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Original Contribution

Noninvasive assessment of localized inflammatory responses

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ABSTRACT

Inflammatory diseases are associated with the accumulation of activated inflammatory cells, particularly polymorphonuclear neutrophils (PMNs), which release reactive oxygen species (ROS) to eradicate foreign bodies and microorganisms. To assess the location and extent of localized inflammatory responses, L-012, a highly sensitive chemiluminescent probe, was employed to noninvasively monitor the production of ROS. We found that L-012-associated chemiluminescence imaging can be used to identify and to quantify the extent of inflammatory responses. Furthermore, regardless of differences among animal models, there is a good linear relationship between chemiluminescence intensity and PMN numbers surrounding inflamed tissue. Depletion of PMNs substantially diminished L-012-associated chemiluminescence in vivo. Finally, L-012-associated chemiluminescence imaging was found to be a powerful tool for assessing implant-mediated inflammatory responses by measuring chemiluminescence intensity at the implantation sites. These results support the use of L-012 for monitoring the kinetics of inflammatory responses in vivo via the detection and quantification of ROS production.

The accumulation of inflammatory phagocytes in tissue is a hallmark of all inflammatory diseases, including atherosclerosis, arthritis, dermatitis, nephritis, ulcerative colitis, psoriasis, inflammatory bowel diseases, and many others [1,2]. It is generally believed that the extent of inflammatory cell accumulation in the wound or surrounding biomaterial implants reflects the degree of inflammatory reactions. However, the numbers of inflammatory cells in tissues are often hard to determine. One of the most common methods to diagnose inflammatory diseases is histological analyses of tissue biopsies, involving sectioning, staining, and microscopic evaluation. In addition to being invasive and unreliable, histological analyses are time consuming, require large numbers of animals, and can provide only semiquantitative assessment of inflammatory reactions at one time point [3–5]. Consequently, there is a need for the development of noninvasive methods to provide continuous and quantitative measurement of inflammatory responses in vivo.

Many cells, including macrophages/monocytes, polymorphonuclear neutrophils (PMNs), mast cells, lymphocytes, and dendritic cells, participate in the pathogenesis of inflammatory diseases [6,7]. Among all immune cells, PMNs are the most abundant type, arriving in large numbers at the injured tissue site only minutes after trauma or infection [8,9]. Therefore, histological evaluations of PMN accumulation in the tissue are often carried out to estimate the extent of acute inflammatory responses [10,11]. It is well documented that recruited activated PMNs activate the respiratory burst, releasing a variety of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hyperchlorous acid [12,13]. Release of ROS may result in oxidative killing of not only foreign microorganisms but also healthy cells [12,13]. The methods used to identify and quantify these ROS include spectrophotometrical measurements, electron spin resonance spectroscopy, ELISA, and chemiluminescence [14]. These methods are well established and have been used extensively to study the extent of ROS production by PMN in vitro. However, most of these methods cannot be used to measure the PMN-associated ROS responses in vivo. Most recently, there has been growing interest in the development of imaging methods to monitor ROS generation in vivo. Several imaging modalities have been established for ROS monitoring. These methods include electron paramagnetic resonance (EPR), fluorescence, and chemiluminescence detection [15–18]. The first two methods have been shown to have many limitations. Specifically, EPR has very low sensitivity [19]. In addition, the signal-to-noise ratio of fluorescent probes is often inadequate because of the autofluorescence generated by tissues and organs [20]. On the other hand, chemiluminescence has many advantages, including high sensitivity and specificity, easy quantitative analysis, and a wide dynamic range, as well as localization and quantification of the light emission at the single-photon level. Furthermore, without the

Abbreviations: PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4(2*H*,3*H*)dione; EPR, electron paramagnetic resonance; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; PMA, phorbol 12-myristate 13-acetate; c48/80, compound 48/80; LPS, lipopolysaccharide; DTPA, diethylenetriaminepentaacetic acid; PU, polyurethane; H-PU, heparin-bonded polyurethane; PLA, polylactic acid; PEG, poly(ethylene glycol); PNIPAM-NH_2, poly(*N*-isopropylacrylamide-co-*N*-(3-aminopropyl)methacrylamide); H&E, hematoxylin–eosin stain.

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requirement of excitation light as in fluorescence, chemiluminescence agents can emit detectable light upon reaction with ROS generated in the biological system with minimal or no background signal [21,22].

Recent developments in optical imaging equipment have made it possible to detect weak chemiluminescence signals in whole animals. Several chemiluminescence probes have been developed for noninvasive real-time imaging in vivo. For example, peroxalate nanoparticles were fabricated and used as a chemiluminescent probe to detect hydrogen peroxide in vivo [23,24]. Oxazine-conjugated nanoparticles have been synthesized and used to detect ex vivo hypochlorous acid and peroxynitrite generation in mouse hearts after myocardial infarction [17]. Luminol, a chemiluminescent probe, has been used in studies to detect myeloperoxidase activity in vivo [25]. Finally, luminol was also used to investigate the role of ROS in the pathogenesis of arthritis and to evaluate the effectiveness of various anti-inflammatory agents as well as to detect biomaterial-induced ROS in vivo [26,27]. Although these chemiluminescent probes (peroxalate nanoparticles and luminol) have shown great promise for in vivo monitoring of ROS activities after severe inflammatory responses, the limited sensitivity of these probes substantially hinders their use in measuring and quantifying localized inflammatory responses. Furthermore, little is known about the relationship between inflammation-mediated ROS production in vivo and histological measurements.

In an effort to improve these detection limits, we have applied a chemiluminescent probe, L-012 (8-amino-5-chloro-7-phenylpyridol [3,4-*d*]pyridazine-1,4(2*H*,3*H*)dione; a luminol derivative), which has much higher sensitivity toward ROS than other probes such as luminol and lucigenin [28,29]. A recent study has demonstrated that L-012 is a sensitive probe for in vivo visualization of ROS production in a model of endotoxin shock [18]. In the present study, a series of in vitro and in vivo experiments were carried out to determine the extent of ROS production. By correlating the ROS imaging results with histological analyses, we evaluated the feasibility of using ROS chemiluminescence to noninvasively monitor the in vivo kinetics of localized inflammatory reactions in real time. Finally, the ROS imaging method was tested for its ability to monitor in real time foreign body responses to various types of biomaterial implants in the same animal.

Materials and methods

Materials

L-012 was purchased from Wako Chemicals. Luminol, casein, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol), phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide, catalase, and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma–Aldrich. Polyurethane (PU) and heparin-bonded PU catheters were obtained from Sentry Medical Products (Green Bay, WI, USA). Polylactic acid (PLA) microparticles (5- to 10-μm diameter), poly(ethylene glycol) (PEG) nanoparticles (100-nm diameter), and amine-rich poly(*N*-isopropylacrylamide-co-*N*-(3-aminopropyl) methacrylamide) (PNIPAM-NH_2; 100-nm diameter) were prepared according to Refs. [30–32]. PMN neutralizing antibody (rabbit anti-mouse PMN) was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY, USA). PMN staining antibody (rat anti-mouse neutrophil antibody) was purchased from Abcam (Cambridge, MA, USA).

Quantification of ROS production by isolated PMNs

PMNs were isolated from mouse peritonea after casein administration as described earlier [33]. In vitro ROS measurements were carried out using both L-012 and luminol as chemiluminescent probes. Various numbers of PMNs (in Hanks' buffered solution) were incubated with L-012 (2 mM) or luminol (4 mM) for 4 min at room temperature. The ROS production was then initiated by adding 10 μl of PMA (6.5 nM). In some experiments, Tempol, a superoxide scavenger, was used to neutralize ROS products in solution. For that, cells were incubated with various concentrations of Tempol before PMA activation. In these in vitro studies, chemiluminescence intensities were recorded continuously for 60 min using a luminescence reader (Infinite M200; Tecan, Männedorf, Switzerland) with a 10-s acquisition time.

In vitro chemiluminescence imaging

In vitro imaging was carried out in black-bottom 96-well plates: 200 μl of H_2O_2 solution of various concentrations was mixed with 10 μl of L-012 (50 mM) [34]. The chemiluminescence images were taken 1 min later using a Kodak *In Vivo* FX Pro system (Kodak, USA) (f/stop, 2.5; no optical filter, 4×4 binning).

In vivo animal model and chemiluminescence imaging

Balb/c mice (female, 20–25 g body wt) were purchased from Taconic Farms (Germantown, NY, USA) and used in all in vivo studies. The animal protocols were approved by the Animal Studies Committee at the University of Texas at Arlington. All animal tests were carried out with six animals per group. To detect H_2O_2-mediated chemiluminescence activities, 200 μl of H_2O_2 solution (0.5 mM) and 100 μl of L-012 (15 mg/ml) were mixed at room temperature. Various volumes of solutions (20, 40, 60, and 80 μl) were injected subcutaneously into the back of anesthetized mice (ketamine/xylazine). Chemiluminescence images were captured with a 5-min acquisition time using a Kodak *In Vivo* FX Pro system (f/stop, 2.5; no optical filter, 4×4 binning) and then quantitated after background correction. Regions of interest were drawn over the implantation locations in the chemiluminescence images and the mean intensities for all pixels in the chemiluminescence images were calculated. All data analyses were performed with the Carestream Molecular Imaging software, network edition 4.5 (Carestream Health, Woodbridge, CT, USA).

Chemiluminescence imaging of localized inflammatory responses in vivo

To induce localized inflammatory responses, PLA microspheres (50 μl, 10% wt in saline), or saline as a control, were implanted into various locations on the back of mice, and then 100 μl of L-012 solution (15 mg/ml) was administered intraperitoneally at various time points. It should be noted that L-012 has very good biocompatibility and low toxicity [18]. Our preliminary experiments showed that L-012 has no apparent effect on the immune responses of animals up to 150 mg/kg (Supplementary Fig. 1). The nontoxic or low-toxicity nature of L-012 permits its use in cell culture study and animal work. Chemiluminescence images were captured sequentially every 5 min up to 1 h. Similar experiments were carried out using PMN-depleted mice, which were produced based on a modified published protocol [35]. In brief, 100 μl of PMN neutralizing antibody (rabbit anti-mouse PMN; Accurate Chemical & Scientific Corp.) was injected intraperitoneally (ip) and 18 h later a second injection of 100 μl antibody was given ip. Four hours after the second injection, 50 μl of PLA particles (10% wt/vol) were implanted subcutaneously on the back of the PMN-depleted mouse and a control mouse. The chemiluminescence images were taken 24 h after PLA implantation. To investigate the effects of either catalase (a scavenger of hydrogen peroxide) or DTPA (a metal chelator) on implant-mediated chemiluminescence [36–38], 50 μl of PLA particles (10% wt) were mixed with catalase (500 units/ml), DTPA (1 mM), or saline (as control) before subcutaneous implantation followed by chemiluminescence imaging after 24-h implantation as described above.