Fibronectin on the surface of myeloma cell-derived exosomes mediates exosome-cell interactions

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Running Title: Fibronectin facilitates exosome-cell interactions

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ABSTRACT

Exosomes regulate cell behavior by binding to and delivering their cargo to target cells, however the mechanisms mediating exosome-cell interactions are poorly understood. Heparan sulfates on target cell surfaces can act as receptors for exosome uptake, but the ligand for heparan sulfate on exosomes has not been identified. Using exosomes isolated from myeloma cell lines and from myeloma patients, we identify exosomal fibronectin as a key heparan sulfate-binding ligand and mediator of exosome-cell interactions. We discovered that heparan sulfate plays a dual role in exosome-cell interaction; heparan sulfate on exosomes captures fibronectin and on target cells it acts as a receptor for fibronectin. Removal of heparan sulfate from the exosome surface releases fibronectin and dramatically inhibits exosome-target cell interaction. Antibody specific for the Hep II heparin-binding domain of fibronectin blocks exosome interaction with tumor cells or with marrow stromal cells. Regarding exosome function, fibronectin-mediated binding of exosomes to myeloma cells activated p38 and pERK signaling and expression of downstream target genes DKK1 and MMP-9, two molecules that promote myeloma progression. Antibody against fibronectin inhibited the ability of myeloma-derived exosomes to stimulate endothelial cell invasion. Heparin or heparin mimetics including Roneparstat, a modified heparin in phase I trials in myeloma patients, significantly inhibited exosome-cell interactions. These studies provide the first evidence that fibronectin binding to heparan sulfate mediates exosome-cell interactions revealing a fundamental mechanism important for exosome-mediated crosstalk within tumor microenvironments. Moreover, these results imply that therapeutic disruption of fibronectin-heparan sulfate interactions will negatively impact myeloma tumor growth and progression.

Multiple myeloma remains incurable, in spite of recent treatment advances (1). There is mounting evidence that targeting intercellular communication between malignant plasma cells, or between malignant cells and host cells may enhance therapeutic outcome. Extracellular vesicles, because they can directly interact with target cells, have emerged as important mediators of intercellular communication. Exosomes are a type of extracellular vesicle derived from endosomes and released by cells into the extracellular space. Exosomes are generally characterized by their size (ranging from
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approximately 30-150 nm) and expression of certain surface markers. However, at present there are no markers specific for exosomes (2). Several studies have shown that these exosome-like particles secreted by tumor cells can affect survival, apoptosis, invasion, angiogenesis, resistance to therapy and pre-metastatic niche formation (3-6). Exosomes influence these processes by mediating signaling at the cell surface and by facilitating the intercellular transfer of tumor-derived proteins and nucleic acids. Recent studies have demonstrated critical roles for myeloma-derived exosomes in mediating intercellular communication and promoting myeloma progression (7-9). For example, myeloma cell-derived exosomes can promote the formation and function of osteoclasts and thereby stimulate osteolysis (7). Another study showed that exosomal micro(mi)RNA 135b released by hypoxic multiple myeloma cells enhances angiogenesis by targeting factor inhibiting hypoxia-inducible factor 1 (8). In addition, we recently discovered that exosomes secreted by aggressive myeloma cells can induce tumor cell spreading and endothelial cell invasion (9).

Heparan sulfate proteoglycans are glycoproteins that contain a core protein with one or more covalently attached negatively charged heparan sulfate glycosaminoglycan chains. Heparan sulfate chains can bind and regulate the function of a wide variety of adhesion molecules, cytokines, growth factors, chemokines, morphogens, various types of viral particles, lipoproteins, etc (10). By interacting with various cell adhesion receptors and extracellular matrix proteins such as fibronectin and collagens, heparan sulfate chains promote cell-cell and cell-ECM interactions (10). Although the functional effects of exosomes rely on their interaction with, and subsequent delivery of their cargo to target cells, the mechanisms mediating exosome-cell interactions remain unclear. Studies have shown that heparan can block interactions between extracellular vesicles and cells and that heparan sulfate proteoglycans on glioblastoma cells facilitates extracellular vesicle uptake (11-14). This uptake was blocked by the addition of exogenous heparin (a highly sulfated glycosaminoglycan mimetic of heparan sulfate), which can compete with cell surface heparan sulfate to bind exosomes. However, the receptor on the exosome surface that binds to heparan sulfate on the target cell has not been identified.

In the present study, we discovered that fibronectin on the exosome surface mediates their interaction with heparan sulfate chains on target cells. Fibronectin is a high affinity ligand for heparin/heparan sulfate and contains a strong heparin/heparan sulfate binding site, designated Hep-II, located within the C-terminal repeat units FN12-14 of fibronectin (15). Several studies have shown that binding of cell surface heparan sulfate proteoglycans such as syndecans, to the heparin binding domain on fibronectin or to a synthetic peptide corresponding to this domain, induces the formation of stress fiber, focal adhesion kinase and cell spreading (16-18). We demonstrate that myeloma-derived exosomes have fibronectin bound on their surface and that, via its Hep-II domain, fibronectin facilitates exosome interaction with heparan sulfate chains present on the surface of target cells. Additionally, we show that fibronectin is bound to exosome surface via interacting with heparan sulfate chains on the exosome surface. These findings provide the first evidence that fibronectin present on the surface of tumor-derived exosomes facilitates exosome-target cell interaction and reveal a novel mechanism whereby the heparan sulfate proteoglycans present on exosomes and on target cells can influence exosome mediated intercellular communication.

EXPERIMENTAL PROCEDURES

Cell Lines- RPMI-8226 and CAG human myeloma cells were cultured as described previously (19). Aggressive (heparanase-high) and less aggressive (heparanase-low) control CAG cells were selected as described previously (20). For preparing cells to secrete fluorescent exosomes, CAG cells were infected with lentivirus particles packaged with pCT-CMV vector encoding CD63 tetraspanin-red fluorescent protein (RFP) fusion protein (System Biosciences). Cells infected were selected using puromycin. Human bone marrow stromal cell line HS-5 and human umbilical vein endothelial cells (HUVEC) were maintained as described previously (21).
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Exosome isolation from cell culture supernatants and myeloma patient serum- Human myeloma cells growing in culture were washed twice with PBS and grown in serum-free medium for 24-42 h. As previously described, exosome-like particles secreted into the medium by CAG myeloma cells were isolated by differential centrifugation, characterized by surface marker expression and size and found to be consistent with the characteristics of exosomes (9). Particle size was measured by nanoparticle tracking analysis using a NanoSight 300 (Malvern Instruments Ltd, UK). For mass spectrometry and flow cytometry analyses, 800 μg of exosomes obtained by ultracentrifugation were layered on the top of a 40% iodixanol cushion (Sigma) and centrifuged for 16 h at 28,000 rpm. The remaining exosome fraction excluded by the cushion was pelleted by ultracentrifugation at 100,000 x g for 70 min and used for analysis. Exosomes were quantified by nanoparticle tracking analysis or by measuring the protein using a BCA protein assay kit (Pierce).

Serum samples were obtained from treatment naïve multiple myeloma patients enrolled in the Molecular and Genetic Epidemiology (iMAGE) study of myeloma who met the revised and updated International Multiple Myeloma Working Group classification criteria for myeloma (22). Approvals from the appropriate Institutional Review Boards were obtained prior to study initiation. Exosomes were isolated from serum using an ExoQuick isolation kit (System Biosciences). Briefly, to 100 μl of serum, 30 μl of ExoQuick solution was added and incubated at 4ºC for 1 h then centrifuged at 1500 x g for 30 min. The pellet was resuspended in PBS and the exosomes further purified using anit-CD63 conjugated to magnetic beads (System Biosciences), according to the manufacturer’s instructions. Particle size and number was assessed using NanoSight 300. The capture settings and analysis settings were performed manually according to the manufacturer’s instructions. For some experiments, exosomes were fluorescently labeled using PKH67 (green) or PKH26 (red) (Sigma), according to manufacturer’s recommendation, followed by extensive washing to remove residual lipid dye.

Flow cytometry analysis of exosomes bound to beads- Flow cytometry analysis to identify molecules on the surface of exosomes was performed after attaching exosomes to either anti-CD63 bound beads or heparin agarose beads (MP Biomedicals Inc). 100 μg of purified exosomes were mixed with the anti-CD63 beads or heparin agarose beads and incubated on a rotating rack at 4ºC overnight. Exosomes bound to beads were suspended in 200 μl of 1% BSA in PBS and stained with antibodies against fibronectin or syndecan-1 prior to analysis with a Becton Dickinson FACSCalibur flow cytometer located in the UAB Comprehensive Flow Cytometry Core. Fibronectin was stained using a mouse monoclonal anti-human fibronectin-PE conjugated antibody (R&D Systems). Mouse isotype matched (IgG1) PE (Thermo Fisher) was used as the control. For detection of syndecan-1, exosomes bound to anti-CD63 beads were treated with bacterial heparitinase (Seikagaku) for 2 h at 37ºC followed by extensive washing. This enzyme treatment, by releasing heparan sulfate and any bound ligands (e.g., fibronectin), exposes the core protein epitope to the antibody. Syndecan-1 was detected using an affinity purified polyclonal goat anti-syndecan-1 IgG (R&D) and PE-conjugated secondary antibody. Normal goat IgG was used for the control (Santa Cruz).

Exosome protein analysis by MS/MS- Exosomes excluded by an iodixanol cushion were solubilized in 1X LDS sample buffer (NuPAGE, Life Technologies) followed by membrane disruption for 10 min in an ultrasonic bath (Thermo Fisher), and heat denaturation as per manufacturer’s instructions for the LDS buffer. Protein extracts were then quantified using the BCA Protein Assay Kit (Pierce, Life Technologies). An aliquot containing 20 μg of protein was reduced, denatured, and loaded onto a 10% Bis-tris gel (NuPAGE reagents, Life Technologies) and separated as a short stack run (~1 cm). The gel was stained with Colloidal Blue Staining Kit (NuPAGE, Life Technologies), de-stained and visualized. The upper gel section containing protein for each sample was cut out and digested using Trypsin Gold (Promega), followed by peptide extraction as per manufacturer’s instructions, and the volumes were reduced using a
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Savant SpinVac Concentrator (Thermo Fisher). One microgram of peptide extract (diluted to ~1 μg/10 ul in 0.1% formic acid) was loaded onto a 100 μm × 13 cm capillary column, packed in-house with C18 Monitor 100 A-spherical silica beads, and eluted over a 90 min gradient (0%–30% acetonitrile in 0.1% TFA). Liquid Chromatography Mass spectrometric analysis was performed in duplicate using an LTQ Velos Pro Orbitrap (Thermo Fisher), and data were analyzed within the UAB Comprehensive Cancer Center Mass Spectrometry/Proteomics Shared Facility as previously described (23).

Analysis of exosome-cell interactions-
Subconfluent RPMI-8226 myeloma cells or HS-5 bone marrow stromal cells were incubated with CD63-RFP or PKH labelled myeloma-derived exosomes (100 μg/ml) for 2 h. Cells were washed in PBS, fixed in 3% paraformaldehyde for 15 min on ice. Myeloma cells grow in suspension and therefore were cytopspun on to slides for analysis. All samples were analyzed using a Nikon A1 confocal microscope. Fluorescent exosomes bound to cells were quantified by flow cytometry using a FACSCalibur instrument. For experiments designed to block the Hep II domain of fibronectin on exosomes, the exosomes were preincubated with monoclonal antibody (A32) (25 μg Ab/100 μg exosome) that binds specifically to the Hep II heparin binding domain of fibronectin (Thermo Fisher). For experiments designed to block heparan sulfate on the surface of target cells, the cells were incubated with a 40 kDa fragment of human fibronectin (purified from chymotryptic digest of human plasma fibronectin) that contains the Hep II binding domain (50 μg/mL) (Millipore). After washing to remove unbound fibronectin fragments, exosome-target cell interaction was analyzed by confocal microscopy or flow cytometry. For experiments in which heparan sulfate was removed from cell surface, cells were treated with 5 μU/mL of bacterial heparinase for 2 h at 37ºC, followed by extensive washing. For removing heparan sulfate from exosomes, 100 μg exosomes were treated with 1.5 μU/mL heparinase for 3 h at 37ºC; new enzyme was added and exosomes were incubated overnight. Exosomes were then pelleted by ultracentrifugation to remove enzyme. In some blocking experiments, exogenous heparin (10 μg/mL), Roneparstat (10 μg/mL) or a synthetic heparin dodecasaccharide (100 μg/mL) was added simultaneously with 100 μg exosomes/mL. Roneparstat (previously known as SST0001) is a proprietary drug of sigma-tau Research Switzerland S.A. that inhibits growth of myeloma tumors in animal models (24) and is currently in phase I trials in advanced multiple myeloma patients (ClinicalTrials.gov Identifier: NCT01764880). The heparin dodecasaccharide is a fully sulfated molecule prepared by chemoenzymatic synthesis (25).

Fibronectin ELISA- Fibronectin levels were quantified using a commercially available human fibronectin ELISA kit (Millipore), according to the manufacturer’s protocol. The assay is in the format of competitive inhibition ELISA. For some experiments, exosomes were treated without or with 1.5 μU/mL heparinase for 3 h at 37ºC followed by addition of fresh enzyme and incubation overnight. Exosomes were then washed and pelleted by ultracentrifugation or ExoQuick exosome precipitation solution. Exosome protein content of the various samples was determined by BCA protein assay and an equal amount of exosome protein (8 μg) from each sample was used for the ELISA.

Western blot- 100 μg of exosomes, based on BCA protein assay, were lysed in RIPA buffer containing protease inhibitors for 10 minutes on ice and proteins were separated by electrophoresis in a 4-12% SDS-PAGE. The proteins were electroblotted to nitrocellulose membrane and probed with primary antibodies that recognize fibronectin (R&D Systems), clathrin (Abcam) or flotillin (Abcam). In some experiments the exosomes were treated with bacterial heparitinase and probed for syndecan-1 (R&D Systems), clathrin (Abcam) or flotillin (Abcam). Primary antibodies were detected using HRP-conjugated secondary antibodies, and bands were visualized by chemiluminescence. In some experiments RPMI-8226 cells were treated with or without heparinase for 2h at 37ºC and then exposed to 100 μg/ml of either control or aggressive exosomes for 20 minutes. Cells were lysed and probed for phosphorylated p38 or total p38 (Cell Signaling).
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Real-time PCR- RNA was isolated from CAG human myeloma cells incubated with or without exosomes and real-time PCR was conducted for MMP-1 and DKK1 using SYBER Green supermix (Biorad). mRNA expression was determined relative to actin.

Human phosphokinase array- A membrane based antibody array (R&D systems) that determines the relative levels of 43 different human phosphorylated protein kinases was utilized according to manufacturer’s instructions. Briefly, equal amount of cell lysates of human RPMI-8226 cells treated without or with 100 μg of exosomes were incubated overnight with the phosphokinase array membrane. The array was washed to remove unbound proteins followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied to visualize the signal produced at each capture spot corresponding to the amount of phosphorylated protein bound.

Endothelial cell invasion- The effect of exosomes on the invasion of HUVEC cells was assessed using Biocoat Matrigel invasion chambers (BD Biosciences), as described (9). To study the relevance of fibronectin-heparan sulfate interactions for the functional activity of exosomes, exosomes were preincubated without or with the anti-fibronectin antibody A32 (25 μg antibody/100 μg exosome protein) before adding to the endothelial cells.

RESULTS

Fibronectin is present on the surface of myeloma-derived exosomes and enhances exosome-cell interactions- Expression of the heparan sulfate degrading enzyme heparanase in CAG human myeloma cells reprograms them toward a highly aggressive phenotype in vivo endowing tumors formed by these heparanase-high cells with increased metastasis, angiogenesis and osteolysis compared to control, heparanase-low expressing cells (26-29). In analyzing exosomes from these aggressive and control cells we found those secreted by the aggressive cells had a higher level of syndecan-1 (CD138), VEGF and HGF and were able to promote tumor cell spreading and endothelial cell migration much better than exosomes from control cells which failed to substantially impact cell behavior (9). To look for other exosomal proteins that regulate exosome function, we compared the protein content of exosomes secreted by the aggressive vs. control cells using mass spectrometry. As expected the level of heparanase detected was much higher in exosomes secreted by the aggressive CAG cells, while the level of immunoglobulin light chain was identical in the exosomes secreted by control and aggressive CAG cells (Fig. 1A). Interestingly, fibronectin was significantly enhanced in abundance in exosomes from aggressive cells compared to control cells. The presence of fibronectin in exosomes was confirmed by western blotting and ELISA (Fig.1B and 1C).

Interaction of exosomes with target cells is a critical step in the process of exosome mediated intercellular communication. Because fibronectin is known to play an important role in adhesion via interactions with integrins, collagens, fibrin and heparan sulfate proteoglycans, we hypothesized that it played a role in exosome-cell interactions. First, to determine if the level of fibronectin on exosomes correlated with their ability to interact with target cells, we transfected the aggressive and control CAG cells with a construct coding for a chimeric protein composed of CD63 and red fluorescent protein (CD63-RFP). CD63 is a tetraspanin protein that is abundant on the exosome membrane. Thus cells transfected with the CD63-RFP secrete exosomes that can easily be tracked by fluorescence microscopy. Interestingly, by both confocal microscopy and flow cytometry, we found that the exosomes secreted by the aggressive cells and containing high levels of fibronectin interacted much better with target cells than did the exosomes having low levels of fibronectin (Fig. 1D and 1E). As shown in Fig. 1F, the RFP levels in the exosomes secreted by aggressive and control cells were equivalent thereby validating their use for quantitative studies. Having established that exosomes having high levels of fibronectin readily interact with target myeloma cells, we utilized these exosomes in all the subsequent in vitro studies to explore the role of fibronectin in exosome-cell interactions.
In initial studies we assessed whether fibronectin was localized to the exosome surface. For this, exosomes were isolated by centrifugation over an iodixanol cushion and bound to either anti-CD63-containing magnetic beads (Fig. 2A, left panel) or heparin-agarose beads (Fig. 2A, right panel), exposed to anti-fibronectin antibody and subjected to flow cytometry. Results demonstrated that fibronectin is abundant on the exosome surface. Because fibronectin can bind to heparan sulfate, and because these exosomes from aggressive CAG cells have a high level of the heparan sulfate-bearing proteoglycan syndecan-1 on their surface (9), we speculated that fibronectin may be bound to the exosome surface through its interaction with heparan sulfate chains. To test this, exosomes were exposed to bacterial heparitinase, an enzyme that extensively degrades the heparan sulfate chains of syndecan-1 on the surface of these exosomes (Fig. 2B, right panel). Following the removal of heparan sulfate from the exosome surface by heparitinase, the fibronectin remaining associated with the exosome was greatly reduced (Fig. 2B, left panel). To determine if exosomes depleted of fibronectin from their surface affected exosome-target cell interaction, CD63-RFP exosomes were treated with heparitinase, pelleted to remove the enzyme and then incubated with RPMI-8226 myeloma cells. Fluorescence microscopy and flow cytometry revealed that removal of fibronectin from the exosome surface dramatically reduced their binding to target cells compared to exosomes having fibronectin on their surface (Fig. 2C). Together these results indicate that fibronectin is present on the surface of exosomes, it is retained there via interaction with heparan sulfate chains, and its removal inhibits interaction of exosomes with target cells.

Because disruption of exosome-target cell interaction could be of therapeutic value, we tested whether heparin mimetics would compete against exosome and/or cell surface heparan sulfate and thereby disrupt the interaction. Two mimetics were tested, a fully sulfated 12-mer oligosaccharide (that was generated by chemoenzymatic synthesis, and Roneparstat, that is 100% N-acetylated and 25% glycol split. Both mimetics inhibited exosome-target cell interaction as did heparin, the positive control for the experiment (Fig. 3C). While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction. While heparin and Roneparstat were effective at a concentration of 10 μg/ml, a dose of 100 μg/ml was required for the heparin 12-mer to effectively block exosome-cell interaction.

Next, to determine if fibronectin on the exosome surface was binding to heparan sulfate on target cells, we utilized reagents that would block that interaction. Fibronectin exists either in a soluble dimeric form or an insoluble, fibrillar multimeric form (15). One monomer is composed by the repeat of modular segments of 40–90 amino acids, termed type I, II and III repeat units (15). The heparin/heparan sulfate binding domain known as Hep-II, which is located in the type III segment, is the strongest heparin/heparan sulfate-binding site within fibronectin. When target cells were pre-incubated with a fragment of the fibronectin protein containing the Hep-II binding site, it greatly diminished binding of CD63-RFP.
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Exosomes as assessed by confocal microscopy and flow cytometry (Fig. 4A). A similar result was found when exosomes were labeled with PKH67, a lipophilic dye that incorporates within lipid membranes of vesicles (Fig. 4A). Next we utilized A32, an anti-fibronectin monoclonal antibody specific for the Hep-II binding domain of fibronectin that blocks binding of fibronectin to heparan sulfate (30). Pre-incubation of this antibody with exosomes inhibited their interaction with target cells thereby confirming the role of the Hep II domain in exosome-target cell interaction (Fig. 4B). It is important to note that antibody A32 binds to fibronectin in a heparin/heparan sulfate dependent manner. Previous work demonstrated that heparin-induced conformational changes in fibronectin increases the binding of A32 to fibronectin (31). Thus it is possible that binding of fibronectin to heparan sulfate on the surface of exosomes increases the availability of epitopes for A32 thereby contributing to the effectiveness of A32 inhibition of exosome-cell interactions.

Interaction of myeloma patient-derived exosomes with target cells is mediated by fibronectin binding to heparan sulfate - To determine if the above in vitro findings are relevant in humans, exosomes from the sera of three treatment naïve multiple myeloma patients were isolated using an ExoQuick isolation kit and further purified using anti-CD63 linked to magnetic beads. Characterization of the purified particles by nanoparticle tracking revealed that they ranged in size from 50-200 nm, with the majority of particles smaller than 150 nm, consistent with the size range of exosomes (Fig. 5A). ELISA revealed that fibronectin was present on the exosomes from all three myeloma patients examined (Fig. 5B). Interestingly, exosomes isolated from the serum of healthy controls also had fibronectin on their surface indicating that the mechanism we describe may be relevant to exosomes secreted by both tumor and normal cells (data not shown). Further characterization by flow cytometry of exosomes from one of the myeloma patients demonstrated that both syndecan-1 and fibronectin were on the exosome surface (Fig. 5C). As was found with exosomes secreted by the CAG myeloma cell line (Fig. 1), enzymatic removal of heparan sulfate from the patient exosomes greatly diminished the amount of fibronectin associated with exosomes (Fig. 5D and 5E).

To determine if fibronectin on exosomes from patients mediates interaction with target cells, serum exosomes were isolated, fluorescently labeled with PKH26 and incubated with cells from a human bone marrow stromal cell line. The exosomes readily interacted with stromal cells and this was inhibited by pretreatment of exosomes with monoclonal antibody A32 that binds to and blocks the Hep II heparin binding domain of fibronectin (Fig. 5F). Together these studies demonstrate that exosomes in the circulation of myeloma patients have fibronectin bound to their surface by heparan sulfate and that the exosomal fibronectin interacts with heparan sulfate chains on target cells to mediate the exosome-target cell interaction.

The fibronectin mediated interaction of exosomes with target cells impacts target cell function - We have previously demonstrated that exosomes secreted by the heparanase-high, aggressive myeloma cells promotes myeloma cell spreading and endothelial cell invasion. To explore this further and to determine if the fibronectin-mediated interaction of exosomes with target cells impacted their function we performed two experiments. First, an antibody array was utilized to determine if specific signaling events were initiated in the target cells shortly after their interaction with exosomes. Exosomes secreted by aggressive (heparanase high) myeloma cells were incubated with RPMI-8226 myeloma cells for two hours, the cells were extracted and the extracts incubated with antibody arrays to determine the level of tyrosine phosphorylation of specific molecules within key signaling pathways. Results demonstrated that cells incubated with exosomes have increased levels of phosphorylated p38 and to a lesser extent phosphorylated ERK (Fig. 6A). Further to determine if the enhanced p38 activation induced by cells is dependent on the fibronectin-heparan sulfate interactions, equal amount of exosomes (100 μg) from control and aggressive myeloma cells were added to RPMI-8226 cells and analyzed for phosphorylated p38 by western blotting. Fig. 6B shows that aggressive exosomes induces p38 activation in RPMI-8226
Fibronectin facilitates exosome-cell interactions and this is inhibited when heparan sulfate chains are removed from RPMI8226 cells. These results demonstrate that enhanced docking of exosomes to cells through a fibronectin-heparan sulfate interaction can activate signaling pathways in myeloma cells. To determine if the upregulation in p38 signaling was sufficient to impact gene expression, we examined its downstream targets DKK1 and MMP-9. Analysis by PCR revealed that transcription of both DKK1 and MMP-9 were elevated in the cells following interaction with exosomes (Fig. 6C). In a second approach, the impact of exosome-endothelial cell interaction on endothelial cell invasion was examined. Exosomes were preincubated with antibody to the Hep II heparin-binding domain of fibronectin and their ability to promote HUVEC invasion through matrigel coated chambers was compared to exosomes not exposed to antibody. Results indicate that blocking of fibronectin with the antibody significantly inhibited endothelial invasion (Fig. 6D). Together these studies indicate that fibronectin mediated interaction of exosomes with target cells can significantly impact target cell behavior.

DISCUSSION

This work demonstrates a novel mechanism through which cancer exosomes interact with target cells. The data indicate that heparan sulfate on the exosome surface captures fibronectin, and when a target cells is encountered, the exosomal fibronectin binds to heparan sulfate proteoglycans present on the target cell surface (Fig. 7). This model is supported by studies utilizing exosomes from both myeloma cell lines and myeloma patient sera. Although we cannot verify that all the vesicles isolated from patients were derived from tumor cells, a relatively high number of the isolated exosomes were CD138 positive (Fig. 5), in line with the fact that most myeloma cells express high levels of this marker (32). In addition, most of the patient-derived exosomes had fibronectin on their surface which could be removed by treatment with heparan sulfate degrading enzyme. Further, blocking the Hep II domain with antibodies inhibited binding of the patient exosomes to target cells (Fig. 5). Together, these data support the notion that fibronectin on circulating exosomes from myeloma patients mediates their binding to heparan sulfate on target cells. Due to its abundance on cell surfaces, within the extracellular matrix and in the serum, we speculate that fibronectin is not restricted to tumor-derived exosomes but is present on the surface of exosomes derived from many cell types in both normal and pathological settings. In addition, it seems likely that exosome-fibronectin interaction may also facilitate exosome binding to extracellular matrix, by binding to collagens or heparan sulfate trapped within the matrix. This is important because tumor-derived exosomes have been shown to bind components of the extracellular matrix where they can promote matrix degradation and tumor cell invasion and motility (33).

Interestingly, and in support of our finding of fibronectin on the surface of exosomes, it was recently demonstrated that exosomes present in blood plasma of normal human subjects can be isolated on heparin-affinity beads (34), clearly demonstrating that a ligand for heparan sulfate exists on the surface of circulating exosomes. Moreover, heparin has been shown to directly interact with extracellular vesicles and heparin inhibits vesicle binding to target cells (11,14). In another study, exogenous heparin or treatment of cells with heparan sulfate degrading enzymes reduced exosome-target cell interaction by about 50 percent (12). However, in contrast to our findings which point to a role for exosomal fibronectin in exosome-target cell interactions, these prior studies failed to identify the ligand present on the exosome that was interacting with heparan sulfate on the cell surface.

Although fibronectin has been detected by mass spectroscopy as cargo within exosomes from many cell sources (35,36), to our knowledge, our data provide the first evidence that fibronectin is mediating exosome-target cell interaction. Fibronectin has been shown to be present on the surface of exosomes secreted by trophoblasts where it associates with macrophages and induces their expression of IL-1β (37). This occurs without exosome internalization and is likely mediated through the interaction of exosome fibronectin with integrin surface receptors on the macrophages. In addition, it was recently
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demonstrated that HT1080 fibrosarcoma cells deposit exosomes to the substrata that are assessable to the basal cell surface. These exosomes have surface fibronectin that helps support cell adhesion and accelerate cell migration (38). In this case, it was demonstrated that about 50 percent of the fibronectin could be displaced from exosomes by incubation with the RGD peptide, indicating at least some of the fibronectin was bound to the exosome via integrins. Similarly, fibronectin was found on the surface of exosomes isolated from ex vivo cultured 4T1 breast cancer cells, and those exosomes could stimulate tumor cell migration via activation of integrins (39).

However, none of the above studies examined whether the fibronectin on the exosomes being studied associated with heparan sulfate present on either the exosome or on the cell surface. Interactions between fibronectin and heparan sulfate could explain why only half the fibronectin was displaced from the fibrosarcoma-derived exosomes by the RGD peptide (38). Interestingly, although we did not explore whether integrins were involved in the targeting of myeloma-derived exosomes to cells, we found that removal of heparan sulfate from exosomes did remove most of the exosome associated fibronectin (Fig. 2 and 5). This suggests that heparan sulfate is responsible for binding most of the fibronectin to the surface of these myeloma-derived exosomes. Notably, removal of heparan sulfate from target cells only reduced exosome binding by ~50 percent (Fig. 3) and interfering with the Hep II domain of fibronectin (Fig. 4) also only partially inhibited binding of exosomes. This raises the possibility that integrin receptors on target cells may also contribute to the fibronectin-mediated targeting.

Our results indicate that heparan sulfate plays a dual role in the interaction between exosomes and cells; first by capturing fibronectin on the exosome surface and then through interaction of exosome fibronectin with heparan sulfate on the surface of cells. Because heparan sulfate is ubiquitously expressed on cell surfaces the mechanism we describe here is likely not mediating targeting of exosomes to specific cell types. Rather, this may be a general mechanism that mediates initial binding of exosomes to most cells. However, some specificity of exosome binding to cells could be conferred through the structure of cell surface heparan sulfate chains. For example, it was demonstrated that cells lacking heparan sulfate 2-O or N-sulfation exhibited reduced exosome binding compared to controls (13). It was previously demonstrated that after binding of exosomes to cells, cell surface heparan sulfate proteoglycans (HSPGs) were internalized by cells thereby mediating exosome uptake (13). Thus cell surface HSPGs appear to be internalizing receptors for exosomes rather than just cell surface attachment sites. Although the mechanism of release of exosome cargo within cells is not clear, once internalized, exosomes may fuse with the delimiting membrane of the endocytic compartment and deliver the cargo to the cytosol of target cells (40). It is not known if HSPGs play a role in exosome cargo release.

In regard to heparan sulfate on exosomes, it has been shown that the syndecan heparan sulfate proteoglycans, through formation of a molecular complex with syntenin and ALIX, help drive exosome formation (41). We found that heparanase stimulated exosome secretion in myeloma cells (9) and further studies by Roucourt and colleagues recently confirmed our findings and demonstrated that heparanase, by trimming the heparan sulfate chains of syndecan, activates the syndecan-syntenin-ALIX pathway (42). Interestingly, heparanase causes more syndecan-1 to be recruited to exosomes (9,42) and in the present work we discovered that this results in a greater amount of fibronectin associating with the exosome surface leading to enhanced exosome-target cell interaction (Fig.1). Thus, our current findings demonstrate that the importance of the heparanase/syndecan axis in regulating the biology of exosomes is not restricted solely to exosome biogenesis and cargo content, but also impacts exosome-target cell interaction, a key step in the ability of exosomes to regulate cell behavior.

Although we find that heparan sulfate on myeloma-derived exosomes plays an important role in their interaction with target cells, this may not be a universal mechanism used by all exosomes. For example, using exosomes isolated from the U-87 MG glioblastoma cell line, it was
concluded that although both syndecan and glypican proteoglycans were associated with the exosomes, heparan sulfate present on the exosome was not involved in interactions with target U-87 MG cells (12). This does not preclude the possibility that fibronectin is involved, as fibronectin in this setting rather than being bound by heparan sulfate chains, could be attached to exosomes predominantly via integrins present on the exosome surface. Another possibility is that myeloma cell-derived exosomes have a higher level of heparan sulfate and/or fibronectin on their surface that drives the exosome-cell interaction as compared to the glioblastoma-derived exosomes. This would be consistent with our observation that exosomes having low levels of heparan sulfate and fibronectin do not interact with cells as avidly as exosomes having higher levels of these molecules (Fig. 1). This suggests, at least in the case of the myeloma cells, that the capacity of exosomes to interact with target cells is regulated by the amount of heparan sulfate on the exosome surface.

We demonstrate that the exosome-target cell interaction can be blocked by enzymatic removal of heparan sulfate from either the exosome or target cell, by exposure to heparin/heparin analogues that interfere with heparan sulfate/fibronectin interactions, by antibody against the Hep II protein domain within fibronectin that binds to heparan sulfate, or by addition of excess fragments of the fibronectin protein that contain the Hep II domain. These findings not only support our model of fibronectin-heparan sulfate mediated interaction of exosomes with cells, but also expose multiple ways in which that interaction could be attacked in patients for therapeutic benefit. Of particular importance is the finding that heparin, a synthetic fully sulfated 12-mer heparin mimic and heparin-derived Roneparstat are all capable of significantly inhibiting targeting of exosomes to cells. Importantly, unlike heparin, both the 12-mer mimic and Roneparstat lack significant anticoagulant activity and thus can delivered to patients at high doses thereby disrupting or preventing exosome-target cell interactions. These findings reveal a potential and previously undescribed mechanism of action for this class of drugs and provide further rationale for their investigation in clinical trials.

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CONFLICT-OF-INTEREST DISCLOSURE

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AUTHOR CONTRIBUTIONS

A.P. and R.D.S. designed the study, analyzed the data and wrote the manuscript; A.P performed the research; S.K.B. characterized patient exosomes and performed flow analysis for syndecan-1; E.E.B. provided samples from multiple myeloma patients; J.L. synthesized the heparin 12-mer; J.A.M. performed mass spectroscopy; and all authors read and agreed to the final version of the manuscript.
Fibronectin facilitates exosome-cell interactions

REFERENCES


Fibronectin facilitates exosome-cell interactions


FOOTNOTES

Abbreviations used: HSPG, heparan sulfate proteoglycan; FN, fibronectin; ELISA, enzyme linked immunosorbent assay; MMP-9, matrix metalloproteinase-9

FIGURE LEGENDS

Figure 1: Fibronectin is present on myeloma-derived exosomes and a high level of fibronectin correlates with enhanced exosome interaction with target cells. A, Result of a single mass spectrometry analysis of selected exosomal proteins from human CAG control (heparanase-low) or aggressive (heparanase-high) myeloma cells. Fibronectin was the protein most enhanced in abundance in exosomes from aggressive cells compared to control. B and C, The amount of fibronectin associated with exosomes secreted by control or aggressive CAG myeloma cells were quantified by western blot (B) and ELISA (C). Results are mean ± SD of three independent experiments. *P<0.05* compared to control. Clathrin, a marker for cell-derived vesicles, serves as the loading control for the western blot. D, To generate cells that secrete CD63-RFP exosomes, CAG control or aggressive cells were transfected with a
lentiviral vector coding for a CD63-RFP chimeric protein. Exosomes (10 μg/ml) secreted by these cells were incubated with human RPMI-8226 myeloma cells. After two hours, cells were washed, fixed with 3.7% formaldehyde, cytospin and imaged by confocal microscopy. RFP exosomes, red; nucleus, blue. E, Quantitative data from an experiment similar to that shown in D was obtained by subjecting the cells with bound exosomes to flow cytometry. Data are presented as fold increase of median fluorescence intensity of cells incubated with exosomes as compared to cells not incubated with exosomes and are mean ± SD from three independent experiments, * $P<0.05$. F, To ensure that results in D and E above were not due to a higher per exosome concentration of CD63-RFP in the exosomes secreted by aggressive cells, the RFP intensity of an equal amount (8 μg or 40 μg) of exosomes (exo) with low (L) and high (H) levels of fibronectin was measured. The fluorescence intensity (excitation 565 nm / emission 610 nm) of exosomes was equivalent.

**Figure 2: Fibronectin is present on the exosome surface and its removal inhibits exosome interaction with target cells.** A, Fibronectin is present on the surface of exosomes. Exosomes from aggressive CAG cells, purified by ultracentrifugation and excluded by an iodixanol cushion, were captured either using anti-CD63 magnetic beads (left panel) or using heparin-agarose beads (right panel) and subjected to flow cytometry analysis using a mouse monoclonal PE-conjugated anti-fibronectin antibody (blue histogram). PE-conjugated isotype-matched antibody was used as a control (orange histogram). B, Removal of heparan sulfate from the exosome surface removes most of the fibronectin associated with the exosome. Exosomes were either not treated or treated with bacterial heparitinase (1.5 mU/mL heparitinase for 3 h at 37°C followed by addition of fresh enzyme and incubation overnight), a heparan sulfate degrading enzyme, and fibronectin levels quantified by ELISA (left panel). Data are expressed as mean ± SD. * $P<0.05$ compared to exosomes not treated with heparitinase. Right panel, western blot for syndecan-1 on exosomes after heparitinase digestion. Flotillin, a marker for cell-derived vesicles, serves as the loading control. C, Depletion of heparan sulfate from exosomes decreases exosome-target cell interaction. CD63-RFP exosomes secreted by CAG cells were either untreated or treated with heparitinase, washed, and their interaction with RPMI-8226 cells analyzed by confocal microscopy. Right panel, quantitative data from a similar experiment analyzed by flow cytometry. Results expressed as fold change of the median fluorescence intensity. * $P<0.05$ compared to no heparitinase treatment.

**Figure 3: Exosome-target cell interaction is mediated by heparan sulfate chains on target cells.** A, Confocal microscopy analysis of polarized or non-polarized CAG myeloma cells following addition of CD63-RFP exosomes. Exosomes preferentially interact with heparan sulfate-rich uropods of polarized cells, but are widely distributed on non-polarized cells. B, Depletion of heparan sulfate chains on myeloma cells decreases exosome-target cell interaction. RPMI-8226 cells were untreated or treated with heparitinase, washed, and their interaction with RPMI-8226 cells analyzed by confocal microscopy. Right panel, quantitative data from a similar experiment analyzed by flow cytometry. * $P<0.05$ compared to no heparitinase treatment. C, Heparin and heparin oligosaccharides inhibit exosome-target cell interaction. CD63-RFP exosomes were incubated with RPMI-8226 cells in the presence of heparin (10 μg/ml), fully sulfated heparin dodecasaccharide (12-mer) (100 μg/ml) or Roneparstat (10 μg/ml). Exosome alone is used as the control. After 2 hours, exosome-target cell interaction was quantified by flow cytometry. Results are mean ± SD from three separate experiments. * $P<0.05$ compared to control.

**Figure 4: Fibronectin on exosomes binds to heparan sulfate on target cells to mediate exosome-target cell interaction.** A, Heparin binding fragment of the fibronectin protein inhibits exosome interaction. RPMI-8226 cells were untreated or treated with a heparin binding (Hep II) 40 kDa fragment of purified human plasma fibronectin for 1 h, washed and then incubated for 2 h with CD63-RFP exosomes or PKH67-labeled exosomes. Cells were then washed, fixed and exosome interactions analyzed by confocal microscopy. Quantitative data from a similar experiment, using CD63-RFP exosomes, analyzed by flow cytometry (right panel). * $P<0.05$ compared to control. B, A monoclonal antibody (A32)
that binds to the Hep II domain of fibronectin decreases exosome-target cell interaction. CD63-RFP exosomes were pre-incubated with A32 antibody for 1 h and then added to RPMI-8226 cells. After 2 h cells were fixed and analyzed by confocal microscopy. Quantitative data from similar experiment analyzed by flow cytometry (right panel). *$P<0.05$ compared to control.

**Figure 5:** Fibronectin on exosomes isolated from multiple myeloma patients facilitates interaction with bone marrow stromal cells. A, Characterization of exosomes from serum of treatment naïve multiple myeloma patients. Exosomes were purified from serum of myeloma patients by ExoQuick precipitation followed by isolation using anti-CD63 conjugated beads. Particle size was analyzed by nanoparticle tracking using a NanoSight 300. Histogram shows two lines representing duplicate analyses. B, ELISA quantification of the levels of fibronectin in exosomes isolated from serum of three myeloma patients. C, Syndecan-1 and fibronectin are present on the surface of myeloma patient derived exosomes. Exosomes purified from the serum of myeloma patients using ExoQuick precipitation followed by anti-CD63 magnetic bead isolation were subjected to flow cytometry analysis using an affinity purified polyclonal goat anti-syndecan-1 IgG antibody (blue) or mouse monoclonal PE-conjugated anti-fibronectin antibody (blue). Normal goat IgG and mouse IgG1 isotype were used as control (red), respectively. Note: syndecan-1 (core protein) was detected on the surface of exosomes only after removal of heparan sulfate chains by heparitinase treatment to expose the epitope. D and E, Removal of heparan sulfate chains removes most of the fibronectin from exosomes isolated from the serum of myeloma patients. Exosomes isolated from patient serum were either treated or not treated with heparitinase and fibronectin levels were quantified by ELISA (D) and compared by western blot (E). *$P<0.05$ compared to heparitinase untreated exosomes. F, A32 antibody against the Hep II domain of fibronectin blocks the interaction of patient-derived exosomes with bone marrow stromal cells. Exosomes isolated from the serum of a myeloma patient were labeled with PKH26 (red) dye, preincubated with or without A32 antibody for 1 h and allowed to bind HS-5 human bone marrow stromal cells. After 2 h cells were fixed and images analyzed by confocal microscopy.

**Figure 6:** Exosome-target cell interaction mediated by fibronectin impacts cell behavior. A, Exosome interaction with RPMI-8226 cells activates p38 and ERK signaling. An antibody array that simultaneously examines the phosphorylation levels of 43 different protein kinases was utilized to determine what signaling pathways were activated when myeloma-derived exosomes interacted with target cells. RPMI-8226 cells were incubated with or without exosomes isolated from aggressive myeloma cells (CAG cells expressing high heparanase) and cell lysates were exposed to membranes and the membranes probed with a phospho-tyrosine specific antibody. Phosphorylated p38 and ERK (circled) were enhanced in cells incubated with the exosomes. The different phospho kinases that are activated in RPMI-8226 cells independent of the addition of exosomes are shown by arrows. Duplicate dots at the corners represent phospho-tyrosine positive controls. B, RPMI-8226 cells were treated with exosomes (100 μg/ml) secreted by control or aggressive myeloma cells for 20 minutes and analyzed for phosphorylated p38 by western blot. Total p38 serves as the loading control. To determine the role of cell surface heparan sulfate in mediating exosome induced signaling, RPMI-8266 cells were treated with bacterial heparitinase for 2 hour prior to the addition of exosomes from aggressive myeloma cells. C, MMP-9 and DKK1, two downstream target genes of activated p38 are upregulated following the interaction of myeloma-derived exosomes with the RPMI 8226 cells. RPMI 8266 myeloma cells were incubated with or without exosomes and DKK1 and MMP-9 mRNA expression in these cells were assessed using real-time PCR and normalized using actin expression. *$P<0.05$ compared to no exosome addition. D, 10 μg CD63-RFP exosomes from aggressive CAG cells were added to endothelial cells in the presence or absence of A32 antibody and the number of cells that invaded through the matrigel coated chamber overnight were determined. Data are mean ± SD from three separate experiments. *$P<0.01$ compared to no exosome addition and $^\gamma P<0.05$ compared to exosomes only.
**Figure 7: Model of exosome-target cell interaction mediated by fibronectin.** 1, Fibronectin (FN) is captured on the surface of exosomes by heparan sulfate proteoglycans (HSPG). 2, Fibronectin, on exosomes, binds to heparan sulfate (HS) on the surface of the target cell. 3, Removal of exosome HS or cell surface HS with heparitinase, addition of exogenous heparin or heparin mimetics, addition of Hep II domain-containing fragment of fibronectin, or exposure to antibody against the Hep II domain of fibronectin dramatically diminishes exosome interaction with target cells.
Figure 1

A

![Bar chart showing peptide count for Fibronectin, Heparanase, and Light chain between control cell exosomes and aggressive cell exosomes.]

B

![Western blots for Fibronectin and Clathrin in control and aggressive cell exosomes.]

C

![Graph showing ng Fibronectin/100 μg exosome protein between control and aggressive cell exosomes.]

D

![Images of FN-low and FN-high exosomes with CD63 and nucleus staining.]

E

![Graph showing fluorescence intensity (fold change) with asterisk indicating significant difference.]

F

![Graph showing fluorescence intensity with 8 μg and 40 μg of exosome protein.]

17
Figure 2

A

B

C

Heparitinase

Heparitinase

Heparitinase

Syndecan-1

Flotillin

CD63/nucleus

Fluorescence Intensity (Fold change)

Heparitinase: - +

0 0.5 1 1.5

0 50 100 150 200 250

ng Fibronectin/10 µg exosome protein

- +
Figure 3

A

Polarized cell with uropod

Non-polarized cell without uropod

B

Heparitinase

+  -

CD63/nucleus

C

Fluorescence Intensity (fold change)

Control  Heparin  Fully sulfated 12-mer  Roneparstat

#
Figure 4

A. Heparin binding fragment (Hep II) of FN

B. Antibody against the heparin binding (Hep II) domain of FN
Figure 5

A. Concentration of F6 particles/mL vs. Size (nm)

B. Fibronectin (μg/10^7 exosome particles) vs. Patient #

C. Flow cytometry plots showing Syndecan-1 and Fibronectin levels.

D. Bar graph showing Fibronectin (μg/mL serum exosome) levels with and without Heparitinase treatment.

E. Western blot showing Fibronectin levels with and without Heparitinase treatment.

F. Immunofluorescence images showing antibody against the heparin binding domain of FN with and without treatment.

Legend:
- +: Heparitinase present
- -: Heparitinase absent
#
Figure 6

A

without exosomes

with exosomes

AMPKα1
CREB
STAT2
Chk2
HSP60

B

Exosomes: None control aggressive aggressive
Heparitinase: - - + -

Phospho-p38
Total -p38

C

mRNA (fold change)

DKK1
MMP-9

D

% endothelial invasion

None Exo Exo+FN Ab

* # ¥
Figure 7

Heparitinase
Heparin/Heparin mimetic
Hep II FN fragment
Anti-Hep II domain antibody

1. Exosome
2. Fibronectin fiber
3. Target cell
Fibronectin on the surface of myeloma cell-derived exosomes mediates exosome-cell interactions
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