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Ultrasound-Mediated Delivery of RNA to Colonic Mucosa of Live Mice

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BACKGROUND & AIMS: It is a challenge to deliver nucleic acids to gastrointestinal (GI) tissues due to their size and need for intracellular delivery. They are also extremely susceptible to degradation by nucleases, which are ubiquitous in the GI tract. We investigated whether ultrasound, which can permeabilize tissue through a phenomenon known as transient cavitation, can be used to deliver RNA to the colonic mucosa of living mice. **METHODS:** We investigated delivery of fluorescently labeled permeants to colon tissues of Yorkshire pigs ex vivo and mice in vivo. Colon tissues were collected and fluorescence was measured by confocal microscopy. We then evaluated whether ultrasound is effective in delivering small interfering (si)RNA to C57BL/6 mice with dextran sodium sulfate–induced colitis. Some mice were given siRNA against tumor necrosis factor (*Tnf*) mRNA for 6 days; colon tissues were collected and analyzed histologically and TNF protein levels measured by enzyme-linked immunosorbent assay. Feces were collected and assessed for consistency and occult bleeding. We delivered mRNA encoding firefly luciferase to colons of healthy C57BL/6 mice. **RESULTS:** Exposure of ex vivo pig colon tissues to 20 kHz ultrasound for 1 minute increased the level of delivery of 3 kDa dextran 7-fold compared with passive diffusion ($P = .037$); 40 kHz ultrasound application for 0.5 seconds increased the delivery 3.3-fold in living mice ($P = .041$). Confocal microscopy analyses of colon tissues from pigs revealed regions of punctuated fluorescent dextran signal, indicating intracellular delivery of macromolecules. In mice with colitis, ultrasound delivery of unencapsulated siRNA against *Tnf* mRNA reduced protein levels of TNF in colon tissues, compared with mice with colitis given siRNA against *Tnf* mRNA without ultrasound ($P \leq .014$), and reduced features of inflammation ($P \leq 4.1 \times 10^{-5}$). Separately, colons of mice administered an mRNA encoding firefly luciferase with ultrasound and the D-luciferin substrate had levels of bioluminescence 11-fold greater than colons of mice given the mRNA alone ($P = .0025$). Ultrasound exposures of 40 kHz ultrasound for 0.5 seconds were well tolerated, even in mice with acute colitis. **CONCLUSIONS:** Ultrasound can be used to deliver mRNAs and siRNAs to the colonic mucosa of mice and knock down expression of target mRNAs.

Keywords: Antisense Therapy; Ulcerative Colitis; Inflammatory Bowel Disease; Ultrasound-Mediated Gastrointestinal Drug Delivery.

The gastrointestinal (GI) tract presents a striking opportunity for the delivery of therapeutics. It is characterized by a large surface area and is designed to absorb material.[1] These features in particular make the GI tract a logical site for drug administration. However, the ability to absorb nutrients is facilitated by a harsh environment well suited to breaking down complex nutrients. Specifically, the low pH and wealth of proteases and nucleases makes the delivery of biologics via the GI tract extremely challenging.[2] This has largely limited GI luminal drug delivery to small molecules.[3] Indeed, the efficient delivery of biologics via the GI tract might present a paradigm shift in clinical and research settings, allowing for the local administration of highly effective biologics in the clinic to treat diseases like inflammatory bowel disease (IBD).[4,5]

More broadly, challenges in delivery have also hampered clinical development of new therapeutics for a host of diseases. The development of new therapeutics necessitates the targeting and validation of new therapeutic targets with an active molecule successfully hitting a new therapeutic target, the delivery of which, is nontrivial. In the case of IBD, for example, these challenges are highlighted by the development times of alicaforsen and mongersen, two new antisense therapies.[6,7] The development times of these molecules, with alicaforsen having been under development for Crohn's disease as early as 1997,[8] underscores the difficulties in

Abbreviations used in this paper: **DAPI, 4′,6-diamidino-2-phenylindole; DSS, dextran sodium sulfate; GI, gastrointestinal; IBD, inflammatory bowel disease; mRNA, messenger RNA; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TNF, tumor necrosis factor.**

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identifying, validating, and evaluating the efficacy of hitting new therapeutic targets. Beyond the targets of these two new drugs, mainly intercellular adhesion molecule-1 and SMAD7, respectively, lie a myriad of potential targets that might be effectively modulated to treat the underlying disease.[9] However, properly identifying a potential target and its function requires targeting it with an active biologic, the delivery of which is challenging. This is particularly true in ulcerative colitis research, where the optimal therapeutic target has yet to be identified, despite decades of intense research.[10]

These limitations highlight the glaring need for drug-independent methods of delivery. A platform that might enable the delivery of biologics and nucleic acids without the need for extensive formulation and nucleic acid modification could represent a paradigm shift in drug-delivery science and have broad clinical impact. A physical enhancer, such as ultrasound, may enable formulation-independent delivery of biologics.

Ultrasound is a pressure wave with frequencies >20 kHz. Clinically, ultrasound is widely used for imaging, lithotripsy, and tumor ablation. Ultrasound has been shown to reversibly permeabilize tissue through a phenomenon known as transient cavitation.[11] This phenomenon has been investigated extensively for facilitating permeabilization of the skin in the context of transdermal drug delivery and received US Food and Drug Administration approval for the topical delivery of lidocaine.[12]

Aside from its use in clinical settings, ultrasound has also recently been noted to facilitate reversible permeabilization of cellular membranes, allowing for intracellular delivery of fluorescent permeants.[13] This has been noted to result from cavitation events creating small defects in the cell membrane, allowing diffusion of species into the cell.[13] However, few studies exist on the use of ultrasound to facilitate intracellular delivery in vivo in complex biologic settings, such as the GI tract.

Building on these observations, we sought to investigate the use of ultrasound to facilitate permeabilization of the GI tract and simultaneously porate individual cells, to allow for intracellular delivery. We chose the delivery of RNAs, given the need for these therapeutics to be delivered into cells and because of the recognized difficulty in delivering this type of molecule currently.[14]

Materials and Methods

Chemicals

Phosphate buffered saline (PBS), lysine-fixable 3 kDa dextran labeled with Texas red, murine small interfering (si) RNA targeting tumor necrosis factor (*Tnf*) messenger RNA (mRNA) (Stealth siRNA MSS211991), and diethylpyrocarbonate-treated water were purchased from Invitrogen (Carlsbad, CA). ^{14}C-labeled inulin was purchased from American Radiolabeled Chemicals (St Louis, MO). Dextran sodium sulfate (DSS) was purchased from Affymetrix Inc (Santa Clara, CA). mRNA coding for firefly luciferase was purchased from TriLink Biotechnologies (San Diego, CA). D-luciferin, Soluene-350, and Hionic-Fluor scintillation cocktail fluid were purchased from Perkin-Elmer (Waltham, MA).

Ex Vivo Experiments

Porcine tissue preparation. The Massachusetts Institute of Technology Committee on Animal Care approved all animal-related research aspects of this study. Colon tissue was procured from Yorkshire pigs within 20 minutes of the animal being euthanized and stored at 4°C. The tissue was used within 6 hours of procurement. Tissue was washed with PBS, sectioned into pieces approximately 2 × 2 cm in size, and mounted in 15 mm-diameter Franz diffusion cells (PermeGear, Hellertown, PA) or 29 mm-diameter custom-made diffusion cells (to accommodate different-sized ultrasound horns) with the luminal side exposed to the donor chamber. After mounting all tissue, Franz cells were randomly assigned to either ultrasound or control experimental groups.

Ultrasound administration. Immediately before administration, the donor chamber was filled with 1.5 mL permeant solution. ^{14}C-labeled inulin was used as received at a concentration of 0.03 mg/mL and 3 kDa dextran tagged with Texas red at a concentration of 0.2 mg/mL in PBS. 20-kHz ultrasound was utilized to maximize transient cavitation events, which have previously been shown to be the primary mechanism of enhancement.[15] The 20-kHz and 60-kHz ultrasound were generated with a 13-mm diameter VCX 500 and a 13-mm diameter custom-ordered probe, respectively (Sonics and Materials, Inc., Newtown, CT). 1 MHz ultrasound was generated using a Dynatron D125 ultrasound probe (Dynatronics Corporation, Salt Lake City, UT). For all applications, the ultrasound probe tip was placed 3 mm away from the surface of the tissue. Ultrasound intensities were calibrated by calorimetry to 5 W/cm^2, 9.6 W/cm^2, and 1.5 W/cm^2 for 20 kHz, 60 kHz, and 1 MHz ultrasound, respectively. For frequency comparison studies, total applied ultrasound power and permeant contact time were kept constant across all three frequencies. For 3 kDa dextran, ultrasound was applied for 1 minute using a 50% duty cycle (5 seconds on, 5 seconds off).

After administration, the permeant was removed and the tissue thoroughly washed with PBS. Delivery into the tissue using ^{14}C-labeled inulin was quantified using a liquid scintillation counter (Perkin-Elmer) by solubilizing the tissue. Delivery of 3 kDa dextran was quantified by imaging the tissue with an IVIS fluorescence imager (Perkin-Elmer).

Multiphoton microscopy. After delivery of 3 kDa dextran, tissue sections were fixed in 10% formalin overnight. They were then stained for 30 minutes with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. After staining, the tissue was washed and imaged by the microscopy core facility in the Swanson Biotechnology Center (Massachusetts Institute of Technology). Specifically, an Olympus FV1000 Multiphoton Laser Scanning Microscope was used. Z-stack images were acquired with a step size of 5 μm to a total depth of 125 μm. The second harmonic (to show tissue architecture), DAPI, and dextran channels were acquired.

In Vivo Experiments

Animals. Fifteen-week-old, female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) for all studies. Each cage (group) of animals was used as received and assigned randomly to a particular experimental group by the researchers performing the work.