Mesenchymal Stromal Cell-Derived Extracellular Vesicles Provide Long-Term Survival After Total Body Irradiation Without Additional Hematopoietic Stem Cell Support

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ABSTRACT

The therapeutic effect of mesenchymal stromal cells (MSC) in tissue regeneration is based mainly on the secretion of bioactive molecules. Here, we report that the radioprotective effect of mouse bone marrow derived mesenchymal stromal cells (mMSC) can be attributed to extracellular vesicles (EV) released from mMSC. The transplantation of mMSC-derived EV into lethally irradiated mice resulted in long-term survival but no improvement in short-term reconstitution of the recipients. Importantly, the radiation rescue was efficient without additional hematopoietic support. In vitro we show a protection by EV of irradiated hematopoietic stem cells but not progenitor cells using stroma-cell cultures and colony-forming assays. After systemic infusion into lethally irradiated recipients, labeled EV traveled freely through the body reaching the bone marrow within 2 hours. We further show that long-term repopulating Sca-1 positive and c-kit low-positive stem cells were directly targeted by EV leading to long-term survival. Collectively, our data suggest EV as an effective first-line treatment to combat radiation-induced hematopoietic failure which might also be helpful in alleviating myelosuppression due to chemotherapy and toxic drug reaction. We suggest the infusion of MSC-derived EV as efficient and immediate treatment option after irradiation injuries. STEM CELLS 2017;35:2379–2389

SIGNIFICANCE STATEMENT

Radiation as used in conditioning of patients prior to hematopoietic stem cell transplantation is associated with severe side effects. Previous research has shown survival-promoting properties of mouse mesenchymal stromal cells in a model of lethal irradiation without support of hematopoietic stem cells. This phenomenon formerly was attributed to “paracrine” effects. In recent years, extracellular vesicles (EVs) have gained increasing interest as mediators of intercellular communication based on their capability to transfer new information to sites of action without loss at physiological barriers. This novel data demonstrate that EV derived from mouse bone marrow-derived mesenchymal stromal cells are the active component of these cells providing a radiation-protective effect. Results show that hematopoietic, long-term repopulating stem cells are targeted rapidly by the EV and thereby rescued from radiation damage. These results open a new line of treatment options for patients suffering from radiotherapy induced myelosuppression without the potential side effects of cell therapies.

INTRODUCTION

Total body irradiation (TBI) at lethal dosage is widely used in conditioning patients prior to hematopoietic stem cells (HSC) transplantation or can occur following major radiation leakage incidents. Radiation often leads to long-lasting bone marrow (BM) suppression connected with high morbidity and mortality. We have previously shown that transplantation of mouse bone marrow derived mesenchymal stromal cells (mMSC) without additional stem cell transplantation provided long-term survival of lethally irradiated recipients [1]. Although mMSC accumulated in the lung, protective properties were evident in the BM as an organ of hematopoiesis. In this experimental setting, peripheral leukocytes and thrombocytes showed a long-term recovery similar to that seen following HSC transplantation. Mesenchymal stromal cells (MSC) are an integral...
part of the hematopoietic niche and have been reported to provide protective effects in several tissue injuries, where their regenerative properties are largely attributed to paracrine effects. Besides biologically active components such as cytokines, extracellular vesicles (EV) released by MSC are promising candidates for mediating the ascribed tissue regeneration.

In the late 1990s, Raposo et al. and Zitvogel et al. published the first reports of shed vesicles as important mediators of intercellular communication [2–5]. Today, it is assumed that most if not all cells actively release diverse types of vesicles of endosomal and plasma membrane origin, respectively, called exosomes and microvesicles, into the extracellular environment. Here, we will use the term EV to describe all classes of extracellular membrane vesicles with a size of 30–1,000 nm since most methods purifying EV from in vitro and in vivo fluids detect a mixed population [6–8]. EV are phospholipid bilayer enclosed particles comprising membrane and cytosolic proteins, lipids, DNA, and RNA, and therefore might have a low susceptibility to degradation when leaving the cells of origin. This assumption is supported by detection of EV in various biological fluids, thus demonstrating their secretion in vivo as well as in vitro [9]. All EV bear surface molecules that allow them to be specifically targeted to recipient cells. After attachment to a target cell, EV can induce a modification of the physiological state of the corresponding cell [3, 10, 11].

In previous work, we attributed the protective properties of mMSC after lethal irradiation to their secretion of EV. Exclusive transplantation of mMSC-derived EV led to similar leukocyte and accelerated thrombocyte recovery in lethally irradiated recipients compared to mMSC transplantation [12]. Here, we show that mMSC-derived EV support HSC recovery in vitro and long-term survival of recipients in vivo by directly targeting reconstituting HSC, thus providing a novel and importantly cell-free tool to combat neutropenia and thrombocytopenia.

**Materials and Methods**

**Expansion of mMSC and EV Generation**

Animal experiments were approved by the local ethical committee (License Department Hamburg) under application No. 25/12 and performed according to their guidelines. Mouse MSCs were isolated from the BM of male C57Bl/6J mice aged 6–8 weeks. At passage 6, a working cell bank was frozen in liquid nitrogen and regularly used as the starting cell population for further expansion and characterization as described [1]. Phenotypical analysis was carried out using the following antibodies: CD9 (clone RAM34), CD45 (clone 30-F11), CD90.2 (clone 53-2.1), CD117 (clone 28B8), CD11b (clone M1/70), Sca-1 (clone E13–161.7) (all from BD Bioscience, Heidelberg, Germany), MHCI (clone M5/114.15.2; Biologend, Koblenz, Germany), CD73 (clone TV/11.8), CD105 (clone M7/18) (both from eBioscience/Thermo Fisher Scientific, Waltham, MA) and appropriate isotype controls. Mouse MSC were expanded in dulbecco’s modified eagle medium (DMEM)/Ham’s F12 medium (Biochrom, Berlin, Germany) supplemented with 20% preselected fetal calf serum (FCS) and 2 mM glutamine (both: Invitrogen/Thermo Fisher Scientific, Waltham, MA). For last feeding before EV generation, FCS was EV-depleted by overnight centrifugation with 120,000g at 4°C for ≥18 hours. The majority of FCS-borne EV thus was removed from FCS [13]. EV were generated in medium supplemented with 10% EV-depleted FCS and 2 mM glutamine for 48 hours. EV were harvested from the supernatant at 4°C using a modified centrifugation protocol published by Thery et al. [14]. Centrifugation was carried out for 20 minutes at 2,000g to deplete cells and debris and 12,000g for 20 minutes to deplete apoptotic bodies and large vesicles. The EV were pelleted for 70 minutes at 100,000g using Beckman Coulter ultracentrifuge equipped with swinging bucket rotor, washed in phosphate-buffered saline (PBS), resuspended in small volumes α-MEM (Minimum Essential Medium) supplemented with 25 mM HEPES (both Invitrogen) and stored at −80°C. Cells were lifted with Trypsin-EDTA (Invitrogen) and counted using Trypan-Blue (Invitrogen) for live-dead discrimination.

**Characterization of EV**

Electron microscopy was carried out as described [15]. For Western blotting, mMSC-derived EV and parental mMSC were lysed in RIPA buffer, the protein concentration determined by Lowry Assay (Bio-Rad Laboratories GmbH, München, Germany) in a NanoDrop 1000 spectrophotometer (PEQLAB Biotechnology, Erlangen, Germany) and 20 μg of protein lysate analyzed by 12% SDS-PAGE electrophoresis sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western transfer onto polyvinylidene difluoride (PVDF) membrane using standard procedures. The following antibodies were used: CD9 (1:100), Tsg101 (1:500), Hsp70 (1:1,000), Calnexin (1:500) (all from Santa Cruz, Heidelberg, Germany) and Flotilltin (1:1,000; Becton Dickinson) as primary antibodies, horseradish peroxidase-coupled goat anti-rat (1:2,500), goat anti-mouse (1:5,000; both from Santa Cruz) or goat anti-rabbit (1:5,000; GE Healthcare, Solingen, Germany) secondary antibody depending on the primary antibody. Detection was done with SuperSignal West Pico Chemoluminescence Reagent (Thermo Fisher Scientific). Exposure time of x-ray films was identical for the same EV and mMSC protein.

**Transplantation of EV**

EV derived from 10⁶ mMSC in 150 μl serum-free medium were transplanted i.v. via the tail vein into female C57Bl6J/Ly5.1 recipients aged 8–12 weeks who received a lethal dose of TBI of 9.5 Gy using a Cs-137 radiation source (mMSC-EV). EV-recipients did not receive any further hematopoietic support. Groups of irradiated recipients without any further treatment (irradiation control) and recipients transplanted with 10⁶ mMSC (mMSC) were included. Survival of recipients in all groups was documented more than 7 months and plotted in a Kaplan-Meier graph.

**Influence of EV on Hematopoietic Progenitor Cells**

BM of unmanipulated female C57Bl6J/Ly5.1 mice was flushed out of femurs and tibiae, erythrocytes lysed with 0.8% NH₄Cl and counted with Trypan blue (Invitrogen) for live-dead discrimination. About 3 × 10⁶ mononuclear cells (MNC) were resuspended in 1 ml of complete methylcellulose for murine cells (Stem Cell Technologies, Köln, Germany) and plated in triplicates of 0.3 ml. The MNC for the radiation control and experimental groups were irradiated with 4 Gy using the RS225 Research System (Gulmay Medical, Camberley, U.K.)
before resuspension in methylcellulose. In preliminary tests, this dose gave rise to approximately 10% residual colony growth in the methylcellulose assay in pretestings compared to non-irradiated cells. To test the influence of EV on rescue of progenitor colony formation, irradiated MNC were mixed with mMSC-derived EV in a ratio of 1 MNC: EV derived from 40 mMSC and assayed for colony growth. The experiment was repeated 11 times in triplicates with different EV preparations.

Influence of EV on Hematopoietic Stem Cells

The stromal cell line MS-5 was cultivated in α-MEM (Invitrogen) supplemented with 20% pretested horse serum (Biological Industries, Kibbutz Beit-Haemek, Israel) and 2 mM glutamine. Stromal layers were irradiated with 30 Gy to inhibit growth of stromal cells. Fresh BM-derived MNC were left untreated, irradiated with 4 Gy or irradiated with 4 Gy and incubated with mMSC-derived EV at a ratio of 1:40. About 10⁶ MNC of each group were seeded into 25 cm² MS-5 flasks in triplicates (n = 8) and incubated for 7 days. Additionally, EV were isolated from NIH3T3 cells and tested in parallel in this system (n = 4 in triplicates, EV preparation derived from three individual cultures). At day 7, pictures were taken of cobblestone areas (CA) and their numbers were counted. The supernatants of all inoculated MS-5 cultures, containing the progenitors derived from CA, were harvested at day 7, resuspended in 1 ml of complete methylcellulose, plated in triplicates of 0.3 ml, and tested for progenitor colony formation. Colonies were counted at day 12–14.

Intravenous Injection of Stained EV

Mouse MSC-derived EV were stained with PKH26 (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer’s instructions. The fluorescently labeled EV derived from 10⁶ mMSC were injected i.v. into lethally (9.5 Gy) irradiated recipients. After 2 and 4 hours, BM was flushed out from tibiae and femurs, 5 × 10⁶ cells cytospun onto slides and fixed with ice-cold acetone. Slides were stained with anti-mouse CD117 (c-kit 1:200; ebioscience/Thermo Fisher Scientific), anti-mouse Sca-1 (BioLegend, 1:200), followed by a secondary Alexa Fluor 488-coupled goat anti-rat antibody (Al488, Invitrogen, 1:500) or left without antibody staining. Spleens and lungs were frozen in liquid nitrogen and cut into 8 μm sections. All slides were counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma) at 1 μg/ml. All cytospins and tissue sections were evaluated for the presence of PKH26-labeled EV (n = 4 per time point in two separate experiments).

Additional experimental groups of irradiated and EV-injected mice as described above were used to evaluate the colocalization of EV with BM cells using ImageStream® Mark II Imaging Flow Cytometer (Merck Millipore, Darmstadt, Germany). BM from recipients was harvested at 2 (n = 9 in three separate experiments) or 4 hours (n = 8 in three separate experiments) after injection of PKH26-labeled EV. PBS-injected irradiated mice were included as controls (n = 3). For each single time point and individual recipient, five samples of 1 × 10⁶ BM cells after hemolysis were prepared. Cells were double-stained with Al488-conjugated Sca-1 (antibodies-online, Aachen, Germany) and Al647-conjugated c-kit (Novus Biologicals, Wiesbaden, Germany), counterstained with Hoechst 33258 (Sigma) at 1 μg/10⁶ cells, and 50,000 cells analyzed with the ImageStream.

Statistical Analysis

Analysis of variance (ANOVA) (Figs. 2A, 4A) or Kruskal-Wallis tests (Fig. 2C, 2D) with post hoc analysis were performed with p < .05 being regarded as statistically significant. Results were presented as mean ± SD. The number of independent experiments is shown in the respective section.

RESULTS

Characterization of mMSC and Derived EV

EV-producing mMSC were regularly characterized using flow cytometry and in vitro differentiation assays. Mouse MSC displayed a fibroblastoid morphology (Supporting Information Fig. S1A). Their phenotype (Supporting Information Fig. S1B) was characterized by negativity for CD34, CD45, CD117 (c-kit), CD90.2, CD11b, MHCII, and positivity for CD105, CD73, and Sca-1 in concordance with findings of others [16–18]. Furthermore, they readily differentiated into adipocytes, chondroblasts, and osteoblasts under appropriate conditions (Supporting Information Fig. S1C). We have previously shown that the prolonged mMSC expansion time of at least six passages is sufficient to remove all hematopoietic cells capable of generating donor chimerism after transplantation into lethally irradiated recipients [1]. EV derived from mMSC were characterized using electron microscopy and Western blot analysis. EV showed the typical spherical morphology (Fig. 1A). Furthermore, they expressed EV characteristic proteins HSP70, flotillin, Tsg101, and CD9 similarly to parental mMSC but lack calnexin (Fig. 1B) in accordance with the minimal experimental requirements for the definition of EV [19].

Mouse MSC-Derived EV Give Rise to Long-Term Survival

Lethally irradiated recipients after i.v. transplantation of mMSC-EV showed a long-term survival similar to that of mice receiving mMSC but EV were unable to improve short-term survival (Fig. 1C). The Kaplan-Meier survival curves display the survival data of EV-recipients (mMSC-EV), mMSC recipients (mMSC), and control group without any transplantation (irradiation control). Comparing the leukocyte and thrombocyte recovery of long-term surviving mMSC recipients (Fig. 1D) to mMSC-derived EV recipients (Fig. 1E), we documented a similar kinetic of white blood cell recovery. Interestingly, the recovery of thrombocytes in EV recipients showed an accelerated kinetic compared to mMSC only reaching normal levels within approximately 60 days. Seven months later, all recipients were hematologically well with a normal distribution of peripheral cell populations (data not shown). Non-reconstituting animals did not recover leukocytes, thrombocytes, or erythrocytes and eventually died from BM failure (Fig. 1C).

EV Rescue Hematopoietic Stem Cells but Not Hematopoietic Progenitors

Next, we assayed the influence of EV on hematopoietic progenitor and stem cells from mouse BM in vitro. First, the
effects of EV on progenitor cells was investigated in colony assays. We chose a proportion of MNC: EV of 1:40 (1 MNC: EV derived from 40 mMSC) after testing several higher EV numbers with no difference in results (data not shown). Further reductions in the number of EV used per MNC generated inconsistent results.

Compared to the irradiated MNC control, the treatment with EV did not result in an improvement of colony growth (Fig. 2A) suggesting that EV cannot rescue colony formation. We then went on to investigate the influence of EV on HSC. To do so, BM-derived MNC were isolated from unmanipulated mice as described above. Three groups of MNC were incubated for 7 days on MS-5 stroma layers: unmanipulated MNC (MNC), MNC after irradiation with 4 Gy (irradiated MNC), MNC after irradiation with 4 Gy, and treatment with EV (irradiated MNC + EV). Within 7 days, we detected a substantial outgrowth of CA in the cultures incubated with EV-treated MNC, compared to only marginal CA formation in cultures seeded with irradiated MNC without EV (Fig. 2B). As controls for biological activity of mMSC-derived EV, we included EV derived from NIH3T3 cells (irradiated MNC + EV NIH) which did not show an improvement of CA formation (data not shown). Enumeration of the total number of CA at day 7 showed a nonsignificant increase in the EV-treated group compared to irradiated only group (Fig. 2C, left). Interestingly, when comparing the number of large CA (>200 cells) a highly significant increase was found in the EV-treated group compared to the irradiation only group (Fig. 2C, right). In contrast, NIH-derived EV failed to support CA formation after irradiation as shown by the very low numbers of both total and large CA (Fig. 2C).

The supernatants of the different MS-5 cultures were harvested at day 7 and tested for colony formation. The number of colonies formed (Fig. 2D) increased markedly in the EV-treated group to achieve an almost identical number of colonies as seen in the group of non-irradiated MNC, representing a highly significant increase compared to irradiated MNC without EV-treatment. Again, no promotion of hematopoiesis was
detected using NIH3T3 fibroblast-derived EV, which gave rise to identical numbers of CA and colonies compared to the irradiation only group (Fig. 2C, 2D).

**Transplanted EV Target Hematopoietic Stem Cells**

To follow the fate and targeting of intravenously injected EV, the localization of fluorescently labeled EV was investigated after injection into lethally irradiated recipients without any additional hematopoietic support. DAPI staining of BM cyto-
spins, which were harvested 2 or 4 hours after EV transplantation, revealed a colocalization of PKH26-positive EV with potential stem cell-like cells characterized by small round nuclei (Fig. 3A) but not progenitors of the myeloid line such as metamyelocytes (for example, see Fig. 3A, rightmost picture with several metamyelocytes). Impressively, the EV were detected in clusters of potential HSC leaving out other clusters of BM cells. The amount of all nuclei compared to the number of PKH26-positive EV indicated a rapid immigration of EV into the BM within 2 hours (approximately 2 EV per nucleus) and no further increase up to 4 hours (approximately 1 EV per nucleus, Fig. 3F). More than 200 cells per group were counted and included into the calculation. At the same time, only minute amounts of PKH26-EV were detected in spleens or lungs of recipients at 2 and 4 hours (not shown).
Figure 3. Intravenously injected EV target potential hematopoietic stem cells. PKH26 stained EV were injected i.v. into lethally irradiated recipients. After the indicated times of 2 and 4 hours, bone marrow (BM) was harvested and stained with indicated antibodies or left without antibody-staining and counterstained with DAPI. Stainings of BM with DAPI (A) or counterstained with Sca-1 (B, C) or c-kit (D, E) 2 and 4 hours after injection of PKH26-stained EV are shown. Bar = 20 μm. n = 8 per time point in two separate experiments. (F): From all slides, nuclei of all cells (gray bars) and PKH26-positive EV (black bars) associated with these cells were counted and shown as summarized numbers for 2 and 4 hours after injection. More than 200 cells per group were counted and included into the calculation. (G): From Sca-1 and c-kit stained slides, antigen positive cells were counted (gray bars, between 140 and 1,100 cells) and compared to the PKH26-positive EV (black bars) colocalizing with antigen-positive cells. In (F) and (G), summarized cell numbers were set as 1 and the number of EV calculated accordingly so that 1 stands for 1 antigen-positive cell targeted by 1 EV, 2 stands for 1 antigen-positive cell targeted by 2 EV, etc. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; PKH-EV, PKH-positive EV.
Further identification of stem cells and progenitors using Sca-1 and c-kit antibodies displayed a primary targeting of Sca-1 and to a lower degree of c-kit positive cells by EV (Fig. 3B–3E) within 2 hours which was not further increased with time (Fig. 3G). Impressively, in c-kit stained samples, primarily the cells with low expression of this antigen were targeted and single EV were found only rarely in clearly positive cells. A demonstrative example is Figure 3E, leftmost picture, where the cluster with c-kit positive cells was not targeted by labeled EV whereas the majority of EV was identified in the low/negative cluster. Enumeration of between 140 and 1,100 antigen-positive cells targeted by labeled EV revealed that a single c-kit-positive cell was targeted by 1.9 EV at 2 hours decreasing to 1.4 at 4 hours whereas a single Sca-1 positive cell contained 3 EV at 2 hours and 2.3 at 4 hours (Fig. 3G). The quantification of targeted c-kit positive cells included those with low expression. PKH26-positive EV had to be evaluated carefully since they appeared as extremely small red dots which also might be located within or below the antigen stains. Our findings were supported by confocal microscopy showing an accumulation of labeled EV within the cytoplasm and/or around the nucleus within 2 hours without further increase (Supporting Information Videos 1, 2).

To quantify the number of EV-targeted potential stem cells, we repeated the transplantation of PKH26-labeled EV into lethally irradiated recipients and harvest of BM 2 and 4 hours post-transplantation. Cell staining and colocalization of PKH26-labeled EV with BM cells were evaluated with ImageStream which allows for parallel flow cytometric quantification of Sca-1 and c-kit stained cells which were targeted by EV, spot counting of PKH26-labeled EV in particular cell populations and parallel microscopic visualization. The gating strategy for quantification of positive populations and PKH26 spot counting was depicted in Supporting Information Figure S2. Figure 4A summarizes the percentages of Sca-1+, c-kit+, Sca-1 + c-kit+ cells as well as PKH26-positive cells in each population. With ImageStream, the Sca-1, c-kit, and Sca-1/c-kit double-positive populations showed a nonsignificant tendency of higher PKH26-positive cells at 4 hours after transplantation. About 0.35% or 0.42% of the of Sca-1 positive population, 0.33% or 0.44% of the c-kit positive population, and 0.16% or 0.24% of the double positive population were targeted by EV, respectively. Importantly, virtually all EV-targeted c-kit+ cells were clearly low antigen-positive. For each population, a representative image is shown with additional examples in Supporting Information Figure S3. The numbers of PKH26-positive spots per cell for Sca-1, c-kit, and Sca-1/c-kit positive populations are summarized in Figure 4B. Here, the majority of antigen-positive cells were targeted by 1–2 EV with no evident differences between the corresponding 2 and 4 hours populations. Note that for spot counting the PKH26 positive populations were set as 100% and the percentage of positive cells with 1–6 spots calculated accordingly.

**DISCUSSION**

MSC have been shown to effectively regenerate injuries of several tissues, for example, in acute kidney injury [20, 21], myocardial infarction [22], and lethal irradiation [1]. A common feature of all these injuries is a profound inflammation often leading to development of multiple organ dysfunctions [23] which can be significantly mitigated by MSC. Ionizing radiation, for example, leads to persistent signs of inflammation with increased plasma levels of tumor necrosis factor-α, interferon-β, interleukin-6, and C-reactive protein, leading to development of multiple organ dysfunctions [23–25]. While low dose irradiation might be managed by supportive care, the situation is more serious after irradiation doses higher than 6 Gy. In our irradiation model, we concentrated on severe irradiation injury similar to the TBI used in conditioning patients before hematopoietic stem cell transplantation and in the realistic range of that experienced as a consequence of radiation accidents. The mechanism(s) by which MSC combat the inflammation preferentially was previously ascribed to paracrine actions. Recent work has since implied that released EV might be an active component of MSC showing a similar efficacy in regeneration as do the whole cells [26–28].

Here, we show that MSC-derived EV have similarly positive effects on survival as the parental MSC when given after lethal irradiation. Interestingly, the Kaplan-Meier survival curve of EV showed a long-term survival of recipients similarly to mMSC but EV were unable to improve short-term survival. Our purification protocol of EV was based on ultracentrifugation excluding apoptotic and other larger particles, resulting in a homogeneous EV preparation with a Gaussian distribution mode size of 131–153 nm (not shown). The observed survival therefore can be attributed to the radiation rescue by rather small vesicles but not necessarily exosomes only, thus mimicking physiological conditions. The notion that “exosome markers” are in fact present in all different EV types [7] supports this assumption. Importantly, to compare the EV and mMSC transplantation outcomes in the absence of further
HSC support, we injected either $10^6$ mMSC or an EV amount derived from $10^6$ mMSC. In order to define which cells were supported by EV, we assayed hematopoietic progenitors from mouse BM in vitro using the colony-forming assay. This assay reliably detects progenitors, whereas stem cells are not able to form colonies without prior differentiation. Interestingly, the assay revealed no improved colony formation of EV-treated irradiated MNC compared to the irradiation control suggesting that progenitors were not rescued by EV. In contrast, the incubation on stromal cells revealed a remarkable outgrowth of CA indicative for a survival-promoting effect of EV on irradiated stem cells. In this system, it was not the number of CA but rather their size and the production of progenitors that were shown to be decisive. We could clearly attribute this effect to MSC-derived EV by lack of hematopoiesis-promoting properties of NIH3T3 fibroblast-derived EV indicating that MSC might transfer their paracrine supportive properties via EV. The test of stroma-derived hematopoietic progenitors in colony assays supported this notion showing significant colony outgrowth.

Figure 4. Quantification of EV-mediated stem cell targeting. Lethally irradiated mice were injected intravenously with PKH26-labeled EV. After 2 and 4 hours, bone marrow cells were harvested, double-stained for Sca-1 and c-kit, nuclei visualized with Hoechst, and analyzed with ImageStream. (A): Shown are representative pictures for each label: BF with size bar, Sca-1 in green, PKH26 in red, Hoechst in violet, c-kit in orange, and merged cells (magnification $\times 60$). Additional pictures were located in Supporting Information Figure S3. The graph depicted the % positive cells for the indicated group. (B): Each PKH26-positive population was analyzed for the number of positive spots per cell. Abbreviations: BF, bright field; EV, extracellular vesicles; ns, not significant.

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after treatment of HSC with EV. Thus, the in vitro results showed that HSC but not progenitors were rescued from irradiation by the effects of mMSC-derived EV.

Systemic inflammation, which is a consequence of TBI in transplant recipients, leads to increased EV transfer [3]. For this reason, we expected a robust dissemination of EV in our in vivo model. Indeed, on BM cytospins, we detected a rapid targeting of BM stem cell-like cells within 2 hours. Potential HSC were characterized by small nuclei of approximately 10 μm and a low cytoplasm/nucleus ratio [29]. Concordantly, the cells targeted by EV 2 and 4 hours after injection met the cytological criteria of the potential HSC. At the same time, we observed no trapping of EV in the lung or spleen as has been documented for MSC [1], revealing that EV traveled freely through the body. The targeted potential HSC were further characterized using stem cell specific antibodies to Sca-1 and c-kit, based on early work defining mouse HSC as Sca-1 and c-kit positive but negative for CD34 [30, 31].

Using the innovative method of ImageStream, we confirmed our results obtained with BM immunocytochemistry, demonstrating preferential targeting of potential HSC by EV. Although c-kit+ cells were also targeted by EV, in both experimental settings virtually all of the c-kit+ cells showed a relatively low c-kit positivity. This was particularly clear in the Sca-1/c-kit double positive population which represents the potential stem cells. This finding is consistent with the observation that reconstituting pluripotent stem cells in mice are Sca-1 positive but c-kit low on those cells [32]. Additional support comes from findings showing that metabolically active c-kit(hi) cells display a more limited expansion capacity [33] and are recruited from a more primitive quiescent c-kit(neg) HSC population [34]. The majority of cells was targeted by 1–2 EV irrespective of the specific populations. The variances in cell targeting of immunocytochemistry and ImageStream methods might be due in part to the different manipulation times involved: for cytospins, the carefully flushed BM was

Figure 5. Hypothetical mode of action of mMSC versus extracellular vesicles (EV) after i.v. injection into lethally irradiated mice. (A): mMSC accumulate in the lung after i.v. injection into lethally irradiated recipients. There, a polarization of tissue resident and immigrating macrophages takes place, changing the pro-inflammatory environment after irradiation into an anti-inflammatory one (see [37]). Although mMSC were trapped in the lung, decreased inflammation, oxidative stress and apoptosis as well as enforced cell cycle in the bone marrow (BM) have been detected (see [1]). (B): Mouse mesenchymal stromal cells-derived extracellular vesicles after i.v. injection into lethally irradiated recipients transport several cargos including proteins and various RNAs directly into the BM without notable accumulation in other tissues. EV target BM-resident hematopoietic stem cells and stimulate management of oxidative stress, cell cycle, and probably other regenerative processes (preliminary unpublished results). Abbreviations: BM, bone marrow; EV, extracellular vesicles; miRNA, microRNA; mMSC, mouse bone marrow derived mesenchymal stromal cells.
spun directly onto slides without strong pipetting and the antibody staining done on fixed slides, while the BM cells for ImageStream went through the procedure of hemolysis, antibody staining with repeated washing steps and measurements of preferentially single cells. The repeated handling of BM samples might lead to loss of not yet firmly attached or incorporated EV, thus underestimating the number of cell-associated EV at 2 hours.

Our results suggest that long-term survival of recipients is mediated by EV targeting to HSC. It has been shown that vesicles were efficiently taken up within minutes as intact single vesicles by surfing on filopodia [35]. Without accumulation at the cell surface, a steady state between uptake and turnover was reached in which EV are transported to the endoplasmatic reticulum-membrane to allow for efficient entry of their cargo into the translation machinery [35]. Thus, our in vivo data also well correlate with the in vitro data showing in both systems a rescue of potential stem cells which were Sca-1 positive and c-kit low-positive but not of progenitors. The loss of recipients within the first weeks of the reconstitution period but long-term survival of approximately 60% of recipients supports the conclusion that activation of HSC occurred, without supporting the progenitors responsible for short-term rescue.

In line with our findings, Wen et al. reported a mitigation of radiation injury by MSC-derived EV in a different transplantation model using low-dose irradiation with competitive reconstitution of donor and recipient HSC [36]. However, in their study, neither the cell type rescued by EV nor the sole action of EV on HSC in vivo was addressed.

Taken together, we propose a mechanistic model of action of both mMSC and the EV derived from them (Fig. 5). On the basis of our previous results [1], we knew that the transplantation of mMSC affects the inflammatory BM milieu after irradiation, possibly mediated by polarization of macrophages in the lung as has been shown by Nemeth et al. [37]. This assumption of an indirect mode of action is supported by findings of Drouet et al. [38] showing that intrafemural MSC therapy affected the inflammatory BM milieu after irradiation injuries. Additionally, the reconstitution model using low-dose irradiation with competitive reconstitution of donor and recipient HSC [36].

In conclusion, we showed a radioprotection and direct stimulation of irradiated cobblestone area forming cells in vitro and a colocalization of i.v. injected EV with Sca-1/c-kit positive potential stem cells in vivo. Importantly, the radioprotection of HSC occurred without additional support of donor HSC. Although further investigations are needed to fully explore the EV cargo for mediating the protective effect, the results already open a new line of treatment options for patients suffering from radiotherapy-induced myelosuppression while avoiding potential negative effects of infused cells.

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