Determination of Chronic Inflammatory States in Cancer Patients Using Assay of Reactive Oxygen Species Production by Neutrophils

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Abstract. *Background: Although the systemic inflammatory condition can be confirmed in cancer patients, the pathophysiological importance of reactive oxygen species (ROS) produced by neutrophils has not yet been defined. Patients and Methods: Twenty-one patients with inoperable, chemoresistant and radioresistant cancer were enrolled in this study. At least 4 weeks prior to sampling, the patients were free from antitumor treatments. Control samples were also obtained from a healthy donor (39-year-old male). Peripheral blood samples were set 150 μl each on the 2 ml tube with 50 μl Mebiol Gel, and the production of ROS from neutrophils was detected by luminol-dependent chemiluminescence (LmCL) in a kinetic mode at 30-minute intervals for 2.5 hours with a luminometer at 37˚C. Results: Each point, peak value and sum of values of LmCL in the patient group was statistically higher than those in the healthy donor. There were no differences in LmCL according to performance status (PS), type of cancer, age, or gender in cancer patients. Conclusion: Our findings suggested that ROS produced by neutrophils universally reflects the systemic inflammatory condition in cancer patients.*

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 leukocyte 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 5×10^{10}-10×10^{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

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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).

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; 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: 589).

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 (Hank's Balanced Salt Solution 1×, 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 micro tubes (2 ml), and spread carefully at 4˚C, and set on block incubators at 37˚C.

In addition, 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 150 μl 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), in kinetic mode at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 hours (Figure 1).

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

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.

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

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 add ($p>0.05$) (Figure 2b).

Sum of value in LmCL. The total LmCL are shown in Figure 2c. The LmCL values of the patients at 1.5 hours and 2.5 hours were significantly higher than those of the control add ($p>0.05$).

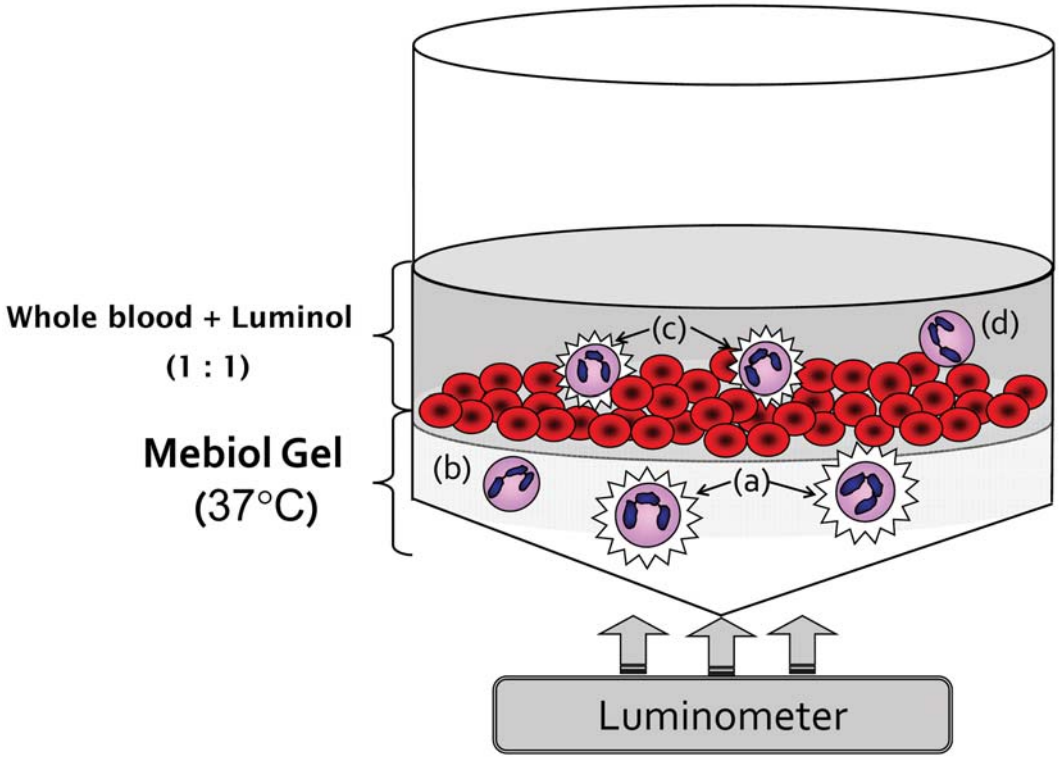

Figure 1. *Peripheral blood samples mixed with 2.5 mM luminol were transferred to microtubes filled with Mebiol Gel to measure the production of reactive oxygen species (ROS). In time-dependent change, neutrophils within microtubes were categorized into four groups regarding ROS production and migratory activity: Neutrophils infiltrating into Mebiol Gel with ROS production (a); neutrophils infiltrating into Mebiol Gel without ROS production (b); neutrophils suspended in blood with ROS production (c); neutrophils suspended in blood without ROS production (d). In this assay system, only ROS produced by neutrophils categorized as in (a) were detected by luminometry.*

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).

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,

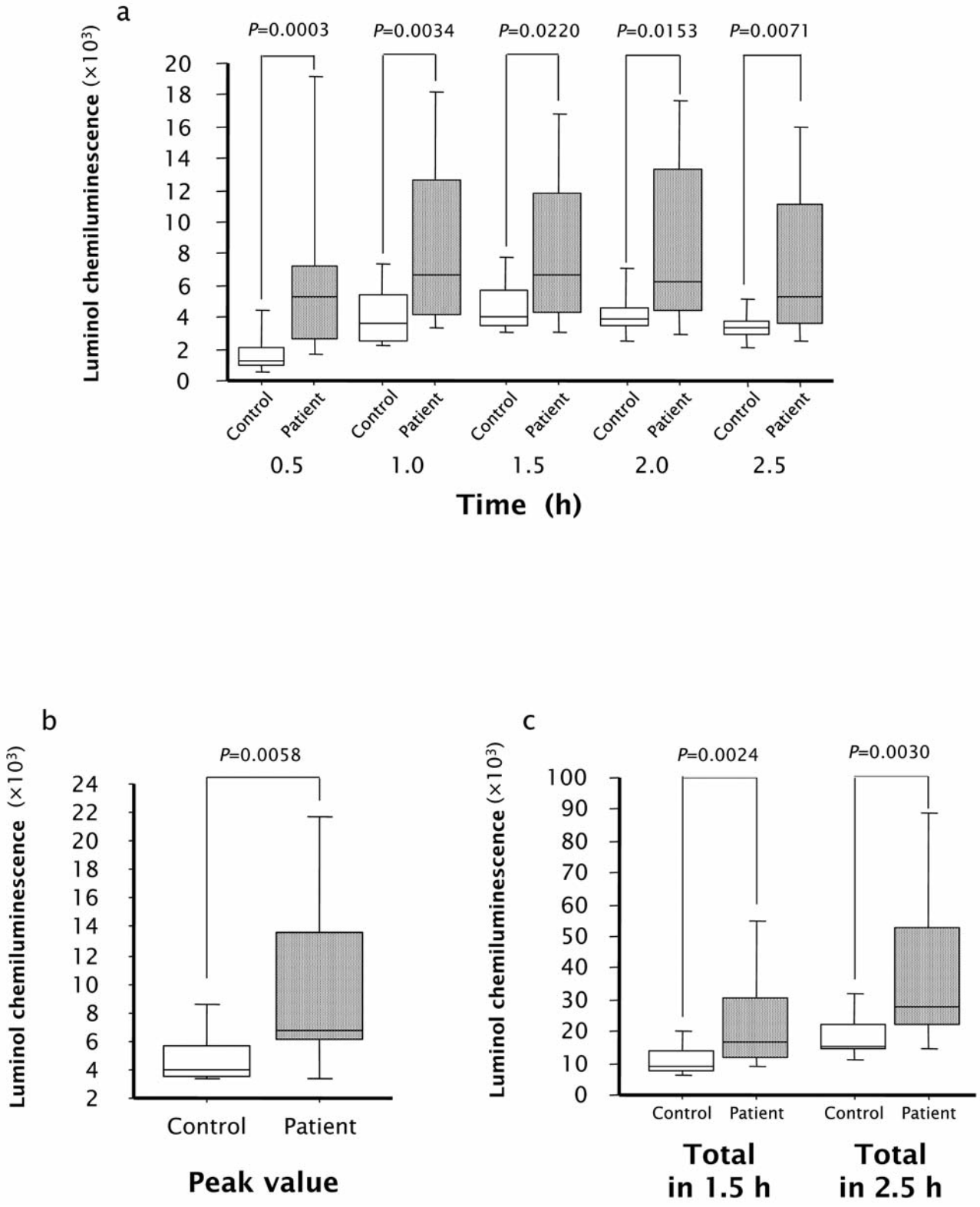

Figure 2. *a-c. Box and whisker plots demonstrating luminol-dependent chemiluminescence (LmCL) samples from patients and control: time-dependent change (a), peak value (b), sum of value (c). Box contains values between 25th and 75th percentiles of LmCL (central line, median). Vertical lines represent the 10th and 90th percentiles.*

Table I. *Patient's characteristics*.

No.	Age (years)	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	Duodenal 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, M: male, F: female.

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 thermoreversible gelation polymer, 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 finding showed 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 1). 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 the 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.

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