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Exosomes secreted from bone marrow-derived mesenchymal stem cells protect the intestines from experimental necrotizing enterocolitis

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ABSTRACT

Purpose: Treatment options for necrotizing enterocolitis (NEC) remain inadequate. Bone marrow-derived mesenchymal stem cells (BM-MSCs) can protect the intestines from NEC. Exosomes are nanoparticle-sized vesicles with important cell signaling capabilities. The objective of this study was to determine whether BM-MSC-derived exosomes can prevent NEC.

Methods: Rat pups were either breast fed (Group 1) or subjected to experimental NEC and randomized to receive either no treatment (Group 2) or an intraperitoneal (IP) injection of PBS (Group 3), BM-MSC (Group 4), or BM-MSC-derived exosomes (Group 5). Histologic injury grade and intestinal permeability were determined. The effect of BM-MSC-derived exosomes on IEC-6 intestinal epithelial cells in an *in vitro* scrape model of wound healing was also determined.

Results: Animals exposed to NEC that were either untreated or received PBS alone had an NEC incidence of 46% and 41%, respectively ($p = 0.61$). Compared to untreated pups, the incidence of NEC was significantly lower in pups treated with either BM-MSC (9%, $p = 0.0003$) or MB-MSC-derived exosomes (13%, $p = 0.0008$). Similar results were found for intestinal permeability. Wound healing in IEC-6 cells was significantly increased by BM-MSC-derived exosomes.

Conclusion: BM-MSC-derived exosomes protect the intestines from NEC and may represent a novel, cell-free, preventative therapy for NEC in the future.

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Necrotizing enterocolitis (NEC), the most common surgical emergency in neonates, has an incidence of 1–3 cases per 1000 live births in the United States, affecting 2000–4000 predominantly premature newborns [1,2]. Severe NEC comprises 27% to 63% of all cases, often requiring substantial surgical resection of nonviable intestine [2]. NEC mortality ranges from 20% to 50%, resulting in approximately 1000 deaths each year [2]. NEC is the leading cause of short bowel syndrome (SBS) in the pediatric population. Although significant research has been performed to identify novel preventive and therapeutic interventions, the incidence, morbidity, and mortality from NEC remain unacceptably high.

Research on mesenchymal stem cell (MSC) therapy for injured and diseased tissues, including the intestines, has been promising [3,4]. MSCs have the ability to engraft into injured tissues and promote healing through paracrine mechanisms [5–9]. Recent studies have shown that MSCs administered either intraperitoneally (IP) or intravenously (IV) engraft into injured intestinal tissue and decrease the incidence of experimental NEC in rats [10,11]. However, the number of MSCs that engraft into injured tissues may not be sufficient to account for their robust overall protective effects. This suggests that additional mechanism(s) mediates these protective effects, including secreted bioactive factors that confer protection in a paracrine fashion [12–14].

Exosomes are nanovesicles produced by the endosomal pathway that are initially contained intracellularly within multivesicular bodies. They are secreted into the extracellular space when these multivesicular bodies fuse with the cell membrane [15]. Once thought to contain cellular material no longer valuable to the cell, research over the last decade has revealed that exosomes are important in cellular communication and signal transduction [16–18]. Exosomes secreted by many different types of cells affect cell signaling by interacting with receptors on target cell membranes, modifying the extracellular milieu surrounding target calls, or fusing with the target cell membrane and releasing their contents into the target cell cytoplasm [19]. The contents of exosomes include micro-, messenger-, and small interfering RNAs, as well as proteins such as growth factors [20–23]. The molecular cargo of exosomes is variable depending upon the nature of the secreting cell. Exosomes secreted by MSCs have been shown to contain antiapoptotic miRNAs, to promote epithelial and endothelial wound healing and angiogenesis, and to contain growth factor receptor mRNAs known to

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promote wound healing [24–29]. The goal of this study was to determine if exosomes secreted from BM-MSCs could protect the intestines from experimental NEC.

1. Materials and methods

1.1. Ethics statement

All animal procedures (Protocol #04203AR) were approved by the Institutional Animal Care and Use Committee (IACUC) of the Research Institute at Nationwide Children's Hospital.

1.2. Isolation and identification of BM-MSCs

These studies utilized a murine bone marrow-derived MSC line that was previously established and characterized [30]. Murine bone marrow-derived MSCs harvested from H2K-GFP transgenic mice were previously subjected to flow cytometry to confirm lack of expression of CD45 and other hematopoietic lineage markers (TER119, CD3, B220, CD11b, and Gr-1) and expression of the MSC markers CD29, CD49e, CD90, CD105, and Sca-1). The trilineage (osteogenic, adipogenic, and chondrogenic) differentiation potential of these cells was previously confirmed.

1.3. Preparation of BM-MSCs for in-vivo administration

Adherent BM-MSCs were grown in D-MEM/F12/Glutamax-I™ (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) in T-75 flasks until 80% confluent. Cells were then trypsinized (0.25% trypsin, Gibco Invitrogen, Carlsbad, CA) for 3 min, followed by trypsin neutralization using media containing 10% FBS. Cells were quantified using a hemacytometer, centrifuged at 180 ×*g* for 5 min at 4 °C, and resuspended in sterile phosphate buffered saline (PBS) at a concentration of 3×10^5 cells per 50 μl. This preparation of cells was then loaded into 0.3 ml low-dose U-100 insulin syringes with 29 gauge needles (Becton Dickinson, Franklin Lakes, NJ) immediately prior to IP injection.

1.4. Isolation and characterization of exosomes

BM-MSCs were cultured until ~80% confluent, and were then cultured in serum-free media (SFM) for 48 h, at which time exosomes were isolated from the BM-MSC-conditioned media (CM) by one of two methods. The first method, used for *in vivo* studies, was performed according to the manufacturer's instructions for the P100 PureExo Exosome Isolation kit (101bio, Palo Alto, CA). Exosomes were isolated from one T-75 flask containing ~80% confluent BM-MSCs, resuspended in 150 μl PBS, and used for three separate IP injections (50 μl/pup). These isolated exosomes were stored at 4 °C and used within 72 h or were frozen at −80 °C. Exosomes isolated using this method were characterized by nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern Instruments Inc., Westborough, MA). Quantification indicated that this isolation method yielded ~2.5×10^9 BM-MSC-derived exosomes/50 μl volume. The second method, used for *in vitro* wound healing studies, used serial centrifugation to obtain both BM-MSC-derived exosomes as well as exosome-depleted BM-MSC-CM for use as a control [31,32]. One T-75 flask containing ~80% confluent BM-MSCs was used to prepare BM-MSC-CM which was collected and centrifuged at 300 ×*g* for 10 min to pellet cells which were discarded. The supernatant was collected and centrifuged at 2000 ×*g* for 20 min, and the resulting supernatant was centrifuged at 10,000 ×*g* for 30 min to pellet contaminating cellular debris, which was discarded. The remaining supernatant was then ultracentrifuged at 100,000 ×*g* for 18 h to obtain both isolated exosomes (pellet) as well as exosome-depleted BM-MSC-CM (supernatant) [32]. The pellet was resuspended in 150 μl PBS, and both isolated exosomes and exosome-depleted BM-MSC-CM were stored at 4 °C and used within 3 days or frozen at −80 °C. These preparations were used for three separate treatments of IEC-6 cells, at a volume of 50 μl each, as described in section 1.9. Exosomes isolated using both methods showed strong Western blot immunostaining to the well-established exosome markers CD-9 and flotillin-1.

1.5. Animal model of NEC

Premature Sprague–Dawley rat pups were delivered by caesarean section under CO_2 anesthesia at E-21 of gestation. Briefly, pups were then subjected to a modification of the experimental NEC protocol initially described by Barlow et al. [33]. Pups were kept in an incubator at 35 °C with 50% humidity and fed by orogastric gavage five times daily using a NICC-NATE® 1.9 French single lumen silicone catheter (Utah Medical Products Inc., Midvale, UT). The hypertonic formula used for feedings [15 g of Similac 60/40 (Ross Pediatrics, Columbus, OH) in 75 ml of Esbilac (Pet-Ag, New Hampshire, IL] provided 836.8 kJ/kg per day. Feeds were started at 0.1 ml of formula per feed with the volume of formula advanced each day by 0.1 ml per feed to a maximum of 0.4 ml per feed by the fifth day of life. Immediately after feeding, pups were exposed twice daily to hypoxia (100% nitrogen for 90 s), followed by hypothermia (4 °C for 10 min) until the end of the experiment at 96 h of life.

1.6. In vivo experimental design

Newborn rat pups were randomized to one of the following groups: Group 1: breast-fed control pups (N = 8) that were placed with a surrogate dam so that they could receive physiologic breast milk feeds; Group 2: NEC only (N = 46); Group 3: NEC plus a single IP dose of 50 μl PBS vehicle (N = 59); Group 4: NEC plus BM-MSCs in 50 μl PBS IP (N = 35); or Group 5: NEC plus BM-MSC-derived exosomes in 50 μl PBS IP (N = 40). Pups were exposed to BM-MSC or BM-MSC-derived exosomes 5 h after delivery. Pups exposed to NEC were sacrificed by decapitation upon the development of clinical signs of NEC. All pups surviving for 96 h of life were sacrificed at that time. After each sacrifice, the small intestine was immediately removed and formalin-fixed for histologic analysis.

1.7. Histologic evaluation of experimental NEC injury

The distal small intestine of each pup was removed upon sacrifice and fixed in 10% formalin for 24 h. The fixed tissue was paraffin-embedded and transverse sections were prepared and stained with hematoxylin and eosin (H&E). H&E stained sections were graded by two independent observers using a standard histological injury scoring system [34] as follows: Grade 0, no villus damage; Grade 1, distal villus enterocyte detachment; Grade 2, sloughing of enterocytes to the mid villus level; Grade 3, loss of the entire villus with preservation of the crypts; and Grade 4, transmural necrosis. Injury grades of 2, 3, or 4 were defined as NEC, while injury grades 3 and 4 were defined as severe NEC.

1.8. Evaluation of intestinal mucosa permeability

Fluorescein isothiocyanate (FITC) labeled dextran (FD70, molecular weight 73,000) (Sigma-Aldrich Inc., St. Louis, MO) was used to assess mucosal permeability as described previously [35]. Briefly, rat pups from each experimental group received 750 mg/kg FD70 suspended in sterile PBS by orogastric gavage 46 h after C-section. Four hours later, pups were sacrificed and serum levels of FD70 were measured by spectrophotofluorometry. The FD70 concentration in the plasma of each pup was calculated from a standard curve of known FD70 concentrations.

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