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● *Original Contribution*

QUANTITATIVE ULTRASOUND MOLECULAR IMAGING

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Abstract—Ultrasound molecular imaging using targeting microbubbles is predominantly a semi-quantitative tool, thus limiting its potential diagnostic power and clinical applications. In the work described here, we developed a novel method for acoustic quantification of molecular expression. E-Selectin expression in the mouse heart was induced by lipopolysaccharide. Real-time ultrasound imaging of E-selectin expression in the heart was performed using E-selectin-targeting microbubbles and a clinical ultrasound scanner in contrast pulse sequencing mode at 14 MHz, with a mechanical index of 0.22–0.26. The level of E-selectin expression was quantified using a novel time–signal intensity curve analytical method based on bubble elimination, which consisted of curve-fitting the bi-exponential equation $I_{\text{tissue}}(t) = A_{\text{f}}e^{-\lambda_{\text{f}}t} + A_{\text{r}}e^{-\lambda_{\text{r}}t}$ to the elimination phase of the myocardial time–signal intensity curve. A_{r} and A_{f} represent the maximum signal intensities of the retained and freely circulating bubbles in the myocardium, respectively; λ_{r} and λ_{f} represent the elimination rate constants of the retained and freely circulating bubbles in the myocardium, respectively. A_{r} correlated strongly with the level of E-selectin expression ($|r|>0.8$), determined using reverse transcriptase real-time quantitative polymerase chain reaction, and the duration of post-lipopolysaccharide treatment—both linearly related to cell surface E-selectin protein (actual bubble target) concentration in the expression range imaged. Compared with a conventional acoustic quantification method (which used retained bubble signal intensity at 20 min post-bubble injection), this new approach exhibited greater dynamic range and sensitivity and was able to simultaneously quantify other useful characteristics (*e.g.*, the microbubble half-life). In conclusion, quantitative determination of the level of molecular expression is feasible acoustically using a time–signal intensity curve analytical method based on bubble elimination. (E-mail: p.nihoyannopoulos@imperial.ac.uk) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Molecular imaging, Targeted microbubbles, Contrast agent, Contrast echocardiography, Echocardiography, Ultrasound imaging, Quantification, Time–signal intensity curve, Microbubble elimination, E-Selectin.**

INTRODUCTION

Ultrasound molecular imaging has been achieved using echogenic microbubbles targeting molecules of interest (Lindner et al. 2001). After intravenous administration, the targeting bubbles circulate and accumulate in regions expressing the molecules of interest, depicted on ultrasound images as areas of bright signals localizing the molecules. This technique has allowed ultrasound molecular imaging of pathophysiological processes such as inflammation, angiogenesis and thrombosis (Yeh 2010), indicating its potential for clinical applications.

The imaging technique, however, remains predominantly a semi-quantitative tool. Acoustic quantification has been assessed against independent (non-acoustic) methods, such as semi-quantitative immunohistochemistry and fluorescence immunohistochemistry (Behm et al. 2008; Kaufmann et al. 2007b; Korpanty et al. 2007; Lee et al. 2008; Leong-Poi et al. 2005; Liu et al. 2011; Mancini et al. 2013; Palmowski et al. 2008, 2009; Weller et al. 2003; Xie et al. 2011), illustrating at best the semi-quantitative capability of the imaging technique. Only a handful of studies have used highly quantitative independent assays to assess acoustic quantification; these included quantitative radioactive assay (Bin et al. 2008), quantitative

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fluorescence imaging (Saini et al. 2013), quantitative chemiluminescence immunoblotting (Deshpande et al. 2011; Lyshchik et al. 2007) and quantitative fluorescence immunohistochemistry (Bachawal et al. 2013). However, the degree of correlation between the acoustic and non-acoustic methods varied from moderate to strong (Bachawal et al. 2013; Bin et al. 2008; Deshpande et al. 2011) or was not calculated (Korpanty et al. 2007; Lyshchik et al. 2007; Saini et al. 2013). Surprisingly, none provided scatterplots that could be used to examine the correlations claimed (Bachawal et al. 2013; Bin et al. 2008; Deshpande et al. 2011; Korpanty et al. 2007; Lyshchik et al. 2007; Saini et al. 2013).

The conventional method of acoustic quantification of molecular expression in a tissue is based on the signal intensity of target-bound (retained) bubbles in the tissue at one time point after bubble administration, when the bubbles have attached to the molecular targets and unbound (freely circulating) bubbles have sufficiently (Lindner et al. 2000; Rychak et al. 2007; Stieger et al. 2008) or almost completely (Hernot et al. 2012; Tlaxca et al. 2013) cleared from the blood pool. Where the freely circulating bubble signal in the tissue remains significant at the time point chosen (more likely at earlier time points or with larger bubble dosages), it can be removed by subtraction to obtain the retained bubble-only signal (Lindner et al. 2000; Rychak et al. 2007; Stieger et al. 2008). This requires the use of high-power ultrasound that destroys all (retained plus freely circulating) bubbles in the acoustic field, followed by further imaging to obtain the freely circulating bubble-only signal as circulating bubbles re-fill the acoustic field. The subtraction step is not mandatory if the residual circulating bubble signal is minimal. Bubble signals can be obtained using bubble-specific or non-bubble specific imaging modes at high or low acoustic powers (Lindner et al. 2000; Rychak et al. 2007; Stieger et al. 2008). Although the conventional quantification method is widely used, it has not been formally validated. Limitations of the method include the arbitrary nature of the time point chosen for signal analysis, which varies widely in practice (ranging from 2 to 15 min post-bubble administration), yielding different values in the same subject. Furthermore, based on one single time point analysis, it is prone to error. Other quantification methods have been described; most are based on analysis of the time–signal intensity curve (TIC) (Behm et al. 2008; Carr et al. 2011; Chen et al. 2012; Fisher et al. 2002; Lindner et al. 1998; Sirsi et al. 2012). However, they remain untested for molecular quantification. Furthermore, they often require curve-fitting the entire TIC, which makes them prone to errors caused by signal saturation/bubble cloud attenuation in the early time points of the TIC, where bubble concentrations are high.

As the level of molecular expression may reflect the state, type, prognosis or response to therapy of a disease, the ability of the imaging technique to quantitatively measure the level of molecular expression would increase its potential diagnostic power and breadth of clinical applications. In this study, we developed and tested a novel TIC-based method for acoustic quantification of molecular expression that does not require curve fitting the entire TIC.

METHODS

Antibodies

MES-1 monoclonal antibody (mAb), a rat IgG2a,κ against mouse Esel (Reynolds et al. 2006) and its $F(ab')_2$ fragments were provided by D. Brown (UCB Celltech, UK). Reduced MES-1 $F(ab')_2$ (containing two thiol groups per $F(ab')_2$ from tris(2-carboxyethyl)phosphine hydrochloride reduction) was prepared as described in the Appendix. MEC13.3 mAb, a rat IgG2a,κ against mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) (BD Biosciences, UK), rat IgG2a,κ isotype-negative control mAb (BD Biosciences) and biotinylated rabbit mAb against rat IgG2a (Vector Laboratories, UK) were purchased.

Animals

Wild-type (WT) mice were adult male C57BL/6J (Charles River, UK). Esel knockout (KO) mice were adult male Esel homozygote KO on C57BL/6J background (Labow et al. 1994), bred locally from mice donated by K. Norman and P. Hellewell (University of Sheffield, UK). All animal work was carried out under licences granted by the Home Office under the Animals (Scientific Procedures) Act 1986, with ethical approval obtained from Imperial College London's Ethical Review Panel.

Mouse model of lipopolysaccharide-induced inflammation (experimental endotoxemia)

Wild-type and Esel KO mice were treated with 50 μg lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, UK), made up to a 200-μL volume in normal saline, by intraperitoneal injection to induce systemic inflammation (Eppihimer et al. 1996).

Immunohistochemistry

Immunohistochemistry was performed on acetone-fixed cryosections of freshly harvested hearts of WT (with or without LPS pre-treatment) and Esel KO (LPS pre-treated) mice, using a standard protocol detailed in the Appendix. The primary antibodies used were MES-1 (for Esel), MEC13.3 (for PECAM-1, an