Short communication

HOMING OF ANNEXIN-LABELED STEM CELLS TO APOPTOTIC CELLS

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Abstract: Ischemic diseases are characterized by the presence of pro-apoptotic stimuli, which initiate a cascade of processes that lead to cell injury and death. Several molecules and events represent detectable indicators of the different stages of apoptosis. Among these indicators is phosphatidylserine (PS) translocation from the inner to the outer leaflet of the plasma membrane, which can be detected by annexinV (ANXA5) conjugation. This is a widely used *in vivo* and *in vitro* assay marking the early stages of apoptosis. We report here on an original method that employs PS-ANXA5 conjugation to target stem cells to apoptotic cells. Mesenchymal stem cells (MSCs) from GFP-positive transgenic rats were biotinylated on membrane surfaces with sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biot) and then bound to avidin. The avidin-biotinylated MSCs were labeled with biotin conjugated ANXA5. Bovine aortic endothelial cells (BAE-1 cells) were exposed to UVC to induce caspase-dependent apoptosis. Finally, we tested the ability of ANXA5-labeled MSCs to

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bind BAE-1 apoptotic cells: suspended ANXA5-labeled MSCs were seeded for 1 hour on a monolayer of UV-treated or control BAE-1 cells. After washing, the number of MSCs bound to BAE-1 cells was evaluated by confocal microscopy. Statistical analysis demonstrated a significant increase in the number of MSCs tagged to apoptotic BAE-1 cells. Therefore, stem cell ANXA5 tagging via biotin-avidin bridges could be a straightforward method of improving homing to apoptotic tissues.

Key words: Stem cell, Apoptosis, Homing, Annexin

INTRODUCTION

Based on the results of the years of work with bone marrow as a treatment for leukemias, cell transplantation and autotransplantation have been recently proposed as possible treatments for several other diseases. In particular, stem cells have been studied in depth as a source for the treatment of ischemic diseases such as stroke, ischemic retinopathy, myocardial infarction, liver disorders, renal failure and limb dysfunction. Several cell types, including embryonic and adult stem cells, have been used with different transplantation procedures to repopulate injured tissues in animal experiments and clinical trials. However, the rate of tissue regeneration depends on multiple variables, including the transdifferentiation and survival potential of the injected cells, and the adequate delivery of a high number of cells to the ischemic region [1, 2].

The physiological targeting of stem cells to the site of homing is a multiple process in which cells invade tissue and proliferate to provide a supportive environment. Stem cell trafficking/homing is regulated by many physiological mechanisms; among these, the predominant role of the chemokine SDF-1 and its receptor CXCR-4 for hematopoietic stem cells is well established [3], and there was a recent report on the effect of different soluble factors on bone marrow mesenchymal stem cells (MSC) including chemokines or growth factors [4].

Strategies aimed to improve the efficacy and tissue specificity of stem cell engraftment were directed to the upregulation of specific chemotactic mechanisms [5] or to the employ of *in vitro* protocols of stem cell homing optimization. Among these protocols, bispecific antibody targeting is a promising technical approach to direct cells to a specific tissue, although further improvements are necessary [6]. Strategies of optimization of stem cell homing could use targets identified in the ischemic regions.

Apoptotic cells are a typical target that have already been used to diagnose the extent of the damage. Ischemia is a complex process with several phases that are characterized in different tissues by the release of several molecules including inflammation markers, growth factors and small molecules. Cell death in the ischemic region is due to a mixture of necrosis and apoptosis, with different time courses and locations [7, 8].

Apoptosis can be *in vivo* detected using the exposition of phosphatidylserine as a simple marker. This is an early event in the apoptotic process, lasting until the

end of the cell death process. Annexin V (ANXA5), a 35.8-kDa phospholipid binding protein, binds specifically with nanomolar affinity (Kd = 7 nM) to membrane-bound, externalized PS [9]. Radiolabeled ANXA5 has been studied extensively for the detection of apoptosis in humans and other animals, with the most clinical experience being gained with a 99mTc-labeled ANXA5 complex modified with a hydrazinenicotinamide ligand (99mTc-HYNIC-ANXA5) [10].

A protocol of stem cell homing to ischemic tissue could therefore use apoptotic areas as a target and ANXA5 and phosphatidylserine as a guiding system. This approach requires a crosslinker between the stem cells and ANXA5. Here, we report on a method to link ANXA5 to biotinylated MSCs through an avidin bridge, and we show that this protocol significantly increases the *in vitro* targeting of MSCs to apoptotic cells.

MATERIALS AND METHODS

Cell culture and media
Mesenchymal stem cells (MSCs) were isolated from the bone marrow of the femurs of 6- to 12-month old rats that had been stably transfected with green fluorescent protein (GFP) [11]. The MSCs were harvested and cultured as described by Javazon *et al.* [12]. BAE-1 cells were purchased from European Collection of Cell Cultures (Salisbury, Wiltshire, UK).

The MSCs were grown in minimum essential Eagle's medium, alpha modified (αMEM), and the BAE-1 cells in Dulbecco's modified Eagle's medium (DMEM), both from Sigma (St. Louis, MO, USA). The cultures were maintained at 37ºC, in a humidified atmosphere of 5% CO_2 in air. The media were supplemented with 10% heat-inactivated fetal calf serum (FCS, Biowhittaker, Verviers, Belgium), 100 U/ml penicillin, 100 μg/ml streptomicin, 2 mM glutamine and 0.25 mg/ml fungizone (BioWhittaker, Europe).

Confocal microscopy
An Olympus IX70 inverted microscope with a Fluoview 200 confocal head and an Ar/Kr laser (488 and 568 nm) was used. Image analysis was performed with ImageJ (Rasband, W.S., US National Institutes of Health, Bethesda, MA, USA, http://rsb.info.nih.gov/ij/, 1997-2005) on a PowerMac G5 (Apple, Cupertino, USA).

Biotinylation of MSCs and avidin binding
Biotinylation of the MSC membrane surface was performed with sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin, Sigma, St. Louis, MO, USA), which is water soluble and cell impermeable. For adherent cells, confluent cultures of MSCs grown on glass bottom dishes (35/22 mm ∅, Willco Wells, Amsterdam) were washed three times with phosphate buffer saline (PBS, pH 7.5) and incubated at 37ºC in 5% CO_2 for 10 minutes with 200 μM sulfo-NHS-LC-biotin freshly dissolved in PBS. After the removal of the reaction mixture, the cells were washed three times with PBS plus 100 mM glutamine to remove the excess of biotin reagent. The cells were then incubated at 37ºC in

5% CO_2 for 10 minutes with 1 μM avidin (Molecular Probes). After three washes with PBS, the cells were either used for ANXA5 conjugation or incubated at 37ºC in 5% CO_2 for 10 minutes with 1 μM Atto 565 biotin (Sigma) in order to assess cell biotinylation. Finally, the MSCs were washed with PBS three times and observed under a confocal microscope immediately and after 24 hours.

For biotinylation of the MSCs in suspension, a 58-cm^2 dish of confluent cells was washed twice with PBS without Ca^{2+} and Mg^{2+}, and the cells were detached using trypsin. Cell treatment was performed as described above in microcentrifuge tubes. During each incubation, the tubes were gently flicked several times to avoid cell adhesion and to facilitate reagent interaction. The reagent concentrations were increased to 2 mM sulfo-NHS-LC-biotin and 10 μM avidin accordingly to the increased number of cells treated in the same reaction volume. After each incubation, the cells were washed twice and collected at every step by centrifugation at 800 rpm for 1.5 minutes at room temperature. After the treatment, cells were either stained with Atto 565 biotin, plated on glass bottom dishes and observed under a confocal microscope immediately and after 24 hours, or used for ANXA5 conjugation.

Conjugation of biotinylated MSCs with ANXA5

After biotinylation and avidin binding of the adherent MSCs, the cells were washed three times with PBS and incubated at 37ºC in 5% CO_2 for 10 minutes with 2 μl/ml of biotin conjugated ANXA5 (Sigma). After incubation, the cells were washed once with PBS and then twice with PBS plus BSA (Sigma, 1 mg/ml).

In order to evaluate ANXA5 conjugation, the cells were then incubated in PBS plus BSA with the primary antibody against ANXA5 (monoclonal mouse IgG1 isotype, clone AN5, Sigma, 1:100) for 1 hour at 37ºC in 5% CO_2. The glass bottom dishes were washed three times with PBS and the MSCs were stained for 30 minutes at 37ºC in 5% CO_2 with the secondary antibody (Cy3 anti-mouse IgG, Sigma, 1:1000). After three washes with PBS, the cells were observed under a confocal microscope.

For the MSCs in suspension, cells from a 58 cm^2 confluent dish were detached as described above, and treatments were performed in micro centrifuge tubes as previously described. The biotin-conjugated ANXA5 concentration was increased to 20 μl/ml accordingly to the increased number of cells treated in the same reaction volume. Biotin-conjugated ANXA5 cells in suspension were used for the binding assay.

Apoptosis induction and detection

Confluent cultures of BAE-1 cells grown on glass bottom dishes (35/22 mm ∅, Willco Wells, Amsterdam) were exposed to UVC irradiation (emission range of 100-280 nm, with an emission peak of 254 nm) using a germicidal lamp (PHILIPS TUV 15W/G15 T8) for 3 minutes. After irradiation, the cells were

further incubated with culture medium for 3 hours before proceeding with apoptosis detection. Apoptotic and non-apoptotic cells were either used for the binding assay or for apoptosis detection.

For apoptosis detection with sulforhodamine caspases 3 & 7 FLICA (SR-DEVD-FMK) from Immunochemistry Technologies, cells were washed twice in PBS and incubated with FLICA reagent for 1 hour at 37ºC, 5% CO_2. After 2 washes with wash buffer, the cells were directly observed under a confocal microscope. To test the ANXA5 binding to apoptotic cells, after irradiation, the cells were washed twice with PBS and incubated with 2 μl/ml ANXA5 biotin conjugated (Sigma) for 10 minutes at 37ºC, 5% CO_2. After one wash in PBS alone and two in PBS plus BSA (Sigma, 1 mg/ml), the cells were incubated in PBS plus BSA with a primary antibody against ANXA5 (monoclonal mouse IgG1 isotype, clone AN5, Sigma, 1:100) for 1 hour at 37ºC, 5% CO_2, washed three times in PBS, and incubated with the secondary antibody (Cy3 anti-mouse IgG, Sigma, 1:1000) for 30 minutes at 37ºC, 5% CO_2. Finally, the cells were washed three times in PBS and directly observed under a confocal microscope.

Binding assay

Confluent cultures of MSCs grown on a 58-cm^2 dish were detached as previously described. The MSCs were biotinylated and conjugated with ANXA5 biotin conjugated as described in the previous sections. After treatment, about 100,000 cells/dish were seeded on BAE-1 cells grown on glass-bottom dishes (35/22 mm ∅) and previously exposed (apoptotic cells) or not (non-apoptotic cells) to UVC irradiation for 3 minutes. For the control experiments, the same quantity of non-functionalized MSCs were seeded on apoptotic and non-apoptotic BAE-1 cells. The glass-bottom dishes were then incubated for 1 hour at 37ºC, 5% CO_2, washed twice with PBS to remove unbound cells, and directly observed under a confocal microscope. For each experiment, at least five random fields were acquired at 20x magnification.

The MSCs in each field from the apoptotic and non-apoptotic BAE-1 cells were counted using ImageJ Software. The mean number of cells counted in each experiment was normalized to the number of cells/field initially seeded on the glass-bottom dish.

RESULTS

MSC biotinylation

The basis of this methodological approach employs biotin-ANXA5 conjugated to avidin-biotinylated MSCs in order to direct ANXA5-bound MSC to apoptotic cells in an *in vitro* model of a damaged tissue. The first step of the protocol is the biotin labeling of the MSC membrane proteins with the biotinylating agent sulfo-NHS-biotin, as described in the Methods section. Biotinylation was performed on both adherent and suspended MSCs. To validate the specific labeling of sulfo-NHS-LC-biotin to the cell surface, MSCs were incubated in

sequence with avidin and with fluorochrome-conjugated biotin (Atto 565 biotin). Labeled MSCs were observed via confocal microscopy: as shown in Fig. 1, a localized red fluorescence was present on the membranes of both adherent (A) and suspended (C) MSCs. The MSCs were observed again after 24 hours of culture to evaluate the duration of membrane biotinylation: red fluorescence was present in the cytoplasmic compartment, indicating internalization of biotin due to membrane turnover (B and D). To visualize the fluorescence signal of the Atto 565 biotin more clearly, the background GFP fluorescence was represented in grayscale. The number of MSCs successfully biotinylated (red fluorescence) was $87 \pm 4.7\%$ (n = 11) above the total MSCs.
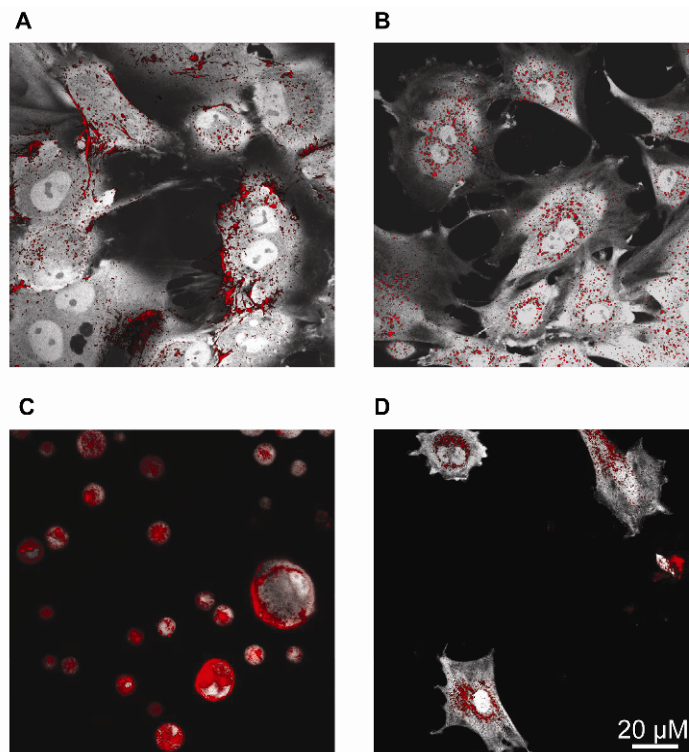

Fig. 1. MSC biotinylation. Biotinylation of MSCs was performed with sulfo-NHS-LC-biotin and detected with avidin and Atto 565 biotin. Localized red fluorescence was present on the membranes of both adherent (A) and suspended (C) MSCs at t_0 and in the cytoplasm after 24 hours (B and D). Confocal microscope images (60x magnification) of live cells; superposition of GFP (grayscale) from MSCs and Atto 565 biotin (red). Scale bar = 20 μm.

ANXA5 conjugation of biotinylated MSCs
The second step was to label the biotinylated MSCs with biotin-conjugated ANXA5, through an avidin bridge, and to verify the specific annexin labeling on

the MSC membranes with an anti-ANXA5 antibody detected with a secondary red fluorescent antibody. Fig. 2 shows a typical experiment of ANXA5 conjugation: the red fluorescence indicates that binding of ANXA5 to MSC membranes occurred, and GFP fluorescence of MSCs is represented in grayscale. The number of MSCs successfully conjugated to ANXA5 (red fluorescence) was $78 \pm 3{,}6$ % (n = 15) above the total MSCs.

ANXA5-labeled MSC homing to apoptotic cells
We tested the ability of ANXA5 labeled MSCs to bind apoptotic cells. This test was developed using BAE-1 cells treated with UVC to induce caspase-dependent apoptosis [13]. The percentage of apoptotic cells evaluated by cell morphology was 77 ± 7% (n = 11). UVC-induced apoptosis was verified with a sulforhodamine FLICA apoptosis detection kit, based on a short aminoacidic inhibitor sequence of caspases linked to a red (sulforhodamine, SR) fluorescent probe (Fig. 3 A, B). The level of red fluorescence, indicating caspase activation, was higher in apoptotic cells (A) than in non-apoptotic cells (B).
Apoptosis was also assessed by labeling UVC-treated and control BAE-1 cells with ANXA5, followed by detection with an anti ANXA5 antibody (Fig. 3 C, D). The level of red fluorescence in UVC-exposed BAE-1 cells (C) relative to the control cells (D) demonstrates that UVC treatment successfully triggered phosphatidylserine exposure on the cell membrane.
The culmination of this protocol was to verify the increased homing of ANXA5-labeled MSCs to apoptotic cells compared to the level of homing to non-apoptotic cells. In these experiments, about 100,000 suspended MSCs prepared as described in the previous sections (biotinylation plus ANXA5 conjugation) were seeded for 1 hour on a glass-bottom dish containing a monolayer of apoptotic or non-apoptotic BAE-1 cells. After washing, the number of MSCs bound to BAE-1 cells was evaluated by confocal microscopy, as described in the Methods section. Fig. 4 A, B, shows a representative experiment of MSC homing: apoptotic BAE-1 cells clearly bind a higher number of ANXA5-labeled MSCs (A) than control cells (B).
In order to verify the dependence of homing on MSCs-ANXA5 conjugation, we performed control experiments with non-functionalized MSCs seeded as previously described on apoptotic or non-apoptotic BAE-1 cells. As shown in Fig. 4 C, D, and in the bar graph of panel E, unlabeled stem cells have a very low level of binding. This experiment supports the specificity of annexin-labeling in the increased homing to apoptotic cells.
Panel C shows a quantitative analysis of MSC homing experiments, demonstrating a significant increase in the number of ANXA5-labeled MSCs tagged to apoptotic BAE-1 cells (64.5 ± 10.7% for non-apoptotic cells and 115.6 ± 20.3% for apoptotic cells, both with respect to the number of cells/field seeded. $p < 0.03$, n = 5), and a low number of non-functionalized MSCs on BAE-1 cells (4.6 ± 0.7% for non-apoptotic cells and 7.1 ± 1.5% for apoptotic cells, n = 2).
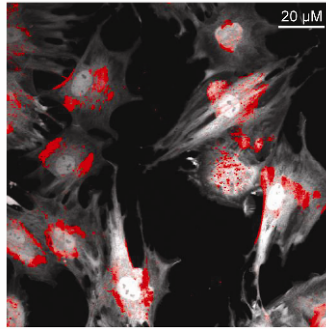
Fig. 2. ANXA5 conjugation of biotinylated MSCs. ANXA5 conjugation to adherent biotinylated MSCs through an avidin bridge was detected using ANXA5 antibody. Red fluorescence indicates that binding of ANXA5 to MSC membranes occurred. Confocal microscope images (60x magnification) of live cells; superposition of GFP (grayscale) from MSCs and CY3 (red). Scale bar = 20 µm.
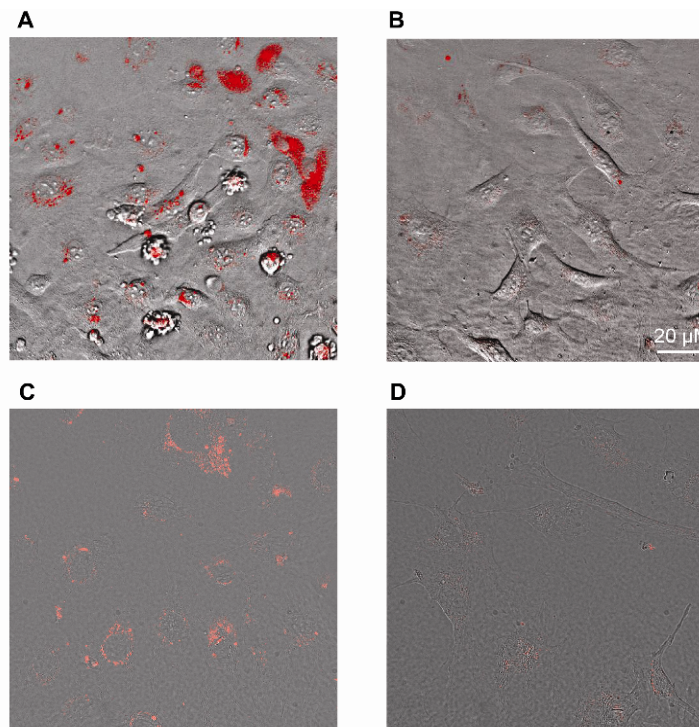

Fig. 3. Apoptosis detection with FLICA (A, B, red fluorescence) and with ANXA5-anti ANXA5 antibody (C, D, red fluorescence). BAE-1 cells were exposed to UVC irradiation for 3 minutes to induce caspase-dependent apoptosis. The level of fluorescence was higher in the apoptotic cells (A, C) than in the non-apoptotic cells (B, D). Confocal microscope images (60x magnification) of live (A, B) and fixed (C, D) cells; superposition of red and bright field microscopy (Nomarsky). Scale bar = 20 µm.
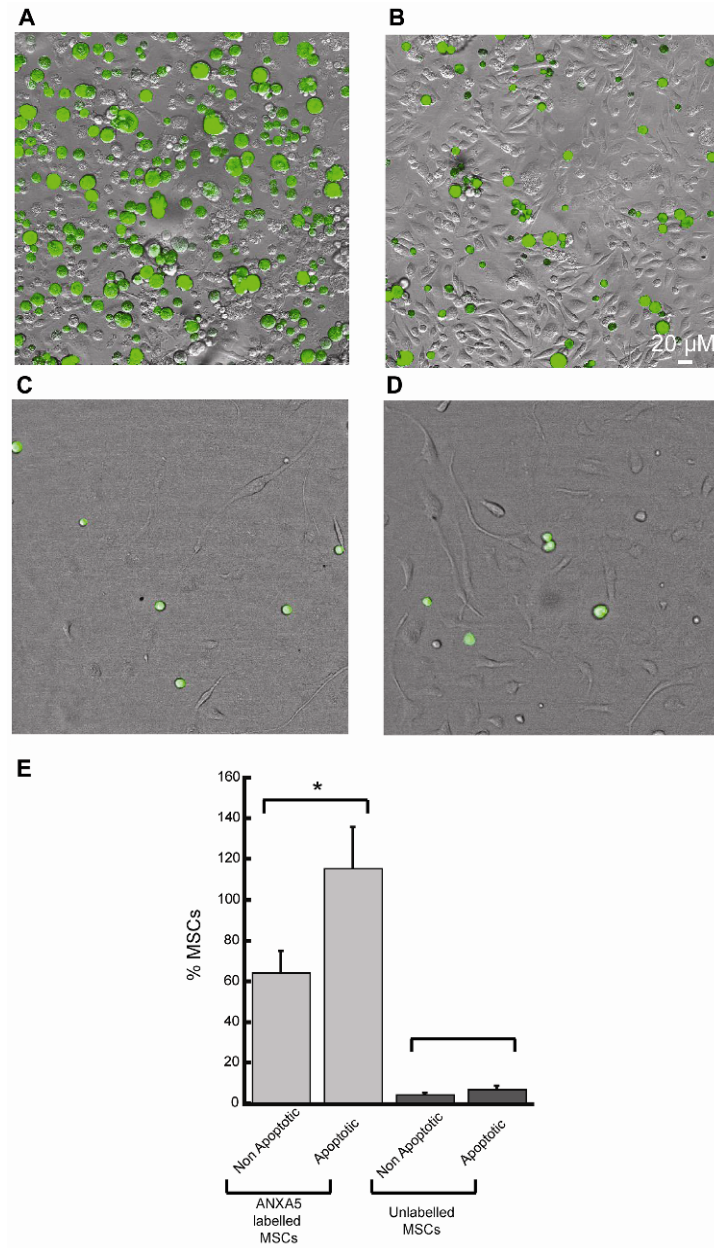
Fig. 4. MSC homing to apoptotic cells. A, B – A representative experiment showing the increased homing of ANXA5-labeled MSCs to apoptotic (A) over non-apoptotic (B) BAE-1 cells. C, D – A representative experiment showing the low homing level of non-functionalized MSCs to apoptotic (C) and non-apoptotic (D) BAE-1 cells. Panel C shows a quantitative analysis of MSC homing experiments (see the main text for details). Confocal microscope images (20x magnification) of live cells; superposition of GFP (green) from MSCs and bright field microscopy (Nomarsky) of BAE-1 cells. Scale bar = 20 μm.

DISCUSSION

In this study, we exploited the ability of ANXA5 to bind PS residues on cell membranes, to design a feasible protocol of cell targeting towards apoptotic cells. AnnexinA5 belongs to a superfamily of phospholipid-binding proteins, the annexins, which share the property of Ca^{++}-dependent binding to negatively charged phospholipid surfaces. In particular, the various biological effects of ANXA5 (anticoagulant and Ca^{++} channel activities, PLA2 regulation, inhibition of phagocytosis of apoptotic cells, immune system modulation), are closely associated to its ability to bind to PS [14].

The discovery of PS exposure by apoptotic cells and the feasibility of conjugating ANXA5 to a wide range of reporter compounds (fluorophores, radioisotopes) led to the birth of the ANXA5 affinity assay to detect apoptosis. This assay was further designed in multiple variants for measuring apoptosis *in vitro*, *in vivo* in animal models, and in patients non-invasively. In particular, Technetium-labeled ANXA5 was used to detect apoptosis in patients with acute myocardial infarction, intracardiac tumor and heart transplantation rejection, and to evaluate atherosclerotic plaques [15].

The properties of ANXA5 allowed its application as a molecular imaging probe to be extended also as a therapeutic targeting vector. The first application in this direction was the preparation of a chemical conjugate of urokinase with ANXA5 to target phospholipid-containing thrombi [16].

The originality of our method concerns the conjugation of ANXA5 to stem cell membranes to enhance their delivery to apoptotic tissues. We used binding between the protein avidin and the vitamin biotin as an extrinsic high affinity receptor-ligand system to conjugate biotinylated ANXA5 and biotinylated mesenchymal stem cells.

Avidin-biotin interaction has been largely exploited for "protein detection" techniques (in immunology, histochemistry, *in situ* hybridization, affinity chromatography and other areas) as well as in pioneer techniques aimed at improving cell adhesion or drug delivery. Of these, biotinylation of TNF-α was proposed as an approach to direct the cytokine to the membrane of melanoma cells [17], and biotinylation of red blood cells as a potential antigen delivery system via the biotin-avidin-biotin bridge [18]. An interesting report demonstrates the safety and specificity of red blood cell delivery of HIV-Tat as a step in the design of a new anti-HIV vaccine [19].

To tweak our technique, we first performed biotin labeling of MSC membrane proteins with the biotinylating agent sulfo-NHS-biotin reagents (Fig. 1). This step was also verified on suspended MSCs (Fig. 1C), in the perspective of an *in vivo* application on autologous bone marrow stem cells. After 24 hours, the biotin was internalized (Fig. 1 B, D), showing the safeguard of the physiological membrane protein turnover. Consistently with this last observation, several investigators reported that the biotinylation reagents do not seem cytotoxic in *in vitro* and *in vivo* experimental models [18, 20, 21].

In the second step of the protocol, biotin-conjugated ANXA5 was labeled to the biotinylated MSCs through an avidin bridge. The ANXA5 binding was verified with an anti-ANXA5 antibody (Fig. 2). For an *in vitro* model of apoptotic cells, we used BAE-1 cells treated with UVC radiation, which induces apoptosis via the intrinsic pathway [13].

The MSC-ANXA5 conjugate clearly shows a significant increase in the MSC tagging to apoptotic BAE-1 cells (Fig. 4). However, a potential limitation of this protocol could be the interference of annexin A5 labelling with the reparative ability of MSCs. Therefore, the current experiments suggest a new strategy that *in vivo* experiments will possibly verify. The potential of MSCs to repair in *in vivo* models of ischemic disorders requires the successful delivery of the MSCs to specific tissues.

In conclusion, this result represents an important preliminary stage for the design of a protocol of enhanced cell homing, alternative to the chemokine-mediated or bispecific antibody-mediated protocols. In particular, our approach offers a potentially non-toxic system of delivery of stem cells, in which apoptosis of the targeted tissue is the fundamental requirement.

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