Chapter 6

Antibody mediated targeting of microbubbles to the vasculature of diabetic kidneys

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Abstract
Drugs can be delivered locally using ultrasound and microbubbles targeted drug delivery. When microbubbles are injected intravenously, local delivery of drugs can be achieved by applying ultrasound to specific tissues or organs. The targeting of microbubbles using antibodies against vascular disease markers would render the concentration of microbubbles in the target tissue dependently on the level of antigen. This allows for a less invasive method for monitoring disease progression without the need for biopsies. Furthermore, this approach may be used therapeutically if the antibodies are able to neutralize factors involved in the progression of disease. In the present study we explored the feasibility of targeting microbubbles bearing neutralizing antibodies to the vasculature of diabetic kidneys in experimental diabetes using neutralizing antibodies against TGF-ß and P-selectin.

Neutralizing antibodies against TGF-ß and P-selectin were attached to Targestar B microbubbles via avidin/biotin coupling chemistry. Targeted microbubbles were injected intravenously in control and diabetic C57/Bi6 mice and accumulation of microbubbles in the kidney was observed using a Siemens Sequoia ultrasound imaging platform in Contrast Pulse Sequencing mode.

TGF-ß and P-selectin targeted microbubbles targeted specifically to the diabetic kidney and were retained in the kidney. A high intensity ultrasound pulse was able to destroy the microbubbles. However, the released neutralizing antibodies were not detectable in the kidney using fluorescent microscopy.

Introduction
It has been demonstrated that ultrasound and microbubble mediated delivery is a promising vector for local drug delivery. A significant body of prior work has established that low-frequency ultrasound energy induces sonoporation in targeted cells in vitro and in vivo; microbubbles have been shown to potentiate the sonoporation effect by several orders of magnitude\(^1\)\(^2\). Examination of the behavior of microbubbles in an acoustic field has revealed the formation of transient microporation and signal transduction events\(^1\)\(^-\)\(^5\). In addition, the involvement of endocytosis in ultrasound and microbubble mediated delivery of large molecules has recently been demonstrated\(^6\).

Microbubble induced delivery has been shown to result in efficient transfection with good cell viability, and can be performed within FDA-approved acoustic safety thresholds. Microbubble-based delivery has primarily been investigated in the context of gene delivery\(^7\)\(^-\)\(^11\), although recent work has explored the feasibility of delivery of drugs\(^12\). Microbubbles have been shown to be useful for delivering drugs in models of thrombosis\(^13\) and cancer\(^14\)\(^\text{-}\)\(^16\).

Local delivery using ultrasound mediated drug therapy can be accomplished by applying ultrasound to specific tissues and organs. This effect can be mediated in a wide variety of settings using most conventional ultrasound imaging platforms and low-frequency therapeutic ultrasound devices.

Intravenous injection of microbubbles is the most convenient route of administration in vascular ultrasound and microbubble delivery therapy. However, when microbubbles
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distribute throughout the total blood volume, the concentration of microbubbles drops dramatically. Furthermore, microbubbles and drugs quickly separate after intravenous injection if both are not directly coupled. For this reason, most in-vivo studies relied on microbubble infusion directly upstream of the target organ.

Recent advances in microbubble technology allows for the coupling of antibodies to the shell of the microbubble (figure 1), enhancing the retention of the microbubbles in specific organs and allowing for the specific delivery of drugs to the target tissue. Destruction of the microbubble shell using locally applied ultrasound releases the drug within the target tissue. This approach would specifically deliver drugs to the target tissue, contributing to the development of a clinical ultrasound and microbubble mediated delivery therapy. In addition, the specific retention of targeted-microbubbles to the diseased tissue, may enable monitoring of the progression of disease without the need for biopsies. In such a strategy, the presence of disease markers can be quantified using targeted microbubbles and conventional low-power ultrasound-imaging platforms.

In the present study, we aimed at evaluating the feasibility of targeting microbubbles to the diabetic kidney using antibodies directed against TGF-β and P-selectin. P-selectin is known to be upregulated in the kidney of diabetic humans and in experimental models of diabetes and thus presents an excellent molecular target for specific renal targeting. Although TGF-β is activated in diabetic nephropathy and is expressed on cells that line the blood vessels, it is not extensively used as a targeting entity. As high concentrations of latent TGF can be found in the blood, targeting would require an antibody that is specific for active TGF-β. The neutralizing monoclonal TGF-β antibody 2G7 is specific for active TGF-β.

TGF-β has been established as an important cytokine intimately involved in tissue fibrosis. TGF-β has been found to be up-regulated in a variety of kidney diseases characterized by excess matrix deposition, including diabetic kidney disease. Further, the involvement of TGF-β as mediator of experimental diabetic kidney disease was clearly demonstrated in experiments using neutralizing TGF-β antibodies. Although the negative effects of TGF-β in kidney have been widely studied, some TGF-β expression may be necessary for normal organ function. The TGF-β1 knockout mouse develops a multi-focal autoimmune process.
which is likely mediated by the effect of TGF-ß to finely modulate T cell subsets, B cells, and macrophages\(^{15, 20}\). Inhibition of TGF-ß signaling through over-expression of Smad7 resulted in severe pathological alterations in multiple epithelial tissues, indicating an important role of TGF-ß signaling in development and maintenance of homeostasis of epithelial tissues\(^{21}\). Therefore, a complete blockade of all TGF-ß signaling pathways may result in unwanted side-effects in non-renal tissues.

Previously, we demonstrated that the chronic type 1 diabetic mouse\(^{22}\) and the db/db mouse overexpress TGF-ß in the glomeruli\(^{17}\). Therefore we hypothesize that administration of TGF-ß antibody complexed with microbubbles will localize to cells that overexpress TGF-ß in the diabetic kidney. The likely target cells are mesangial cells as these cells have clearly been demonstrated to stimulate TGF-ß protein production in the diabetic kidney and they are exposed to the luminal surface of the circulation. Given their unique location, the mesangial cell would be in a prime location to have the circulating TGF-ß antibody-microbubble complex localize to this site. As the microbubble can be visualized with ultrasound, the trapping of microbubbles will provide a real time, non-invasive readout of TGF-ß production in the diabetic glomeruli. Destruction of the microbubbles will release the neutralizing TGF antibody from the microbubble, increasing the availability of the neutralizing antibody within the target tissue. A similar approach can be taken with P-selectin antibodies. An optimization of this approach would provide a potential clinical application to identify patients at high risk for progressive diabetic nephropathy, even if on standard of care therapy.

The endothelial selectin P-selectin is a biomarker for various inflammatory disorders and has been found to be upregulated in both diabetic patients and in experimental models of diabetes\(^{23, 24}\). Previously, we have used the Targetstar-B coupling scheme to conjugate biotinylated P-selectin monoclonal antibodies, and several selectin binding glycoconjugates to the surface of biotin-bearing microbubbles\(^{25-28}\). We have shown that these ligands mediate specific adhesion to P-selectin in vitro using the flow chamber assay. Furthermore, these selectin-avid microbubbles were subsequently used to image post-ischemic kidney injury in a mouse\(^{25}\). Selectin-targeting strategies have also been utilized to image ischemic injury in several mouse models of myocardial injury\(^{25, 26}\). Further, neutralizing P-selectin antibody therapy has been effective in reducing the influx of neutrophils in experimental models of myocardial ischemia-reperfusion injury\(^{23}\). However, the efficacy of P-selectin antibody therapy in attenuating injury in diabetic nephropathy remains to be established.

In the present study we explore the feasibility of targeting microbubbles to the vasculature of diabetic kidneys in experimental diabetes by coating them with neutralizing antibodies against TGF-ß and P-selectin.

**Materials and methods**

*Microbubble Synthesis and Analysis*

Microbubbles were synthesized at Targeson (Charlottesville, VA, USA) using established protocols and existing equipment. A mixture of 1 mg/mL poly(ethylene glycol stearate) (Sigma), 2 mg/mL disteroyl phosphatidylcholine (Avanti), 0.4 mg/mL 1,2-distearoyl-3-
trimethylammoniumpropane (Avanti), and 0.05 mg/mL biotin-PEG-distearoyl phosphoethanolamine (Avanti) was sonicated to clarity in normal saline using a probe-type sonicator (Misonix; Farmingdale, NY), while bubbling perfluorocarbon gas (Flura; Newport, TN) through the dispersion. This procedure generated lipid-stabilized microbubbles bearing a cationic charge. Approximately 2-5% of the lipid molecules bear a biotin residue, which formed the basis for coupling the TGF and P-selectin antibodies.

Unreacted components not incorporated into microbubbles were removed by centrifugal washing for 2 minutes at 400g. Microbubbles of the desired diameter were isolated from the raw dispersion by floatation. The dispersion was placed in a 100 mL syringe and positioned upright. Large microbubbles possess a greater buoyant force, and will travel to the top of the syringe more rapidly than smaller microbubbles; unwanted large microbubbles (10-30 μm diameter) were thus isolated from the bulk by collecting the infranatant after allowing sufficient time for the large particles to float to the top of the syringe. Microbubbles were packaged into crimp-seal vials containing 1-2 mL of microbubble dispersion and the balance perfluorocarbon gas. Microbubble synthesis, sizing, and packaging were performed under aseptic conditions in a laminar flow cabinet.

Targeting Ligand Conjugation
Microbubbles were targeted to P-selectin and TGF-β by means of a surface-bound targeting ligand. The anti-P-selectin antibody R40.34 has been shown to mediate specific retention of microbubbles to P-selectin in vitro and in vivo28. As high concentrations of latent TGF can be found in the blood, targeting requires an antibody that is specific for active TGF-β. The neutralizing monoclonal TGF-β antibody 2G7 is specific for active TGF-β. Approximately 10% of the neutralizing antibodies were labeled with a Fitc-fluorescent dye to enable detection of the delivered antibodies in the kidney. Microbubbles were incubated with 3 μg streptavidin per 10^7 microbubbles for 30 minutes, then centrifugally washed at 400g for 2 minutes to remove unreacted streptavidin. Biotinylated antibody was conjugated to the microbubble surface by incubation at 8 μg antibody per 10^7 microbubbles, as in Rychak26. Antibody conjugation was assessed spectrophotometrically.

Animals
Diabetes in C57/Bl6 mice (18 weeks) was induced by treatment with streptozotocin (STZ 50mg/kg body weight) over five days. This protocol induces hyperglycemia and glomerular hypertrophy, and is an established model for type 1 diabetes. Two weeks after induction of diabetes, mice were anesthetized with an intraperitoneal injection of 125 mg/kg body mass ketamine (Parke-Davis; Morris Plains, NJ), 12.5 mg/kg xylazine (Phoenix Scientific; St. Joseph, MO) and 0.025 mg/kg atropine sulfate (Elkins-Sinn; Cherry Hill, NJ) and the ventral surface was clipped and depilated. Body temperature was maintained using a heat pad. Microbubbles bearing antibody were administered at a dose of 4x10^4 microbubbles per gram body mass via a tail-vein injection.
Ultrasound Imaging and Destruction of Targeted Microbubbles

A Siemens Sequoia ultrasound imaging platform was used to observe microbubble accumulation in the mouse kidney. The scanner was configured for small-animal imaging by the manufacturer, and a small parts transducer (1SL8 operating at 7 – 14 MHz) was used for all experiments. The kidney and adjacent tissues (liver, gallbladder, bowel) were visualized in B-mode (greyscale) at 14 MHz. The kidney was imaged in the long axis with the mouse placed in the left lateral position. In some animals, the kidney was imaged at a slightly oblique angle to permit visualization of adjacent liver. Contrast Pulse Sequencing (CPS), an imaging scheme with high sensitivity to microbubbles, was used to image adherent microbubbles at 8 MHz. The microbubble signal was visualized using a color map in shades of orange. Microbubble wash-in was imaged at low mechanical index (non-destructive imaging) to verify delivery to the kidney; imaging was re-commenced 10 minutes after administration. After collecting several images, a low-frequency destructive pulse was applied, which served to destroy the microbubbles within the beam. Imaging was continued for 1 minute after microbubble destruction.

Fluorescent microscopy

Immediately after imaging, the abdomen of the mouse was opened by a mid-line incision and both kidneys were excised. Kidneys were cut into smaller fragments, embedded in tissue-tek and frozen in liquid nitrogen. 7 µm sections were cut and the presence of the fitc-labeled antibodies was evaluated using confocal-microscopy.

Western-blotting

Kidney fragments were lysed in 300 µl of ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM 8-mercapto-ethanol, 40 µg/ml PMSF, 100 µg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml aprotinin in PBS). Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, The Netherlands). On a 5-20 % SDS-PAGE gel, 20 µg of total protein was run and transferred to nitrocellulose.

Polyclonal HRP conjugated anti-mouse IgG secondary antibody, HRP-conjugated streptavidin and HRP-conjugated anti-mouse-IgG2b antibody (sc-2062, Santa Cruz) were used for detection of the mouse 2g7 TGF-ß IgG2b antibody.

Results

Prior to microbubble administration, the kidney and adjacent tissues (liver, gallbladder, bowel) were visualized in B-mode (greyscale) at 14 MHz (figure 2). Furthermore, Color Doppler showed arterial blood flow and arterial branching in the mouse kidney. CPS imaging prior to microbubble injection demonstrated some artifacts in the skin, but not in liver or kidney.
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Figure 2: Pre-contrast images of mouse kidney. Pre-contrast images of mouse kidney in long axis at 14 MHz in B-mode (A) and with liver and regions of kidney indicated (B). Color Doppler image of kidney in long axis shows arterial flow in kidney (C). A representative pre-contrast CPS image shows good tissue signal suppression with some artifact from tissue at skin line (arrows) before microbubble administration (D).

Figure 3: Representative images of adherent microbubbles in healthy and diabetic mice 10 minutes after microbubble injection. Organ positions were outlined from B-mode images. Liver is outlined in green and kidney is outlined in red. Accumulation of targeted microbubbles in liver, but not kidney, is apparent in healthy mice. In diabetic mice, additional specific accumulation of P-selectin and TGF-β targeted microbubbles was observed in kidney.
Accumulation of targeted microbubbles in the diabetic kidney

Ten minutes after microbubble injection, diabetic mice showed appreciable accumulation of microbubbles bearing anti-P-selectin and anti-TGF-β mAb in the kidney, while healthy control animals showed minimal microbubble accumulation (figure 3). In animals imaged at an oblique angle in which the liver was visible, focal accumulation of microbubbles was observed in the adjacent liver. Microbubble destruction was verified by the disappearance of the microbubble contrast signal after the application of the destructive sequence. No replenishment of the microbubble signal was observed after destruction, suggesting that circulating microbubbles were cleared during the 10 minute dwell time.

Detection of anti-TGF-β antibodies in the kidney

Using fluorescent microscopy we aimed at detecting the fluorescently labeled anti-TGF-β antibody in both the kidney exposed to the destructive ultrasound pulse and in the contralateral kidney that had not been exposed to ultrasound. In both kidneys, we occasionally observed staining in the glomerulus and in the tubulointerstitial space (figure 4). No intact microbubbles could be detected in the kidney that had not been exposed to ultrasound.

Next, we aimed at detecting the anti-TGF-β antibodies in the kidney by Western-blotting using a polyclonal HRP conjugated anti-mouse IgG secondary antibody, HRP-conjugated streptavidin or a HRP-conjugated anti-mouse-IgG2b antibody. For this, both diabetic and control animals were injected with either anti-TGF-β-microbubbles or non-conjugated
Antibody mediated targeting of microbubbles. All blots showed strong signals in the lanes of mice injected with non-conjugated microbubbles, indicating the presence of high background levels of mouse IgG, biotin-like epitopes and mouse IgG2b, respectively (figure 5). Injection of anti-TGF-ß microbubbles did not result in a further increase of these signals.

Figure 5: Detection of anti-TGF-ß antibody in the kidney by Western blot. Diabetic and control mice were injected with either anti-TGF-ß-microbubbles (T) or non-conjugated microbubbles (C). Approximately 10 minutes after injection, animals were killed and the kidneys were snap frozen in liquid nitrogen. Kidneys homogenates were analyzed by Western blot. A. The polyclonal HRP conjugated anti-mouse IgG secondary antibody detected both the light and heavy chains of IgG molecules present in the kidney. No increase in IgG’s could be observed in mice injected with the anti-TGF-ß-microbubbles. B. HRP-conjugated streptavidin reacted with molecules larger than 100kDa, indicating that...
molecules other than biotin-labeled IgG's were detected. Similar results were obtained in mice that were not injected with microbubbles (data not shown). No increase in signal could be observed in mice injected with anti-TGF-ß-microbubbles. C) The HRP-conjugated anti-mouse-IgG2b antibody detected the heavy chain of mouse IgG2b molecules present in the kidney. No increase in IgG2b molecules could be observed in mice injected with the anti-TGF-ß-microbubbles.

Discussion
In the present study we demonstrate that microbubbles can be targeted to the vasculature of diabetic kidneys in experimental diabetes using neutralizing antibodies against TGF-ß and P-selectin. Therefore, these microbubbles may be used in a diagnostic setting. Targeted microbubbles can be used to quantify disease progression as has been demonstrated with microbubbles targeted to mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) in experimental ileitis. As the involvement of TGF-ß has been demonstrated in several (renal) diseases, anti-TGF-ß microbubbles may be used to detect and monitor the progression of kidney disease.

Previously we have shown that neutralizing antibodies directed against TGF-ß attenuated kidney hypertrophy and decreased extracellular matrix gene expression in experimental models of diabetic kidney disease. Furthermore, combination therapy of anti-TGF-ß antibody with an ACE inhibitor completely arrested progressive diabetic nephropathy in the rat. For these studies in small rodents, large amounts of anti-TGF-ß antibody were needed. The implementation of the anti-TGF-ß antibody therapy in humans would be extremely costly, due to even larger amounts of antibody needed. Local delivery of anti-TGF-ß antibody directly to the kidney would require lower amounts of antibody, while reducing unwanted side-effects. Recently several large biotech and pharmaceutical companies have developed humanized anti-TGF-ß antibodies. Their use has been limited by the potential for systemic side effects. The major concern has been an effect on the immune system and in potentially disrupting the beneficial wound healing process in non-renal sites. Therefore our approach to deliver anti-TGF-ß antibodies to the kidney using targeted microbubbles would be a major advance towards a personalized and targeted approach.

Although P-selectin is upregulated in diabetes, it is unclear whether neutralizing P-selectin antibody therapy would be beneficial in diabetic kidney disease. Neutralizing P-selectin antibody therapy in the kidney has been successfully employed in models of kidney ischemia and glomerulonephritis. In these models, the influx of neutrophils and platelets could be inhibited by P-selectin antibodies. Although diabetic nephropathy was not considered an inflammatory disease in the past, recent studies have demonstrated the role of neutrophils and other inflammatory cells in diabetic nephropathy (reviewed by Galkina et al.), indicating that neutralizing P-selectin antibody therapy may also be useful in diabetic kidney disease.
In the present study, we targeted molecules that were exposed to the lumen of blood vessels. However, the delivery of antibodies may not be confined to the endothelium as gene delivery of perivascular cells has been demonstrated in vivo following intravenous administration of plasmid-bearing microbubbles. This suggests that extravascular deposition of antibodies is possible, and may be related to microbubble fragmentation due to applied high-power ultrasound. Microbubble-potentiated drug delivery is thus not limited to the endothelium, although microbubbles remain confined (due to the several-micron diameter of the microbubbles) within the intravascular compartment until release by ultrasound.

Although ultrasound imaging of the kidney demonstrated the accumulation of anti-TGF-ß microbubbles in the kidney, we only occasionally detected fluorescently labeled antibodies in the kidney after microbubble destruction. In addition, no intact microbubbles could be detected in the kidney that was not exposed to the destructive ultrasound pulse. As individual microbubbles could be easily visualized by fluorescent microscopy prior to injection, these results suggest that microbubbles are destroyed by the procedure of snap-freezing and subsequent manipulations.

At present, it is unclear why the fluorescently labeled antibodies could not be detected well in the kidney. Microbubble destruction by the high intensity ultrasound pulse may have released the attached antibodies with subsequent flushing of labeled antibodies out of the kidney by the blood stream. However, labeled antibodies were also poorly detectable in kidneys that were not exposed to the high intensity ultrasound pulse. As the microbubbles were still present in the kidney at the moment of excision, the destruction of microbubbles by the freezing procedure may have resulted in dispersion of the attached antibodies and the fluorescent properties of the individual Ftc-labeled antibodies may have been too low for detection. For future research, alternative strategies for antibody labeling should be employed. Possibly, conjugation of antibodies with quantum dots would enable the detection of individual antibodies.

Detection of the anti-TGF-ß antibody (mouse monoclonal IgG2b) in mouse kidney using Western blotting was hindered by high background levels in control kidneys. High levels of mouse IgG and IgG2b molecules are present in both diabetic and non-diabetic animals. Furthermore, the biotin-groups on the labeled antibody could not be used for specific detection of the anti-TGF-ß antibody as high background levels of biotin-like epitopes are present in the kidney. Radioactive or quantum dot labeling could improve the specific detection and quantification of microbubble delivered antibodies in the kidney.

In addition to the specific targeting of microbubbles to the kidney, we observed some accumulation of microbubbles in the liver. Non-specific delivery in the liver and spleen is reportedly on the order of 0.1% of that of the targeted organs. It is possible that this microbubble accumulation in the liver is due to non-specific mechanisms, such as retention by resident Kupfer cells or via entrapment in liver sinusoids. Future approaches of microbubble mediated antibody therapy should therefore consider possible side effects in the liver.
At present, it is unclear whether the microbubble delivered antibodies could have a local therapeutic effect in the kidney. Preliminary data presented at the Fourteenth European Symposium on Ultrasound Contrast Imaging (2009) demonstrate that plasmids can be delivered locally to the intestines using MadCam-1 targeted microbubbles\textsuperscript{41}, indicating that the microbubbles can carry a substantial load of large molecules. Furthermore, several strategies may be taken should microbubbles fail to deliver a therapeutic dose of antibody to the targeted organ. As microbubbles are well tolerated, increased local delivery of antibodies may be achieved by repeated treatments. Alternatively, local therapeutic concentrations of antibodies could be achieved by supporting microbubble antibody delivery with systemic administration of a sub-therapeutic dose of antibody.

In summary, in the present study we demonstrated that microbubbles can be targeted to the vasculature of diabetic kidneys in experimental diabetes using neutralizing antibodies against TGF-\(\beta\) and P-selectin. This approach may reduce the amount of antibodies needed for neutralizing antibody therapy while limiting possible side-effects.
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References


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