TGF-β1–Containing Exosomes from Injured Epithelial Cells Activate Fibroblasts to Initiate Tissue Regenerative Responses and Fibrosis

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ABSTRACT

Hypoxia is associated with tissue injury and fibrosis but its functional role in fibroblast activation and tissue repair/regeneration is unknown. Using kidney injury as a model system, we demonstrate that injured epithelial cells produce an increased number of exosomes with defined genetic information to activate fibroblasts. Exosomes released by injured epithelial cells promote proliferation, α-smooth muscle actin expression, F-actin expression, and type I collagen production in fibroblasts. Fibroblast activation is dependent on exosomes delivering TGF-β1 mRNA among other yet to be identified moieties. This study suggests that TGF-β1 mRNA transported by exosomes constitutes a rapid response to initiate tissue repair/regenerative responses and activation of fibroblasts when resident parenchyma is injured. The results also inform potential utility of exosome-targeted therapies to control tissue fibrosis.

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Tissue injury can result from various stimuli, including infections, autoimmune reactions, toxins, radiation, and trauma. The repair process after parenchymal injury typically involves a regenerative phase, in which injured cells are repaired or replaced, without leaving a lasting evidence of damage.1 Although initially beneficial, the repair process can become pathogenic when it is not appropriately regulated and such chronic unproductive fibrosis replaces normal parenchyma with scar material consisting of connective tissue, which eventually leads to loss of organ function.2 Myofibroblasts are contractile cells that likely play a key role in wound healing and fibrosis.2,3

Kidney fibrosis is responsible for most types of chronic progressive kidney failure, affecting approximately 8%–10% of individuals in the developed world.5,6 AKI can result in incomplete repair response, persistent tubulointerstitial inflammation, and tissue hypoxia.7,8 In many types of kidney injury, the tubular epithelial cells (TECs) are damaged, in part due to hypoxic injury.9–11 Injured and hypoxic TECs likely undergo many cellular and molecular changes, and secrete soluble factors in an effort to initiate repair events.12 Here, we explored the potential mechanistic role of exosomes in the activation of fibroblasts and initiation of hypoxia-related fibrosis after obstruction.

Exosomes are small extracellular membrane vesicles secreted by various cell types, including platelets,13 cytotoxic T lymphocytes,14 dendritic cells,15 and mast cells.16 These 30- to 100-nm vesicles are formed by inward invagination in endosomal compartments known as multivesicular bodies that can fuse with plasma membranes, resulting in the release of entrapped vesicles into the extracellular space. Exosomes are enriched in CD63, a tetraspanin protein that is implicated in adhesive and costimulatory functions and is widely used as their marker protein.17 These small vesicles...
have been reported to carry proteins, mRNA, and microRNAs and to facilitate transfer of genetic information between cells.\textsuperscript{18}

Prominent kidney fibrosis induced due to unilateral ureteral obstruction (UUO) is observed by day 10 after surgery, with accumulation of myofibroblasts seen as early as day 2 after UUO. End stage fibrosis is mainly associated with fibroblast activation and collagen I deposition.\textsuperscript{4,19} Here, in a model of hypoxic fibrosis after obstruction,\textsuperscript{19,20} we confirm that the initial events are associated with increased proliferation of fibroblasts with expression of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) (day 2 of UUO) (Figure 1A). As early as day 2, post-UUO, wrinkling and rupture of the tubular basement membrane is observed, as demonstrated by discontinuous laminin staining (Figure 1B). Such basement membrane disruption may provide easy transit route for exosomes from injured TECs to the interstititial space. We isolated, characterized, and quantified exosomes from contralateral control kidneys and fibrotic UUO kidneys using transmission electron microscopy (TEM), Western blotting (using CD63 as an exosome marker), and total protein quantification (Figure 1, C–E). On day 2 after UUO, these kidneys exhibited increased exosome production compared with control kidneys, as measured by CD63 expression and protein quantification (Figure 1, D and E). In addition, we demonstrate that fibrotic kidneys secrete exosomes highly enriched in TGF-\(\beta\)1 mRNA compared with contralateral control kidneys (Figure 1F).

Mouse and human TEC lines, MCT and HK2, were cultured under normoxic and hypoxic conditions for 24 and 48 hours. A significant increase in hypoxia inducible factor 1\(\alpha\) (HIF-1\(\alpha\)) protein was observed after 24 and 48 hours of hypoxia (Figure 2A). Lactate production was also significantly increased in TECs after 48 hours of culture in hypoxic conditions (Figure 2B). Exosomes were extracted from TECs and evaluated by TEM. Nanovesicles with a size of \(\leq 100\) nm were identified (Figure 2C). To

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\caption{An increased number of exosomes are released by fibrotic kidneys that contain TGF-\(\beta\)1 mRNA. (A) Hypoxyprobe, bromodeoxyuridine, and \(\alpha\)-SMA staining in kidney cortex after 2 days of UUO surgery using the contralateral kidney as control. Graphs on the right represent the respective quantifications using the number of positive intersections per field or the number of positive nuclei. (B) Immunofluorescence staining with laminin antibody at UUO day 2. Arrows point to the site of tubular basement membrane disruption. (C) Morphologic characterization using electronic microscopy of exosomes extracted from whole contralateral or UUO kidneys of operated mice. The size of exosomes is quantified and expressed in nanometers. (D) Protein blot of exosomes extracted from contralateral and UUO kidneys of operated mice using CD63 antibody and \(\beta\)-actin as loading control with densitometry quantification. Protein extracts prepared from exosomes are normalized by protein extract volume/tissue weight. (E) Protein extracts prepared from exosomes are normalized by protein extract volume/tissue weight and expressed in micrograms per milligram of weight. (F) TGF-\(\beta\)1 mRNA content in exosomes extracted from mouse kidneys after 2 days of UUO. Results are depicted in percentages of TGF-\(\beta\)1 mRNA expression in UUO compared with CL kidney. CL, kidney from contralateral side. UUO, kidney after UUO surgery. Original magnification, \(\times 200\). *\(P<0.05\).}
\end{figure}
Hypoxia induces increased production of exosomes by TECs. (A) HIF-1α Western blot in MCT cellular extracts cultured under normoxic or hypoxic conditions. The bands are quantified and the HIF-1α and β-actin ratio is expressed in relative units (RUs). (B) Lactate quantification in culture medium from cells cultured under normoxic (N) and hypoxic (H) conditions. (C) Morphologic characterization of MCT exosomes by electronic microscopy. The size of exosomes is measured and expressed in nanometers. (D) Total exosome protein quantification (right graph). Protein extracts prepared from exosomes are normalized by protein extract volume per cell number and expressed in micrograms per 106 cells. CD63 expression in hypoxia (left panel). Protein extracts prepared from exosomes are normalized by protein extract volume per cell culture number (micrograms per 106 cells; upper panel) or by concentration (10 μg/ml; lower panel) and are assessed using antibody against exosomal protein marker CD63. (E) Western blot using CD63 and GFP antibodies of protein extract from MCT CD63-GFP cells using β-actin expression as loading control. (F) Confocal microscopy for GFP presence in MCT CD63-GFP cells cultured under normoxic or hypoxic conditions. Green signal represents the fusion protein CD63-GFP.

Figure 2. Hypoxia induces increased production of exosomes by TECs. (A) HIF-1α Western blot in MCT cellular extracts cultured under normoxic or hypoxic conditions. The bands are quantified and the HIF-1α and β-actin ratio is expressed in relative units (RUs). (B) Lactate quantification in culture medium from cells cultured under normoxic (N) and hypoxic (H) conditions. (C) Morphologic characterization of MCT exosomes by electronic microscopy. The size of exosomes is measured and expressed in nanometers. (D) Total exosome protein quantification (right graph). Protein extracts prepared from exosomes are normalized by protein extract volume per cell number and expressed in micrograms per 106 cells. CD63 protein quantification (left panel). Protein extracts prepared from exosomes are normalized by protein volume per cell culture number (micrograms per 106 cells; upper panel) or by concentration (10 μg/ml; lower panel) and are assessed using antibody against exosomal protein marker CD63. (E) Western blot using CD63 and GFP antibodies of protein extract from MCT CD63-GFP cells using β-actin expression as loading control. (F) Confocal microscopy for GFP presence in MCT CD63-GFP cells cultured under normoxic or hypoxic conditions. Green signal represents the fusion protein CD63-GFP.

We next investigated whether the exosomes secreted by hypoxic TECs carry TGF-β1 mRNA to engage fibroblasts in the repair/fibrotic response. Using a Boyden chamber assay coated with basement membrane preparation, we show that GFP+ exosomes released from CD63-GFP TECs cross the basement membrane material and reach fibroblasts (Supplemental Figure 2). Next, equal amounts of exosomes extracted from normoxic and hypoxic cells were added to cultures of fibroblasts (murine embryonic-NIH-3T3 and renal tubulointerstitial-TFB). Exosomes from hypoxic TECs specifically induced proliferation, TGF-β1 expression, α-SMA expression, F-actin expression, and type I collagen (α1 chain) production upon 24 and 48 hours of incubation, as assessed by quantitative RT-PCR and/or immunofluorescence (Figure 3, A–C). Direct culture of fibroblasts in hypoxic conditions did not induce expression of genes associated with their activation including TGF-β1 mRNA, suggesting that exosomes produced by hypoxic TECs specifically mediate this action rapidly in fibroblasts (Figure 3, A–C and Supplemental Figure 3).

Brefeldin A (BFA), a chemical reagent shown to inhibit exosome secretion from cells,23 was used to pretreat TECs. Exosome-deficient conditioned media from these pretreated cells were collected and used to culture 3T3 and TFB fibroblasts. The conditioned media without exosomes did not alter proliferation or TGF-β1 mRNA content of 3T3 and TFB fibroblasts (Supplemental Figure 4, A and B). Next, hypoxic TECs were pretreated with actinomycin-D (ACT-D) to inhibit general transcription including that of TGF-β1 mRNA and its subsequent accumulation in exosomes. An increase in TGF-β1 mRNA was not observed in 3T3 and TFB fibroblasts incubated with exosomes from ACT-D pretreated cells (Supplemental Figure 4C).

CD63 protein localizes within intraluminal bodies of cells, a site of exosome biogenesis before secretion, and it directly correlates with exosome production and quantity.21,22 Therefore, a stable TEC line expressing CD63-GFP fusion protein was generated to quantitatively measure exosome production (Figure 2E). After exposing CD63-GFP cells to hypoxic conditions for 48 hours, there was an increase in CD63-GFP protein (per cell) compared with CD63-GFP TECs under normoxic conditions (Figure 2F). Collectively, these results indicate that hypoxic TECs secrete a higher number of exosomes compared with normoxic TECs.

Evaluate the number of exosomes released by TECs in hypoxic and normoxic conditions, relative total protein content was measured and expression of membrane-associated CD63 protein was assessed17 (Figure 2D and Supplemental Figure 1, A and B). Expression of CD63 was higher in exosome preparations from hypoxic TECs at 24 and 48 hours compared with normoxic TECs (Figure 2D blot and Supplemental Figure 1A). Total exosome protein normalized by cell number was also significantly higher when isolated from hypoxic TECs (Figure 2D graph and Supplemental Figure 1B).

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Cell death was similar in normoxic and hypoxic TEC cells at 24 and 48 hours of culture (Supplemental Figure 4D) and in TECs treated with Etoposide (0.6 mg/ml), a proapoptotic agent.24 TGF-β1 mRNA levels remained unchanged (Supplemental Figure 4E).

Next, TGF-β1 small interfering RNA (siRNA) was used to silence TGF-β1 in TECs. In addition, TGF-β1 siRNA was also used to silence target TGF-β1 mRNA directly in exosomes (Figure 4A). A significant decrease in TGF-β1 mRNA expression was observed in cells with TGF-β1 siRNA (Figure 4A). Direct electroporation of exosomes with TGF-β1 siRNA also significantly decreased the total amount of TGF-β1 mRNA in the exosomes (Figure 4A). When fibroblasts were treated with exosomes silenced for TGF-β1, α-SMA expression, TGF-β1 expression, and type I collagen expression remained unchanged, supporting the fact that fibroblast activation did not occur in this setting (Figure 4, A–C). A possibility that TGF-β1 mRNA targeting could occur due to direct transfer of TGF-β1 siRNA from exosomes to fibroblasts was ruled out because expression of TGF-β1 mRNA in the exosomes incubated with fibroblasts remained unchanged and did not decrease (Figure 4, A and B). This was accomplished by using stoichiometrically limited amounts of siRNA in our transfections (Figure 4A).

Collectively, our study demonstrates that hypoxia-relevant fibrotic kidneys and hypoxic epithelial cells produce a significantly higher number of exosomes with specific genetic information that has the capacity to initiate proliferation and activation of fibroblasts. We demonstrate that exosomes released by the injured TECs can mediate fibroblast activation specifically by the transfer of TGF-β1 mRNA.

Although exosomes are actively being studied in the context of cancer progression, their potential functional role in tissue repair/regeneration and fibrosis is completely unknown.1,24–26 Here, we show that hypoxic injury/stress causes TECs to increase the production of exosomes and to also alter their composition to facilitate activation of fibroblasts. We propose that exosome-mediated cell–cell communication is likely an important

Figure 3. Hypoxic TEC-derived exosomes lead to fibroblast activation. (A) MTT assay shows proliferation of 3T3 and TFB fibroblasts in normoxia (N), hypoxia (H24h), or exposed to exosomes extracted from MCT cells cultured under hypoxia for 48 hours (E_{MCT} H48h). (B) TGF-β1, α-SMA, and collagen-1 expression in normoxic (N) 3T3 and TFB fibroblasts that are treated with hypoxic exosomes secreted from MCT cells after 24 hours (E_{MCT} H24h) and 48 hours (E_{MCT} H48h) of hypoxia. Results are expressed in arbitrary units (AU). (C) Immunofluorescence of α-SMA, collagen-1 (Col-1), and F-actin in 3T3 and TFB fibroblasts in normoxia (N), hypoxia (H24h), or treated with exosomes from MCT hypoxic cells (E_{MCT} H48h). Fluorescence intensity per nuclei (FI/Nuclei) is quantified and expressed in relative units (RUs). *Significantly different from normoxic control cells (P<0.05).
Figure 4. Hypoxic TEC exosome-derived TGF-β1 mRNA is functionally important for the activation of fibroblasts. (A) TGF-β1 expression in MCT cells under different experimental conditions. In EMCT/H48h, MCT cells are exposed to hypoxia for 48 hours, the culture medium is collected, and exosomes are obtained for RNA extraction. In EMCT 48h + siRNA, MCT cells are exposed to hypoxia for 48 hours, and exosomes are extracted and treated with TGF-β1 siRNA. In MCT+ siRNA/EH48h, MCT cells are treated with siRNA, exposed to hypoxia for 48 hours, the culture medium is collected, and exosomes are obtained for RNA extraction; after this period, RNA is extracted for TGF-β1 expression analyses. *P<0.05 compared with MCT cells under normoxia (MCT-N); + P<0.05 compared with EMCT H48h. (B) TGF-β1, α-SMA,
early response of injured parenchyma engaging in repair. Interestingly, while many reports suggest that exosomes can facilitate intercellular communication by protein transfer; this study demonstrates that exosomes can also mediate such communication specifically by mRNA transfer. Nonetheless, we cannot rule out the existence of other hypoxia-driven miRNAs or microRNAs in the exosomes that could potentially play a role in cellular cross-talk. The mRNA for TGF-β1 is delivered to the fibroblasts by exosomes, and fibroblasts in turn translate it to initiate acute/rapid production of TGF-β1 protein to facilitate proliferation and activation utilizing an autocrine-signaling loop. Therefore, hypoxia plays a role in exosome biogenesis as well as packaging of TGF-β1 mRNA into exosomes that, in contrast to TGF-β1 protein, can be translated to generate larger quantities of protein in a rapid fashion in cells that fuse with such exosomes. Such production of TGF-β1 protein can initiate an autocrine process that ultimately leads to proliferation and activation of fibroblasts. This process is likely most efficient in early stages of injury before significant inflammation is encountered.

CONCISE METHODS

Cell Culture
Mouse proximal tubular cells, NIH-3T3 mouse embryonic fibroblasts, and kidney interstitial fibroblasts (TFBs) were maintained in DMEM and proximal human TECs (HK-2) were maintained in DMEM/F12, cultured with 5% CO₂ and 95% humidity, supplemented with FBS, centrifuged, and filtered in a 0.1-μm filter to remove exosomes contained in the FBS. Human or mouse TECs were divided into normoxia and hypoxia groups.

Exosome Extraction
Hypoxic conditions were created by flushing 5% CO₂ and 95% N₂ through a modified chamber. The culture system was sealed and incubated at 37°C for 24 or 48 hours. Exosomes were isolated. Briefly, collected culture medium from normoxia and hypoxia groups was centrifuged. The resultant supernatants were subjected to filtration on 0.1-μm pore filters, followed by ultracentrifugation. The resulting pellets were resuspended in culture medium for fibroblast treatment for 24 or 48 hours or suspended in PBS, pooled, and again ultracentrifuged at 100,000 × g for 60 minutes. The final pellets of exosomes were re-suspended in radioluminoprecipitation assay buffer for protein extraction or Trizol for RNA extraction.

Kidneys Exosome Extraction
For kidney exosome extraction, 100 mg of kidney cortex was collected and submitted to mechanical and enzymatic digestion with collagenase and trypsin for 4 hours at 37°C. After this period, the sample was collected and centrifuged, and the supernatant was filtrated and ultracentrifuged as previously described for protein and RNA extraction.

Treatment of Fibroblasts with Exosomes
For fibroblast treatment, the exosome content of one of the flasks was analyzed using the microbicinchoninic acid assay and the remnant was used for treatment after being resuspended in culture media. Fibroblasts were treated with 30 μg/ml of exosomes for a period of 24 hours.

ACT-D and BFA Treatments
To deplete RNA from exosomes, MCT cells were treated with ACT-D (0.1 μg/ml; Sigma) or BFA (0.1 μg/ml; Sigma) and submitted to hypoxic conditions for 24 hours for exosome extraction and fibroblast treatment. In addition, in some experiments, 3T3 and TFB fibroblasts were treated with 1.0 μg/ml and 0.1 μg/ml of ACT-D for 24 hours, respectively. After this period, the culture medium was collected and exosomes were extracted. In both experiments, the concentration and exposition period were chosen based on the lowest dose showing biologic significance and low cell death.

Cell Viability Assays
MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium; Sigma) was used to detect cell viability and proliferation.

RNA Extraction and Quantitative PCR
Total RNA from exosome preparations and cells was extracted using Trizol, according to the manufacturer’s instructions. RT was done using a High Capacity cDNA Reverse Transcription Kit for real-time PCR. Primers for mouse TGF-β1, α-SMA, collagen I, and human TGF-β were designed and synthesized by Eurofin Operon. QuantumRNA Universal 18S ribosomal RNA was used as endogenous control. Real-time analysis was performed using SYBR Green PCR Master Mix for real-time PCR.

Lactate Production
Lactate accumulation in the culture medium was determined using a colorimetric L-lactate assay kit from Eton Bioscience Inc, according to the manufacturer’s instructions.

Western Blotting
For Western blotting, aliquots of total protein extracts (10 μg) from cells or exosomes or the volume of exosome lysate corrected by the cell number were loaded and separated by 10% SDS-PAGE and the resulting proteins were then transferred onto a nitrocellulose membrane. After treatment with blocking solution, the membranes were incubated with rabbit polyclonal antibodies of CD63, rabbit polyclonal antibody for HIF-1α, or rabbit polyclonal antibody to actin. The membrane was then incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibodies and was examined by enhanced chemiluminescence. The membranes were then scanned, and the signal intensity of each band was quantified.
Immunofluorescence
For immunofluorescence, cells were grown on Labtek II glass slides and were fixed and permeabilized. After blocking with 5% BSA/PBS, FITC conjugated antibody to α-SMA, Cy5 conjugated antibody to phalloidin, and primary goat antibody to collagen I were added overnight at 4°C in 5% BSA/PBS. The FITC-labeled secondary anti-goat was added and cells were incubated with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on glass slides with glycerin and analyzed using Nikon fluorescence microscopy.

TGF-β siRNA
RNAiFect reagent was used to deliver TGF-β pool siRNA or individual siRNA molecules to MCT cells. siRNA duplex oligonucleotides were transfected per the manufacturer’s instruction. In brief, cells were plated the day before transfection, and were transfected and incubated for an additional 48 hours before harvesting. After this period, the culture medium was collected; exosomes were extracted for protein quantification and treatment of 3T3 and TFB fibroblasts. Exosomes at a total protein concentration of 150 μg/ml were electroporated with siRNA, pooled together for ultracentrifugation, and used to treat fibroblasts and/or RNA extraction.

Transfection of CD63-GFP
For constitutive expression of CD63 in MCT cells, transfection with pCT-CD63-GFP (SB) was followed by selection of stable clones with 1.5 μg/ml of puromycin.

TEM
The pelleted exosomes were mixed freshly prepared with paraformaldehyde/glutaraldehyde, fixed with osmium tetroxide, and dehydrated. Samples were embedded in resin. Ultrathin sections were cut with a diamond knife and sections were then placed on either copper or nickel mesh grids. The sections were stained with the heavy metal uranyl acetate for contrast and viewed on a Tecnai BioTwin.

UUO
Male C57BL6/6j mice were submitted to UUO and mice were euthanized on day 2 after surgery. The mice were injected with bromodeoxyuridine and hypoxyprobe, as previously described. The kidney cortex was extracted and weight for exosome extraction as described previously.

Immunohistochemistry
Masson’s trichrome staining was performed on paraffin-embedded tissue to detect collagen fibers according to standard protocol and the amount of collagen deposition was then digitally quantified. Formalin-fixed paraffin-embedded tissue sections were dehydrated and processed using MoM Kit (Vector Labs). Incubation of sections with primary hypoxyprobe, α-SMA antibodies was carried out overnight. Immunohistochemistry for bromodeoxyuridine was performed according to the manufacturer’s recommendations. Staining was quantified in renal sections of all mice in eight randomly chosen microscopic fields (cortex, inner medulla) at low magnification (×20).

Statistical Analyses
Data were expressed as mean ± SEM. Experimental and control groups were compared using the t-test. Significance level for nullity hypothesis was set at 5% (P<0.05).

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DISCLOSURES
None.

REFERENCES


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