CXCL12/SDF-1-dependent retinal migration of endogenous bone marrow-derived stem cells improves visual function after pharmacologically induced retinal degeneration

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Abstract

Mobilized bone marrow-derived stem cells (BMSC) have been discussed as an alternative strategy for endogenous repair. Thereby, different approaches for BMSC mobilization have been pursued. Herein, the role of a newly discovered oligonucleotide for retinal homing and regeneration capability of BMSCs was investigated in the sodium iodate ($NaIO_3$) model of retinal degeneration. Mobilization was achieved in GFP-chimera with NOX-A12, a CXC-motif chemokine ligand 12 (CXCL12)/stromal cell-derived factor 1 (SDF-1)-neutralizing L-aptamer. BMSC homing was directed by intravitreal SDF-1 injection. Visual acuity was measured using the optokinetic reflex. Paraffin cross sections were stained with hematoxylin and eosin for retinal thickness measurements. Immunohistochemistry was performed to investigate the expression of cell-specific markers after mobilization. A single dose of NOX-A12 induced significant mobilization of GFP^+ cells which were found in all layers within the degenerating retina. An additional intravitreal injection of SDF-1 increased migration towards the site of injury. Thereby, the number of BMSCs ($Sca\text{-}1^+$) found in the damaged retina increased whereas a decrease of activated microglia ($Iba\text{-}1^+$) was found. The mobilization led to significantly increased visual acuity. However, no significant changes in retinal thickness or differentiation towards retinal cell types were detected. Systemic mobilization by a single dose of NOX-A12 showed increased homing of BMSCs into the degenerated retina, which was associated with improved visual function when injection of SDF-1 was additionally performed. The redistribution of the cells to the site of injury combined with their observed beneficial effects support the endogenous therapeutic strategy for retinal repair.

Keywords

Bone marrow-derived stem cells (BMSC), sodium iodate, NOX-A12, olaptesed pegol, L-aptamer, Spiegelmer, mobilization, retinal degeneration

Introduction

Replacement of the degenerated retinal pigment epithelium (RPE) before irreversible degeneration of the foveal photoreceptors was considered as a potential therapy for age-related macular degeneration (AMD) twenty years ago (1). However, experimental and clinical trials since then have shown that allogeneic RPE cells do not attach to senescent Bruch's membrane efficiently, and do not undergo proliferation or spreading to fill in the defect (2, 3). Furthermore, rejection of the allogeneic transplant hinders successful replacement of the damaged tissue (4, 5). Autologous RPE cell transplantation has also been explored in animals and humans, without evidence of functional recovery (6). As a consequence, there has been increasing interest in the use of stem cells (SC) as means to regenerate lost retinal cells and to rescue vision more effectively and safely.

New findings have recently contradicted central dogmas of commitment of adult SC including bone marrow-derived stem cells (BMSCs) by showing their plasticity to differentiate across tissue lineage boundaries, irrespective of classical germ layer designations (7). In fact, non-hematopoietic, including retinal, transdifferentiation of BMSCs has been shown (8, 9). Several subsets of SCs have been found in the bone marrow: 1) hematopoietic stem cells, the source of all blood cells which are characterized by expression of CD45; 2) mesenchymal stem cells, adherent, non-hematopoietic stromal cells that differentiate into mesenchymal tissues; 3) SC populations already committed to neural, cardiac, and other lineages, known as tissue-committed stem cells. Several publications showing the existence of the latter have led to renewed interest in adult BMSCs as a source for tissue repair *in vivo* (10, 11). Indeed, a subset of Lineage $(Lin)^-$ $Sca\text{-}1^+$ $CD45^-$ BMSCs that express markers of ocular (retina/RPE) progenitors has been identified and isolated in mice (12). Furthermore, BMSCs can be triggered to commit to and progress along the RPE lineage in culture (13). In addition, BMSCs home to the altered subretinal space if transferred systemically. The cells express RPE lineage markers as shown in a mouse model of pharmacologically induced retinal degeneration (14, 15). BM-derived

progenitor cells can also regenerate RPE and improve retinal function utilizing intravitreal injection (16). Predifferentiation of SC *in vitro* using extrinsic factors and/or coculturing is also a possible way to direct the cells towards a distinctive fate (17, 18). A similar concept is pursued by the recent approaches using RPE cells derived from embryonic SCs (19) and induced pluripotent SCs (20) for the treatment of degenerative eye diseases.

An alternative approach is the employment of the endogenous population of BMSCs by mobilizing it out of the bone marrow (BM) into the periphery and directing its migration towards the site of injury (21). Therefore, we have investigated the potential of mobilized endogenous BMSCs to morphologically and functionally regenerate damaged retinal tissue. Endogenous BMSC can be mobilized by granulocyte colony-stimulating factor (G-CSF) and migrate towards the retinal damage (22). Recently, new compounds with similar effects on the mobilization of BMSCs have been developed. One of these is the PEGylated, structured mirror-image oligonucleotide NOX-A12 (olaptesed pegol), that specifically antagonizes stromal cell-derived factor 1 (SDF-1, also known as CXC-motif chemokine ligand 12, CXCL12) and leads to dose-dependent mobilization of BMSCs and differentiated leukocytes. NOX-A12 has a benign safety profile and is well tolerated. The mobilizing effect has been induced by a single injection in mice, monkeys and humans as opposed to G-CSF for which several injections are necessary (23). NOX-A12 is a so-called Spiegelmer®, a class of oligonucleotide therapeutics made from the L-stereoisomer acting like a “chemical antibody” with very high stability in biological fluids and no immunogenicity (24). We have now used NOX-A12 to induce a redistribution of BMSCs from the bone marrow to retinal lesions that had been induced by a single dose of sodium iodate ($NaIO_3$) in mice.

$NaIO_3$ is specifically toxic for the RPE, triggering damage to photoreceptors and the choriocapillaries in a dose- and time-dependent manner (25, 26). The deleterious effects of $NaIO_3$ could also be linked to the impairment of visual function (27, 28). As shown earlier, $NaIO_3$-induced retinal degeneration led to a locally increased secretion of SDF-1 in the eye (14). SDF-1

is a key regulatory element in the homing of HSCs (29) and an SDF-1 gradient is needed to guide the migration of the mobilized BMSCs towards the site of injury (30, 31). However, the $NaIO_3$-induced retinal damage did not cause significantly higher plasma SDF-1 levels (21). In order to increase the directed migration of the mobilized BMSCs, the weak, damage-induced endogenous SDF-1 gradient can be strengthened by intravitreal injection of recombinant SDF-1. This artificial homing has been proven to be effective in the recruitment of various stem/progenitor cells via different techniques (32). Besides mobilizing BMSCs using the SDF-1 inhibitor NOX-A12, we therefore tried to enhance their redistribution to the retina by injecting SDF-1 intravitreally when mobilization is accomplished. This would provide a stronger, time-delayed SDF-1 gradient that may facilitate the redistribution of the BMSCs once SDF-1 inhibition by the Spiegelmer ceases. The visual acuity was assessed by optokinetic reflex measurements and BMSC infiltration and differentiation was visualized using IHC.

Taken together we present evidence that mobilized endogenous BMSCs can significantly improve visual function but do not rebuilt retinal structures in retinal degeneration.

Materials and Methods

Development of GFP chimeras and directed BMSC mobilization: The experiments were carried out in green fluorescent protein (GFP)-chimeras in order to track migration of the mobilized BM-derived cells after NOX-A12 injection. In order to develop chimerism, $2x10^6$ BM cells from GFP^+ donors (C57BL/6-Tg(UBC-GFP)30Scha/J, #4353, Jackson Laboratory, Bar Harbor, ME, USA), were i.v. injected into irradiated (10 Gy) C57BL/6 hosts. After 8 weeks of BM reconstitution retinal degeneration was initiated by $NaIO_3$ (25 mg/kg, i.v.). BMSC were mobilized by a single administration of the Spiegelmer NOX-A12 (13.4 mg/kg, i.v.; NOXXON in-house synthesis) three days after $NaIO_3$ injection. This time point has been chosen as RPE damage can be observed already at that but the cells are not completely destroyed (28). To potentially further boost the migration, an additional intraocular injection of 100 ng, 200 ng, or 500 ng SDF-

1 four hours after induction of mobilization was performed. Intravenous glucose (vehicle) and intraocular BSS (SDF-1 solvent) injections served as controls for NOX-A12 and SDF-1, respectively.

Functional measurement: Visual function was assessed using the OptoMotry system (CerebralMechanics, Lethbridge, AB, Canada) as described previously (28). The optokinetic reflex (OKR) was thereby measured at baseline (BL), three days after $NaIO_3$ injection and on days 7, 14, 21, and 28 after mobilization. In brief, a virtual cylinder comprising a vertical sine wave grating was projected in three-dimensional (3-D) coordinate space on computer monitors arranged in a square around a testing arena. Visual stimuli were drawn on the walls of the cylinder. Software also controlled the speed of rotation and geometry of the cylinder and the spatial frequency of the stimuli and enabled live video feedback of the testing arena. The direction of grating rotation (clock wise vs. counter-clock wise) was randomly chosen by the software and allowed for the separate measurement of the left and the right eye, respectively. Mice standing unrestrained on the centered platform tracked the grating with reflexive head movements. Two independent observers assessed whether the animals tracked the cylinder by monitoring the image of the cylinder and the animal in the video window. Using a staircase procedure, the spatial frequency of the grating was randomly increased until the animal no longer responded. The process of changing the spatial frequency of the test grating was repeated several times until the highest spatial frequency that the mouse could track was identified as the threshold by the software.

Thickness measurement: For cross sections, eyes were enucleated seven days after the last OKR measurement, dehydrated, embedded in paraffin, and 5-μm thick transverse sections were cut and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, Buchs, Switzerland). The thickness of the outer nuclear layer (ONL) was measured (ImagePro plus©; Media Cybernetics, Bethesda MD, USA) at six different locations (400, 1000 and 1600 µm inferior and superior from the center of the eye), and the mean was calculated on the level of the optic nerve head (ONH).

Immunohistochemistry: IHC was performed to characterize GFP^+ BM-derived cells that had migrated into the degenerated tissue and to evaluate BMSC differentiation *in vivo* 28 days after mobilization. For this, paraffin cross sections (5 μm) of the retina were cut and then heated in Tris EDTA pH 9.0 + 0.05% Tween 20 in a pressure cooker for antigen retrieval for 3 min. Nonspecific binding was blocked with 10% normal goat serum + 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) + 0.025% Triton 100 (ThermoFisher, Waltham, MA, USA) at pH 7.6 in a humidity chamber at RT for 2 h. The sections were then incubated with marker-specific primary antibodies at 4 °C overnight (Table 1). After washing with TBS the sections were incubated with secondary antibodies [anti-mouse / rabbit / rat Alexa594 (ThermoFisher) 1:500] at RT for 45 min. This was followed by consecutive staining for GFP, beginning with blocking with 3% normal mouse serum at RT for 1 h and followed by primary antibody [chicken anti-GFP (Abcam, Cambridge, UK) 1:500] incubation at 4 °C overnight. After washing with TBS the sections were incubated with secondary antibodies [goat anti-chicken Alexa488 (ThermoFisher) 1:1000] at RT for 45 min and mounted with Vectashield with DAPI (Vector Labs, Burlingame, CA, USA).

Quantification:

Migration of GFP^+ BM-derived cells into the retina and changes in the distribution of distinct cell populations therein was quantified using fluorescence microscopy (Nikon Eclipse 80i; Nikon AG, Egg, Switzerland). The quantification was performed after IHC (see above and Table 1) on paraffin sections of eyes from the different treatment groups (Glc + BSS, Glc + SDF-1, NOX-A12 + BSS, NOX-A12 + SDF-1). The number of positive cells in all retinal layers per visual field (visible microscopic area of 1.14 μm^2) at six respectively three different locations was counted manually by one blinded observer.

Statistics: The data were presented as mean ± SD/SEM and compared using SigmaPlot 12.0 (Systat Software, Erkrath, Germany). One Way ANOVA with post hoc analysis by the Student-

Newman-Keuls method or the Student's t-test were applied. Differences were considered statistically significant at $P \leq 0.05$.

Results

Chimerism and Mobilization: In order to visualize the BM-derived cells we have developed GFP-chimeras with an average 63.8 ± 0.1% chimerism. The migrated GFP^+ BM-derived cells in the retina were visualized and quantified on day 7 after NOX-A12 and/or SDF-1 injection (n=3). Thereby, GFP^+ cells were found in all layers within the retina with an increase after NOX-A12 treatment (Figure 1A-D). The migration into the $NaIO_3$-altered sensory retina could be significantly increased by systemic NOX-A12 plus intraocular injection of 500 ng SDF-1 compared to Glucose/BSS and Glucose/500 ng SDF-1 (67% increase) or NOX-A12/BSS (46% increase). Injection of lower SDF-1 concentrations in conjunction with BMSC mobilization had no comparable effects (Figure 1E). Thus the concentration of 500 ng SDF-1 was used for the following experiments.

Characterization of the migrated cells: GFP^+ BM-derived cells that had migrated into the degenerated tissue were characterized by immunohistochemistry (n=5; Figure 2). Here, BMSCs (Sca-1), microglia (Iba-1) as well as macrophages (F4/80) were specifically stained and counted in retinal sections prepared 7 days after mobilization (see Table 2 for details). BMSC mobilization by NOX-A12 followed by ocular SDF-1 injection led to an increase in BMSC (Sca-1; 226.7% compared to Glc + BSS) but a decrease of activated microglia (Iba-1; 73.0% compared to Glc + BSS) in the degenerated retina. The number of detectable macrophages (F4/80) increased with NOX-A12 (224.1%) but remained at a constant level after additional SDF-1 injection (106.2%). Furthermore, SDF-1 injection into the eye led to a distinct increase of BMSCs in the retina, even without NOX-A12-induced BMSC mobilization (249.2% compared to Glc + BSS).

Functional measurements after BMSC mobilization: Visual function was quantified using OKR measurements at baseline, three days after $NaIO_3$ injection as well as 7, 14, 21 and 28 days after mobilization (NOX-A12; n=8) or vehicle (Glc; n=5). The results revealed that the combination of systemic NOX-A12 with ocular SDF-1 (500 ng) yielded significant results over NOX-A12 alone (days 14 - 28) or glucose/BSS (day 28; Figure 3).

Thickness measurement: Despite an improved visual function after treatment (see above), no rescue of retinal structures in the $NaIO_3$-induced retinal degeneration was seen after injection of NOX-A12 with or without additional intraocular injection of SDF-1 (n=3 (Glc) / n=5 (NOX-A12)). Thereby, non-significant general thickening of the whole retina was observed (Figure 4). This could be also found in the most affected layer, the ONL (data not shown). In all cases, injection of SDF-1 alone did not have a significant influence on the retinal layer thickness.

Differentiation: The expression of retina-specific markers on GFP^+ cells in the subretinal space was studied at the end of the observation period (day 28 after mobilization). The RPE marker bestrophin, the glia marker GFAP as well as the neuronal marker βIII tubulin were investigated. However, no double labeling indicating retina-specific differentiation of the migrated BMSC was found. Representative images depicting positively stained retinal cells and GFP^+ BM-derived cells are shown in Figure 5.

Discussion

Endogenous BMSC mobilization using the SDF-1-neutralizing Spiegelmer NOX-A12 was induced in the murine $NaIO_3$-model of retinal degeneration. SDF-1 is a key regulatory element in the homing (33) and mobilization (29) of BMSCs. We had established in previous experiments that injections of NOX-A12 dose-dependently mobilized leukocytes including colony-forming BMSCs in mice, cynomolgus monkeys and healthy volunteers (23). Moreover, others have shown that a chemokine gradient is needed to guide the migration of the mobilized BMSCs towards the site of injury (30, 31). Here we show that upon mobilization more GFP^+ BM-derived

cells migrated to the area of interest, the damaged subretinal space. Similar results have been found by using systemic BMSC mobilization by administration of GM-CSF and Flt3 ligand (22). In order to optimize this migration further, an intraocular SDF-1 injection was performed additionally at the estimated peak of the mobilization 4 h after NOX-A12 administration. The SDF-1 treatment led to a significant increase of BM-derived cells in the subretinal space and herewith to a reservoir of potential candidates for repair and regeneration. This is in line with a novel strategy involving mobilization of large numbers of endogenous stem and progenitor cells from bone marrow into the circulation which has been shown to have positive healing effects in other tissues (34).

In order to investigate this further, we characterized the specific cell type of the migrated BM-derived cells. An increase of BMSCs (Sca-1) in the subretinal space was found after NOX-A12-induced mobilization and/or SDF-1 injection. Sca-1^+ cells are primitive murine hematopoietic cells (35). These cells are known to migrate in response to chemotactic signals expressed by the damaged RPE and may therefore have the potential to serve as an endogenous source for tissue regeneration after RPE damage (14). On the other hand, the number of activated microglia (Iba-1^+ cells) in the degenerated retina decreased after mobilization and SDF-1 treatment. This is in contrast to the observation that BM-derived cells that migrated to the retina in response to pharmacologically induced specific photoreceptor damage (methylnitrosourea) differentiated mainly into microglia (36). Our results may reflect the circumstances of the $NaIO_3$ model or indicate that the presence of redistributed BMSCs in the subretinal space inhibits microglia formation. This is especially important as microglia cells are immunoreactive and might hinder retina repair by exacerbating undesirable inflammatory processes (37). In our experimental setup the level of macrophages (F4/80) identified in the retina after SDF-1 treatment remained constant but increased after NOX-A12 mediated mobilization compared to the relevant controls. Others have found that damage to the RPE corresponded with the accumulation of macrophages phagocytizing melanin granules and cell debris (38). As we have

not included animals without $NaIO_3$-induced retinal damage an analogy to this report cannot be drawn. But our results are in line with immune suppressive effects of the recruited BMSCs reported after BMSC transplantation in a spinal cord injury model (39). There, BMSC transplants prevented hematogenous monocyte/macrophage recruitment by restoration of the blood-spinal cord barrier which was associated with decreased levels of (a) inflammatory cytokines (tumor necrosis factor-α, interleukin-6); (b) mediators of early secondary vascular pathogenesis (matrix metallopeptidase 9); (c) macrophage recruiting factors (CCL2, CCL5, and CXCL10), but increased levels of a microglial stimulating factor (granulocyte-macrophage colony-stimulating factor).

Visual acuity was measured at different time points in order to quantify the potential restorative effects of the mobilization and directed migration of the BM-derived cells. A significantly increased visual function was found beginning on day 14 after treatment. The positive effect was detectable when comparing the directed mobilization (NOX-A12 + SDF-1) with mobilization alone (NOX-A12 + BSS). A similar outcome was found after NOX-A12-induced mobilization compared to non-mobilized animals (glucose injection) at the latest time point (d28 after mobilization) only. This indicates that the ocular injection of SDF-1 promotes directing the mobilized BMSCs to the site of injury (retina) where they can accelerate functional repair. Therefore, topical delivery of SDF-1 may improve repair of the degenerated retina as successfully performed for dermal wounds (40).

However, the visual acuity baseline values were not reached after treatment, indicating that the number of migrated cells might not be sufficient to further promote functional recovery. In the literature a direct correlation between the number of autologous BMSCs applied and the subsequent repair effect has been described for dermal lesions in mouse and human (41). Therefore, modified interventions, e.g. repeated dosing of NOX-A12 and potentially SDF-1 in order to increase the number of BMSC in the retina might further enhance their regenerative effect.

It has been published that BMSCs can protect and repair damaged neurons through multiple mechanisms, including transdifferentiation, cell fusion, and production of growth factors (42). In order to identify the mechanism behind the improved visual function, we have investigated a possible differentiation of the BMSC into retinal cell types. However, none of the retinal markers, including bestrophin for RPE, the glia marker GFAP and the neuronal marker βIII tubulin, were found to be expressed on the migrated GFP^+ BM-derived cells. In the absence of retinal markers, the transdifferentiation of BMSC is very unlikely. Therefore, our results point towards the release of secreted factors by the BMSC promoting the protective effects in our model. Similar paracrine cell-cell communication from migrated or transplanted SCs has been found in other animal models as well (43-45).

In summary, using NOX-A12 and intravitreal SDF-1 we were able to enhance the homing of BMSCs to the damaged retina and improve visual acuity. The paracrine actions of migrated endogenous BMSCs might thereby open new possibilities for retinal repair. Our results encouraging additional experiments with endogenous BMSC mobilization as a possible therapeutic tool for retinal degenerations.

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Compliance with Ethical Standards

Conflict of Interest: AV and AK are employees of NOXXON Pharma which holds or has applied for patents relating to the content of the manuscript.

Ethics approval**:** All animals were treated according to the ARVO statement for the use of animals in vision und ophthalmic research and after governmental approval.

Figure legends

Figure 1: A-D) Representative examples of positive staining for GFP indicating the migrated BM-derived cells in the damaged retina on day 7 after mobilization. Scale bar = 100 μm. E) Number of GFP^+ cells per visual field in the subretinal space after treatment with 25 mg/ kg $NaIO_3$, NOX-A12 and different concentrations of SDF-1 (n = 3, mean ± SD). Thereby, the injection of 500 ng SDF-1 increased the homing of GFP^+ cells significantly ($P \leq 0.05$; *****) compared to NOX-A12 + BSS, glucose + BSS, glucose + SDF-1 as well as the lower SDF-1 concentrations.

Figure 2: Cell type-specific staining of retina sections of animals with $NaIO_3$-induced retinal degeneration followed by systemic NOX-A12 and intraocular SDF-1 injection (day 7 after mobilization). GFP^+ BM-derived cells are shown in green in all panels. Specific labeling for the stem cell marker Sca-1 (A), the microglia marker Iba-1 (B) or the macrophage marker F4/80 (C) is depicted in red. Arrows indicate double positive BM-derived cells (yellow). Scale bar = 100 μm.

Figure 3: Visual acuity measurement by using the optokinetic reflex after $NaIO_3$ induced retinal degeneration with and without NOX-A12 (n = 8 or 5, respectively) and/or intraocular injection of SDF-1. Thereby, significant increase ($P \leq 0.05$) in visual acuity was found after treatment with NOX-A12 + SDF-1 vs. treatment with NOX-A12 + BSS (*; mean ± SD) beginning on day 14 after mobilization. Furthermore, there is also a significant improvement of the visual acuity after NOX-A12 treatment compared to glucose (Glc) injection on day 28 after mobilization (#).

Figure 4: Morphometric measurements of the retinal thickness in paraffin sections from eyes with $NaIO_3$-induced degeneration followed by treatment with NOX-A12 and/or SDF-1 (n=3). No significant differences between the groups were found (mean ± SD).

Figure 5: Cell type-specific staining of retina sections of animals with $NaIO_3$ induced retinal degeneration followed by systemic NOX-A12 and intraocular SDF-1 injection. Migrated BM-derived cells could be identified by GFP (green) in all panels. Specific markers (red) depict the RPE marker bestrophin (A), the glia marker GFAP (B) and the early neuronal marker βIII tubulin (C). No double labeling was found. Scale bar = 100 μm.

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