EFFECTS OF GaAlAs DIODE LASER ON SERUM OPSONIC ACTIVITY ASSESSED BY NEUTROPHIL-ASSOCIATED CHEMILUMINESCENCE

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The purpose of this study was to investigate the effects of low level laser therapy (LLLT: GaAlAs diode laser: 830 nm) on serum opsonic activity, which was assessed by neutrophil-associated chemiluminescence (CL) responses to zymosan opsonized with sera irradiated in vitro with various doses of LLL. We used both lucigenin-dependent CL (LgCL) for superoxide (O_2^-) detection and luminol-dependent CL (LmCL) which detects myeloperoxidase (MPO)-dependent formation of hypochlorous acid in combination with an MPO inhibitor, sodium azide (NaN_3). When serum opsonic activity was assessed by LgCL, NaN_3 markedly enhanced the responses, suggesting that O_2^- accumulates due to the MPO blockade, leading to the excitation of LgCL. However, LLL-irradiation had no effects on serum opsonic activity. On the other hand, when serum opsonic activity was assessed by LmCL, NaN_3 strongly inhibited the response. The effects of LLLT at different output powers were characterized by similar values, but significantly higher values were observed at the highest dose tested (60 mW for 1 min) in the absence of NaN_3 . Since this enhancement effect disappeared in the presence of NaN_3 , it was suggested that high dose LLL-irradiation probably activates opsonic activity by facilitating the degranulation of MPO from neutrophils. However, lower doses similar to those used for therapeutic purposes had no effects at any output powers tested.

Key Words: GaAlAs diode laser, serum opsonic activity, neutrophils, reactive oxygen species, chemiluminescence, luminol, lucigenin, myeloperoxidase.

Introduction

In higher organisms, neutrophils are principally responsible for monitoring the host's first line of defense against invading microorganisms. Their ability to kill bacteria depends to a large extent on a sequence of events whereby neutrophils first leave the blood and migrate to site of infection, then recognize and bind targets, ingest them and finally destroy them in phagolysosomes with toxic agents derived from a burst of oxidative metabolisms.⁽¹⁾

The efficient recognition and binding of microorganisms by neutrophils depends on opsonins, repre-

sented as immunoglobulins and the C3 component of the complement system.⁽²⁾ Opsonically active C3 molecules can be formed from C3 by direct activation of the alternative complement pathway or by immunoglobulin activation of the classical complement pathway.⁽³⁾ In the process of microbial killing during infections, the production of reactive oxygen species (ROS) by phagocytic cells is recognized to be a key event. The initial product of oxygen reduction is superoxide (O_2^-), generating during a respiratory burst. Following this O_2^- generation, other oxygen metabolites may be then formed including H_2O_2 , $\bullet OH$, 1O_2 , and $HOCl$, the latter being formed in a reaction catalyzed by myeloperoxidase (MPO).⁽⁴⁾

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In the last decade, it has been proved that low level laser therapy (LLLT: GaAlAs diode laser) can induce pain attenuation and pain removal in different acute and chronic pathological illness.^(5,6) Using neutrophils as a model for a therapeutic modality at the cellular level, experimental approaches and clinical investigations reported enhanced chemotaxis, oxidative metabolism (generation of ROS) and phagocytic activity of normal human neutrophils after LLL-irradiation (LLI) *in vitro*.⁽⁷⁾ Our laboratory⁽⁷⁻⁹⁾ has demonstrated that low levels of laser irradiation activate the ROS-production capacity of human neutrophils. On the other hand, Karu *et al.*⁽¹⁰⁾ observed suppression of chemiluminescence of human peripheral blood after irradiation and concluded that these effects depended on dose, wavelength and the pulse repetition rate. Since they did not separate neutrophils from the whole blood, it is necessary to determine whether the effect is attributed to neutrophil activity itself or opsonic activity which encourages and assists neutrophils to attack foreign materials. Indeed, no study on the effects of LLLT on opsonic activity has been reported so far. The aim of the present study was thus to investigate the *in vitro* effects of GaAlAs diode laser on serum opsonic activity using as a marker neutrophil-associated chemiluminescence.

Materials and Methods

Preparation of serum samples

Peripheral venous blood was collected from 8 healthy human male subjects. Serum samples were separated from blood using Vacutainer blood-collection tubes (Becton Dickinson, Franklin Lakes, USA) followed by centrifugation at 1000g for 10 min after allowing the collected blood to clot for 30 min at room temperature. These samples were stored frozen at -80°C until analysis and rapidly thawed in a thermostatically controlled heater to 37°C immediately before the laser irradiation.

Laser Irradiation

We used a GaAlAs diode laser Model FL 370 (Meiji Labax Co. Ltd. Tokyo, Japan). This machine emits in continuous wave at 830 nm, and the output power can be varied from 1 to 60 mW. The output power of the diode laser was checked by a type AQ-2101 optical power meter (Ando Electronics, Tokyo, Japan). The laser output powers were set at 15, 30 and 60 mW with exposure times at each output power of 10, 30 and 60 sec. The nonirradiated control serum was handled in exactly the same manner as the irradiated specimens with the exception of the actual laser irradiation.

Opsonization of particles

In this experiment we used zymosan from *Saccharomyces cerevisiae* that is a well known activator of the alternative

pathway of the complement system. Zymosan A (Sigma, St Louis, USA) was suspended in Hank's balanced salt solution (HBSS) at a concentration of 5 mg/ml and then opsonization was performed by adding the final concentration of 13% of the control and laser irradiation serum and incubating at 37°C for 30 min. The particles were then washed twice with HBSS and re-suspended to 5 mg/ml for IgCL⁽¹¹⁾ and 1 mg/ml for LmCL.⁽¹²⁾

Chemiluminescence probes

In this study, we used two chemiluminogenic probes, lucigenin and luminol, for the detection of the reactive oxygen species (ROS). Lucigenin was prepared by dissolving bis-N-methylacridinium nitrate (Sigma, Co. Ltd.) in HBSS at a final concentration of 0.5 mM at pH 7.4⁽¹¹⁾. Luminol was prepared by dissolving 5-amino-2,3-dihydro-1, 4-phtalazinedione (Sigma) initially in 1N NaOH to give a clear solution, and then adjusting using HCl and HBSS to give a final concentration of 2 mM and pH 7.4.

Myeloperoxidase inhibitor

Sodium azide (NaN₃; Sigma) at a concentration of 1 mM was added to the cells to inhibit catalase and MPO activity. The solution of this inhibitor (50 µl) was added to the neutrophil suspension prior to cell stimulation. Control wells were made up to the same volume as the experimental wells with HBSS in place of NaN₃ solution.

Isolation of neutrophils

Standard neutrophils were obtained from peripheral blood of a healthy male adult, and separated using Histopaque (Sigma) density gradient centrifugation according to the method previously described.⁽¹¹⁾ Briefly, human blood was diluted with the same volume of HBSS containing heparin (5 U/ml) and this heparinized diluted blood was then settled onto Histopaque 1077, layered over Histopaque 1119 and then centrifuged at 500 g at 4°C for 30 min. The neutrophil rich layer was collected and washed twice in HBSS and the neutrophils were suspended to 3 x 10⁶ cells/ml using an automatic blood cell counter (Sysmex Microcell counter F-300, TOA Medical Electronics Inc., Kobe, Japan) to prepare the neutrophil suspension.

Chemiluminescence

Ten microlitres of opsonized zymosan (OZ) suspension prepared from the irradiated and nonirradiated serum as described above was added to each well of black flat-bottom plastic microplates (Greiner Japan, Tokyo, Japan). Then, 50 µl of luminol or lucigenin solution, 50 µl of NaN₃ solution or HBSS and 50 µl of the standard neutrophils was added. The plates were transferred to the imaging box (dark box) and the CL responses were

Fig 1: Effect of GaAlAs diode laser irradiation on serum opsonic activity measured by lucigenin-dependent chemiluminescence in the absence and presence of myeloperoxidase inhibitor, sodium azide (NaN₃).

Measurement conditions: lucigenin: 50 μ l (0.5 mM) pH 7.4; neutrophil concentration: 50 μ l of 3.0×10^6 cells/ml; zymosan: 100 μ l (5 mg/ml) opsonized with nonirradiated and irradiated serum; sodium azide (1 mM) or HBSS: 50 μ l. Details of measurement conditions are described in the text.

Fig 2: Effect of GaAlAs diode laser irradiation on serum opsonic activity measured by luminol-dependent chemiluminescence in the absence and presence of myeloperoxidase inhibitor, sodium azide (NaN_3).

Measurement conditions: luminol: 50 μl (2 mM) pH 7.4; neutrophil concentration: 50 μl of 6.0×10^5 cells/ml; zymosan: 100 μl (1 mg/ml) opsonized by non-irradiated and irradiated serum; sodium azide (1 mM) or HBSS: 50 μl . Details of measurement conditions are described in the text.

automatically measured with the Lumi Box H-1000 system (Funabashi, Japan) as described before in detail.^(11,12) All measurements were performed at 37°C. The peak value in the response curve was expressed as peak height (PH) and the reaction time taken to reach PH was defined as peak time (PT). Each sample was run in duplicate and values were averaged.

Statistics

All the results were presented as means $\pm$ standard deviation (S.D). The statistical significance of the differences in the values of each condition was calculated using Student's t-test and a probability $p < 0.05$ was regarded as significant. Control wells of serum unirradiated by the diode laser were used for comparison of each sample.

Results

Figure 1 shows that the serum opsonic activity expressed by PH of LgCL was enhanced in the presence of NaN_3 and statistical differences ($p < 0.01$) were observed in comparison with the values found in the absence of NaN_3 . However, LLLI had no effects on serum opsonic activity at any of the laser doses tested.

Figure 2 shows that the serum opsonic activity evaluated by PH of LmCL was lowered in the presence of NaN_3 . This lowering of values could not be correlated either with output powers of LLLI or with exposure time. The effects of LLLI at different doses varying between 15–45 mW on serum opsonic activity assessed by LmCL were characterized by similar values of PH. Markedly higher PH with a significant statistical difference ($p < 0.01$) was observed between the control and the highest laser dose (60 mW for 60 sec), but the enhancement disappeared when the MPO inhibitor NaN_3 was used. PT values of LgCL and LmCL were not changed by any laser irradiation conditions compared with control values (data not shown).

Discussion

Phagocytosis as part of the defense mechanisms of organisms is designed to ingest foreign materials and then to kill them. This process is accompanied by a burst of respiratory activity which leads to the formation of reactive oxygen species (ROS). The generation of these toxic oxidants by stimulated neutrophils and macrophages is considered to play an important role in antimicrobial and anti-cancerous activity, in repair of tissue injury and also in production of CL.⁽¹³⁾ LgCL has been used to measure O_2^- production by NADPH-oxidase, whereas LmCL mainly depends on the MPO-mediated formation of hypochlorous acid.^(11,13,14) This was

confirmed by the fact that LmCL is inhibited by MPO-inhibitors such as NaN_3 and is markedly reduced in patients with MPO-deficiencies.⁽¹⁵⁾

As was shown in our previous study,⁽¹¹⁾ the present study showed that the LgCL response was markedly enhanced by treatment with NaN_3 , suggesting that O_2^- is accumulated due to inhibition of MPO, leading to the enhancement of LgCL because NaN_3 inhibited both MPO and catalase activities, and the consumption of H_2O_2 was blocked. Taking this ROS metabolic change into account, it must be pointed out that the usual methods for O_2^- and H_2O_2 assays in the absence of MPO inhibitors measure the remaining O_2^- and H_2O_2 which survives from the degradation due to MPO and catalase, and therefore do not reflect the 'true production' by NADPH oxidase activity.

In contrast, serum opsonic activity evaluated by LmCL was remarkably depressed in the presence of NaN_3 . Our results concerning the effects of NaN_3 on the LgCL and LmCL responses are in full agreement with experimental observations of other researchers.^(4,11,13-15) Based on these findings (the increase of LgCL and inhibition of LmCL in the presence of NaN_3), we attempted to evaluate serum opsonic activity with multiple assay conditions for the detection of O_2^- production (NADPH oxidase activity) and HOCl acid formation (MPO activity due to degranulation).

The serum opsonic activity promotes phagocytosis by adsorption of opsonins to the surface of particles such as bacteria and foreign material and is recognized by separate but complementary receptors on the neutrophil cell membrane. Two types of opsonins, one heat-labile and the other heat-stable, are present in the serum. It is generally accepted that the specific opsonins which are heat-stable are immunoglobulins (Fc moiety of IgG subclasses IgG₁ and IgG₃) and that the specific heat-labile opsonins are components of the complement (for example C3b, a degradation product of the third component of complement). C3 binding promotes efficient particle attachment while Fc binding triggers ingestion.⁽²⁾ These two systems generally act in a synergistic manner to prepare particles for ingestion.

In this work, the serum opsonic activity was measured by neutrophil-associated LmCL and LgCL responses in combination with NaN_3 . In LgCL, diode laser irradiation had no effects on serum opsonic activity and was correlated neither with output powers of GaAlAs diode laser irradiation nor with exposure time. On the other hand, in LmCL, the effects of LLLI on opsonic activity at different output powers were characterized by similar values, but marked higher values were observed at the highest laser dose tested (60mW

for 60 sec). Since this enhancement effect disappeared in the presence of an MPO inhibitor, it was suggested that LLLI at higher doses probably activates the opsonic activity in terms of enhanced degranulation of MPO from neutrophils (phagosome-lysosome fusion). Lower doses, however, which are generally applied for therapeutic purposes failed to produce any statistically significant effects. The mechanism for the suppression of whole blood chemiluminescence reported by Karu *et al.*⁽¹⁰⁾ remained, however, unclear and further investigations are necessary.

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