Indocyanine Green and Lymphatic Imaging: Current Problems

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Abstract

Background: Near-infrared (NIR) fluorescence imaging using indocyanine green (ICG) has recently been presented as a comparatively easy and informative technique to image lymphatic channels *in vivo*. However, no data or references have been provided concerning the impact of ICG application on normal lymphatic contractility and lymph transport. Thus, the imaging agent and/or the method of administration may introduce a significant artifact. **Methods and Results:** Standard pharmacological tests were performed to investigate the influence of ICG on the spontaneous contractility of isolated, cannulated, and pressurized rat mesenteric lymphatic vessels. The data demonstrate that non-irradiated ICG dramatically and dynamically influences the contractility of rat lymphatic vessels in both a dose- and diluent-dependent manner with low ICG concentrations principally altering contractile frequency and higher ICG concentrations completely blocking lymphatic contractility. **Conclusions:** Currently, both researchers and doctors should exercise caution in extrapolating the data obtained with ICG imaging to normal lymphatic function regardless of whether it was obtained in mice, pigs, or humans. Careful and extended pharmacological tests must be performed to evaluate the mechanism of action of ICG on the contractility and physiology of lymphatic vessels with consideration of dose, diluent, and duration of irradiation.

Introduction

SEVERAL RECENT PUBLICATIONS HAVE described near-infrared (NIR) fluorescence imaging using indocyanine green (ICG) as a comparatively easy and informative technique to image lymphatic channels *in vivo*.^{1–4} This topic was widely discussed at several lymphatic conferences, including the Gordon Conference “Molecular Mechanisms In Lymphatic Function & Disease” in 2006 and 2008 and the NIDDK/NIH workshop “Lymphatics in the Digestive System: Physiology, Health and Disease”, November 3–4, 2009, Bethesda, MD. In the December 2007 issue of *Lymphatic Research and Biology*, Kwon and Sevic—Muraca claimed that they developed a “new method to noninvasively and quantitatively reveal the mechanism for intrinsic lymph pumping, generated by the spontaneous contractions/relaxations of lymphatic smooth muscle” in mice.² Briefly, this study used intradermal injections of ICG in mice to perform subsequent visualization of mouse lymphatic vessels using the NIR technique. The authors stated that they found pulsive

lymph flow in mice and concluded that it is “likely related to the active propagation of lymphatic smooth muscle contraction between lymphatic valves along the channel length.”² The authors further concluded that “their ability to image contractile motion in mice provides new opportunities to (1) assess lymphatic function in transgenic mice models to better understand the role that specific gene expression has on lymphatic function, and (2) to investigate pharmacologic agents that stimulate the formation of functional lymphatics as well as stimulate the contractile apparatus of existing lymphatics.”³ While the promise of such a tool would be indisputable in both research and clinical settings, we, after careful review, have identified several significant questions and concerns surrounding these data which have lead us to believe that the conclusions cited above are, at a minimum, too preliminary. We have already expressed some of our concerns about this technique while discussing the utility of genetically modified mouse models to investigate the regulatory mechanisms of lymphatic contractility.⁵

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This work was supported in part by Texas A&M Health Science Center College of Medicine and the Department of Systems Biology and Translational Medicine, and by the National Institutes of Health (NIH RO1 AG030578).

First, the authors attempt to attribute the presented lymph transport to normal phasic contractions of the collecting lymphatic vessels; however, the presented technique does not offer sufficient resolution to accurately monitor lymphatic vessel diameter and therefore to perform quantitative analysis of lymphatic pumping. Furthermore, the presented lymphatic motility, particularly in the mouse,² is not necessarily consistent with the phasic contractile activity documented in most species and described by experts in the field. On the contrary, some of the behaviors of the mouse lymphatic vessels presented in Ref. 2 are more indicative of single groups of slow contractions of potentially overdilated lymphatic vessels. Second, the authors did not provide any literature and/or experimental information on how ICG can influence the rate of lymph formation and/or the extent to which the injection volume disrupts and/or exceeds the ranges of physiological interstitial fluid pressures (a documented potent stimulus of lymph formation and, subsequently, collecting lymphatic contractility⁶⁻⁹). Finally, the authors have presented no data or references concerning the impact of ICG application on normal lymphatic contractility and lymph transport. Thus, the imaging agent and/or the method of administration may introduce a significant artifact that precludes the use of this technique to observe "normal" phasic lymphatic contractility or "normal" lymph transport, particularly in the mouse where the volume and concentration of dye are significantly large relative to the studied tissue.

Because the development of viable *in vivo* imaging tools is invaluable to furthering our knowledge of lymphatic biology, we began evaluating *in vivo* lymphatic imaging methodologies in our own laboratory and, given the potential benefits of NIR imaging with ICG, attempted to examine some of the issues presented above. We performed multiple pharmacological tests to investigate the influence of ICG on the spontaneous contractility of isolated, cannulated, and pressurized rat mesenteric lymphatic vessels. As a result, we have acquired data that seriously question the value of this technique in the research and clinical settings.

Materials and Methods

The contractile activity of rat mesenteric lymphatic vessels was evaluated using an isobaric isolated vessel preparation that has been described numerous times in our publications.^{5,9-13} Utilizing male Sprague-Dawley rats (weighing between 250 and 350 g) that underwent vessel isolation for our ongoing research projects using protocols and procedures approved by our Institutional Animal Care and Use Committee, we isolated additional segments of mesenteric lymphatic vessels to test the influence of non-irradiated ICG on the lymphatic contractile behavior. Briefly, the rat mesenteric lymphatic vessels were isolated and carefully cannulated onto diameter- and resistance-matched glass pipettes. Lymphatic vessel diameter was digitally recorded using established video microscopy techniques to monitor contractile function.^{5,9-11,13} Indocyanine Green was purchased from Akorn Inc. (Lake Forest, IL) (catalog # NDC-17478-701-25) as part of the ICG kit (catalog # NDC-17478-70102). ICG was perfused through isolated lymphatic vessels at a transmural pressure of 5 cm H₂O and an imposed flow of 5 cm H₂O (a pressure/imposed flow profile for which we have performed numerous control and pharmacological treatment studies.⁹⁻¹¹ The presence of

imposed flow allowed passive passage of ICG through the lymphatic vessel without the influence of syringes or pumps, therefore eliminating potential overdilation of the lymphatic vessel segment. ICG was initially diluted in distilled water as recommended by the manufacturer and then subsequently diluted to the experimental concentration using albumin-containing physiological salt solution (APSS), which is usually used for our isolated lymphatic vessel experiments (in mM: 145.0 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5.0 dextrose, 2.0 sodium pyruvate, 0.02 EDTA, 3.0 MOPS, and 10 g/l bovine serum albumin; pH adjusted to 7.36 at 38°C). APSS is designed to mimic interstitial fluid and is thus an ideal diluent to mimic the dilution of ICG by interstitial fluid upon subcutaneous administration as described in the currently discussed publications.¹⁻⁴ The extent to which ICG is diluted upon interstitial administration and subsequent lymphatic uptake is currently unknown, and thus, it is not possible for us to estimate the exact concentration present in the lymphatic vessels during the authors' observations. We thus selected for our studies a range of concentrations of ICG that represented a 1- to >40-fold dilution from the concentration of 1.3 mM administered to the mouse interstitium in Reference 2. Such ICG concentrations were used as injected concentrations in studies described in References 1-4.

Results

We found that APSS-diluted, non-NIR-irradiated ICG alters frequency of lymphatic contractions predominantly. At a concentration of 32 μ M ($n=4$) ICG diminished lymphatic contraction frequency an average of 30% below control with the maximal negative chronotropic effect observed after approximately 7 min. At the same time, the contraction amplitude fell 10% while lymphatic tone (i.e., diastolic diameter) remained unchanged. At a 10 times higher concentration of ICG (320 μ M; $n=6$) contraction frequency surprisingly increased to 50% above control over the course of approximately 5 min. At the same time, the contraction amplitude fell 5% and lymphatic tone again remained unchanged. A further increase in ICG concentration to 1.3 mM (as used in Ref. 2) induced profound inhibition of lymphatic contractility. Contraction frequency progressively declined and reached complete inhibition after an average of 8 min ($n=5$). Vessels were slightly relaxed (3% increase in diastolic diameter). This inhibition was reversible in 3 of the 5 vessels with normal contractions resuming 20-30 min after washout; however, the two remaining vessels failed to regain contractility after 30 min of APSS washout. It is interesting to note that in all cases, the vessels retained visible green ICG coloration through at least 10 min of washout indicating that ICG was indeed binding to the lymphatic endothelial cell membrane.

For a subset of experiments, we also diluted our stock solution of ICG to its final concentration in normal saline (0.9% NaCl) as described in References 1-4. We found that perfusion of saline-diluted ICG at a concentration of 32 μ M resulted in a progressive increase of both contraction frequency and amplitude over the course of 5 min (40% and 15%, respectively). Subsequent increase in saline-diluted ICG concentration to 320 μ M (after washout by APSS) induced inhibition of contraction frequency to 55% below control after 4 min of ICG perfusion, while contraction amplitude increased 18%. Perfusion of 1.3 mM saline-diluted ICG induced progressive

inhibition of lymphatic contractions with complete cessation occurring after 8 min. Lymphatic tone remained unchanged in all cases.

Discussion

We conclude that non-irradiated ICG dramatically and dynamically influences the contractility of rat lymphatic vessels in both a dose- and diluent-dependent manner, with low ICG concentrations principally altering contractile frequency and higher ICG concentrations completely blocking lymphatic contractility. Because the exact concentration and diluent composition for ICG present in lymphatic vessels *in vivo* cannot be determined or estimated from the present publications,^{1–4} it is impossible to specifically translate the results of our studies to the previously published *in vivo* observations. Thus, it is impossible to determine the extent to which the imaging agent disturbed normal lymphatic contractility and lymph transport in these studies.^{1–4} In this respect it is important to note that lymphatic vessels exhibit high levels of sensitivity to biologically active substances—sometime even in nanomolar ranges (e.g., see Ref. 14). Additionally, the influences of ICG irradiated by fluorescent excitation source on lymphatic contractility remain unknown and may further complicate the interpretation of the published *in vivo* data. It is well known that near-infrared fluorophores can distribute in the body freely, including in the intracellular space.¹⁵ In particular, the extremely cumulative cellular uptake of ICG and its intensive laser-induced photo-oxidation have been demonstrated.¹⁶ Even within 1 h after ICG-mediated phototherapy, this publication demonstrated evidence of cytoplasmic vesiculation, dilation of the endoplasmic reticulum, Golgi complex, and perinuclear cisternae, and the beginning of chromatin condensation in the nucleus. While the laser irradiation in studies by Abels et al.¹⁶ was more intense than that used in the presently discussed work,² these additional points raise further concern about the utility of ICG to study normal lymphatic functioning. It is not clear whether excitation of ICG would ameliorate or aggravate the abnormal functioning we observed in our studies.

We would like to acknowledge that the authors of the presently discussed NIR lymphatic imaging publications have expressed criticism of the established technique we used here to evaluate the effects of ICG on lymphatic contractility. They state that “whereas these *in vitro/ex vivo* studies may reflect physiological lymph function, this invasive method may not recapitulate “normal” functioning lymphatics, creating variable physiological factors and the relative obstruction due to the preparation model.”² While their concern is not without merit, we must note that, with the isolated rat mesenteric lymphatic vessel preparation in particular, the lymphatic contractile behavior observed in these *in vitro/ex vivo* studies (e.g., isobaric, isolated vessel preparations) exhibits excellent correlation with the contractile behavior observed in these vessels *in vivo* using the well-established mesenteric microcirculation preparation.^{8,14,17–19} While this *in vivo* preparation does require incision of the abdominal wall, it is important to note that the mesenteric microcirculation preparation does not disturb the tissue bed of interest, its blood supply, nor its lymphatic drainage. Furthermore, the mesenteric tissue remains submerged in a fluid designed to recapitulate interstitial fluid and there are no alterations in

interstitial fluid pressures due to the experimental manipulation. We thus feel confident that the ICG-mediated alterations in lymphatic function observed in our isolated, isobaric lymphatic vessel preparation would be recapitulated *in vivo* with similar magnitude and influence. It is, therefore, reasonable to conclude that, currently, ICG as a lymphatic imaging agent cannot be used to study “normal” lymphatic function.

As a final remark, we want to underline the following. Our present data indicate a profound dose- and diluent-dependent influence of ICG on lymphatic contractions that would directly translate to alterations in lymph transport *in vivo*. These data do not suggest terminating the usage of ICG for NIR imaging of lymphatic vessels in the clinical or research setting; however, any current or emerging conclusions about mechanisms of lymphatic function based upon data obtained with this technique must be cautiously reevaluated, with consideration for artifacts potentially introduced through the use of ICG. We understand that this technique may provide tremendously useful information about the dissemination of metastatic cancer cells and the general ability of lymphatic vessels to support lymph flow in different parts of body affected by lymphatic-related pathologies. However, we believe that both researchers and doctors should use extreme caution in extrapolating the data obtained with ICG imaging to normal lymphatic function regardless of whether it was obtained in mice, pigs, or humans. Careful and extended pharmacological tests must be performed to evaluate the mechanism of action of ICG on the contractility and physiology of lymphatic vessels with consideration of dose, diluent, and duration of irradiation. Additionally, adequate procedures and/or analyses to compensate for these influences of ICG must be developed. We hope that part of the research funds recently allocated for development of ICG-related NIR lymphatic imaging soon will be used for research tests mentioned above with a goal of eliminating current problems with this application.

Author Disclosure Statement

No competing financial interests exist.

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