancer	0
21	62	F	unknown	0

*PS:Performance Status

Time-dependent change in LmCL.

The responses compared in a kinetic mode are shown in Figure 2a-c. At each time of measurement, the median of LmCL in patients was significantly elevated compared to that of the control ($p<0.05$).
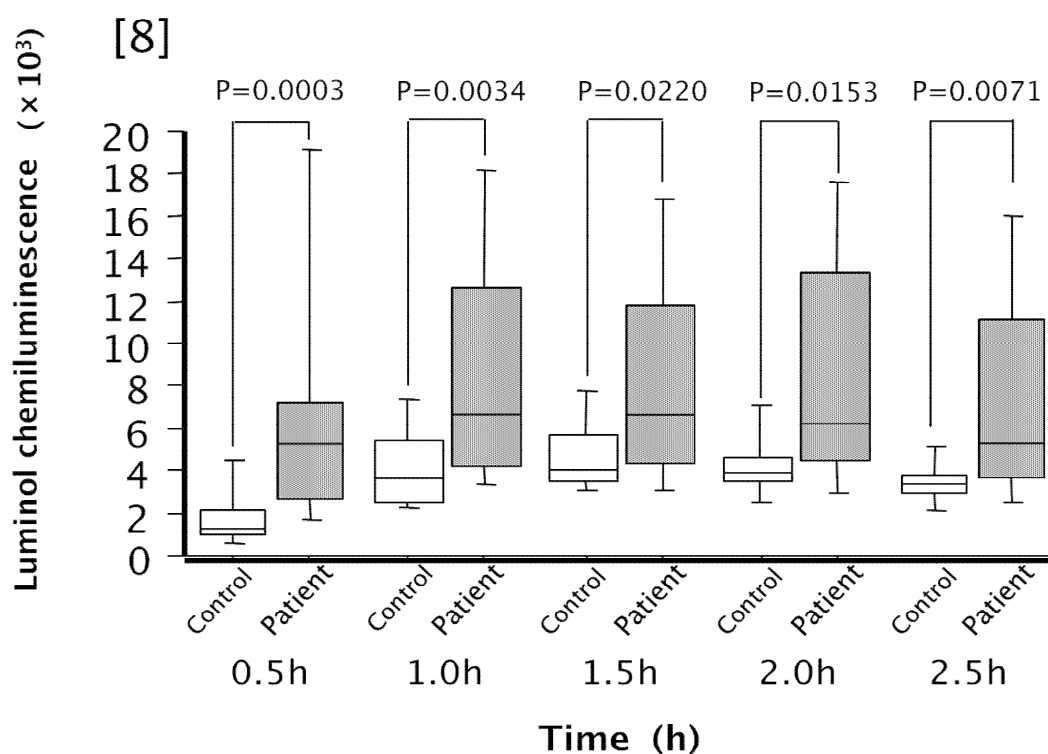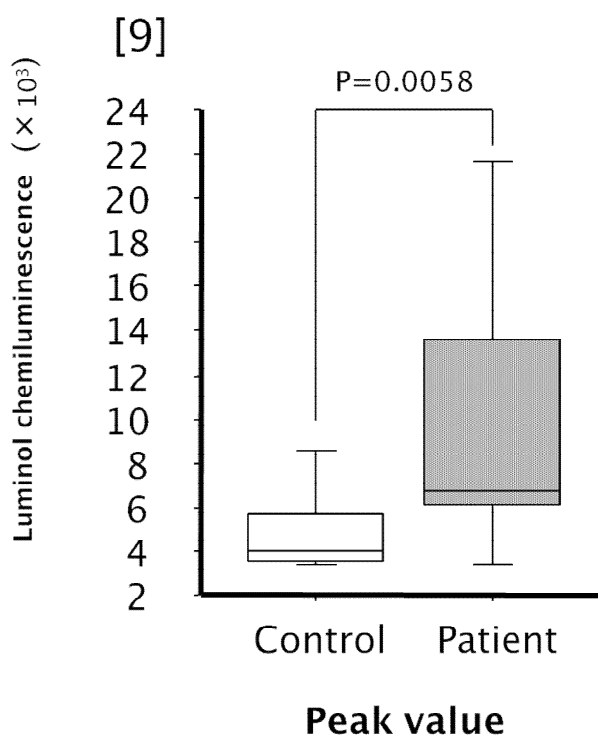

Figure 8. *Box and whisker plots demonstrating luminol-dependent chemiluminescence (LmCL) samples from patients and control: time-dependent change. Box contains values between 25th and 75th percentiles of LmCL (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Peak value in LmCL.

The peak value was assessed in the patients and control during measurement (0.5–2.5 hours). The peak value of the patient group was statistically higher than that of the control (p<0.05) (Figure 9).
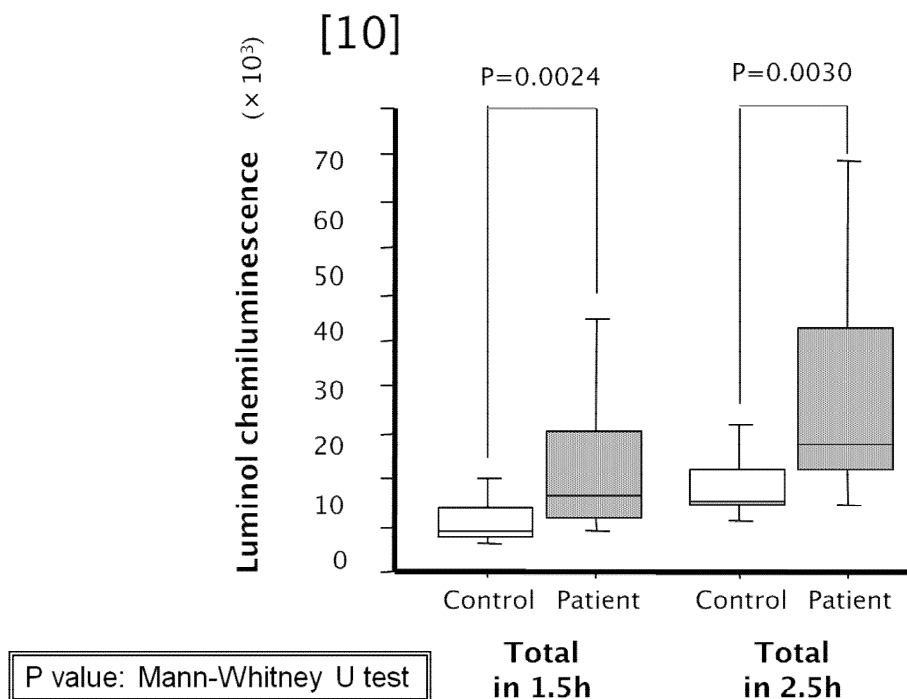

Figure 9. *Box and whisker plots demonstrating luminol-dependent chemiluminescence (LmCL) samples from patients and control: peak value. Box contains values between 25th and 75th percentiles of LmCL (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Sum of value in LmCL.

The total LmCL are shown in Figure 10. The LmCL values of the patients at 1.5

hours and 2.5 hours were significantly higher than those of the control (p<0.05).

Figure 10. *Box and whisker plots demonstrating luminol-dependent chemiluminescence (LmCL) samples from patients and control: sum of value. Box contains values between 25th and 75th percentiles of LmCL (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Number of the migratory neutrophil.

The number of migrated cells are shown in Figure 11. The migration cells counts tend to be higher in patients than control.
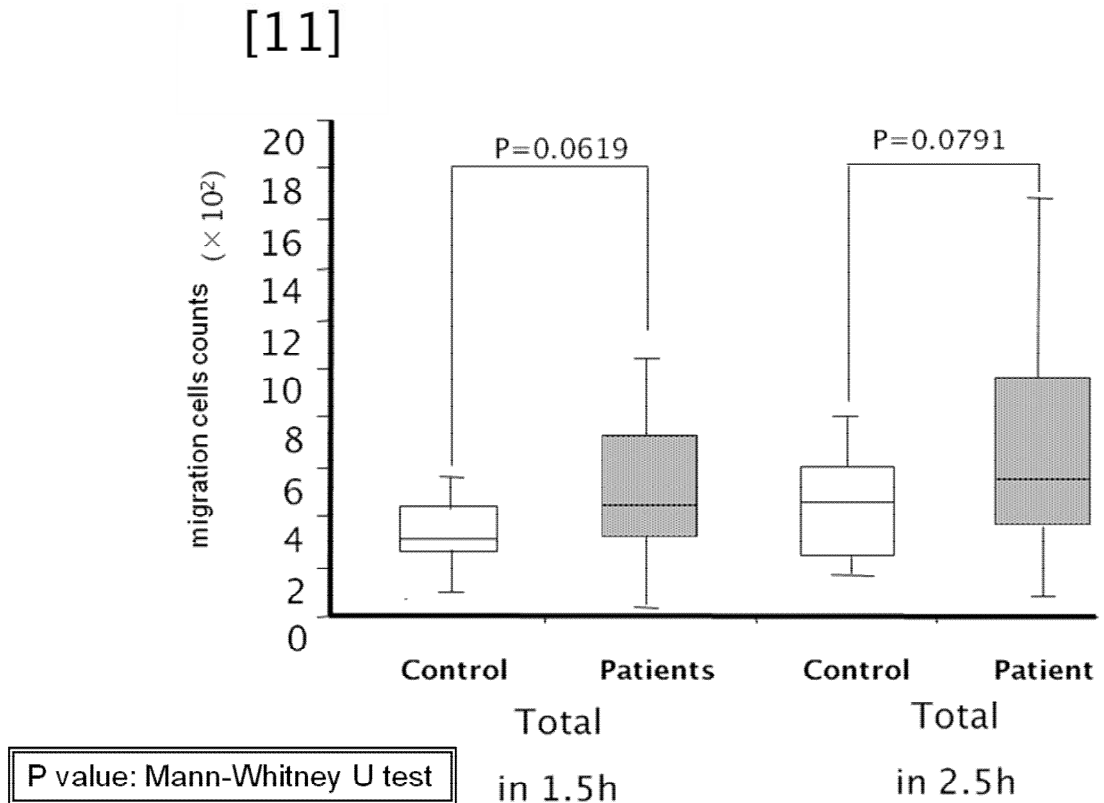
[11]

Figure 10. *Count of cells migrating into gel at 1.5 h and 2.5h. Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Sum of value per a cell.

The LmCL values per cell basis of the patients were significantly higher than those of the control (p<0.05) (Figure 12).
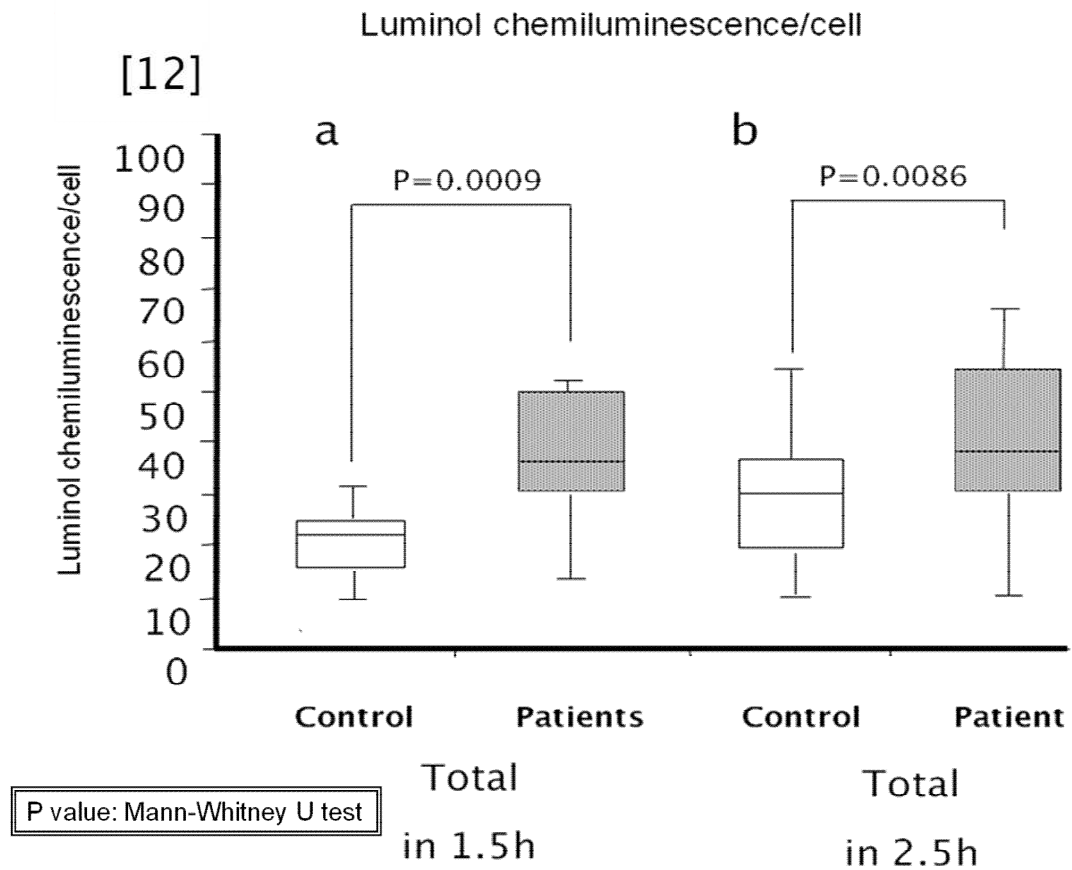

Figure 12. *Sum of value per a cell at 1.5h and 2.5 h. Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Comparison according to other parameters.

There were no differences of LmCL according to performance status (PS), type

of cancer, age or gender (data not shown).

I-4 Discussion

It is well known that the existence of the systemic inflammatory condition can be confirmed in patients with cancer. Satomi *et al.* compared the white blood cell (WBC), lymphocyte and neutrophil counts between the different stages of colorectal cancer and a control group. WBC and neutrophils (granulocytes) increased according to cancer progression. Conversely, lymphocytes decreased in association with stage. The G/L ratio increased sharply as stage advanced, and was highest in the terminal stage; the ratio in stage IIIb, stage IV and the terminal stage were statistically different from those in the controls (18). Furthermore, Shkapova *et al.* reported that high basal production of primary active oxygen forms was detected in the peripheral blood neutrophils of patients with renal cell cancer. *In vitro* stimulation of neutrophils led to more rapid release of superoxide radicals into extracellular space and to a reduction of cell capacity to more intense production of primary active oxygen forms (19).

In assessment of the activity of immune cells such as neutrophils, there are many different ways of measuring ROS: colorimetric, fluorescence, chemiluminescence (CL) and etc. CL has traditionally been used to study the nature of the oxidative bactericidal activity of neutrophils and monocytes, and a considerable number of

patients suffering from immunological disorders have benefited from recent applications of CL assays. Luminol is a very lipid-soluble substance that can penetrate cells and tissue easily, and it is used in various ways to measure luminescence in single phagocytic cells, groups of cells, and cells bound to or located within tissue. The use of LmCL may prove valuable as a method to measure the earliest events in the inflammatory process and may facilitate studying the mechanisms of inflammation. LmCL predominantly reflects the production of HOCl together with nitric oxide/peroxynitrite formation (20).

These methods, however, needed a relatively large quantity of blood for isolating neutrophils from whole blood, and require a fair amount of time to adjust the neutrophil concentrations. The LmCL method developed by our laboratory needs only a small amount of blood, and a short time to measure ROS produced by neutrophils without delay. The scaffold-thermoreversible gelation polymer, S-TGP (Mebiol Gel), was used here. Mebiol Gel is liquid at low temperature but turns to gel immediately upon warming and returns to a liquid state again when cooled. Simply, the sol-gel transformation can be varied by temperature control. By using this characteristic, our method can reliably measure ROS producing activity in neutrophils. With the present method, we found that each point, peak value and sum of values of LmCL in the patient group, reflecting ROS from neutrophils, was

statistically higher than that in the healthy control. Our results were consistent with previous research reports (1, 2, 4, 5, 7, 8).

Satomi *et al.* reported that high levels of ROS in patients with cancer were caused by reduced antioxidative activity (18). In our assay system, the production of ROS from neutrophils was detected as the values of LmCL from whole blood. Therefore, the values of LmCL reflected the surplus of ROS, *i.e.* the ROS neutralized by antioxidative activity (Figure 6). Since this assay system used whole blood at 37°C, the results reflected *in vivo* cellular milieu conditions more accurately. The values of ROS in the patient group were statistically higher than that in the healthy control, which suggests that antioxidant activities are reduced in the cancer patient group.

The greatest benefit of our method is its rapidity and simplicity of analysis using an analytical protocol which does not require any cell separation procedure. In addition, our assay system using the cellular response of neutrophils to assess the state of neutrophil activation within the milieu of the patient's immunologic profile may be applicable for screening antioxidant material for estimating neutrophil activity. These insights are fostering new anti-inflammatory therapeutic approaches to cancer.

In conclusion, although using a small cohort study, we showed that oxidative

stress, which was evaluated using ROS produced by neutrophils, was greater in

patients with cancer than in a healthy individual.

CHAPTER II

Effects of Green Tea on Reactive Oxygen Species

Produced by Neutrophils from Cancer Patients

II-1 Introduction

Chronic inflammation is associated with cancer development and may advance disease progression and negatively affect prognosis in patients with cancer (1, 21). Inflammatory cells infiltrate into the tumor microenvironment and play an important role in the neoplastic process, fostering proliferation, survival and migration (1). The main inflammatory cells which infiltrate tumor tissues are neutrophils and macrophages. Neutrophils are a primary source of production of reactive oxygen species (ROS), and cellular oxidative stress has long been associated with carcinogenesis. Furthermore, the role of chronic inflammation and oxidative stress in the onset of cancer cachexia has been reported (22, 23), and we have demonstrated that oxidative stress, as evaluated using ROS production by neutrophils, is greater in patients with advanced cancer (24).

Recent research has focused on the effectiveness of anti- inflammatory drugs for the treatment of cancer-related cachexia. Various anti-inflammatory drugs and strategies against cancer-related cachexia have been reported in vitro and in vivo (22, 23). Furthermore, clinical trials have been performed with the cyclooxygenase (COX)-2 inhibitor celecoxib, infliximab, various antioxidants, megestrolacetate and dexamethasone (25-29). Among these studies, anti-inflammatory treatment of

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patients with cancer reduced inflammation and oxidative stress parameters, such as

interleukin (IL)-6, tumor necrosis factor (TNF)−α, C-reactive protein (CRP) and ROS,

and were also demonstrated to be useful for improving both cachexia and the

quality of life of patients with cancer.

Green tea and its major constituent epigallocatechin-3-gallate (EGCG) have been

extensively studied as potential therapeutic agents for a variety of diseases,

including cancer. Numerous investigations have indicated that green tea and EGCG

possess antioxidant, anti-inflammatory, anti-mutagenic and anticarcinogenic

properties (30, 31). Moreover, certain studies demonstrate that EGCG has a potent

preventive effect against cancer-related cachexia. Wang et al. demonstrated that

EGCG effectively attenuates skeletal muscle atrophy caused by cancer cachexia in

mice (22). EGCG has been reported to inhibit inflammatory responses mediated by

neutrophils (32), however, the effects of green tea or EGCG on neutrophil activation

in cancer and the related cachexia have not been investigated, to date.

Although the effectiveness of green tea against cancer has been reported, there is

no report of the effect of green tea with respect to neutrophil activation in patients

with advanced cancer. To address this issue, we investigated the effect of green tea

extract on blood neutrophils from patients with cancer, by application of

luminol-dependent chemiluminescence (LmCL), which largely detects

myeloperoxidase (MPO)-dependent formation of highly toxic ROS such as hypochlorous acid (HOCl) (15).

II-2 Materials and Methods

Patients.

The entry criteria were the same of the CHAPTER I: 16-79 years of age; the presence of inoperable, chemoresistant and radioresistant cancer; estimated survival of more than 3 months; performance status 0-3 (Table I); no severe organ function impairment and the written informed consent of the patient. At least 4 weeks prior to sampling, the patients were free from antitumor treatments, such as surgery, chemotherapy and radiation. Control samples were also obtained from a healthy donor (39-year-old male). The protocol was approved by the Ethical Committee at Tokyo Women's Medical University (approval number: 1692) and Waseda University (approval number: 2010-221).

Green tea extract.

The green tea-leaf was first extracted using hot water for filtration, the extract was then applied to a column (polyvinyl alcohol) and eluted using 80% ethanol. This fraction was concentrated in a rotary evaporator and the total green tea fraction (GREEN TEA PE, BHN Co., Tokyo, Japan) was formed using a spray drier. Forty milligrams of GREEN TEA PE were dissolved with 30 ml distilled water, and

transferred to 100 ml volumetric flask. After pipetting 0.4ml of the GREEN TEA PE

solution to the flask, 2 ml of ferrous tartrate were added and mixed before adding 2

ml buffer solution of 1/15 M H_3PO_4 (pH 7.5). The solution was incubated for 5 min

at room temperature and was further clarified by centrifugation at 3000 rpm for 10

min. The absorbance was determined at 540 nm using a standard solution (ethyl

gallate) as a reference. The samples were analyzed by high performance liquid

chromatography (HPLC) and the catechins and (–)-epigallocatechin-3-gallate

(EGCG: Nacalai Tesque Co. Ltd. Kyoto, Japan) were quantified against (+)-catechin

(Sigma-Aldrich Co. Ltd., Tokyo, Japan), (–)-epicatechin (EC: Sigma-Aldrich Co. Ltd.,

Tokyo, Japan), (–)-epicatechin gallate (ECG: Nagara Science Co. Ltd., Gifu, Japan),

(–)-epigallocatechin (EGC: Nagara Science Co. Ltd.), and gallocatechin-3-gallate

(GCG: Nagara Science Co. Ltd.), used as external standards. The HPLC column was

a capcellpak C18, UG120 4.6×250 mm (Shiseido Co. Ltd., Tokyo, Japan), and the

mobile phase (A=distilled water, B=*N-N* dimethylformamide: methanol: acetic acid,

40:2:1.5) was used in a gradient elution. Detection was set at 278 nm. It was

confirmed that the polyphenol content was more than 98%, and catechin content

was more than 80% (EGCG: more than 50%, EGC: 0.6%, Catechin: 0.3%, EC: 0.3%,

GCG: 5%, ECG: 9%) in the GREEN TEA PE.

Dilution of green tea extract.

In a clean-air laminar hood workbench, 40 mg of GREEN TEA PE (BHN Co., Ltd., Tokyo, Japan) were dissolved in 10 ml of Hanks' balanced salt solution (HBSS) with calcium chloride and magnesium chloride. The solution was then filtered using a membrane with 0.22 μm pore size (TPP; Millipore, MA, USA). The concentration of green tea extract was adjusted to 0 μg/ml (HBSS only), 10 μg/ml, 100 μg/ml, and 1000 μg/ml in HBSS and stored at -20 ˚C for further use in experiments.

Synthesis of peptide-bound temperature-responsive polymer (G-TRP).

Described previously (CHAPTER I).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel.

Described previously (CHAPTER I).

LmCL assay.

Peripheral blood samples were obtained from patients using 2 ml Na-heparin tubes (Venoject II, Terumo Co, Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Aldrich, MO,

USA) at a ratio of 1:1. Then, 150 μl-luminol-blood samples were set on 50 μl S-TGP prepared in a tube at 37°C, and chemiluminescence was promptly measured as 0 point using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan), then 50 μl of green tea extract solution were added. The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored by a luminometer in a kinetic mode at 0.5, 1.0, 1.5, 2.0 and 2.5 h.

Neutrophils migrate from the blood into the S-TGP gel in the tube at 37°C, and LmCL can be detected through the transmissive gel, thereby there is no need to separate neutrophils from blood to determine ROS production, reducing any delay in sample processing that is associated with conventional methods (15). After LmCL was measured at 2.5 h, luminol-blood samples in the tubes were removed and the tubes with 50 μl S-TGP, in which neutrophils migrated, were washed three times with PBS warmed at 37°C. Tubes with gel were then cooled on ice, and 50 μl reagent A and 50 μl reagent B (Chemometec A/S, Allerød, Denmark) were added and mixed well. This process effectively makes the cell membrane permeable to the DNA staining dye, and is effective in the dispersion of cell aggregates. The samples were aspirated into a NucleoCassett and the cell number was counted by the NucleoCounter (Chemometec A/S) (Figure 13).

Figure 13. *LmCL assay and* migration cells count.

Statistical analysis.

Differences between the control, medium (HBSS: 0 μg/ml) and green tea extract (10 μg/ml, 100 μg/ml, and 1000 μg/ml) were tested with the Friedman repeated measures analysis of variance on ranks and with post-hoc multiple pairwise comparison for Friedman. Calculations were performed using the statistical software package IBM SPSS Statistics ver. 19 (SPSS Japan Inc., Tokyo, Japan). Statistical significance was accepted at the 5 % level.

II-3 Results

Patients' characteristics.

Eighteen patients were enrolled in this study. The mean age of the 18 enrolled patients was 57.4 years (range 35-76 years). Gender, diagnosis and the Eastern Cooperative Oncology Group performance status of patients are shown in Table II.

Table II. Patients' characteristics

No.	Age (years)	Gender	Diagnosis	PS
1	55	F	Colorectal cancer	2
2	45	F	Pancreatic cancer	2
3	65	M	Pancreatic cancer	1
4	61	F	Colorectal cancer	2
5	63	F	Colorectal cancer	2
6	61	F	Lung cancer	1
7	71	M	Lung cancer	2
8	54	F	Duodenal papilla cancer	0
9	52	F	Ovarian cancer	0
10	35	M	Pancreatic cancer	0
11	47	M	Gastric cancer	3
12	53	M	Colorectal cancer	2
13	74	M	Colorectal cancer	2
14	63	F	Pancreatic cancer	3
15	46	M	Lung cancer	0
16	76	M	Gastric cancer	2
17	50	F	Ovarian cancer	0
18	62	F	Unknown	0

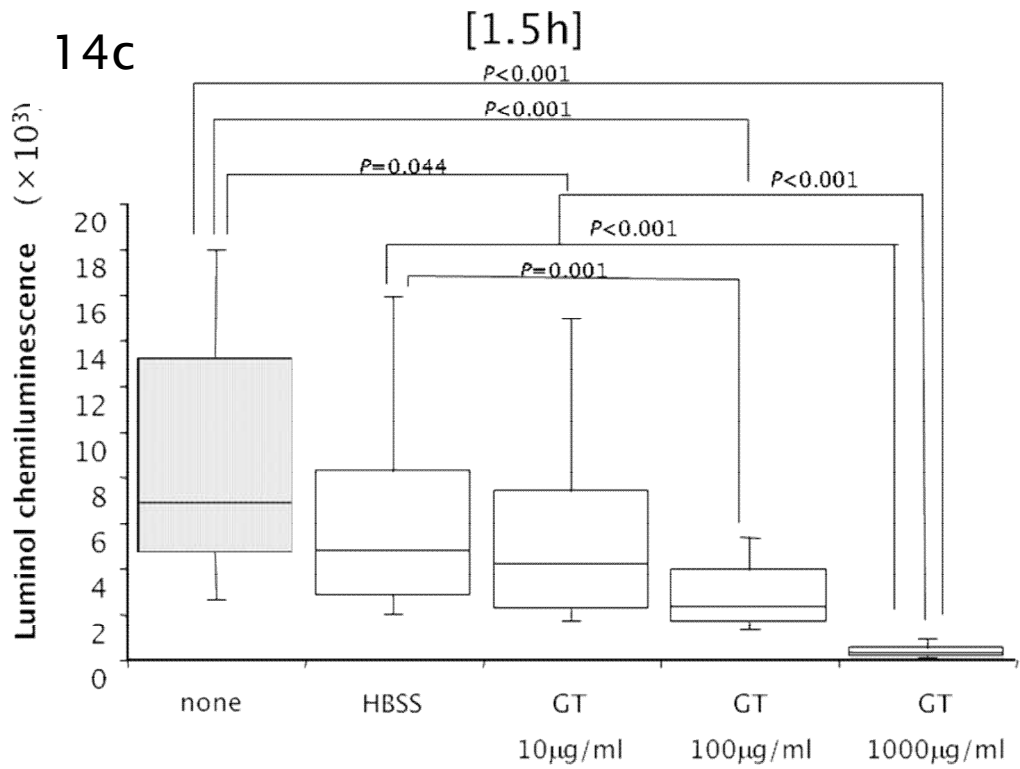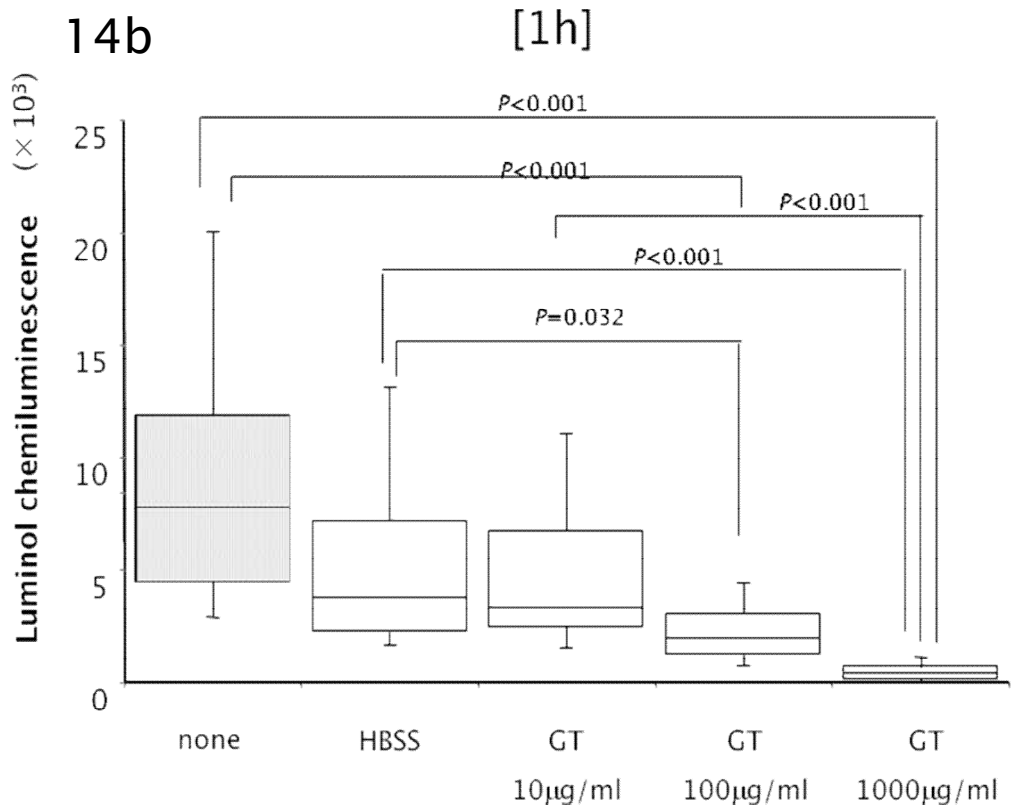PS: Performance status, M: Male, F: Female.
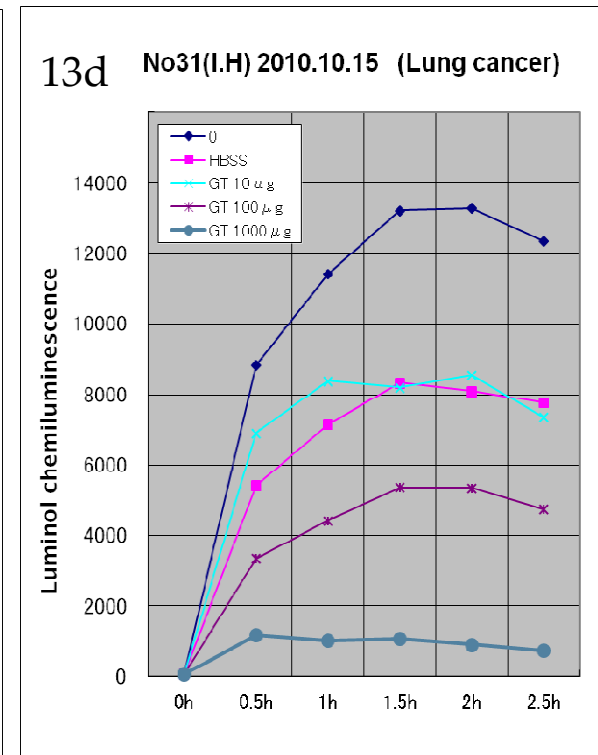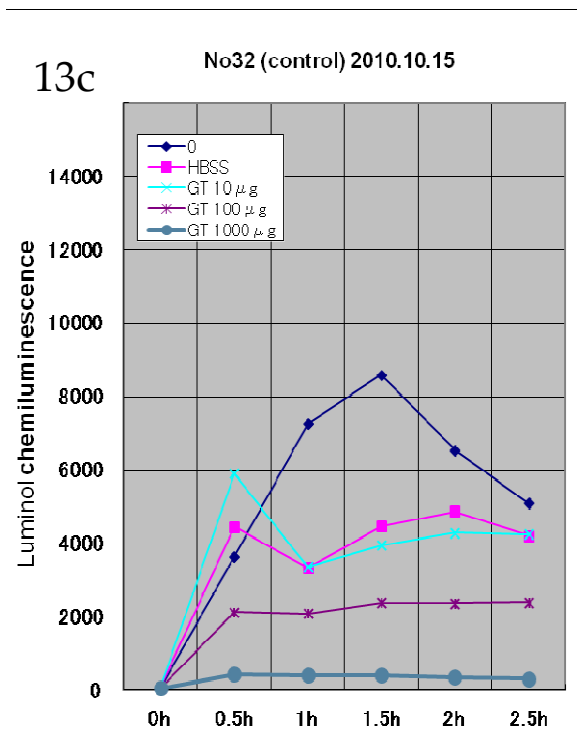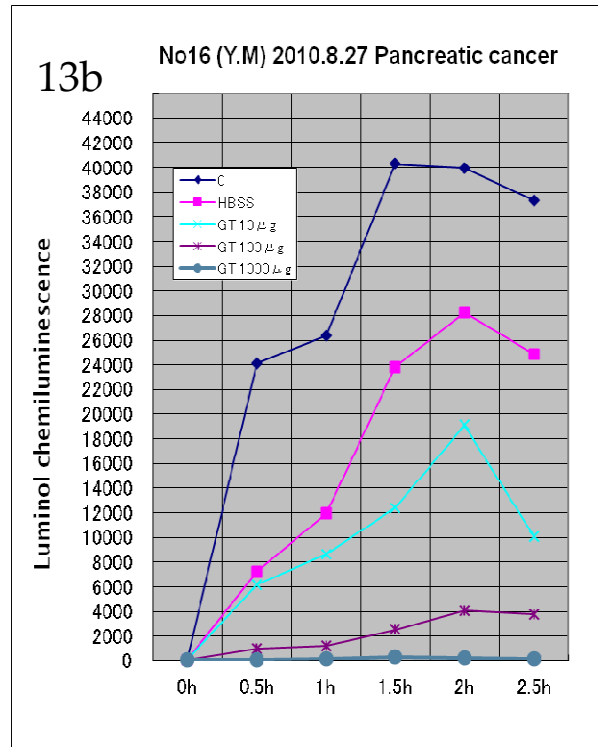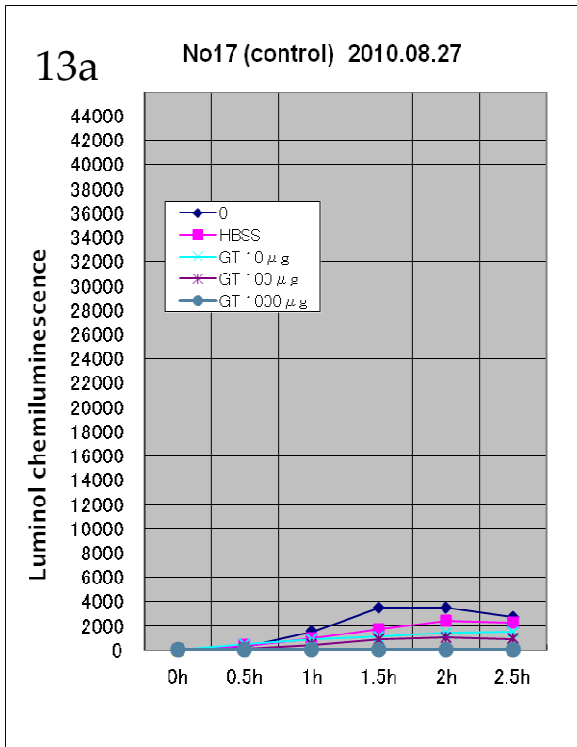
Figure 13a-d. *Effect of GreenTea on reactive oxygen Species*

The LmCL values at each point.

The LmCL values measured at 0.5, 1.0, 1.5, 2.0 and 2.5 h increased up to 1.0 h and plateaued thereafter (Figure 14a-e). The LmCL value of samples was markedly ($p<0.001$) reduced by adding the green tea extract, in a concentration-dependent manner at each time point. At all time points, the LmCL values of the samples with green tea extract at 100 μg/ml and 1000 μg/ml were significantly lower than those of samples without green tea extract or those with HBSS (medium) (Figure 14a-e). At 1.5 h, the LmCL values of the samples with green tea extract at a concentration of 10 μg/ml were significantly lower than samples without it ($p=0.044$) (Figure 14c).

14b

[1h]

14c

[1.5h]

14d

[2h]

P<0.001

P<0.001

P<0.001

P<0.001

P<0.001

Luminol chemiluminescence (×10³)

none   HBSS   GT 10µg/ml   GT 100µg/ml   GT 1000µg/ml

14e

[2.5h]

P<0.001

P<0.001

P=0.001

P<0.001

P<0.001

Luminol chemiluminescence (×10³)

none   HBSS   GT 10µg/ml   GT 100µg/ml   GT 1000µg/ml
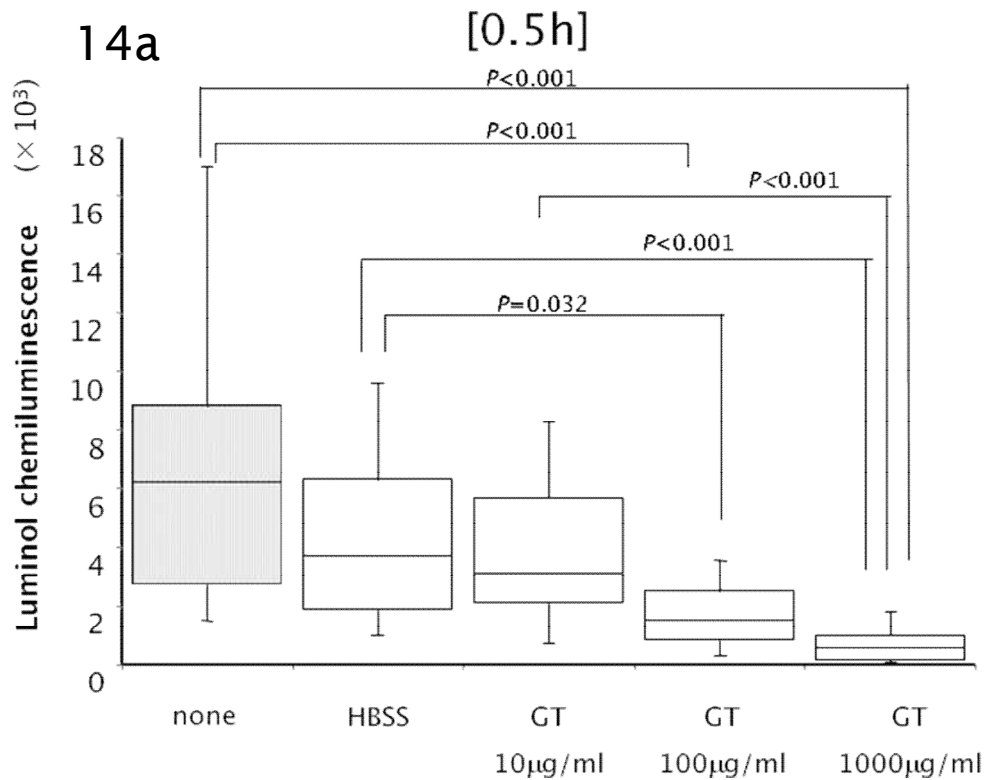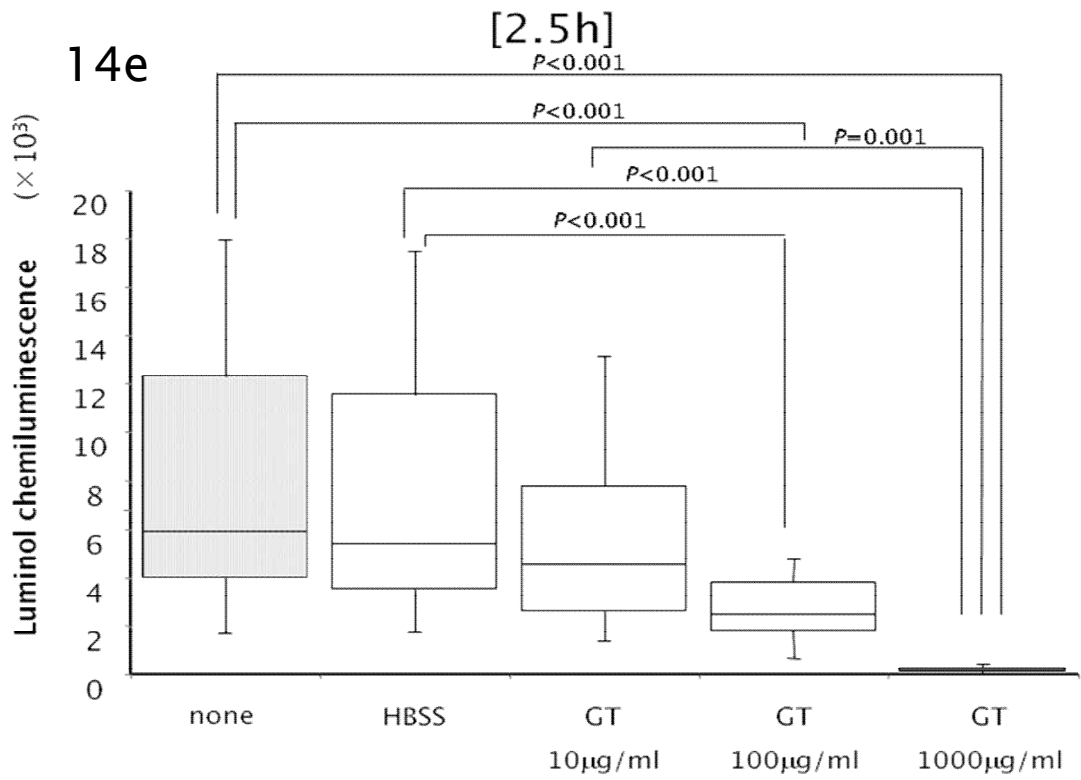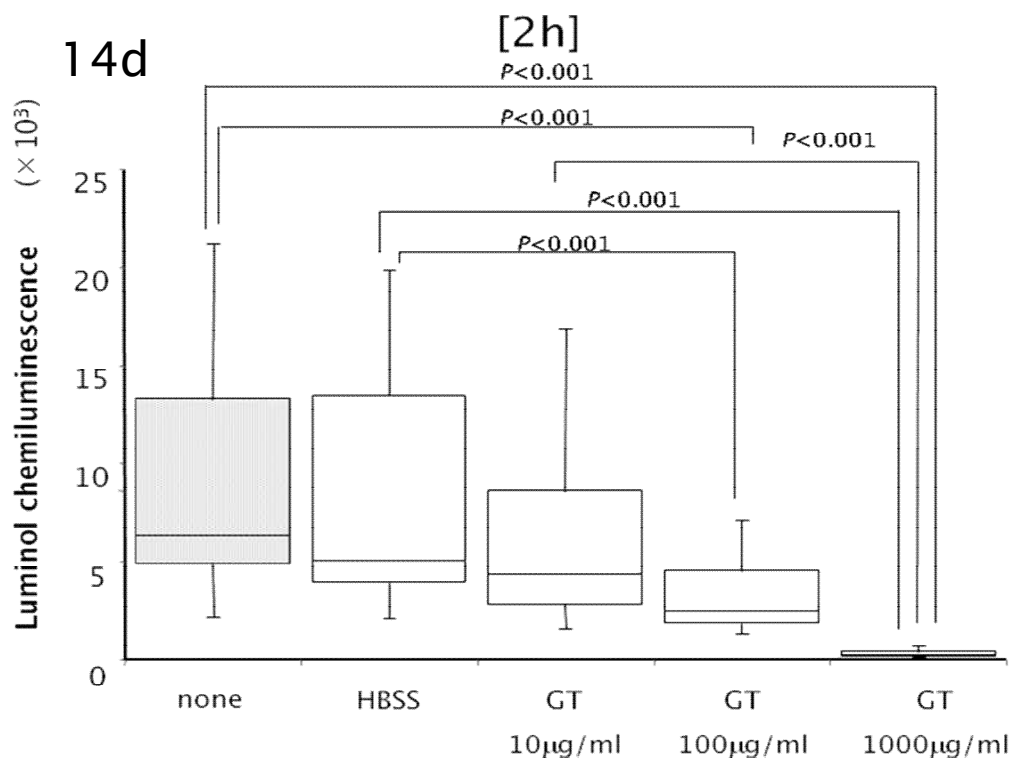
Figure 14a-e. *Box and whisker plots demonstrating neutrophil luminol-dependent chemiluminescence (LmCL) (14a-e), according to the concentrations of green tea extract: LmCL at 0.5 h (14a), 1.0 h (14b), 1.5 h (14c), 2.0 h (14d), 2.5 h (14e) after addition of green tea extract; Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles. HBSS: Hanks' balanced salt solution. GT: green tea extract.*

Peak values in LmCL.

The peak LmCL value of samples was markedly reduced by adding the green tea extract, in a concentration-dependent manner ($p<0.001$). The peak values of the samples with green tea extract (at 10 μg/ml, 100 μg/ml, and 1000 μg/ml) were significantly lower than those of the samples without it (Figure 15). The peak values of the samples with green tea extract at 100 μg/ml and 1000 μg/ml were significantly lower than those of samples with HBSS only.
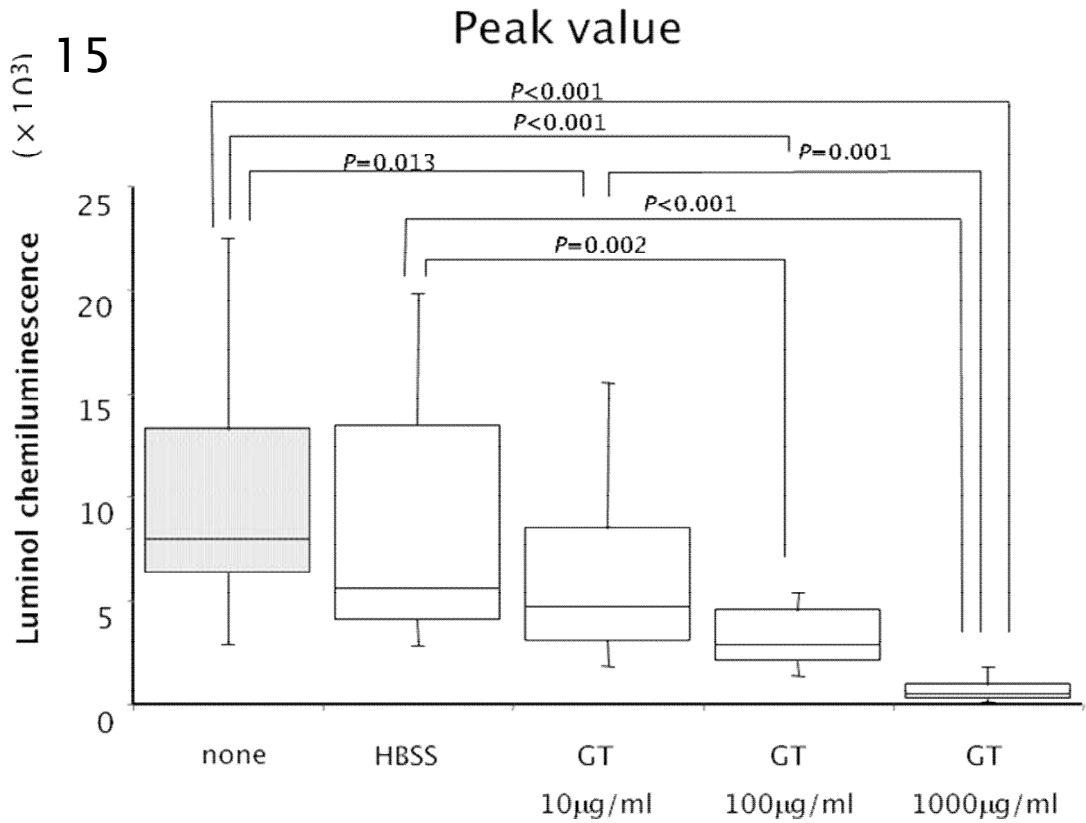
Figure 15. *Box and whisker plots demonstrating neutrophil luminol-dependent chemiluminescence (LmCL); Peak value during measurement , Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles. HBSS: Hanks' balanced salt solution.*

Sum of values in LmCL.

The total LmCL was determined from the sum of LmCL values at 0.5-1.5 h and at 0.5-2.5 h. Total LmCL at 1.5 h and 2.5 h was significantly reduced by adding the green tea extract, in a concentration-dependent manner (p<0.001). Total LmCL at 1.5h and 2.5h, of the samples with green tea extract (at 10 µg/ml, 100 µg/ml, and 1000 µg/ml), was significantly lower than those without it (Figure 16a and 16b). Total LmCL at 1.5 h and 2.5 h was significantly lower with green tea extract at 100 µg/ml and 1000 µg/ml when compared to those with HBSS only.
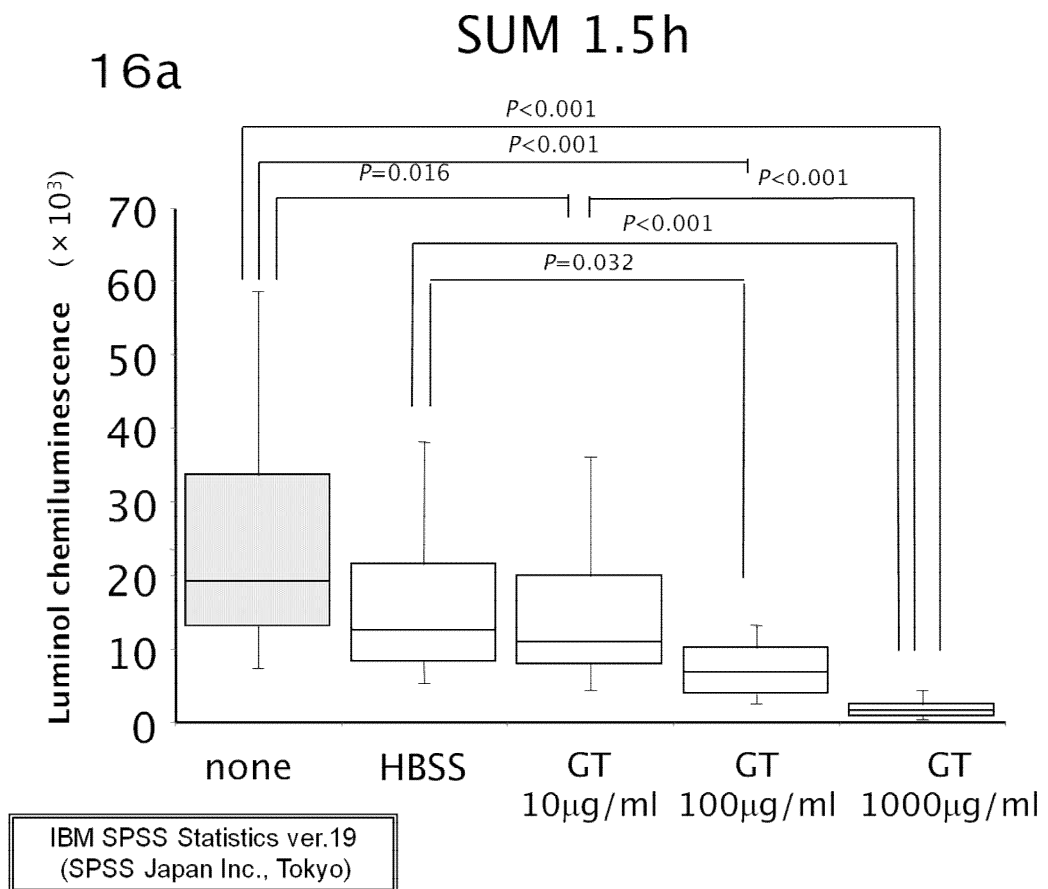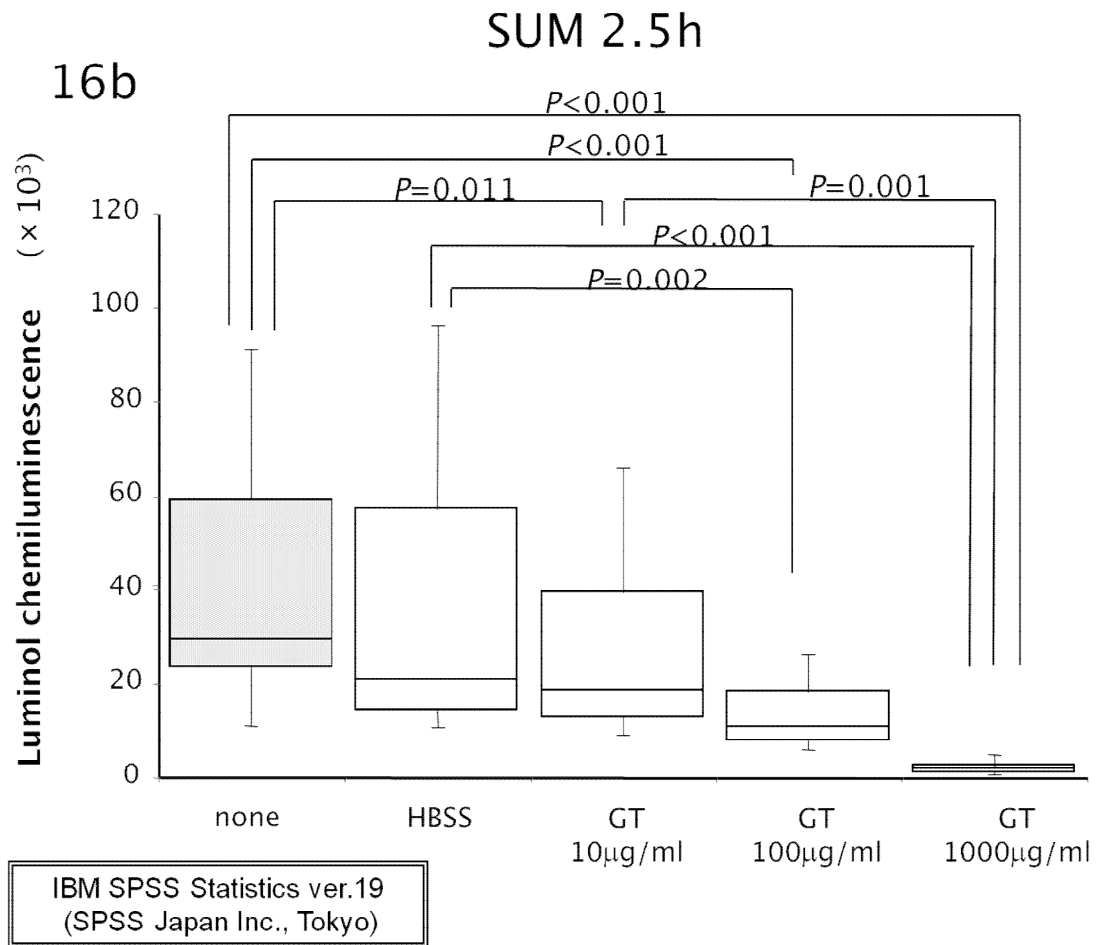
Figure 16a-b. *Sum of value at 1.5 h (16a) and 2.5 h (16b); Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles. HBSS: Hanks' balanced salt solution.*

Number of migratory neutrophils.

The number of migrated cells was not significantly influenced by adding the green tea extract per se (Figure 17a). When adjusted by migrated cell count, the LmCL values per cell basis were still inhibited by the green tea extract (at 100 μg/ml and 1000 μg/ml) (Figure 17b).
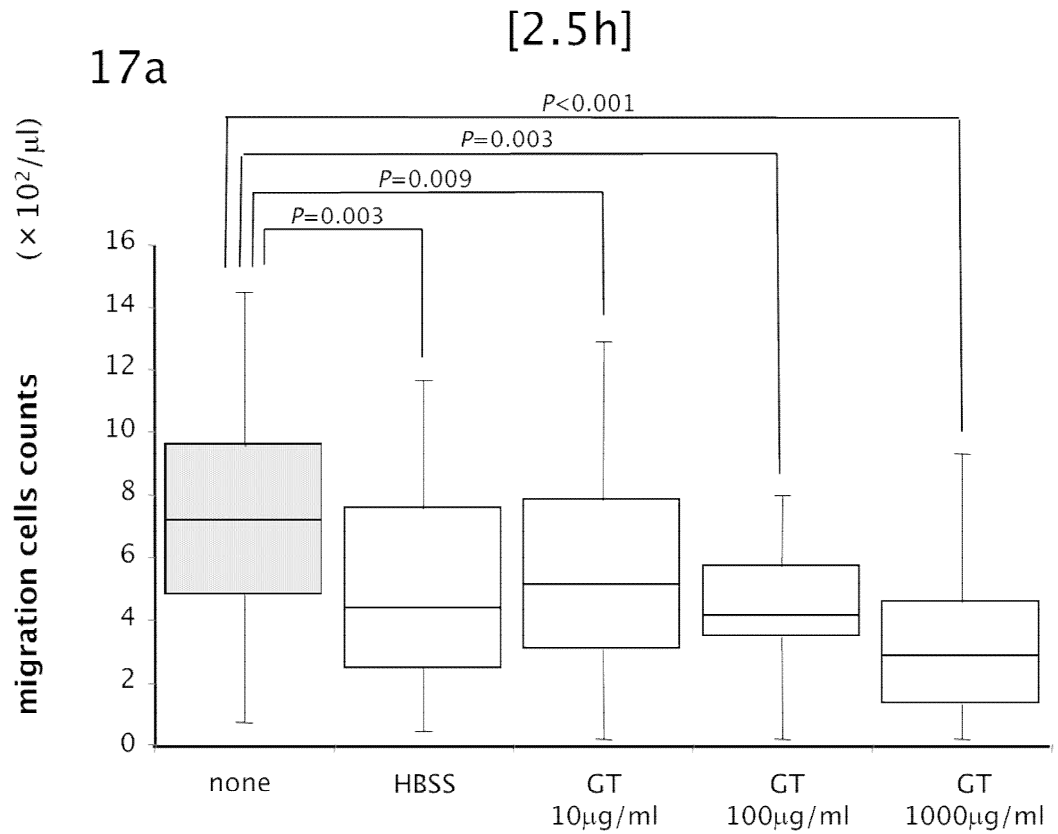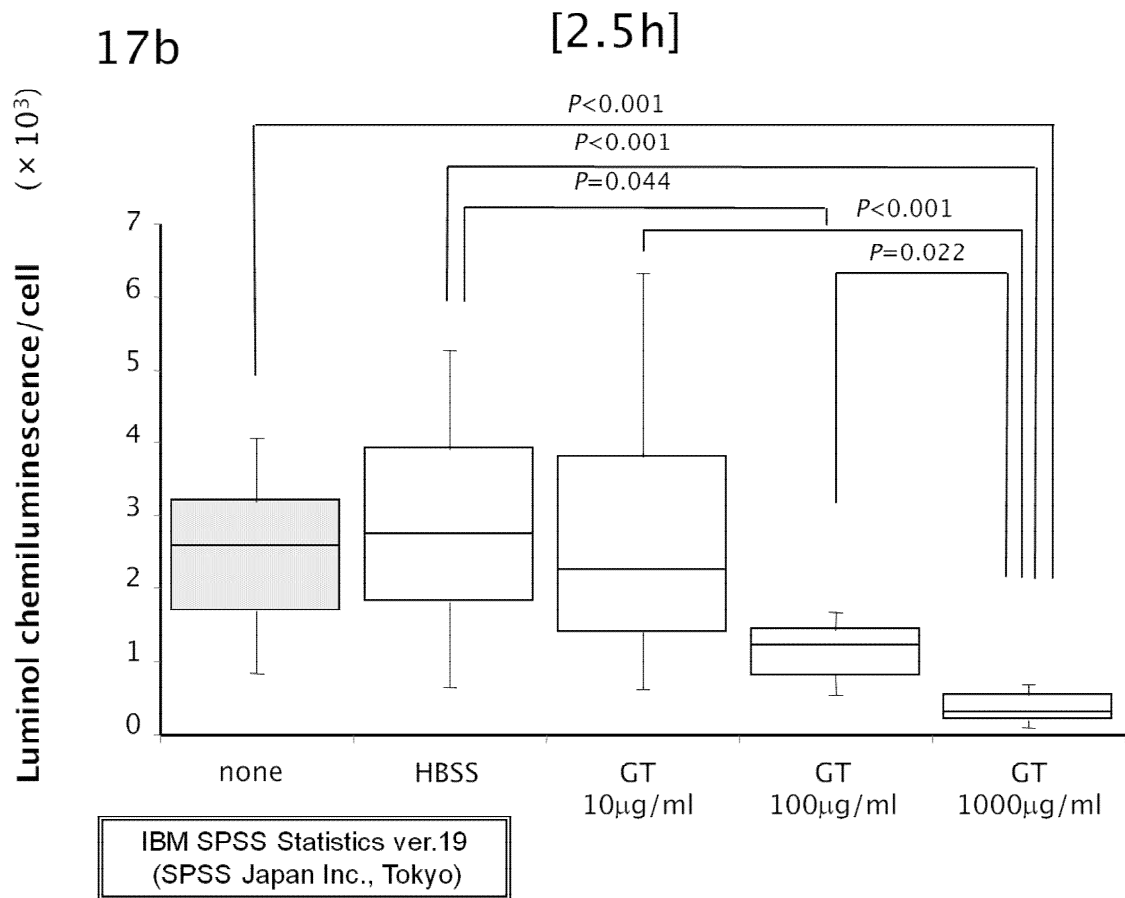
Figure 17a-b. *Count of cells migrating into gel at 2.5 h (17a), and sum of value per a cell at 2.5 h (17b). Box contains values between 25th and 75th percentiles (central line, median). Vertical lines represent the 10th and 90th percentiles. HBSS: Hanks' balanced salt solution. GT: green tea extract.*

II-4 Discussion

This study demonstrated that the green tea extract significantly reduced markers of ROS production from neutrophils from cancer patients in a concentration-dependent manner. Neutrophil activation may be affected by green tea extract based on the antioxidative or anti-inflammatory actions of the green tea extract. Numerous studies have reported anti-inflammatory effects of EGCG, and several studies demonstrated an anti-inflammatory effect of EGCG on cancer (30, 31). Our findings that green tea extract reduced the neutrophil production of ROS are consistent with previous research, supporting antioxidant and anti-inflammatory effects of EGCG.

The production of ROS, not only of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also of MPO-dependent HOCl production of neutrophils, can be measured by LmCL (14, 15). In the present study, green tea extract suppressed the neutrophil LmCL response, suggesting that ROS production was reduced. The major polyphenolic compound of green tea, EGCG, has demonstrated antioxidant potential, especially in detoxifying HOCl (33). The antioxidant effect of EGCG is well known and various *in vitro and in vivo* studies suggest that EGCG is associated with decreased risk and/or slower cancer progression (31, 36, 37). It is plausible

that the beneficial effects of EGCG in cancer may be related partly to a reduction of neutrophil production of ROS.

Green tea is manufactured by drying fresh tea leaves. It contains characteristic polyphenolic compounds such as EGCG, EGC, ECG and EC. Catechin, gallocatechin, epigallocatechin digallates, epicatechin digallate, 3-O-methyl EC, catechin gallate, and gallocatechin gallate are present in smaller quantities (31). However, Kürbitz *et al.* reported that ECG and catechin gallate are superior to EGCG in anti-inflammatory activity in pancreatic tumor cells, and can have an equivalent action (34). Although the differential activity of these compounds is a matter of discussion, we did not fractionate the green tea extract into each component when assaying the effects on neutrophil functions. Thus, the results of our study might support that routine intake of green tea as a dietary supplement and drink is beneficial for the oxidative property of neutrophils in patients with cancer.

In combination with antioxidant properties, extracts of green tea have anti-inflammatory effects and this may have contributed to the suppression of ROS. EGCG suppresses melanoma growth by inhibiting inflammasome and IL-1β secretion (30), and Gutierrez-Orozco *et al*. reported that green tea inhibited cytokine-induced IL-8 production and secretion in gastric cancer cells *via* inhibition

of NF-κB activity (35). Although unrelated to cancer, certain reports have demonstrated that EGCG suppressed neutrophil activation. Zhu *et al.* demonstrated that EGCG inhibits leukocyte activation by bacterial formyl peptide through the formyl peptide receptor (32). The combined antioxidant and anti-inflammatory properties of green tea may have contributed to suppression of ROS production by neutrophils in our study.

Migration of neutrophils to the tissue microenvironment is a first step to evoking local inflammation. Green tea extract did not inhibit neutrophil migration, but ROS production from migrated cells was significantly suppressed. Various neutrophil functions, such as ROS production and/or chemotaxis, could work as a dual-edged sword on both sides of host defense and tissue injury (14). Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly modulated. Considering that chronic oxidative stress occurs in cancer patients, suppression of ROS production from migrated neutrophils is advantageous. In addition, the ability of migration was not affected by the green tea extract, suggesting that the normal host defense response is retained in the presence of green tea extract. Although LmCL reflects oxidative stress based on the balance of production of oxidants and their removal by antioxidants, it can be used as a screening method for effective antioxidants for patients, in order to control

oxidative stress in vitro, as well as for clinical monitoring of interventions *ex vivo*. The use of Mebiol gel made it possible to mimic the *in vivo* microenvironment of neutrophil infiltration into tissues, and this approach may be used for the assessment of the antioxidant and anti-inflammatory actions of bioactive substances, such as green tea extract, using a small amount of blood, as demonstrated in the present study.

Cachexia in patients with terminal cancer is associated with pain, muscle atrophy, fatigue, anorexia, nausea, anemia, and immunodepression, resulting in a marked decrease in the patients' quality of life. Drugs with antioxidant and anti-inflammatory actions have been used in clinical trials. Mantovani *et al*. demonstrated their effectiveness for patients with cancer cachexia, by randomized phase III clinical trial of five different arms of treatment, which included polyphenols plus antioxidant agents (25). Our results in the present study might support the usefulness of green tea for patients with cancer cachexia in terms of increased protection against oxidative stress by reducing ROS production from neutrophils. Further studies are needed to investigate which constituents and regimens are more effective to reduce oxidative stress and risks of cancer development and related symptoms, by taking advantage of appropriate clinical monitoring systems.

CHAPTER III

CONCLUDING REMARKS

LmCL predominantly reflects the production of HOCl. In our assay system, the production of ROS from neutrophils was detected as the values of LmCL from whole blood. The values of LmCL reflected the surplus of ROS, *i.e.* the ROS neutralized by antioxidative activity.

This method developed by our laboratory needs only a small amount of blood, and a short time to measure ROS produced by neutrophils without delay. Since this assay system used whole blood at 37°C, the results reflected *in vivo* cellular milieu conditions more accurately.

The values of ROS in the patient group were statistically higher than that in the healthy control, which suggests that antioxidant activities are reduced in the cancer patient group. With the present method, we found that each point, peak value and sum of values of LmCL in the patient group, reflecting ROS from neutrophils, was statistically higher than that in the healthy control. Our results were consistent with previous research reports.

We also confirmed for the first time the antioxidant potential of green tea extract when it is applied to whole blood from patients with cancer. The major polyphenolic compound of green tea, EGCG, has demonstrated antioxidant potential, especially in detoxifying HOCl (33). The antioxidant effect of EGCG is well known and various *in vitro* and *in vivo* studies suggest that EGCG is associated

with decreased risk and/or slower cancer progression.

Thus, the results of our study might support that routine intake of green tea as a dietary supplement and drink is beneficial for the oxidative property of neutrophils in patients with cancer.

CHAPTER IV

ACKNOWLEGEMENTS

instruction.

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研究業績書

種 類 別	題名、　　　発表・発行掲載誌名、　　　発表・発行年月、　　　連名者（申請者含む）
○ a論文	Determination of Chronic Inflammatory States in Cancer Patients Using Assay of Reactive Oxygen Species Production by Neutrophils ANTICANCER RESEARCH 32: 565-570, 2012. YOKO SUZUKI, SATOSHI OHNO, RYUJI OKUYAMA, ATSUSHI ARUGA, MASAKAZU YAMAMOTO, SHIGEKI MIURA,HIROSHIYOSHIOKA,YUICHI MORI and KATSUHIKO SUZUKI
a.論文	Effect of Green Tea Extract on Reactive Oxygen Species Produced by Neutrophils from Cancer Patients ANTICANCER RESEARCH 32: 2369-2376, 2012. KATSUHIKO SUZUKI, SATOSHI OHNO, YOKO SUZUKI, YUMIKO OHNO, RYUJI OKUYAMA,ATSUSHI ARUGA,MASAKAZU YAMAMOTO, KEN-O ISHIHARA, TSUTOMU NOZAKI,SHIGEKI MIURA, HIROSHI YOSHIOKA and YUICHI MORI
a.論文	新規好中球機能検査法を応用した植物抽出物の機能性評価 Functional Assessment of Plant Extracts by Application of Novel Neutrophil Activity Measurement System 日本補完代替医療学会誌第9巻第2号2012年9月 鈴木克彦、駒場裕太、泊美樹、鈴木洋子、菅間薫、高橋将記，三浦茂樹，吉岡 浩，森 有一
c. 学会発表	癌患者における好中球活性酸素種産生能に対する緑茶抽出物の影響 Effect of green tea extract on reactive oxygen species (ROS) produced by neutrophils in cancer patients 第71回日本癌学会学術総会　2012年9月 Satoshi OHNO, Katsuhiko SUZUKI, Yoko SUZUKI, Yumiko OHNO, Ryuji OKUYAMA, Atsushi ARUGA, Masakazu YAMAMOTO, Ken-O ISHIHARA, Tsutomu NOZAKI, Shigeki MIURA, Hiroshi YOSHIOKA, Yuichi MORI
c. 学会発表	悪性腫瘍患者における酸化ストレスを指標とした慢性炎症の評価 第15回バイオ治療法研究会学術集会 2011年12月 鈴木洋子、大野智、奥山隆二、有賀淳、山本雅一、鈴木克彦
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研究業績書

種 類 別	題名、　　発表・発行掲載誌名、　　発表・発行年月、　　連名者（申請者含む）
c. 学会発表	Screening for factors affecting the immune response from metagenome of sponge-associated bacteria International Union of Microbiological Societies 2011 Yoshiko Okamura, Katsuhiko Suzuki, Yoko Suzuki, Kiyotaka Hara, Haruko Takeyama
c. 学会発表	メタゲノムデータベースを利用した細菌性ペプチドのスクリーニング 第63回日本生物工学会大会　2011年9月 岡村好子、鈴木克彦、鈴木洋子、原 清敬、竹山春子
c. 学会発表	ヒト白血球活性酸素種産生系を応用した各種植物抽出成分の作用解析・評価 Functional Analysis and Evaluation of Vegetables' Extracts by Application of Reactive Oxygen Species (ROS) Production by Human Leukocytes 第11回 日本補完代替医療学学術集会 2008年11月 鈴木洋子、鈴木克彦、石原健夫、高下崇、佐藤裕子
c. 学会発表	ヒト白血球活性酸素種産生系を応用した各種物質作用のハイスループット解析・評価法 High-throughput Analysis System for Screening Materials' Actions by Application of Reactive Oxygen Species (ROS) production by Human Leukocytes 第10回 日本補完代替医療学学術集会 2007年11月 鈴木洋子、鈴木克彦
c. 学会発表	Effect of Interferon beta on Survival for Murine Polymicrobial Sepsis American Thoracic Society International Conference, May, 2013 Yoshiomi Kusakabe, Kanji Uchida, Yoshiro Yamamura, Yoko Suzuki, Takahiro Hiruma, Yoshitsugu Yamada
e.その他 （研究報告書)	好中球機能サイトカイン産生能からみた各種人工血小板素材の in vitro 評価. H12(ADP)リポゾームの人工血小板としての前臨床評価(効力と安全性). 平成21年度 厚生労働科学研究費補助金(創薬基盤推進研究事業:政策創薬総合研究事業)分担研究報告書. 鈴木 克彦, 鈴木 洋子, 菅間 薫, 神田 和江, 沢田 秀司, 勝野 俊介, 武岡 真司