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Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome

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Abstract

Background Recent studies have suggested that ex vivo expansion of autologous hematopoietic cells could be a therapy of choice for the treatment of bone marrow failure. We investigated the potential of a combined infusion of autologous ex vivo expanded hematopoietic cells with mesenchymal (MSCs) for the treatment of multi-organ failure syndrome following irradiation in a non-human primate model.

Methods Hematopoietic cells and MSCs were expanded from bone marrow aspirates. MSCs were transduced with the gene encoding for the green fluorescent protein (e-GFP), in order to track them following infusion. Twelve animals were studied. Nine animals received total-body irradiation at 8 Gy from a neutron/gamma source thus resulting in heterogeneous exposure; three animals were sham-irradiated. The animals were treated with expanded hematopoietic stem cells and MSCs, expanded hematopoietic stem cells alone, or MSCs alone. Unmanipulated bone marrow cell transplants were used as controls.

Results Depending on the neutron/gamma ratio, an acute radiation sickness of varying severity but of similar nature resulted. GFP-labeled cells were found in the injured muscle, skin, bone marrow and gut of the treated animals via PCR up to 82 days post-infusion.

Conclusions This is the first evidence of expanded MSCs homing in numerous tissues following a severe multi-organ injury in primates. Localization of the transduced MSCs correlated to the severity and geometry of irradiation. A repair process was observed in various tissues. The plasticity potential of the MSCs and their contribution to the repair process in vivo remains to be studied.

Keywords ex vivo expansion; hematopoietic cell; mesenchymal stem cell; irradiation; homing; aplasia

Introduction

The therapeutic potential of bone marrow derived mesenchymal stem cells (MSCs) has recently been brought into the spotlight of many fields of research. The therapeutic potential of bone marrow derived mesenchymal stem cells (MSCs) has recently been brought into the spotlight of many fields of research. Not only are these

cells able to migrate to injured or defective tissues of different origin, but their progenies are also able to acquire a different phenotype in accordance with tissues to which they home [1]. Furthermore, it has been shown that MSCs of bone marrow origin could hasten the hematopoietic recovery when infused simultaneously with hematopoietic stem cells in non-human primates [2] and in humans [3–5]. Altogether, the therapeutic potential associated with these properties appears significant and may have many applications. One such application is the repair of injured tissues arising from side effects of radiation treatments or accidental radiation exposure. Accidental acute radiation sickness may be a model to study both the transdifferentiation potential of MSCs and the power to accelerate hematopoietic recovery. Indeed, following a radiation accident, a multi-organ failure occurs. Depending on the energy, the dose, the dose rate and the geometry of the exposure, bone marrow aplasia can be combined with gastrointestinal syndrome, skin burns, muscle radiolysis, lung injury and/or central nervous system failure among other problems [6]. Until now, each of the main aspects of these disorders has been handled separately as a single disease. Bone marrow transplantation or administration of hematopoietic growth factor has been used with mitigated success to treat radiation-induced bone marrow aplasia, the first organ failure to appear due to the high radiation sensitivity of the hematopoietic system. Recent works have suggested that *ex vivo* expansion of hematopoietic stem cells (HSCs) could be a therapeutic option in this setting [7–9]. However, no therapeutic strategy taking into account the injuries to the other organs has proven successful. We hypothesized that using MSCs as a source of cells able to home to various tissues and to repair the widespread damage induced by irradiation might be an interesting therapeutic strategy. We therefore investigated the potential of combining *ex vivo* expansion of HSCs with *ex vivo* expanded MSCs for the treatment of acute radiation syndrome in a non-human primate (NHP) model. HSCs and MSCs were taken from the bone marrow and expanded. A sample of MSCs was transduced with the gene encoding for the enhanced green fluorescent protein (eGFP), in order to track them in the organism. Twelve animals were studied. Nine animals received total-body irradiation at 8 Gy from a neutron/gamma source, thus resulting in heterogeneous exposure; three animals were sham-irradiated. Depending on the neutron/gamma ratio, an acute radiation sickness of varying severity, but of the same nature, could be produced. Three animals received expanded HSCs alone, one animal received expanded MSCs alone, two animals received unmanipulated bone marrow, and three animals received expanded HSCs and MSCs. We report on the results of this study, which showed that both therapeutic objectives were reached, namely the presence of MSCs in the injured tissues and a better hematopoietic recovery for the surviving animals treated with expanded cells than for animals treated with unmanipulated bone marrow cells.

Materials and methods

Animals

A group of 12 healthy young male macaques (*Macaca fascicularis*), mean weight 5.2 ± 1.5 kg (range 3.5–7.9 kg), was used as previously described [8]. All experimental procedures for NHP were approved by the Animal Care Committee of the Institute of Nuclear Radioprotection and Safety (IRSN) and conformed to the French regulations for animal experimentation (Ministry of Agriculture Act no 87-848, October 19th, 1987, modified May, 2001).

Ex vivo expansion of MSCs

Isolation of NHP-MSCs from bone marrow aspirate samples

Bone marrow aspirates were drawn bilaterally into sodium heparin (final concentration of 200 U/ml) from the iliac crest of animals anesthetized under Ketamine/Xylazine anesthesia. Samples were shipped at room temperature (between 19–23 °C) via overnight service for processing in the cell culture laboratory within 48 h. The bone marrow was processed according to procedures previously described [2] with some modifications. Briefly, marrows were diluted 1:2 with ~25 ml of Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA) and centrifuged at ~900 g (2000 rpm) for 10 min at 21 °C. Pellets were collected, resuspended at 1:20 with ~25 ml DPBS and counted. The resulting cell pellet was layered onto an ACT density gradient (1.072 gm/ml; Dendreon, Seattle, WA, USA) with up to 3.5×10^8 nucleated cells and centrifuged at 1100 g (2200 rpm) for 30 min at 21 °C. Light density layers were collected and rinsed again in DPBS as described above and then nucleated cells were counted on a haemocytometer. The cells were plated onto culture plastic (Nunc, Naperville, IL, USA) at $\sim 1.6 \times 10^5$ nucleated cells per cm^2 in 'complete' medium, which consisted of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin solution (Gibco) and 10% FBS (Hyclone, Logan, UT, USA), which was prescreened for MSCs according to previously described methods [10]. Medium was replaced every third or fourth day for approximately 3 weeks or until primary (P0) confluence, when swirling colonies had occupied most space on the culture plastic.

Culture and subculture of NHP marrow

Primary cultures (P0) were released from culture with 0.25% trypsin in 1.0 mM (Gibco). The cell suspension was collected off the plates, centrifuged, and rinsed again as described above. Cells were resuspended in serum-containing medium and counted, then replated onto culture plastic at $5\text{--}6 \times 10^3$ cells/cm [2]. Complete medium was replaced every 3 or 4 days for 7–10 days, until the culture surface comprised a confluent monolayer of cells.

At that time the trypsinization process was repeated and cultures were cryopreserved for shipping [2,10].

Viral vector and packaging cell line

The pSF-EGFP plasmid was kindly provided by H. de Verneuil (Laboratoire de biochimie médicale et biologie moléculaire, université de Bordeaux II). The pseudotyped retrovirus producer cell line PG13/pSF-EGFP was developed by transducing the PG13 packaging cell line (ATCC-CRL 10686) [11]. The producer cell line was cloned and subcloned as previously described [12]. PG13/SF-EGFP subclone was propagated in culture in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal calf serum (FCS) and kept at 37 °C with 5% CO₂ in a humidified atmosphere. The viral titer of the cell lines used was 10⁵ to 10⁶ infectious particles/ml as determined by supernatant titration on cultured human HeLa cells.

Retrovirus-mediated transductions of MSCs

A fraction of expanded MSCs was thawed 2 weeks before NHP irradiations. The MSCs were seeded at 0.25×10^6 cells in T 75-cm² flasks in 10 ml of DMEM supplemented with 10% FCS (Invitrogen). MSCs were transduced 2 days following plating, when a standard of 40% confluence of MSCs was reached. An untransduced control was performed. Supernatants containing recombinant retroviral particles were generated by culturing 80% confluent producer cells for 12 h. The cultures were maintained at 37 °C with 5% CO₂ in a humidified atmosphere. The culture supernatant was subsequently produced and filtered through a 0.45- μ m filter. The MSCs were incubated with the supernatant for 5 h in the presence of 10 μ g/ml of Polybrene (Sigma-Aldrich, St Louis, MI, USA). The supernatant was then removed and replaced by fresh DMEM supplemented with 10% FCS for 19 h of culture. This procedure was repeated daily for 4 days. The expression of the eGFP transgene was evaluated daily by *in situ* visualization of the cells with an inverted fluorescence microscope. Following transduction, MSCs were harvested and the transduction efficiency was assessed by flow cytometry. Counterstaining of the cells with monoclonal antibodies against CD73 (SH3, CD73 PE, Becton-Dickinson) and CD105 (SH2, CD-105 PE, Argene SA) was performed. Lack of hematopoietic cell contaminants was controlled by counterstaining the cells with monoclonal antibodies against CD45 (CD-45 PE, Argene SA).

Ex vivo expansion of BMMNCs

Bone marrow mononuclear cells (BMMNCs, 2×10^5 cells/ml) were cultured as previously described [8]. Briefly, cells were cultured in the presence of recombinant

human (rh) stem cell factor (SCF, 100 ng/ml), Flt3-ligand (FL, 100 ng/ml), thrombopoietin (TPO, 100 ng/ml), Interleukin-6 (IL-6, 5 ng/ml) (all from R&D Systems, Abingdon, UK), and recombinant primate IL-3 (5 ng/ml, a generous gift from G. Wagemaker) [12]. Cultures were incubated for 7 days at 37 °C in humidified atmosphere with 5% CO₂ without any medium change. The cells were stained with CFSE (5-(and 6-)carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Leiden, The Netherlands) by incubating for 15 min at 37 °C in the presence of 5 μ M final concentration of CFSE, washed and immediately reinjected intravenously (i.v.) into irradiated animals.

Apoptosis estimate procedures, phenotyping of hematopoietic cells, hematopoietic progenitor and long-term culture-initiating cell (LTC-IC) assays were performed as previously described [8].

Animal irradiation

Animals were given a single dose of 8 Gy total-body irradiation from an experimental nuclear reactor (Silene reactor, Valduc, France) delivering a mixed field of either 90% gamma rays and 10% neutrons, or 50% gamma rays and 50% neutrons, with a mean dose rate of 6.7 Gy/min. Physical dosimetry by a thermoluminescence dosimeter [13] indicated that the expected doses were obtained in the median plane of the animal body. This was confirmed with biological dosimetry by scoring of dicentric chromosomes [14], which indeed gave estimated whole body doses of irradiation in the range of ± 5 to 10% as compared with the expected dose.

Experimental schedule

A summary of the experimental schedule is presented in Table 1. Five treatment groups were used. Group I

Table 1. Weight, age, and irradiation conditions for each animal used in this study

Treatment group	Animal identification	Weight	Age	γ/η ratio	Irradiation dose*
Sham	M383	6.55	7**	–	0 Gy
	1735	4.3	3.5**	–	0 Gy
	Y856	4.05	2	–	0 Gy
Unmanipulated BMMNCs	1682	5.15	3.5**	1:1	8 Gy
	Y182	3.75	2.5	9:1	8 Gy
7-day expanded HSCs	Q554	7.05	6**	1:1	8 Gy
	1597	4.80	3.5**	1:1	8 Gy
	AA212	3.450	2	9:1	8 Gy
7-day expanded HSCs and MSCs	Q720	7.450	6**	1:1	8 Gy
	1760	4.1	3.5**	1:1	8 Gy
	Y636	4.05	2.5	9:1	8 Gy
MSCs alone	S076	6.650	6**	1:1	8 Gy
M \pm SD		5.15 \pm 1.49	4 \pm 1.77	n.a.	n.a.
Range		3.45–7.85	2–7		

*Irradiation dose indicated is the mean dose obtained in the median plane of the body according to physical dosimetry (not shown).

**Estimated age, on the basis of animal morphology.

consisted of three sham-irradiated animals that did not receive any graft. Group II consisted of two animals that received 3×10^7 /kg unmanipulated BMMNCs after TBI; group III comprised three animals that after TBI received the 7 days *ex vivo* expansion product of 3×10^7 BMMNC per kg of body weight. Group IV consisted of three animals receiving *ex vivo* expanded BMMNCs and expanded MSCs post TBI. Group V included one animal that received only an MSC transplantation post TBI. Bone marrow sampling for *ex vivo* expanded BMMNCs and unmanipulated BMMNC transplantation was made 6 days before irradiation, and bone marrow sampling for MSC expansion was made 8 weeks before irradiation.

Follow-up

Animals were treated post-irradiation as previously described [8]. At autopsy, organ samples were harvested and fixed in formalin for 24 h and paraffin-embedded. Samples were then cut in 3- μ m sections, fixed on histological slides and stained with hematoxylin-eosin-safran (HES). Organ samples were also frozen rapidly in liquid nitrogen for PCR detection of MSCs.

PCR analysis

Genomic DNA for PCR analysis was prepared from tissues using a QIAmp tissue kit (Qiagen, Hilden, Germany). Amplification was performed using manufacturer-provided reagents following the standard recommended amplification conditions (Applied Biosystems, Foster City, CA, USA) as previously described [15,16]. Purified DNA (100 ng) from various tissues was amplified using the TaqMan universal PCR master mix 4304437 (Applied Biosystems), with eGFP and beta-actin primers and probe (Taq man beta-actin control reagent 401846; Applied Biosystems). The primers and probe were designed with Primer Express software (Applied Biosystems). The primers and probe for eGFP were forward primer 5'ctcgtgaccaccctgacctac 3' and 3' reverse primer 5'aagaagatggtgcgctccg3'. The probe labeled with fluorescent reporter and quencher was: 5'FAM-cccgaccacatgaagcagcagc-TAMRA-3'. FAM (6-carboxyfluorescein) was used as a reporter fluorochrome and TAMRA (6-carboxytetramethylrhodamine) was used as quencher. The probe was chosen in order to hybridize with an internal sequence of the PCR target sequence. With each cycle, the fluorescence intensity of additional reporter dye molecules was monitored. Threshold cycles (Ct) were selected in the line in which all samples were in logarithmic phase. The quantity of the PCR product was calculated by the Ct value.

Negative controls consisted of normal tissues from control NHPs extracted concurrently with each set of test samples and a reagent control. In order to determine the efficiency of amplification and the assay precision, calibration curves were constructed with a 0.99 correlation (r^2) and efficiencies greater than 98%.

Titration curves were established by making serial dilutions of eGFP plasmid (pEGFP, Clontech) or serial dilutions of genomic NHP DNA. The copy number of eGFP plasmid was assessed following 260 nm optical density (OD) measurement. Ten-fold dilution series were performed for $1-5 \times 10^6$ eGFP plasmid copies per assay in PCR-grade water containing 100 ng of salmon sperm DNA per assay as carrier. No cross reactivity with NHP DNA was observed when amplification of eGFP was performed with our primers-probe set. The NHP DNA concentration was estimated by OD measurement and a 10-fold dilution was performed in PCR-grade water containing 100 ng of salmon sperm DNA per assay as carrier.

Hematological evaluation of irradiated animals

Peripheral blood (PB) sampling was performed using EDTA-coated tubes for daily blood counts on an ADVIA120 (Bayer Diagnostics, Puteaux, France). The remaining blood was centrifuged at 800 g for 10 min and plasma was collected and cryopreserved at -80°C . White blood cells were then subjected to phenotypic analysis, as previously described. Flt3-ligand (FL) concentration was measured by ELISA, according to the manufacturer's recommendations (R&D Systems) [17].

Statistical analysis

Data from *in vivo* irradiation experiments are data from individual animals. Results of *ex vivo* expansion are expressed as fold increase. Values are reported as mean \pm standard deviation (SD). Statistical significance, calculated using the Mann-Whitney rank sum test, was set for a value of $p < 0.05$.

Results

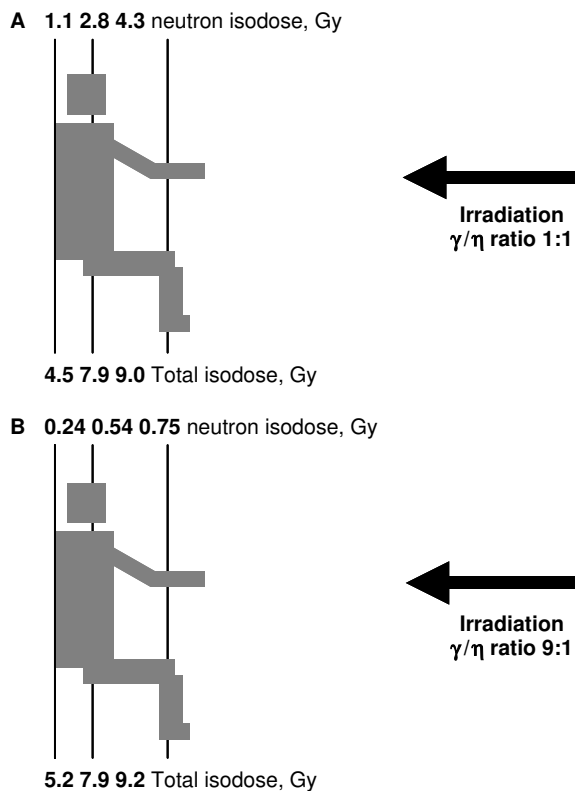
Irradiation and HSC infusion

A total of 12 animals were used for these experiments. Table 1 summarizes age, weight and irradiation conditions for each animal for all treatment groups. Three animals were sham-irradiated, two animals received an unmanipulated BMMNC graft of 3×10^7 MNC/kg of body weight, three animals received the *ex vivo* expansion of 3×10^7 marrow MNC/kg of body weight, three animals received both MSCs and the *ex vivo* expansion product of 3×10^7 MNCs, and one animal received only a graft of MSCs. Two irradiation protocols were used, the first one with a gamma/neutron ratio of 1, and the second one with a gamma/neutron ratio of 9. A mean dose of 8 Gy in the body mid-plane was obtained according to the physical dosimetry (Figure 1). However, since irradiation consisted of either 50 or 10% neutrons, which are strongly attenuated by live tissues, the front of the animals received a higher neutron irradiation dose as compared

Table 2. Results of *ex vivo* expansion of BMMNCs and number of hematopoietic cells infused according to the treatment groups

Treatment group	Animal identification	BMMNC		CFU-GM		BFU-E		LTC-IC	
		Expansion fold	Number infused/kg	Expansion fold	Number infused/kg	Expansion fold	Number infused/kg	Expansion fold	Number infused/kg
Sham irradiated	M383	-	-	-	-	-	-	-	-
	1735	-	-	-	-	-	-	-	-
	Y856	-	-	-	-	-	-	-	-
Unmanipulated BMMNCs	1682	-	30×10^6	-	0.46×10^5	-	0.32×10^5	-	0.55×10^4
	Y182	-	30×10^6	-	0.57×10^5	-	0.33×10^5	-	0.47×10^4
mean			30×10^6		0.515×10^5		0.325×10^5		0.51×10^4
7-day expanded HSCs	Q554	3.5	79.4×10^6	15.1	0.62×10^6	0.97	0.16×10^5	nd*	nd
	1597	4.8	151×10^6	17.2	0.96×10^6	4.03	1.36×10^5	0.85	1.02×10^4
	AA212	6.27	178×10^6	16.6	1.91×10^6	8.96	3.66×10^5	0.27	0.93×10^4
7-day expanded HSCs and MSCs	Q720	3.73	114×10^6	13.6	0.83×10^6	2.23	0.91×10^5	nd	nd
	1760	6.04	184×10^6	15.6	1.17×10^6	3.42	1.1×10^5	nd	nd
Y636	8.74	269×10^6	19.9	2.07×10^6	3.89	3.77×10^5	0.64	1.71×10^4	
MSCs alone	S076	-	-	-	-	-	-	-	-
Mean \pm SD		5.51	162.6×10^6	16.3	1.26×10^6	3.92	1.83×10^5	0.59	1.22×10^4
Expanded HSCs		± 1.95	$\pm 65.5 \times 10^6$	± 2.15	$\pm 0.6 \times 10^6$	± 2.73	$\pm 1.52 \times 10^5$	± 0.29	$\pm 0.43 \times 10^4$

*nd: not determined.

**Figure 1. Results of physical dosimetry, indicating the estimated isodoses for neutron irradiation and for total irradiation for (A) animal Q554, with a γ/η ratio of 1 : 1, and (B) animal Y182, with a γ/η ratio of 9 : 1**

with the back, resulting in an heterogeneity of neutron irradiation dose of 50% when comparing animal front and back (Figure 1).

Bone marrow sampling was made 6 days before irradiation for all animals. BMMNCs were either frozen or used for *ex vivo* expansion protocols as previously

described [8]. Results of *ex vivo* expansion protocols as well as evaluation of progenitors contents in unmanipulated BMMNCs are presented in Table 2. The mean \pm SD fold expansion were of 5.51 ± 1.95 for MNC, 16.3 ± 2.15 for CFU-GM, 3.92 ± 2.73 for BFU-E and 0.59 ± 0.29 for LTC-IC. These results are in accordance with previous results obtained in our laboratory [17]. As a result, a mean of 162.6×10^6 MNC, 1.26×10^6 CFU-GM, 1.86×10^5 BFU-E and 1.22×10^4 LTC-IC per kg of body weight were reinfused to animals (Table 2). For comparison, animals receiving unmanipulated BMMNCs received 30×10^6 MNC, 0.515×10^5 CFU-GM, 0.325×10^5 BFU-E and 0.51×10^4 LTC-IC per kg of body weight. The phenotypic analysis of expanded cells indicated an expansion of $CD34^+$ hematopoietic cells (mean of 21.5 ± 8.3 fold expansion), together with the expansion of $CD11b^+$ $CD14^+$ monocytic cells and $CD11b^+$ $CD16^+$ granulocytic cells (data not shown). Thus animals receiving expanded cells were infused with a mean of $19.1 \pm 8.3 \times 10^6$ $CD34^+$ cells per kg of body weight, vs. $0.79 \pm 0.45 \times 10^6$ $CD34^+$ cells for animals engrafted with unmanipulated BMMNCs.

MSC expansion, transfection and reinfusion

Table 3 summarizes the number of MSCs grafted. Three animals received both *ex vivo* expansion of 3×10^7 CMN/kg of body weight and 5.36–18.1 $\times 10^6$ MSC/kg of body weight. One animal received only a graft of 7.51×10^6 MSC/kg of body weight. In order to follow MSCs after reinjection, NHPs received a fraction of expanded MSC eGFP transduced. EGFP MSCs represented 0.60–4.94 $\times 10^6$ cells/kg of body weight. Flow cytometric analysis of MSCs before transplant demonstrated that 0–41% of cells were SH2+ and 57.26–69% were SH3+.

Table 3. Results of *ex vivo* expansion, gene transfection, and number of MSCs reinfused to the animals

Treatment group	Animal identification	MSC phenotype			MSC transfection			Total number of MSCs
		SH2 %	SH3 %	CFU-F ×1000 cells	CFU-F eGFP %	MSC eGFP %	MSC eGFP infused ×10 ⁶ /kg	Number infused ×10 ⁶ /kg
7-day expanded HSCs and MSCs	Q720	41	ND	7	38	4.34	2.32	5.36
	1760	0	57.26	ND	ND	5.58	0.39	7.07
	Y636	1.7	69	ND	ND	27.28	4.94	18.1
MSCs alone	S076	40	ND	7.5	40	8.01	0.60	7.51
Mean		14.23	63.13	7.25	39	12.40	2.55	10.18
Range		0–41	57.26–69	7–7.5	38–40	4.34–27.28	0.60–4.94	7.07–18.1

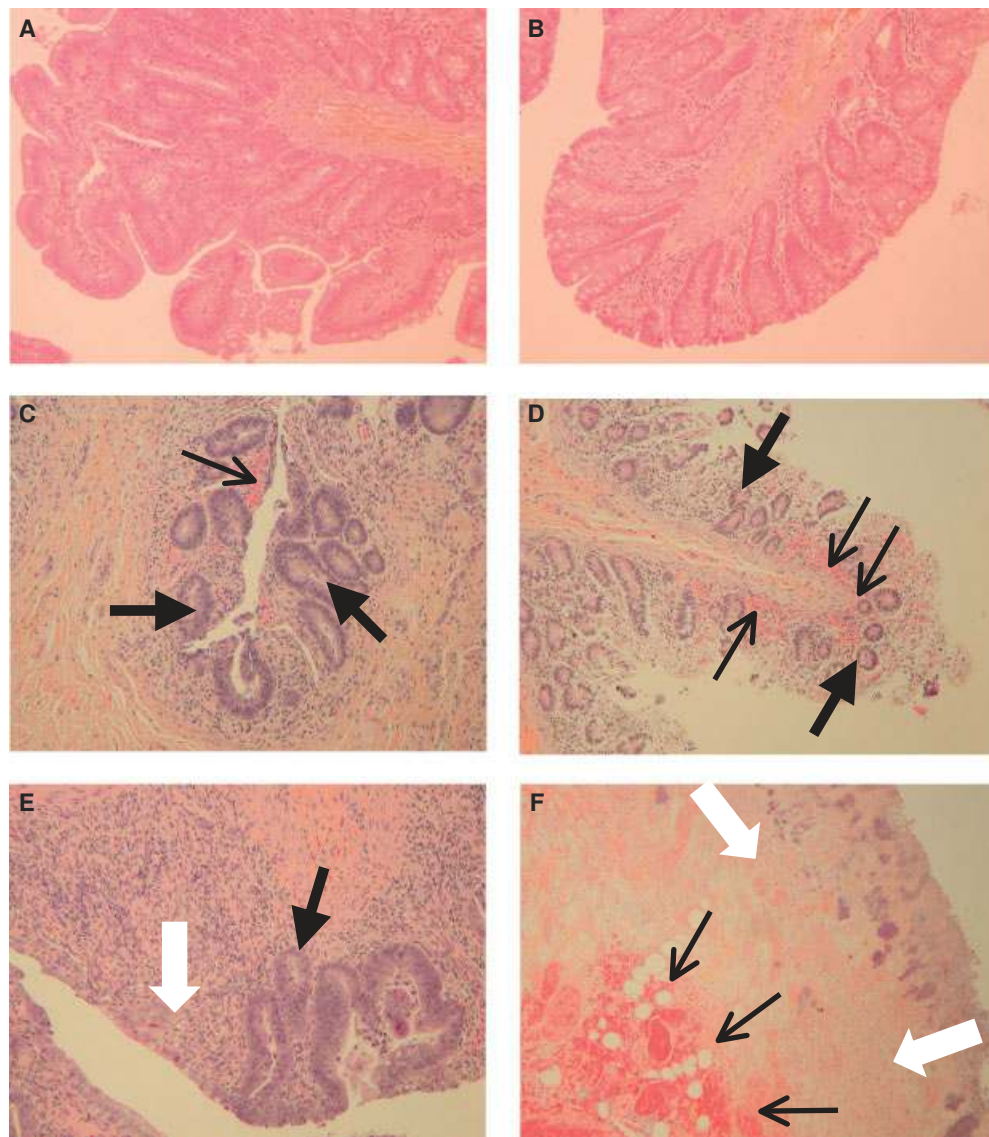


Figure 2. Representative histological imaging of animals Q554 (A and B), 1597 (C and E), 1760 (D) and S076 (F) for small intestine (A, C and E) and large intestine (B, D and F). Normal appearance of small (A) and large (B) intestine on day 111. On days 11 (C, E and F) and 23 (D), a disorganization of intestinal epithelium was observed with haemorrhages (thin arrows) and regenerative crypts (bold arrows). Areas of necrosis (white arrows) were also observed on day 11 (E and F)

Table 4. Clinical outcome for individual animals

Treatment group	Animal identification	γ/η ratio	Irradiation dose	Survival (days)	Cause of death*
Sham	M383	–	Sham	>1 year	
	1735	–	Sham	>1 year	
	Y856	–	Sham	>1 year	
Unmanipulated BMMNCs	1682	1:1	8 Gy	23	Hemorrhages
	Y182	9:1	8 Gy	82	Sacrificed
7-day expanded BMMNCs	Q554	1:1	8 Gy	111	Lung fibrosis and inflammatory disease
	1597	1:1	8 Gy	11	Digestive disease
	AA212	9:1	8 Gy	82	Sacrificed
7-day expanded BMMNCs and MSCs	Q720	1:1	8 Gy	12	Digestive disease
	1760	1:1	8 Gy	23	digestive and liver disease, diffuse hemorrhages
MSCs	Y636	9:1	8 Gy	82	Sacrificed
MSCs alone	S076	1:1	8 Gy	12	Diffuse hemorrhages, infection, digestive disease

*As determined by autopsy.

Frequencies of CFU-F were 7–7.5 CFU-F per 1000 MSCs, of which 38–40% were eGFP+. Flow cytometric analysis showed that CD45+ cells were not present in MSCs, thus excluding the presence of hematopoietic cells as a source of transduced cells in MSC grafts.

Clinical outcome

All animals showed an initial radiation syndrome during the 24 h post-irradiation. All animals experienced gastro-intestinal disease between days 5 and 15, with diarrhea and reduced food and water intake during this period. This early phase of the gastro-intestinal disease was treated with Granisetron (0.2 mg/kg of body weight SQ; Kitril, Roche, France), which in fact reduced diarrhea.

Survival of animals and causes of death are summarized in Table 4. Most animals irradiated with a γ/η ratio of 1 : 1 died between days 11 and 23, with a general picture of digestive disease, including a disorganization of intestinal epithelium, a disappearance of colonic and small bowel crypts (Figures 2C and 2D) and necrotic areas along small and large bowels (Figures 2E and 2F), generally associated with hemorrhage, especially in animals that died on day 23. However, clear-cut evidence of crypt regeneration was observed as soon as day 11 (Figure 2C) but also on day 23 (Figure 2D), with a complete regeneration of the intestinal epithelium on day 82 (not shown) and day 111 (Figures 2A and 2B). By contrast, all three animals irradiated with a γ/η ratio of 9:1 survived the acute phase of radiation syndrome, despite a moderate gastro-intestinal disease with reduced drinking and food intake and mild diarrhea between days 5 and 13 post-irradiation.

Follow-up of hematological parameters

All animals including sham-irradiated animals were followed daily for WBC and Plt counts (Figures 3A

and 3B, respectively). Irradiated animals showed a rapid decrease in WBC numbers during the first 7 days after irradiation. Thereafter, some differences appeared between the treatment groups, whatever the γ/η ratio used. Animals receiving the MSC graft alone showed a rapid and continuous decrease in WBC until day 12. Animals receiving the unmanipulated BMMNC graft alone showed a similar initial decrease in WBC counts during the first 10 days. However, the nadir in WBC took place on day 11 for the two animals, and a progressive recovery of WBC numbers was observed on day 11, until complete recovery by day 40 for the surviving animal (which was sacrificed on day 82 for tissue analyses). In contrast, with *ex vivo* expanded BMMNCs, an initial decrease in WBC counts was observed, but stopped as soon as day 6. A progressive recovery was observed from day 15 in parallel with animals receiving unmanipulated BMMNCs. These results indicated that *ex vivo* expanded BMMNCs were effective in the treatment of radiation-induced bone marrow aplasia, in accordance with our previous results [8]. Animals receiving both expanded BMMNCs and MSCs behaved as those receiving expanded BMMNCs alone but the early WBC peak as well as the secondary count drop were more pronounced. Similar results were observed on platelet counts. The amount of blood products transfused could be compared in the three animals that received a 9:1 gamma/neutron ratio irradiation and surviving long enough for evaluation. Animal Y636 who received both expanded BMMNCs and MSCs received 14 platelet transfusions and 4 red cell transfusions, animal AA212 who received expanded HSCs alone received 11 platelet transfusions and 2 red cell transfusions, whereas animal Y182 who received unmanipulated BMMNCs had 23 platelet transfusions and 4 red cell transfusions. Obviously, the number of animals was too small to observe statistically significant differences in these kinetics of hematopoietic recovery. The plasma FL concentration was monitored in all

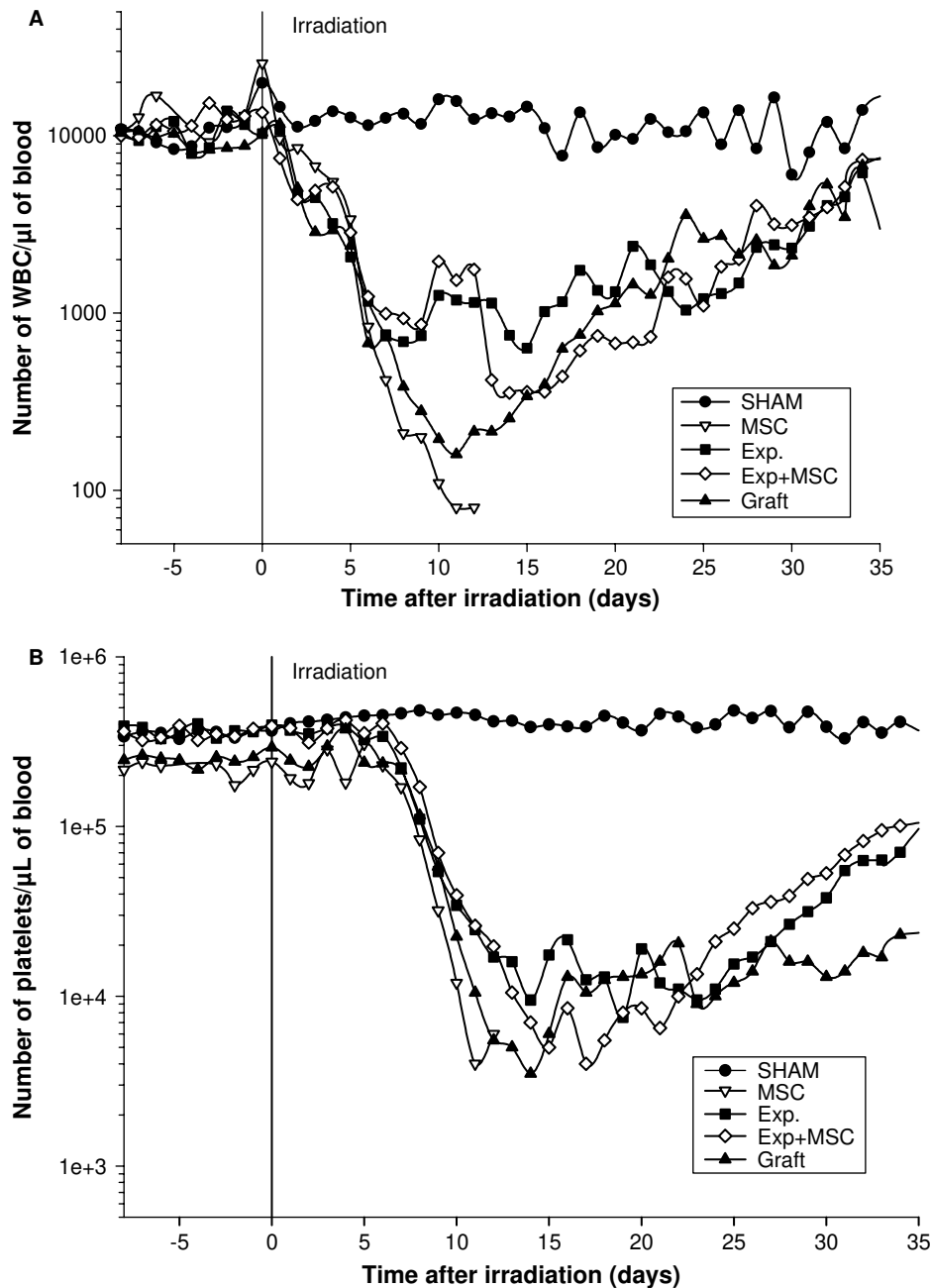


Figure 3. Follow-up of (A) WBC and (B) Plt for each treatment group. Results are given as mean for sham-irradiated animals (closed circles), BMMNC expansion-treated animals (closed squares), BMMNC expansion- and MSC-treated animals (open diamonds), and unmanipulated BMMNC grafts (closed triangles), and as an individual value for the MSC-treated animal (open triangles)

irradiated animals. Sham-irradiated animals showed plasma FL levels ranging from 100.2 to 196.9 pg/ml during the experimental period (Figure 4), which was in accordance with previously defined FL levels under normal physiological conditions. All irradiated animals showed an initial increase in plasma FL concentrations during the first 4 days after irradiation. However, marked differences appeared thereafter, with the stabilization of plasma FL levels between approximately 400 and 1200 pg/ml of plasma for animals that received expanded BMMNCs alone (Figure 4) until day 18, after which a progressive decrease in plasma FL levels was observed, in parallel with the recovery of blood cell populations.

Higher levels of plasma FL concentrations were observed in all other treatment groups.

Detection of eGFP in NHP tissues

A part of the MSCs was transduced with the gene encoding for the green fluorescent protein (eGFP) in order to track where the MSCs had engrafted. Tissues from multiple organs were collected and frozen after euthanasia except for Y636 where biopsies of skin and muscle were performed 2 weeks after graft. Because a gradient of doses occurred during radiation that induced higher damage

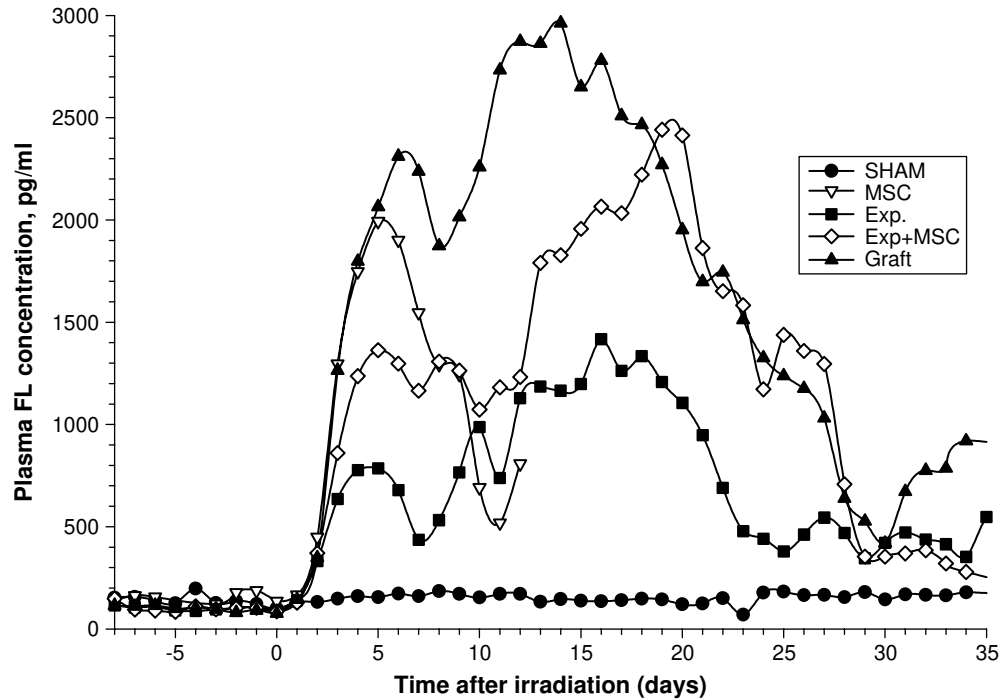


Figure 4. Follow-up of plasma FL concentration for each treatment group. Results are given as mean for sham-irradiated animals (closed circles), BMMNC expansion-treated animals (closed squares), BMMNC expansion and MSC-treated animals (open diamonds), and unmanipulated BMMNC grafts (closed triangles), and as individual values for MSC-treated animals (open triangles)

Table 5. Biodistribution of MSCs in tissues by the detection of MSC engraftment (eGFP detection by real-time PCR)

Tissues	eGFP copies/100 ng DNA					Tissues	eGFP copies/100 ng DNA			
	Animals						Animals			
	Q720	1760	S076	Y636			Q720	1760	S076	Y636
	Day after radiation						Day after radiation			
	12	23	12	15*	86		12	23	12	86
Gut jejunum	nd**	0	nd	nd	400	Spleen	0	0	0	1
Bone marrow, femur	376	0	0	nd	1	Lung	0	0	0	0
Muscle, shoulders	370	0	11	10	1	Kidney	0	0	0	0
Skin, legs	116	0	63	nd	120	Brain	0	0	0	0
Tendon, legs	105	0	0	nd	0	Thymus	0	0	0	0
Bone marrow, humerus	64	0	1	1	1	Hearth	0	0	0	0
Skin, shoulders	262	165	54	1	0	Liver	0	0	0	0
Muscle, legs	0	0	12	nd	0	Tendon, thorax	nd	nd	nd	0
Stomach	0	0	7	nd	0	Tendon, shoulders	nd	nd	nd	0
Muscle, thorax	nd	nd	nd	0	7	Bone, humerus	nd	nd	nd	0
Proximal colon	nd	0	nd	nd	7	Gut duodenum	nd	0	nd	0
Cecum	nd	0	nd	nd	7	Gut transverse colon	nd	0	nd	0
Skin, thorax	nd	nd	nd	0	5	Gut distal colon	nd	0	nd	0
Ileum	nd	0	nd	nd	4	Rectum	nd	0	nd	0
Bone, femur	nd	nd	nd	nd	2	Testicle	nd	0	nd	0

*On day 15 post-irradiation in animal Y636 skin, muscle and bone marrow biopsies were obtained and analyzed for the presence of eGFP DNA.

**nd: not determined.

in front tissues than in the back, we investigated the potential spatial distribution of MSCs by taking samples of skin, skeletal muscle and tendon from leg, thorax and shoulders. A quantitative analysis of MSCs engrafted in each of the organs examined is presented in Table 5a. Significant engraftment occurred in hematopoietic tissues (bones 2.2 copies/100 ng DNA, bone marrow 22–188 copies/100 ng DNA, spleen 1 copy/100 ng DNA), in the

skin (5–110 copies/100 ng DNA), skeletal muscle (7–11 copies/100 ng DNA), tendon (105 copies/100 ng DNA), stomach (7.15 copies/100 ng DNA) and gut (4–400 copies/100 ng DNA) tissues. No GFP presence was detected in the brain, liver, kidney, lung, cardiac muscle, thymus, rectum, and testicle of any recipient animals.

At 12–23 days post-irradiation, the highest engraftment (16.5–262 eGFP copies/100 ng DNA, or up to

13.1% cells) occurred in the skin of the shoulders, which corresponded to the anatomic aspect least exposed to the neutron effects.

Skin samples had the highest frequency of eGFP+ cells of all the tissues in all subjects. Biopsies from NHP Y636 at 2 weeks revealed that MSCs were engrafted in the skin (1 copy/100 ng DNA) and the skeletal muscle (10 copies/100 ng) of the shoulder (Table 5b). This was consistent with necropsy results of skin and skeletal muscle in those animals (NHP Q720 & 1760) sampled at 2 weeks post-infusion. When comparing skin and skeletal muscle from one anatomical site with another, a spatial distribution pattern of MSCs is revealed at 2 weeks post-engraftment in which these tissues over the shoulder were more positive than the same tissues from the legs in those NHPs grafted with both BMMNCs and MSCs. This distribution might be explained by a preferential implantation of MSCs to regenerative areas rather than areas of necrosis.

In NHP1760, only the skin was positive; this may be due to the low number of eGFP+ MSCs (0.39×10^6 cells) infused into NHP1760 compared with the other NHPs ($2.32\text{--}4.94 \times 10^6$ cells). The animal that received only a MSC graft (S076) had MSCs engrafted in stomach (7.15 copies/100 ng), skin (54–63 copies/100 ng), skeletal muscle (11 copies/100 ng), although there were no significant differences between the levels in legs and shoulders. NHP S076 also had a lower percentage of MSCs in humeral bone marrow (1.45 copies) than in animals transplanted with expanded MSCs and bone marrow (4–376 copies/100 ng). At 2 months post-infusion, eGFP+ MSCs were still detectable by PCR in bone marrow (1 copy/100 ng) and in femoral bone (2.2 copies/100 ng). Considering that only a fraction of the MSCs transplanted was eGFP+, and assuming that there is one copy of the eGFP gene per transduced cells, MSCs engrafted from 0.11% in bone up to 7% of the cells in gut jejunum in this animal (Y636).

Further analysis was performed on mononucleated cells from peripheral blood in order to investigate MSC migrations. We were never able, at any time post-infusion, to detect MSCs in peripheral blood from days 1 to 82. Finally, to characterize the frequency of MSCs present in regenerating bone marrow, CFU-F cultures from bone marrow aspirates were established. In all cases these cultures were PCR+ (as high as 10 copies/100 ng DNA for NHP Y636 at 82 days post-irradiation). Also, the presence of eGFP+ cells in CFU-F assays was demonstrated by fluorescent microscopy at 82 days post-irradiation for Y636.

Discussion

Combined HSC and MSC infusion has been proposed as therapy for radio-chemotherapy induced aplasia [2]. It has been shown in humans that, after autologous as well as allogenic HSC transplantation, hematological parameters can be improved after infusion of MSCs [3–5]. HSC transplantations in primates are difficult to realize and are very expensive. All studies performed so far have therefore been based on a limited number of animals and statistical analyses are usually impossible. However, when combining our present observations with our previous studies [8] and comparing with reports from other teams [2], the data suggested that animals receiving expanded HSCs and MSCs or expanded HSCs alone did better than animals receiving unmanipulated bone marrow. No conclusion can however be drawn from the survival data since three animals surviving on day 82 were sacrificed for tissue analyses.

We have previously shown that it is possible to use the follow-up of plasma FL concentration as a biologic indicator of marrow function [17]. The unmanipulated BMMNC-treated group showed an initial increase higher than the group receiving expanded BMMNCs and MSCs, suggesting that the co-infusion of MSCs with expanded

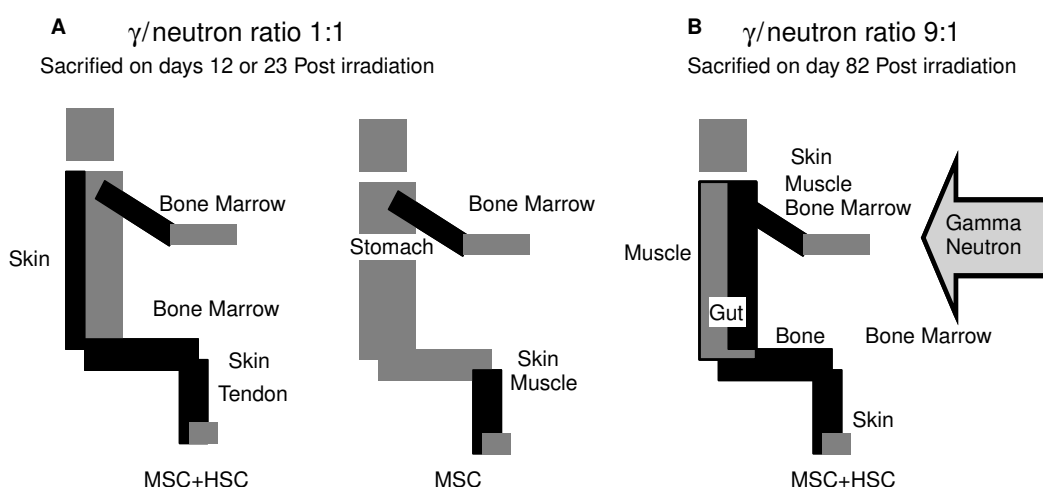


Figure 5. Spatial distribution of MSCs (in black) from days 12 to 82 post-irradiation according to the position of the animals in the gamma/neutron irradiation field. Neutrons are strongly attenuated by live tissue, thus the front of the animals received a higher neutron irradiation as compared with the back

BMMNCs was contributing to correction of bone marrow aplasia. From day 20 until recovery in surviving animals, higher plasma FL levels were observed for animals receiving expanded MSCs and HSCs than for those receiving HSCs alone. This is in accordance with results demonstrating the production of FL by marrow stromal cells [18]. This may be explained by homing of FL-producing MSCs to the marrow and the restoration of the bone marrow stromal function. The animals receiving expanded HSCs and MSCs had a hematopoietic recovery similar to animals receiving expanded HSCs alone. This might reflect that the homing of MSCs over a large territory rather than limited to the bone marrow may have relatively impaired their ability to enhance hematopoiesis. Indeed, in previous reports involving NHPs but with milder irradiation, infused MSCs were demonstrated at very limited numbers in the bone marrow [2]. Furthermore, in clinical settings, expanded MSCs were found in scarce quantities in the recipients [2]. This current study demonstrates that, following a very severe multi-organ injury involving neutron/gamma irradiation, transplanted MSCs can be found in numerous injured tissues up to 81 days post-transplant. In some organs the grafted MSCs represent a large part of the assayed samples (up to 7% for jejunum, taking into account that only a part of MSCs are EGFP-labeled). Our report is the first evidence of the long-term ability of MSCs to home in numerous tissues of a primate as a response to a severe radiation injury.

Our results might suggest a MSC distribution kinetic as a function of the degree of severity of the lesions (and the state of regeneration of damaged tissues). The kinetics of MSC biodistribution might be explained by a specific migration of MSCs to tissues in the process of regeneration rather than to necrotic tissues. Our data supports this hypothesis since tissues receiving the higher level of neutron radiation exhibited less homing of MSCs than other tissues (Figure 5). For the animal receiving MSCs alone, eGFP+ MSCs were limited to those tissues receiving highest radiation doses. For the animal irradiated with a neutron/gamma ratio of 1 : 1 receiving MSCs and HSCs, MSCs were mostly found in the less severely irradiated tissues whereas the inverse was observed for the animal receiving less neutrons. This discrepancy might indicate that MSC homing is related to the dose of irradiation and the time post-transplant. These findings may indicate the contribution of MSCs to tissue repair at 2 weeks and after more than 2 months. The presence of eGFP+ cells in the CFU-F assays from the bone marrow of an animal receiving expanded HSCs and MSCs suggests that the transplanted MSCs may directly contribute to the restoration of bone marrow functions. Whether the homing of MSCs in numerous other tissues involves transdifferentiation or plasticity of the cells remains to be studied.

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