Increasing the Endothelial Layer Permeability Through Ultrasound-Activated Microbubbles

Klazina Kooiman*, *Student Member, IEEE*, Marcia Emmer, Miranda Foppen-Harteveld, Annemieke van Wamel, *Member, IEEE*, and Nico de Jong, *Associate Member, IEEE*

***Abstract*—Drug delivery to a diseased tissue will be more efficient if the vascular endothelial permeability is increased. Recent studies have shown that the permeability of single cell membranes is increased by ultrasound in combination with contrast agents. It is not known whether this combination can also increase the permeability of an endothelial layer in the absence of cell damage. To investigate the feasibility of controlled increased endothelial layer permeability, we treated monolayers of human umbilical vein endothelial cells with ultrasound and the contrast agent BR14. Barrier function was assessed by measuring transendothelial electrical resistance (TEER). Ultrasound-activated BR14 significantly decreased TEER by $40.3\% \pm 3.7\%$ ($p < 0.01$). After treatment, no cell detachment or damage was observed. In conclusion, ultrasound-activated BR14 microbubbles increased the endothelial layer permeability. This feature can be used for future ultrasound-guided drug delivery systems.**

***Index Terms*—Endothelial layer, transendothelial electrical resistance (TEER), ultrasound contrast agent.**

I. Introduction

THE VASCULAR endothelium maintains the barrier between the blood and the extravascular tissues. It does not form a passive barrier, but actively controls the extravasation of fluids, solutes, and cells into the surrounding tissues [1], [2]. Its integrity depends largely upon the presence of specialized junctions between adjacent endothelial cells [3]. On the other hand, the endothelial layer forms a major barrier for drug delivery into the extravascular tissue. A local increase in permeability of the endothelial layer may have important clinical implications as more efficient delivery of drugs to a diseased tissue would be expected [1]–[3]. To enhance drug delivery to extravascular tissues, a controlled, temporal, and local increase in endothelial layer permeability is needed. At the same time, transient opening of the vascular barrier should also make it possible to reduce drug doses and side effects.

Recent studies have established that ultrasound, when combined with a contrast agent, locally and transiently increases the permeability of membranes of single cells *in vitro*, as well as *in vivo* [4]–[7]. *In vivo* drug delivery to the extravascular tissue of nonblood–brain barrier (BBB) has also been reported, but mostly with hemorrhage as a bioeffect [8], [9]. This study focuses on increasing the endothelial layer permeability of non-BBB by means of ultrasound-activated microbubbles in the absence of cell damage. Permeability was determined by transendothelial electrical resistance (TEER), which is a sensitive measure of endothelial layer integrity that effectively reflects rapidly occurring changes in endothelial permeability, well ahead of any measurable changes in macromolecular transport across the monolayer [10].

Manuscript received April 18, 2009; revised June 30, 2009. First published August 25, 2009; current version published January 4, 2010. This work was supported by the Dutch Ministry of Economic Affairs under Innovation Subsidies Collaborative Project IS042035. *Asterisk indicates corresponding author.*

*K. Kooiman is with the Department of Biomedical Engineering, Erasmus MC, P.O. Box 2040, Rotterdam 3000 CA, The Netherlands (e-mail: k.kooiman@erasmusmc.nl).

M. Emmer, M. Foppen-Harteveld, A. van Wamel, and N. de Jong are with the Department of Biomedical Engineering, Erasmus MC, Rotterdam 3000 CA, The Netherlands.

Digital Object Identifier 10.1109/TBME.2009.2030335

II. Materials and Methods

A. Endothelial Monolayers

Primary human umbilical vein endothelial cells (HUVECs, C2519A, Lonza, Verviers, Belgium) were cultured in endothelial cell culture medium (EGM-2, CC-3162, Lonza), and maintained at 37 °C in a humidified incubator with 5% CO_2. HUVECs (passages 2–9) were detached by 0.25% trypsin in ethylenediaminetetraacetic acid (EDTA, Lonza) and replated onto ultrasound transparent membranes of cell culture inserts (23.1 mm diameter, polyethylene terephthalate (PET) membrane, 353090, BD Falcon, Alphen aan den Rijn, The Netherlands). Membranes were pretreated with 1 mL Biomatrix I (90 μg/mL, Biomedical Technologies, Inc., Stoughton, MA) and left to air-dry overnight on a rotating shaker (250 rpm, LaboTech RS 300, Ochten, The Netherlands). EGM-2 was added to both inserts (i.e., apical, 2 mL) and wells (i.e., basolateral, 3 mL), and the membrane was equilibrated for 5 h in the incubator before adding 1.25×10^6 cells per insert. Fluid volumes were selected to yield no hydrostatic pressure gradient across the cells.

B. Treatment of Endothelial Monolayer

Before treatment, culture medium was replaced with an equal volume of Krebs (NaCl 118 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 2.4 mM, D-glucose 5 mM, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 mM, at pH 7.4), and the monolayers were allowed to equilibrate in the incubator. Endothelial monolayers were treated by positioning the inserts upside down in a custom-made micropositioner in front of the ultrasound beam, at a standoff distance of 60 mm in a 4-L Krebs-filled tank (see Fig. 1). The experimental ultrasound contrast agent BR14 (Bracco Research SA, Geneva, Switzerland) was added to the monolayer with a 1 mL syringe and customly curved blunt 19-gauge needle. BR14 is composed of perfluorobutane-containing microbubbles stabilized by a

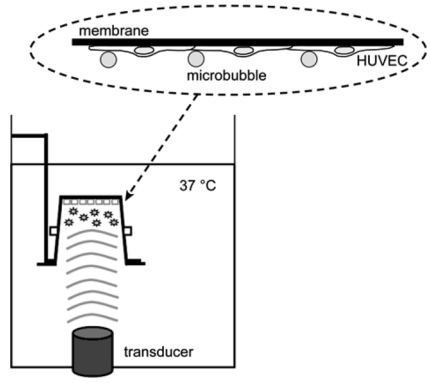

Fig. 1. Experimental setup (not drawn to scale).

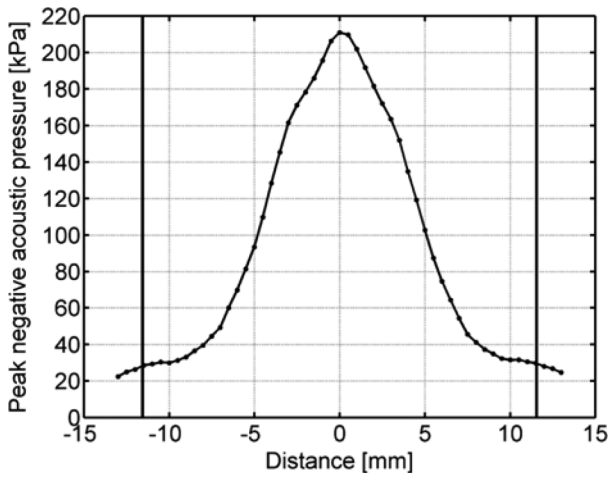

Fig. 2. Lateral beam profile of the 1-MHz unfocused single-element transducer at a standoff distance of 60 mm, i.e., where the endothelial monolayer was positioned. The two vertical lines represent the border of the insert, 23.1 mm in diameter.

phospholipid monolayer coating. It was added in a microbubble–cell ratio of 1:1. To determine the number of microbubbles needed, cells of three inserts were counted in a hemacytometer before each experiment. After the addition of the BR14, ultrasound (1 MHz) was given for 30 s. Thereafter, BR14 was added again to the monolayer and ultrasound was given for another 30 s. As this was again repeated two times, BR14 was added four times and ultrasound was given for 2 min in total. The unfocused single-element transducer (1.27 cm in diameter, V303SU, Panametrics, Waltham, MA) was driven by an arbitrary waveform generator (Agilent 33220A, Agilent Technologies, Amstelveen, The Netherlands) connected to a 60-dB power amplifier (model 150A100B, emv Benelux, Nieuwkoop, The Netherlands). The peak negative acoustic pressures (P_) distribution at the endothelial monolayer, as verified with a calibrated hydrophone (0.2-mm polyvinylidene difluoride (PVDF) needle hydrophone, Precision Acoustics, Ltd., Dorchester, U.K.), is shown in Fig. 2. The highest peak negative acoustic pressure was 210 kPa, which corresponded to a mechanical index (MI) of 0.21. BR14 and monolayers were exposed to an ultrasonic burst consisting of 10 000 cycles of a sine wave with a repetition rate of 20 Hz.

Sham, ultrasound alone, and BR14 alone were used as control treatments. After treatment, the inserts were taken out of the tank and Krebs was reapplied apically. As a positive control, 12 mM EDTA (apically only) was used, as it increases endothelial layer permeability [11].

C. Transendothelial Electrical Resistance (TEER)

Electrical resistances were measured by transferring the inserts to an Endohm-snap chamber (World Precision Instruments, Berlin, Germany). The chamber, filled with 5 mL of EGM-2 (during growth) or Krebs (during treatment), was coupled to an EVOMX resistance meter (World Precision Instruments). TEER (in $\Omega{\cdot}\text{cm}^2$) was calculated using (resistance of experimental insert $-$ resistance of corresponding blank insert) $\times$ 4.2, where 4.2 is the area (in cm^2) of the insert membrane. After plating the HUVECs, TEER was monitored daily (n =18 inserts, resistances of corresponding blank inserts were measured just before plating). During treatments, TEER was determined after preincubation in Krebs and immediately after treatment. Resistances of corresponding blank inserts were measured at the end of the treatment by scraping off the cells with a rubber policeman (VWR, Amsterdam, The Netherlands). TEER was reported as percentage of the TEER measured after preincubation in Krebs.

D. Determination of Cytotoxicity

To determine the cytotoxic effect of the treatment, release of lactate dehydrogenase (LDH) was measured. Immediately after treatment and TEER measurement, EGM-2 medium was added to the cells. At 15, 30, 60, 90, and 120 min, 50 μL medium was taken from the apical chamber and replaced by an equal volume of prewarmed EGM-2 to maintain fluid volumes. Samples were diluted 1:1 with EGM-2 without fetal bovine serum (FBS) to get a 1% FBS concentration, which was required for the LDH diagnostic kit (Roche Diagnostics, The Netherlands). LDH release was measured according to the manufacture's instructions. Absorbance was measured at 490 and 655 nm in a Model680 microplate reader (Bio-Rad, Veenendaal, The Netherlands). Released LDH was expressed as a percentage of sham treatment and corrected for repetitive sampling. Monolayers treated with 1% Triton X-100 (Sigma, Zwijndrecht, The Netherlands) in EGM-2 were used as positive controls. Experiments were done twice in triplicate.

E. Morphology

Immediately after treatment, EGM-2 medium was added to the cells and morphology was studied up to 3 h after treatment. Pictures were taken using a Zeiss AxioVert 100M inverted microscope with an Epiplan NeoFluar 10$\times$ lens and AxioCam camera (Carl Zeiss, Sliedrecht, The Netherlands).

F. Statistical Analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). Comparisons among multiple groups were performed using a one-way analysis of variance (ANOVA) followed by

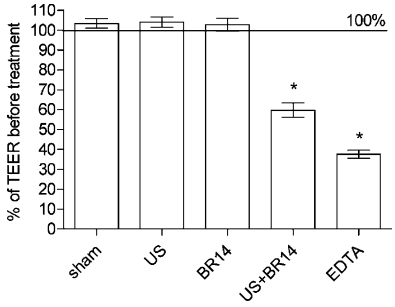

Fig. 3. TEER expressed as percentage of TEER after 30 min preincubation in Krebs for sham, ultrasound (US), BR14, US in combination with BR14, and EDTA treatment ($*$: significantly lower than sham, $p < 0.01$). Columns, means; bars, $\pm$ SEM; $n = 15$–22 inserts.

Dunnett's multiple comparison test (GraphPad InStat version 4.0, GraphPad Software, San Diego, CA). Differences were considered significant, if $p < 0.05$.

III. Results

After plating, TEER was used to assess the integrity of the endothelial monolayer and the formation of tight junctions. TEER reached maximum values after three days postplating, was stabilized for two days, and then decreased daily, as previously reported by others [12]. Maximum TEER at day 3 was $23.1 \pm 0.2\ \Omega{\cdot}\mathrm{cm}^2$, indicating that this time was required for monolayers to develop well-formed intercellular junctions, as established by others [12], [13]. Accordingly, all experiments were performed on inserts three days postplating. Three days postplating, the monolayer consisted of $0.61 \pm 0.02 \times 10^6$ cells ($n = 15$ inserts), which is 49% of the initially plated amount. Plating fewer cells, however, did not result in a visible confluent monolayer nor were maximum TEER values reached.

On the day of the experiments (i.e., three days postplating), culture medium was replaced with Krebs, and monolayers were allowed to equilibrate 30 min in the incubator. This was found to be sufficient to reestablish baseline TEER.

Ultrasound in combination with the contrast agent BR14 decreased TEER significantly to $59.7\% \pm 3.7\%$ ($p < 0.01$) (see Fig. 3). Sham, ultrasound alone, and BR14 treatment alone did not significantly change TEER. As expected, EDTA significantly decreased TEER to $37.6\% \pm 2.0\%$ ($p < 0.01$).

LDH release measurements were done to define whether direct cytotoxicity contributed to the lower TEER. No significant differences from sham-treated monolayers were found for monolayers treated with ultrasound in combination with BR14, ultrasound alone, or BR14 alone, indicating that these treatments had no toxic effect on the HUVECs. Monolayers treated with 1% Triton X-100 showed a 4.3-fold increase in LDH release compared to sham treatment.

Microscopic examinations revealed no apparent cell loss directly after treatment and up to 3 h after treatment for all treatment groups, except for the EDTA positive control group (see Fig. 4). Three hours after EDTA treatment, cells rounded up and detached from the insert, indicating loss of contact and initiation of intercellular gap formation.

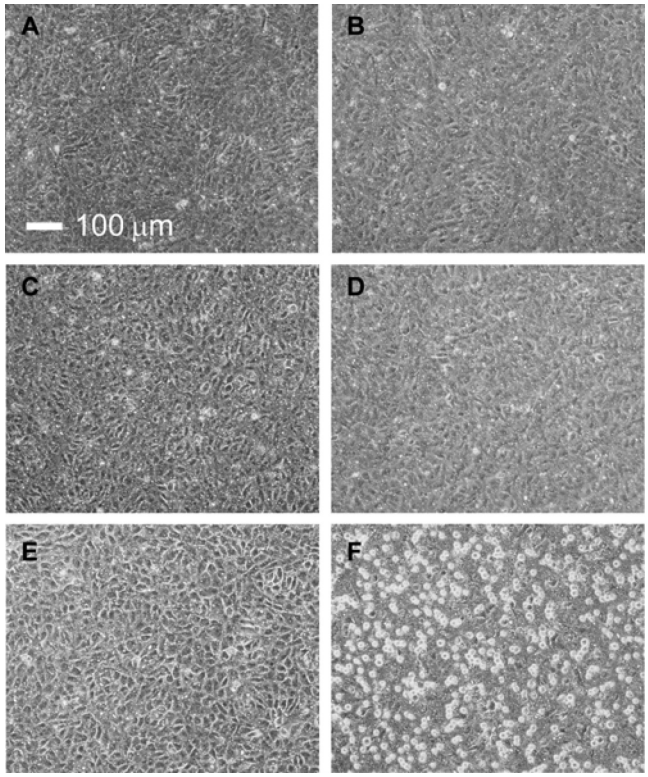

Fig. 4. Microscopic examination of monolayers [(a), (c), and (e)] directly after treatment and [(b), (d), and (f)] 3 h after treatment for [(a) and (b)] sham-treated, [(c) and (d)] ultrasound- and BR14-treated, and [(e) and (f)] EDTA-treated endothelial monolayers.

IV. Discussion

Endothelial monolayers treated with the combination of ultrasound and BR14 significantly decreased TEER values to $59.7\% \pm 3.7\%$ of initial values. This was not a consequence of cell loss or direct cytotoxicity. In addition, ultrasound or BR14 treatments alone were not able to decrease TEER.

HUVECs are widely used to study endothelial barrier function [10], [12]–[14]. In our study, we used these cells as an *in vitro* model system to simulate microbubble interactions with the vessel wall and study effects of ultrasound-activated microbubbles on endothelial barrier function. EDTA treatment resulted in a lower TEER than treatment with ultrasound-activated BR14. Whereas EDTA chelates all Ca^{2+}, and can thereby open all cell–cell junctions [11], only BR14 present within the ultrasound beam may have been able to affect cells. In addition, the lateral beam profile of the transducer used in this study (see Fig. 2) indicates that different parts of the monolayer were insonified at different P_. So, not all microbubbles may therefore have been activated by the ultrasound, or their oscillation amplitude might not have been the same [15]. The range of pressures at which ultrasound-activated microbubbles influence endothelial layer permeability and whether there may be a threshold needs further investigation.

It is known that ultrasound facilitates the delivery of drugs and genes through a complex interplay of the therapeutic agent, microbubble characteristics, target tissue, and ultrasound

energy [16]. Our study revealed that ultrasound-activated BR14 increased endothelial layer permeability without cell loss or cytotoxicity. This is in contrast to another *in vitro* study, which showed disruption of HUVEC monolayers using Definity as the contrast agent [17]. The difference may be explained by the ultrasound energy (continuous wave at MI of 0.4 versus pulsed wave of 0.2 in our study) and the use of different ultrasound contrast agents (Definity versus BR14 in our study).

The mechanism by which ultrasound-activated BR14 increase endothelial layer permeability will be the subject of our future studies, and may involve opening of the cell–cell junctions, just as reported for *in vivo* BBB opening using ultrasound-activated Optison microbubbles [18], although the BBB has much tighter cell–cell junctions than other parts of the vasculature [19]. We will also study whether permeability is transient and assess whether drug delivery is increased. Further studies may also enable us to understand the complex interplay of microbubbles and the endothelial layer. These findings are important for agents that have difficulty in crossing the endothelial barrier. An example is chemotherapeutic drugs, as a high interstitial fluid pressure and a chaotic vasculature hinders the traverse of chemotherapeutic drugs from the bloodstream into the tumor tissue. ten Hage *et al.* [20] use vasoactive compounds, like tumor necrosis factor alpha, histamine, and interleukin-2 to increase permeability of the tumor-associated vasculature, resulting in an increased accumulation of chemotherapeutic drug in the tumor tissue. They have recently called this process tumor vessel abnormalization [20], [21]. With the use of locally applied ultrasound in combination with a contrast agent that manipulates the vasculature to become more permeable, we may also be able to deliver drugs more efficiently to tumors.

ACKNOWLEDGMENT

The authors would like to thank Bracco Research SA, Geneva, Switzerland, for kindly providing the BR14 samples, L. Bekkering (Department of Biomedical Engineering, Erasmus MC, The Netherlands) for his technical assistance, and Dr. A. L. B. Seynhaeve (Department of Surgical Oncology, Erasmus MC) for her assistance in the morphology studies and insightful suggestions.

REFERENCES

[1] J. R. Gudgeon and W. Martin, "Modulation of arterial endothelial permeability: Studies on an *in vitro* model," *Br. J. Pharmacol.*, vol. 98, pp. 1267–1274, 1989.

[2] G. P. van Nieuw Amerongen and V. W. van Hinsbergh, "Targets for pharmacological intervention of endothelial hyperpermeability and barrier function," *Vasc. Pharmacol.*, vol. 39, pp. 257–272, 2002.

[3] P. Telo, S. Lostaglio, and E. Dejana, "Structure of intercellular junctions in the endothelium," *Therapie*, vol. 52, pp. 395–398, 1997.

[4] S. Mehier-Humbert, T. Bettinger, F. Yan, and R. H. Guy, "Plasma membrane poration induced by ultrasound exposure: Implication for drug delivery," *J. Control Release*, vol. 104, pp. 213–222, 2005.

[5] H. Pan, Y. Zhou, O. Izadnegahdar, J. Cui, and C. X. Deng, "Study of sonoporation dynamics affected by ultrasound duty cycle," *Ultrasound Med. Biol.*, vol. 31, pp. 849–856, 2005.

[6] A. van Wamel, K. Kooiman, M. Harteveld, M. Emmer, F. J. ten Cate, M. Versluis, and N. de Jong, "Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation," *J. Control Release*, vol. 112, pp. 149–155, 2006.

[7] B. D. Meijering, L. J. Juffermans, A. van Wamel, R. H. Henning, I. S. Zuhorn, M. Emmer, A. M. Versteilen, W. Paulus, W. H. van Gilst, K. Kooiman, N. de Jong, R. J. Musters, L. E. Deelman, and O. Kamp, "Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation," *Circ. Res.*, vol. 104, pp. 679–687, 2009.

[8] D. L. Miller and J. Quddus, "Diagnostic ultrasound activation of contrast agent gas bodies induces capillary rupture in mice," *Proc. Nat. Acad. Sci. USA*, vol. 97, pp. 10179–10184, 2000.

[9] R. J. Price, D. M. Skyba, S. Kaul, and T. C. Skalak, "Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound," *Circulation*, vol. 98, pp. 1264–1267, 1998.

[10] N. Gautam, P. Hedqvist, and L. Lindbom, "Kinetics of leukocyte-induced changes in endothelial barrier function," *Br. J. Pharmacol.*, vol. 125, pp. 1109–1114, 1998.

[11] W. Tschugguel, Z. Zhegu, L. Gajdzik, M. Maier, B. R. Binder, and J. Graf, "High precision measurement of electrical resistance across endothelial cell monolayers," *Pflugers Arch.*, vol. 430, pp. 145–147, 1995.

[12] P. G. Bannon, M. J. Kim, R. T. Dean, and J. Dawes, "Augmentation of vascular endothelial barrier function by heparin and low molecular weight heparin," *Thromb. Haemost.*, vol. 73, pp. 706–712, 1995.

[13] M. H. Ali, S. A. Schlidt, N. S. Chandel, K. L. Hynes, P. T. Schumacker, and B. L. Gewertz, "Endothelial permeability and IL-6 production during hypoxia: Role of ROS in signal transduction," *Amer. J. Physiol.*, vol. 277, pp. L1057–L1065, 1999.

[14] A. L. Seynhaeve, C. E. Vermeulen, A. M. Eggermont, and T. L. ten Hagen, "Cytokines and vascular permeability: An *in vitro* study on human endothelial cells in relation to tumor necrosis factor-alpha-primed peripheral blood mononuclear cells," *Cell Biochem. Biophys.*, vol. 44, pp. 157–169, 2006.

[15] M. Emmer, A. van Wamel, D. E. Goertz, and N. de Jong, "The onset of microbubble vibration," *Ultrasound Med. Biol.*, vol. 33, pp. 941–949, 2007.

[16] J. M. Tsutsui, F. Xie, and R. T. Porter, "The use of microbubbles to target drug delivery," *Cardiovasc. Ultrasound*, vol. 2, p. 23, 2004.

[17] N. R. Soman, J. N. Marsh, M. S. Hughes, G. M. Lanza, and S. A. Wickline, "Acoustic activation of targeted liquid perfluorocarbon nanoparticles does not compromise endothelial integrity," *IEEE Trans. Nanobiosci.*, vol. 5, no. 2, pp. 69–75, Jun. 2006.

[18] N. Sheikov, N. McDannold, S. Sharma, and K. Hynynen, "Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium," *Ultrasound Med. Biol.*, vol. 34, pp. 1093–1104, 2008.

[19] G. Bazzoni, O. M. Estrada, and E. Dejana, "Molecular structure and functional role of vascular tight junctions," *Trends Cardiovasc. Med.*, vol. 9, pp. 147–152, 1999.

[20] T. L. ten Hagen, A. L. Seynhaeve, and A. M. Eggermont, "Tumor necrosis factor-mediated interactions between inflammatory response and tumor vascular bed," *Immunol. Rev.*, vol. 222, pp. 299–315, 2008.

[21] A. L. Seynhaeve, A. M. Eggermont, and T. L. ten Hagen, "TNF and manipulation of the tumor cell-stromal interface: "Ways to make chemotherapy effective"," *Front. Biosci.*, vol. 13, pp. 3034–3045, 2008.