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***In Vivo* Bioluminescence Imaging of Inducible Nitric Oxide Synthase Gene Expression in Vascular Inflammation**

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Abstract

Purpose—Inflammation plays a critical role in atherosclerosis and is associated with upregulation of inducible nitric oxide synthase (iNOS). We studied bioluminescence imaging (BLI) to track iNOS gene expression in a murine model of vascular inflammation.

Procedures—Macrophage-rich vascular lesions were induced by carotid ligation plus high-fat diet and streptozotocin-induced diabetes in 18 *iNOS-luc* reporter mice. *In vivo* iNOS expression was imaged serially by BLI over 14 days, followed by *in situ* BLI and histology.

Results—BLI signal from ligated carotids increased over 14 days ($9.7 \pm 4.4 \times 10^3$ vs. $4.4 \pm 1.7 \times 10^3$ photons/s/cm²/sr at baseline, $p < 0.001$ vs. baseline, $p < 0.05$ vs. sham controls). Histology confirmed substantial macrophage infiltration, with iNOS and luciferase expression, only in ligated left carotid arteries and not controls.

Conclusions—BLI allows *in vivo* detection of iNOS expression in murine carotid lesions and may provide a valuable approach for monitoring vascular gene expression and inflammation in small animal models.

Keywords

Vascular inflammation; Inducible nitric oxide synthase; Bioluminescence; Macrophages; Atherosclerosis

Accumulating evidence has established that vascular inflammation plays an important role in atherosclerosis [1,2]. Inflammation is involved not only in the initiation of atherosclerosis but also its progression and complications. Thus, visualizing active inflammation within the vessel wall may help to identify atherosclerosis at an early stage as well as characterize disease activity and risk.

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This work is significant for demonstrating serial *in vivo* imaging of iNOS expression in vascular inflammation.

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Nitric oxide (NO) is thought to be a key regulator in the development of atherosclerosis. NO is synthesized from L-arginine by three NO synthase (NOS) isoforms—one inducible form (iNOS) and two constitutive-type isoforms: neuronal (nNOS) and endothelial (eNOS) enzymes. iNOS is barely detectable in the normal vasculature, but it is abundantly expressed in atherosclerosis, primarily by macrophages [3]. Previous studies show that iNOS expression is associated with advanced atherosclerosis and instability of atherosclerotic plaques on histology [4,5]. Therefore, visualizing iNOS expression *in vivo* may provide an important approach to detect and follow vascular inflammation and monitor the effects of therapeutic interventions.

In vivo bioluminescence imaging (BLI) is a sensitive technique for small animal molecular imaging. It allows for real-time assessment of gene induction *in vivo* in models where luciferase expression is controlled by promoter elements of specific genes [6], including iNOS [7]. In this study, we investigated BLI for monitoring iNOS expression in a murine model of vascular inflammation.

Methods

iNOS-luc Mice

The iNOS promoter-luciferase transgenic mouse (*iNOS-luc*; a Xenogen product from Caliper Life Sciences [7]) was used ($n=18$, 8–12 weeks old). This has a white coat on the FVB/N background and has been shown to have high BLI signal from activated macrophages *in vitro* and in a model of arthritis *in vivo* [7].

Macrophage-Rich Vascular Lesion

To induce a macrophage-rich vascular lesion on the FVB/N background, animals were fed a high-fat diet, containing 40% kcal fat, 1.25% (by weight) cholesterol and 0.5% (by weight) sodium cholate (D12109, Research Diets, Inc. New Brunswick, NJ, USA; Fig. 1) [8]. After 4 weeks of high-fat diet, mice were rendered diabetic by administration of five daily intraperitoneal injections of streptozotocin (STZ), 40 mg/kg in citrate buffers (0.05 mol/L, pH 4.5, Sigma Aldrich) [9]. STZ was given for an additional 3 days if the day 5 serum glucose level was below 200 mg/dL. At day 14, after initiation of STZ injection, the left common carotid artery was ligated ($n=14$) below the bifurcation with the use of 5–0 silk ligature (Ethicon) under 2% inhaled isoflurane, as previously described [10]. In sham-operated mice ($n=4$), the suture was passed around the exposed left carotid artery but not tightened. The wound was closed by suture and the animals were recovered on a warming blanket. Animals were followed by serial BLI up to 14 days after ligation. All procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

In Vivo and In Situ Bioluminescence Imaging

BLI was performed serially at days 0 (pre), 3, 5, 7, 10, and 14, as previously described [6,11]. Briefly, animals were anesthetized (2% inhaled isoflurane) and luciferin (10 $\mu\text{g/g}$) was administered intraperitoneally. Seven to ten minutes later, anesthetized animals were imaged for 5 min using an IVIS200 charge-coupled device imaging system (Xenogen Corp. part of Caliper Life Sciences, USA). At day 14, *in situ* BLI was also performed: mice were injected with an additional dose of luciferin (10 $\mu\text{g/g}$, intraperitoneally), then euthanized, and both carotid arteries rapidly exposed and imaged for 5 min beginning at 7–10 min after luciferin injection. Imaging data were analyzed by LivingImage software (Xenogen Corporation, USA) and IgorPro software (WaveMetrics, USA). For quantitative analysis, a region of interest over the left common carotid artery was outlined and the total number of photons in the region was measured, expressed as photons(p)/s/cm²/sr.[11]

Tissue Processing, Histology, and Immunohistochemistry

After *in situ* BLI on day 14, animals were perfusion fixed with 10% phosphate-buffered formalin at physiological pressure. Both right and left common carotid arteries were removed with the heart and further fixed in 10% formalin for 24 h. The left common carotid artery from 1 to 4 mm below the ligation was removed, cut into three portions (1-mm intervals), and embedded into paraffin for serial sectioning (10 μ m). The right common carotid artery, as an internal control, was processed similarly. Macrophages were immunostained by rat anti-mouse Mac-3 monoclonal antibody (#550292, BD Pharmingen). iNOS expression was immunostained by rabbit polyclonal antibody against iNOS (sc-651, Santa Cruz Biotechnology, Inc. CA, USA). Luciferase was immunostained by rabbit polyclonal antibody against luciferase (sc-32896, Santa Cruz Biotechnology, Inc. CA, USA).

Statistical Analysis

Data are expressed as mean $\pm$ SD. One-way repeated measures ANOVA with Fisher's post-hoc test was used for analyzing the serial BLI data of iNOS expression. Two-way factorial ANOVA with Fisher's post-hoc test was used to compare BLI data between ligated and sham-operated mice. $P<0.05$ was considered to be statistically significant.

Results

Macrophage-Rich Vascular Lesions in iNOS-luc Mice

The ligated left common carotid arteries demonstrated macrophage-rich lesions proximal to the suture (Fig. 2), which were not evident in the sham or contralateral (non-ligated) controls. Immunohistochemistry in ligated arteries showed substantial macrophage infiltration of both the neointima and adjacent to the adventitia with increased iNOS expression in the same regions, in contrast to only a small number of adventitial macrophages and no significant iNOS expression in the sham controls. Immunohistochemistry of luciferase (Fig. 3) confirmed a similar pattern of *iNOS-luc* gene expression in the ligated carotid but not in control.

In Vivo Detection of Increased iNOS Expression by Bioluminescence Imaging

The BLI signal from the neck of ligated mice localized to the left common carotid artery (Fig. 4a), which was further confirmed by *in situ* BLI at day 14 (Fig. 4b). Sham-operated mice had only transient BLI signal noted over the midline suture (Fig. 4c). Quantitative BLI analysis (Fig. 5) showed increasing BLI signal from the ligated mice over the 14 days compared to pre-ligation (pre: $4.4\pm1.7\times10^3$ p/s/cm²/sr; day 5: $7.6\pm2.8\times10^3$ p/s/cm²/sr, $p=0.0007$; day 14: $9.7\pm4.4\times10^3$ p/s/cm²/sr, $p<0.0001$). Sham-operated mice (Fig. 5b), in contrast, showed a transient increase in BLI signal peaking at day 5 ($5.8\pm1.5\times10^3$ p/s/cm²/sr, $p=0.02$ vs. pre), then returning to baseline (day 7: $4.6\pm0.2\times10^3$ p/s/cm²/sr, $p=0.4$ vs. pre). Comparing ligated to sham-operated mice, BLI signal was significantly higher in ligated arteries after day 7 ($p<0.05$, Fig. 5).

Discussion

Our primary novel finding is the ability to serially image upregulation of iNOS gene expression, *in vivo*, in vascular inflammation. BLI showed increasing iNOS gene expression over 2 weeks in murine macrophage-rich carotid lesions. We also present a method for inducing vascular inflammation in FVB mice.

Imaging Inflammation in Atherosclerosis by In Vivo BLI

Molecular imaging of vascular inflammation in atherosclerosis has been performed previously *in vivo* using single-photon emission computed tomography [12], fluorescence imaging [13], positron emission tomography [14], MRI [15], and CT [16]. To our knowledge, BLI of vascular inflammation has not been shown previously. *In vivo* BLI has several advantages [6]: it does not need external light excitation, which offers a high signal to background ratio; its rapid chemical reaction to generate light allows for real-time detection of biological processes; and it can provide a quantitative measure.

The Role of iNOS in Atherosclerosis

iNOS is induced by a variety of inflammatory cytokines and has been shown to be abundantly expressed by macrophages in atherosclerotic lesions [5]. Several studies have shown that reducing iNOS expression in animal models reduces atherosclerosis [17,18]. Based on these and other data, it is thought that upregulation of iNOS in vascular macrophages plays an important role in the progression of atherosclerosis [19]. In the present study, we have demonstrated serial increase in vascular iNOS expression with the development of a macrophage-rich carotid lesion. Further studies are needed to evaluate *in vivo* BLI for monitoring the responses to therapeutic interventions.

Macrophage-Rich Atherosclerotic Lesions in iNOS-luc Mice with Diabetes and High-Fat Diet

There are strain-dependent differences in the vascular response to injury [20]. The white coat of FVB mice is advantageous for BLI and many existing transgenic *luc* mice are on the FVB background. However, this strain typically develops proliferative lesions in response to carotid ligation without significant macrophage infiltration [20,21]. Several researchers reported that STZ-induced diabetes can accelerate atherosclerosis in mice mainly through the augmented inflammatory reaction [22,23]. The combination of high-fat diet and STZ-induced diabetes successfully generated macrophage-rich carotid-ligation lesions in FVB mice, broadening the available murine models of vascular inflammation.

Study Limitations

The model of vascular inflammation we used develops much more rapidly than human atherosclerosis. While this is useful as a preclinical model, it does not fully replicate the complex lesions of human atherosclerotic disease. Our data show an increase in iNOS expression by BLI and histology over 14 days, but further studies of BLI over a longer time course and the response to interventions would be valuable. The effects of age on iNOS expression and this vascular inflammation model also warrant further investigation.

BLI itself has several limitations. It does not directly image iNOS, rather the transcriptional activation of the iNOS promoter. Thus, the BLI signal would be expected to correlate with the number of macrophages and their degree of iNOS activation. It is primarily a 2D projection imaging technique, however, without depth information and with decreased sensitivity to detect deeper structures, which could affect the BLI signal independent of vascular macrophage volume. Newly developed CCD cameras that can reconstruct 3D views or fluorescence molecular tomography systems may be helpful. Furthermore, the limited penetration hinders the use of BLI for human application. However, as shown in this study, it does provide a rapid, high-throughput method to study disease *in vivo* over time in relevant pre-clinical models.

Conclusions

BLI can image increased iNOS gene expression *in vivo* in murine vascular inflammation. BLI has the potential to noninvasively monitor gene expression and inflammation in experimental atherosclerosis, which may aid in the development and evaluation of novel therapies.

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Fig. 1.

Experimental protocol. After 4 weeks of high-fat diet, diabetes was induced by intraperitoneal injections of streptozotocin (STZ). Two weeks later, ligation of the left common carotid artery was performed. Serial bioluminescence imaging (BLI) was performed out to 14 days after ligation.

Fig. 2. Colocalization of macrophage infiltration and iNOS expression. Immunohistochemistry showed positive (*brown*) staining for macrophages in both the neointima (*I*) and adjacent to the adventitia (*A*), but not the inner media (*M*), of ligated (**a**) compared to sham-operated controls (**c**). Moreover, enhanced iNOS expression (in *light brown*) appears to colocalize to the macrophage-rich areas in ligated arteries (**b**), not evident in controls (**d**).

Fig. 3. Luciferase staining of carotid arteries. Immunohistochemistry also luciferase staining (*brown*) of the neointima (*I*) and adjacent to the adventitia (*A*) with relative sparing of the inner media (*M*), in the ligated left carotid artery (**a**) but not the contralateral control right carotid artery (**b**).

Fig. 4.

Serial BLI for monitoring iNOS expression *in vivo*. Serial BLI of ligated (a) and sham-operated (c) *iNOS-luc* mice up to day 14. In ligated mice, there was a gradual increase in signal from the left (ligated) side of the neck. At day 14, both left and right carotid arteries were exposed, confirming *in situ* that the light signal was coming from the ligated left carotid artery (b, white arrows indicate the location of the ligated left carotid artery). In contrast, sham-operated mice had only a small signal at the midline suture site, which disappeared by day 14 (c), further confirmed *in situ* (d). Note the scale for *in vivo* BLI is lower than *in situ*, reflecting reduced signal penetration.

Fig. 5. Quantitative analysis of *in vivo* serial BLI. *In vivo* BLI signal in the ligated mice increased significantly compared to pre-ligation by day 5, increasing further by day 14. There was only transient increase in signal in the sham-operated controls, which returned to baseline by day 14. Error bars indicate standard deviations.