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The effect of delivering the chemokine SDF-1 α in a matrix-bound manner on myogenesis

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Abstract

Several chemokines are important in muscle myogenesis and in the recruitment of muscle precursors during muscle regeneration. Among these, the SDF-1 α chemokine (CXCL12) is a potent chemoattractant known to be involved in muscle repair. SDF-1 α was loaded in polyelectrolyte multilayer films made of poly(L-lysine) and hyaluronan to be delivered locally to myoblast cells in a matrix-bound manner. The adsorbed amounts of SDF-1 α were tuned over a large range from 100 ng/cm² to 5 μ g/cm², depending on the initial concentration of SDF-1 α in solution, its pH, and on the film crosslinking extent. Matrix-bound SDF-1 α induced a striking increase in myoblast spreading, which was revealed when it was delivered from weakly crosslinked films. It also significantly enhanced cell migration in a dose-dependent manner, which again depended on its presentation by the biopolymeric film. The low-crosslinked film was the most efficient in boosting cell migration. Furthermore, matrix-bound SDF-1 α also increased the expression of myogenic markers but the fusion index decreased in a dose-dependent manner with the adsorbed amount of SDF-1 α . At high adsorbed amounts of SDF-1 α , a large number of Troponin T-positive cells had only one nucleus. Overall, this work reveals the importance of the

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presentation mode of SDF-1 α to emphasize its effect on myogenic processes. These films may be further used to provide insight into the role of SDF-1 α presented by a biomaterial in physiological or pathological processes.

Keywords

Polyelectrolyte multilayer film; Drug delivery; SDF-1 α /CXCL12; CXCR4; Myoblast; Migration

1. Introduction

Differentiation and commitment of precursor cells such as stem cells or satellite cells is a spatially and temporally controlled process, which occurs over several days. In this process, every step is important, from the initial adhesion to chemoattraction and migration of specific cell populations. Indeed, migration is a key process during the development of several tissues, including muscles [1,2]. During myogenesis, cell–cell adhesion is crucial and necessary for myoblast differentiation as well as for the formation and growth of myotubes *in vitro* [3,1].

Chemokines are secreted proteins that share both leukocyte chemoattractant and cytokine-like properties [4,5]. They are important for the migration of muscle precursor cells during embryonic myogenesis [6] and for macrophage infiltration into damaged muscle tissue [7]. Among these, the stromal cell-derived factor-1 (SDF-1 α) (also named CXCL12) and its receptor, CXCR4, play key roles in trafficking and repopulation of several types of stem cells [8] including muscle progenitors [9,10]. It also controls the development of the hematopoietic and cardiovascular systems as well as the brain [11]. The major biological effects of SDF-1 α are to induce motility, chemotactic responses, adhesion and secretion of matrix metalloproteases (MMPs) [12] and angiopoietic factors in the cells. Importantly, the extracellular matrix, i.e. the cell microenvironment, plays a crucial role in the presentation of these active molecules [13]. In the case of SDF-1 α , its interactions with extracellular matrix components such as heparan sulfates [14] have been shown to be important for an appropriate tissue revascularization after induced acute ischemia [15].

Recently, biomaterial scientists have been working to deliver various growth factors to cells in a “matrix-bound” or immobilized fashion [16–18]. The underlying idea is that the biomaterial surface offers the potential to concentrate the growth factor and deliver it locally, in contrast to a topical administration. The growth factor would also be more protected from degradation by enzymes from tissue fluids. In this context, studies on SDF-1 α presentation by- and local delivery from-biomaterials have begun to emerge [19,20]. These studies mostly focused on the recruitment of immune cells and mesenchymal stem cells using SDF-1 α delivered by poly(lactic glycolic acid) (PLGA) scaffolds or polysaccharide microspheres [20–22]. Interestingly, Burdick and coworkers showed recently that hyaluronan (HA) hydrogels with degradable crosslinks were able to sustain the release of recombinant SDF1 α for up to 7 days [23], SDF-1 α binding to HA via electrostatic interactions. This SDF-1 α -containing HA hydrogel improved the repair of an injured

myocardium, as compared to SDF-1 α in solution [23], suggesting that binding of SDF-1 α to the matrix is important for its function.

Although it is widely acknowledged that SDF-1 α plays a key role in muscle development and regeneration [24,9,10,25] as well as *in vitro* in the migration of myoblasts, its role on myoblast differentiation remains controversial [26,6,27]. To date, all the *in vitro* studies on the role of SDF-1 α in muscle development have been performed by delivering SDF-1 α in solution to muscle precursor cells and for cells grown on rigid substrate (tissue culture polystyrene, TCPS). Engineering of biomaterials for controlling myogenic processes *in vitro* is of prime interest [28] but no study aimed at investigating the effect of SDF-1 α delivered in a matrix-bound manner on the different steps of myogenesis, including adhesion, migration and differentiation.

The layer-by-layer technique appears to be an interesting strategy to build thin biopolymeric coatings presenting matrix-bound SDF-1 α . This technique allows for the precise control of various parameters such as film architecture [29,30], thickness, chemistry and internal crosslinking [31,32]. As the deposition is achieved in aqueous solutions, adsorption or incorporation of sensitive biomolecules, i.e. growth factors, is possible [33] and their bioactivity is preserved. Recently, SDF-1 α loaded in layer-by-layer chitosan/poly(δ -glutamic acid) films was shown to attract human mesenchymal stem cells [21].

In the present study, we investigated the *in vitro* potential of polyelectrolyte multilayer films (PEM) films to present SDF-1 α in a matrix-bound manner and studied its effects on myogenic processes. PEM films made of poly(L-lysine) and Hyaluronan (PLL/HA) were selected in view of their potential as growth factor reservoirs [34]. We first determined if (PLL/HA) films could control SDF-1 α delivery with tunable amounts, and then evaluated C2C12 myoblast responses to matrix-bound SDF-1 α on cytoskeleton organization, migration and differentiation. This study would pave the way towards future *in vitro* culture of engineered tissues and *in vivo* regeneration strategies.

2. Materials and methods

2.1. SDF-1 α production and labeling

Murine SDF-1 α was cloned into a pET17b vector, expressed and purified as described previously [35]. For fluorescent labeling, the pH of the protein solution was raised to 8.5 with a 50 mM bicarbonate buffer. SDF-1 α was reacted for 1 h at room temperature with 1:4 (mol/mol) of Alexafluor 488 carboxylic acid succinimidyl ester (Invitrogen, Cergy-Pontoise, France). The reaction was stopped with Tris buffer (50 mM, pH 8.0). The labeled protein (Alexa-SDF-1 α) was separated from the reagents using a Sephadex G25 column (GE Healthcare, France) eluted with a sodium phosphate buffer (50 mM Na-Phosphate at pH 6.0, 150 mM NaCl). The molar concentrations of the dye and protein were quantified by absorbance spectroscopy and the corresponding labeling ratio was calculated.

2.2. Multilayer film preparation and crosslinking

HA (sodium hyaluronate, MW 360,000 g/mol) was purchased from Lifecore (Chaska, MN, USA) and PLL (P2636) was purchased from Sigma (Saint-Quentin Fallavier, France). PLL

(0.5 µg/mL) and HA (1 µg/mL) were dissolved in a Hepes-NaCl buffer (20 mM Hepes at pH 7.4, 0.15 M NaCl). Film deposition on 14 µm glass slides was performed with an automated dipping machine as previously described [36]. For 96-well plates, films were manually constructed starting with a first layer of poly(ethyleneimine) (7×10^4 g/mol, Sigma, France) at 5 µg/mL [34]. The sequence was repeated until the buildup of a (PLL/HA)_i, *i* being the number of layer pairs, was achieved. Films were crosslinked for 18 h at 4 °C following the protocol previously described [32] using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) at 30 µg/mL or 70 µg/mL and N-hydrosulfosuccinimide (sulfo-NHS) at 11 µg/mL (both purchased from Sigma, France). Final washing was performed with 0.15 M NaCl at pH 8 for 1 h. The multilayer films will be named hereafter film@EDC30 and film@EDC70, respectively.

2.3. Quantification of loading of SDF-1α in PEM films

Incorporation and initial release studies were performed on (PLL/HA)₁₂ films constructed in 96-well plates as previously described [34]. The films were always pre-equilibrated for 30 min in the medium in which SDF-1α was suspended (either 1 mM HCl, or Hepes-NaCl buffer). 50 mL of Alexa-SDF-1α at increasing concentrations from 2.5 to 100 mg/mL were deposited onto the films and adsorbed overnight at 4 °C. 150 mL of Hepes-NaCl were then added in each well and incubated at room temperature for 10 min. The solution was then removed and replaced by fresh Hepes-NaCl buffer before measuring fluorescence (Ex485/Em535) using a fluorescence microplate reader (Infinite1000, Tecan France, Lyon). For release studies, the wells were washed with the Hepes-NaCl solution and the fluorescence was measured at regular time intervals. The incorporated amount was calculated based on a calibration curve obtained with known amounts of Alexa-SDF-1α in solution. SDF-1α loading on film-coated glass slides for cell culture studies was achieved in a similar way, the coated slides being sterilized for 15 min under UV light. Experiments were performed at least in triplicate, with three independent samples per condition in each experiment. Long term SDF-1α release was quantified using an Elisa test (Quantikine, R&D systems, Abingdon, United Kingdom).

2.4. AFM imaging

PEM films in Hepes/NaCl were imaged using a BioCatalyst (Bruker AXS SAS, Palaiseau, France) equipped with a 150 µm scanner in the intermittent-contact mode (512×512 pixels² at line rates of 1 Hz). We used a ScanAsyst-Fluid cantilever (Bruker, resonance frequency of 150 kHz, spring constant of 0.7 N/m). The root mean squared roughness R_{RMS} was calculated from 5×5 µm² AFM images. The PEM films without SDF-1α were dipped into the 1 mM HCl solution prior to imaging and then thoroughly rinsed in the Hepes-NaCl buffer.

2.5. Cell culture

The mouse myoblast cell line C2C12 (<20 passages obtained from ATCC) was cultured as previously described [36]. For all experiments, C2C12 cells seeded on films at 4.5×10^4 cells/cm² in growth medium GM, 1:1 DMEM/F12 supplemented with 10% foetal bovine serum (FBS, PAA Laboratories, Les Mureaux, France 2%) were allowed to grow for 2 days

and were then switched to the differentiation medium (DM, 1:1 DMEM/F12 supplemented with 2% horse serum (HS), PAA Laboratories, Les Mureaux, France) for 3 days. Cell fusion index was calculated by determining the ratio of the number of nuclei in myotubes (>2 nuclei) over the total number of nuclei counted [37].

2.6. Immuno-stainings

Cells were fixed with 3.7% formaldehyde for 20 min and permeabilized in 0.2% Triton X-100 for 4 min. They were labeled with rhodamine-phalloidin (1:800, Sigma, France) or monoclonal mouse anti-troponin T (1:100) (Sigma, St Quentin-Fallavier, France). Primary antibodies were detected using Alexa Fluor 488-conjugated goat anti-mouse (1:1000, Molecular Probes, Invitrogen, France). Cell observation was performed using an Axiovert 200 M microscope and a LSM 700 confocal microscope (both from Carl Zeiss SAS, Le Pecq, France).

2.7. Chemotaxis assay

The chemotactic response of C2C12 cells was evaluated using a 24-well Transwell plate with a 6.5 µm polycarbonate filter of 8 µm pore size (Costar, Cambridge, MA). 2×10^4 cells in DM were seeded in the upper compartment of the Transwell inserts and SDF-1α was added at the bottom of the wells. For the control condition, DM was introduced in both the upper and bottom chamber of the Transwell. After 4 h at 37 °C, non-migrated cells of the top chamber were removed with a cotton swab and migrated cells were fixed and stained with Hoechst 33342 (Invitrogen). Cell migration was quantified by counting the total number of cells in 10 different microscopic fields at 10 × magnification. Cell migration is expressed as a % in comparison to the control condition.

2.8. Polyacrylamide gel electrophoresis and immunoblotting

Film@EDC70 was prepared on 32 µm diameter glass slides with an automated dipping machine and SDF-1α was loaded at 10 mg/mL to get the final loaded concentration of 470 ng/cm². C2C12 cells were seeded at 20,000 cells/cm² in 6-well plates with or without films, allowed to grow for 3 days and switched to DM for 0, 3 and 5 days incubation. In the control TCPS, soluble SDF-1α was added in DM at 200 ng/mL. At each time point, cells were scraped, rinsed in PBS, and then lysed in 2× Laemmli sample buffer (Sigma, France). After boiling, 10 mL of total protein samples were loaded and run on 12% polyacrylamide gels before transfer onto PVDF membranes (GE Healthcare, United Kingdom). Membranes were then saturated in 5% milk in TBS that contained 0.1% Tween 20 for 1 h and subsequently incubated with monoclonal antibodies against myogenin (1:500, BD Biosciences, Pont-deClaix, France), troponin T (1:5000, Sigma, France) and β Tubulin (1:1000, Sigma, France). Membranes were washed and incubated with a peroxidase-conjugated anti-mouse secondary antibody (1:20,000, Amersham). Peroxidase activity was visualized by ECL (West pico signal, Pierce) using a ChemiDoc MP imaging system (Bio-Rad).

2.9. Flow cytometry

5×10^5 cells (at ~80% confluence) were washed with PBS, detached with Versene (Invitrogen) and resuspended in PBS, containing 1% BSA, 0.02% azide and 1 mM EDTA.

Cells were incubated for 60 min at 4 °C with anti-mouse or human CXCR4-FITC antibody (R&D systems, Lille, France) at the final concentration of 10 mg/mL. Finally, cells were washed twice in the same buffer, fixed in 4% paraformaldehyde, washed twice and analyzed by flow cytometry using a FACS scan (Becton Dickinson, Pont de Claix, France).

2.10. Generation of matrix-bound gradients of SDF-1 α

Matrix-bound SDF-1 α gradients were generated on crosslinked (PLL/HA)₁₂ films previously described [38]. Briefly, (PLL/HA)₁₂ films were constructed on glass slides (75 μ m $\times$ 25 μ m) using an automated dipping robot, crosslinked using EDC at 30 μ g/mL and dried. The dried films were placed in contact with a polydimethylsiloxane (PDMS) microfluidic mold consisting of multiple straight channels (50 μ m $\times$ 2.0 μ m $\times$ 100 μ m) with inlet and outlet ports [39], which were pre-filled with 1 mM HCl. Then, a drop of HCl was placed in the outlet and 5 mL of Alexa-SDF-1 α at 100 mg/mL was introduced in the inlet and let for 40 min. The SDF-1 α diffused into the microchannel via passive pumping (due to the difference in surface tension between inlet and outlet drops), followed by a gradient formation due to evaporation. Afterwards, the inlet and outlet ports were sealed with PDMS and the sample was incubated at 37 °C for 90 min to allow SDF-1 α adsorption on the films. Lastly the PDMS device was removed and the sample was rinsed with the HEPES-NaCl buffer leaving only the matrix-bound SDF-1 α gradient. The Alexa-SDF-1 α gradients were visualized using an LSM 700 microscope (Carl Zeiss Sas, Le Pecq, France) and the fluorescence intensity profile was quantified using ImageJ 1.46c (NIH, USA).

2.11. Time-lapse imaging of C2C12 cells on PEM films

For quantification of migration on homogeneous PEM films, 5×10^3 cells were seeded in GM on plastic or on the films with or without SDF-1 α . After 4 h at 37 °C, cells were imaged every 5 min for 15 h on an LSM 700 confocal microscope (Carl Zeiss Sas, Le Pecq, France) equipped with a controlled environmental chamber (37 °C and 5% CO₂). For the quantification of C2C12 myoblast migration on the SDF-1 α gradients, 5×10^4 cells were seeded and allowed to adhere for 4 h on a sample containing three SDF-1 α gradients. Time-lapse images were recorded every 10 min for 6 h along the length of the gradients. Time-lapse images and movies were assembled using Image J software (<http://rsbweb.nih.gov/ij/>). Individual cell tracking was performed using the “Manual tracking” Plugin, which allows to select a cell and record its movement by following its position on each frame. For each experiment, there were 2 samples per condition for homogeneous films and three different gradients for the matrix-bound SDF-1 α . Approximately 20 cells were tracked for each experimental condition. Experiments were performed in triplicate. All data were analyzed using “Chemotaxis tool”, which provides graphic and statistical analysis of the dataset. X/Y Calibration and Time interval were set up based on the parameters of time-lapse images. Finally, a summary of distances and velocities was generated under “Statistic feature”.

2.12. Statistical analysis

Statistical comparisons were performed using SigmaPlot Version 11.0 software. The nonparametric Mann–Whitney *U* test was used to analyze data from two independent groups. When comparing data between more than two groups, analysis of variance

(ANOVA) followed by an appropriate pairwise comparison or comparison versus control group was performed ($p < 0.05$ was considered significant). All experiments were repeated at least 3 times with at least three samples per condition in each experiment. Error bars represent standard errors of the mean value.

3. Results

3.1. Bioactivity of labeled SDF-1 α

In order to quantify the SDF-1 α loading efficiency of (PLL/HA) multilayer films by fluorescence spectroscopy, SDF-1 α was labeled with Alexa488 (Alexa-SDF-1 α). The grafting ratio was found to be $24 \times 6\%$ (mean of three independent experiments). Next, the bioactivity of the labeled SDF-1 α was assessed using human Jurkat T cells that express the CXCR4 receptor at a very high level [40]. These cells were selected for this purpose as they are known to respond to SDF-1 α in a dose-dependent manner [41]. The high expression of CXCR4 in Jurkat cells was confirmed by FACS analysis (Fig. SI1). Jurkat cell migration was assessed by the well-established chemotaxis assay in Transwell chambers (Fig. SI1(B)). In the presence of Alexa-SDF-1 α , cell migration was very similar to that observed with native, unlabeled SDF-1 α . The maximum chemoattraction was observed for an Alexa-SDF-1 α concentration of 25 ng/mL (about 3 nM). This result indicated that the fluorescence labeling of Alexa-SDF-1 α had not altered its bioactivity and validated its use for quantitative measurements.

3.2. Loading and release properties of (PLL/HA) films for SDF-1 α

We first established the experimental conditions for SDF-1 α loading in crosslinked (PLL/HA) films made of 12 pairs of layers. The crosslinking levels EDC30 and EDC70 were selected based on our previous physico-chemical characterization of such films and our study of myoblast response to film crosslinking [42]. This number of layer pairs was chosen as our previous studies, using the BMP-2 growth factor, indicated that a controllable amount of BMP-2 could be loaded into such $\sim 1.2 \mu\text{m}$ thick films [34]. In a first step, the effect of the pH on loading of SDF-1 α was investigated. SDF-1 α was deposited at a fixed concentration of 5 $\mu\text{g/mL}$ onto (PLL/HA) films@EDC30 and EDC70. Their release properties and the absorbed amount of Alexa-SDF-1 α were studied at pH 3 and 7.4, the calculated isoelectric point of SDF-1 α being ~ 9.8 . Both film@EDC30 and EDC70 exhibited a “burst release” after SDF-1 α loading in a physiological buffer during the first hour. For the film@EDC30, SDF-1 α release exhibited some variations depending on the initial loading pH (3 versus 7.4) and the release percentages were in the range of 40%–55%. In contrary, no difference was observed for the film@EDC70 when SDF-1 α was incorporated at pH 3 or 7.4, both showing a release in the order of 25% (Fig. 1(A)). Release of SDF-1 α from the film@EDC70 was systematically lower than that of the film@EDC30. Of note, the equilibrium was rapidly reached after about 4 h of regular rinsing and no further evolution was noted over 24 h (data not shown). Thus, the amount of SDF-1 α remaining in the film after 4 h of release was taken as the effective incorporated amount (Fig. 1(B)) as no subsequent release was noted. The final amount of SDF-1 α loaded was systematically higher for the film@EDC70 than for the film@EDC30. It was also higher for pH 3 as compared to pH 7.4 (Fig. 1(B)).

Next, the initial concentration of SDF-1 α in solution was varied in order to investigate whether it was possible to tune the SDF-1 α loaded amount in the PEM films. For both film@EDC30 and EDC70, the % of released SDF-1 α and the release kinetics did not change significantly for initial concentrations ranging from 2.5 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$. For the film@EDC30 and film@EDC70, the released percentages were in the range of 50–60% and 20–30%, respectively. For both films, a plateau value was reached in about 60–90 min (Fig. 1(C)). Plotting the effective SDF-1 α amount loaded in the films (Fig. 1(D)) revealed a linear increase as a function of the initial concentration of SDF-1 α in solution, no plateau being observed over the investigated concentration range, up to 100 $\mu\text{g/mL}$. The maximum adsorbed amounts were $\sim 6 \mu\text{g/cm}^2$ for film@EDC70 and $\sim 3 \mu\text{g/cm}^2$ for film@EDC30.

Furthermore, as longer term experiments are needed to study myogenic differentiation, we investigated SDF-1 α release from the film into the culture medium over several days. To this end, SDF-1 α release in the culture medium was quantified using an Elisa assay (Fig. SI2). This was achieved in the absence and in the presence of C2C12 cells to determine whether the presence of media containing 10% serum and/or cells were able to induce SDF-1 α release. Notably, the released amounts in the medium were in the range ~ 0.25 – 2.5 ng/mL in the absence of C2C12 cells (Fig. SI2(A)). They were slightly lower ($< 1 \text{ ng/mL}$) when C2C12 myoblasts were cultured on the films (Fig. SI2(B)), suggesting that C2C12 cells might internalize the released SDF-1 α or that SDF-1 α might be degraded by cell secreted proteases.

Altogether, these results indicate that it is possible to load SDF-1 α in PEM films and that the loaded amounts depend both on the initial concentration of SDF-1 α in solution, on the pH of the SDF-1 α solution and on the film crosslinking extent. The data also indicate that SDF-1 α release from the films was always below 1 ng/mL in the presence of C2C12 myoblasts.

AFM imaging of the (PLL/HA)₁₂ films were performed before and after loading with SDF-1 α (Fig. 2) in conditions corresponding to the plateau value of Fig. 1, i.e. after thorough rinsing of the films. In the absence of SDF-1 α , film roughness was $5.7 \pm 0.9 \text{ nm}$ for the film@EDC30 and $9.9 \pm 1.3 \text{ nm}$ for the film@EDC70. After SDF-1 α loading, the film roughnesses were respectively $7.3 \pm 1.6 \text{ nm}$ for the film@EDC30 and $7.2 \pm 1.1 \text{ nm}$ for the film@EDC70. Thus, no significant difference was noted for the film of different crosslinking extent in the presence of SDF-1 α . Few very small aggregates were visible, which may be attributed to the presence of the cytokine.

3.3. Receptor expression and response to soluble SDF-1 α in C2C12 cells

To investigate the activity of matrix-bound SDF-1 α on myogenesis, we first confirmed CXCR4 expression in C2C12 cells by FACS analysis (Fig. 3(A)). The response of C2C12 cells to a soluble gradient of SDF-1 α was also assessed by quantifying C2C12 cell migration in a Transwell assay in response to increasing concentrations of SDF-1 α (Fig. 3(B)). Migration of C2C12 cells was found to increase with the increase of SDF-1 α in solution up to 50% more than the control value. Maximal migration was observed in the range of 125–500 ng/mL of soluble SDF-1 α . Furthermore, immunolabeling of CXCR4 in C2C12 cells, cultured on an SDF-1 α -loaded film@EDC30, revealed the presence of the receptor in

C2C12 cells (data not shown). A punctuate staining was visible. This is consistent with other staining results of CXCR4 receptors found in the literature [43,44].

Besides CXCR4, it was recently reported that CXCR7 is another SDF-1 α receptor involved in C2C12 myoblast response to SDF-1 α [27,25]. However, their expression on C2C12 cells in growth medium and differentiation medium remains controversial to date. Melkiona et al. [27] found that both CXCR4 and CXCR7 are expressed in GM conditions and that their levels decreased in differentiated cells. However, Hunger et al. [25] found that CXCR7 expression levels gradually increased upon switching C2C12 cells to differentiation conditions, whereas CXCR4 expression levels remained unchanged [25]. In our experimental conditions, we confirmed by Western blot that CXCR4 is expressed (Fig. SI3(A)). On the contrary, CXCR7 was not clearly expressed in C2C12 cells (as probed by two different antibodies) (Fig. SI3(B)), in comparison to Jurkat cells [45].

All together, these results showed that C2C12 cells express the SDF-1 α receptor CXCR4 and that they respond to a soluble gradient of SDF-1 α , although their response is less important than that of Jurkat cells (Fig. SI1).

3.4. Effect of matrix-bound SDF-1 α on C2C12 cell spreading and cytoskeleton organization

Next, we investigated the effects of matrix-bound SDF-1 α on the initial adhesion of C2C12 cells. In the following, all the experiments were done after a thorough rinsing of the film in the HEPES-NaCl buffer such as to obtain SDF-1 α concentrations near the plateau value (Fig. 2(A) and (B), i.e. with no more release of SDF-1 α). We observed cell morphology by fluorescence microscopy and cytoskeleton organization by confocal microscopy after actin staining (Fig. 4). On films@EDC30, cells were round and poorly spread in the absence of SDF-1 α ($505 \pm 35 \mu\text{m}^2$), whereas they were significantly more spread on the films with matrix-bound SDF-1 α ($1135 \pm 30 \mu\text{m}^2$) (Fig. 4(A) and (A')). Cells on the film@EDC70 exhibited a large spreading area even in the absence of SDF-1 α ($1220 \pm 25 \mu\text{m}^2$) which was only slightly increased by the presence of matrix-bound SDF-1 α ($1360 \pm 35 \mu\text{m}^2$) (Fig. 4(B) and (B')). As expected, the cell circularity was significantly higher for both films@EDC30 and @EDC70 in the absence of SDF-1 α . Thus, matrix-bound presentation of SDF-1 α increased cell spreading in conditions where cells are otherwise round and poorly spread, i.e. for cells on the film@EDC30.

3.5. Effect of matrix-bound SDF-1 α on C2C12 cell migration

As SDF-1 α is known to regulate the migration of both proliferative and terminally differentiated muscle cells [6], we next addressed the question whether matrix-bound SDF-1 α affected cell migration. Time-lapse experiments were performed for cells plated on films loaded at increasing concentrations of SDF-1 α and cell migration was quantified (Fig. 5). Representative traces of the cell movements are also shown for various adsorbed amounts of SDF-1 α (Fig. 5(A)). Of note, soluble SDF-1 α did not increase the migration speed of cells plated on the films (Fig. 5(A)). In the absence of matrix-bound SDF-1 α , C2C12 cells migrated at $15 \pm 2 \mu\text{m/h}$ on film@EDC30 and at $28 \pm 2 \mu\text{m/h}$ on film@EDC70 (Fig. 5(B)). In contrast, matrix-bound SDF-1 α notably increased cell migration, which reached a similar plateau at $65 \pm 6 \mu\text{m/h}$ for cells on film@EDC30 and at $62 \pm 3 \mu\text{m/h}$ for

cells on film@EDC70. Fits of the migration speed versus adsorbed SDF-1 α amounts show inflection points at 0.085 $\mu\text{g}/\text{cm}^2$ for film@EDC30 and 0.19 $\mu\text{g}/\text{cm}^2$ for film@EDC70 (Fig. 5(B)).

These results indicated that myoblasts responded to matrix-bound SDF-1 α whatever the film crosslinking, but that they were more sensitive to matrix-bound SDF-1 α when it was presented from the film@EDC30 as compared to film@EDC70.

We also showed, using siRNA knockdown against CXCR4, that CXCR4 is involved in C2C12 migration on matrix-bound SDF-1 α (Fig. SI4). All together, these results prove the bioactivity of SDF-1 α as documented by the increase in migration speed for cells in contact with matrix-bound SDF-1 α .

Finally, in order to assess cell migration on films presenting a continuous gradient of matrix-bound SDF-1 α , stable SDF-1 α gradients were generated on (PLL/HA) $_{12}$ films@EDC30 (Fig. 5(C)). These gradients were obtained over a relatively long distance of $\sim 20\text{ }\mu\text{m}$. The SDF-1 α fluorescence intensity profile confirmed that the adsorbed amount of SDF-1 α decreased throughout the length of the channel (Fig. 5(C)). C2C12 cell migration speed was found to decrease when the concentration of matrix-bound SDF-1 α decreased, which further confirms the “accelerating” effect of matrix-bound SDF-1 α on myoblast migration.

3.6. Effect of matrix-bound SDF-1 α on C2C12 cell differentiation

C2C12 myoblast cells normally differentiate into myotubes in low serum-containing medium (DM) on TCPS [46]. However, the role of SDF-1 α in myogenic differentiation remains controversial as soluble SDF-1 α was reported on one hand to fully inhibit cell differentiation [26] and on the other, to decrease cell fusion without having a noticeable effect on terminal differentiation [6,47]. Thus, we investigated the role of soluble versus matrix-bound SDF-1 α in our experimental conditions. To this end, C2C12 cells were differentiated on TCPS in the presence of soluble SDF-1 α or on the film@EDC70 with matrix-bound SDF-1 α . These films were selected as myogenic differentiation was found to be better at high crosslinking levels [36]. On the control TCPS, C2C12 cells aligned and fused to form thick myotubes and expressed troponin T, a marker of myogenic differentiation (Fig. 6). When soluble SDF-1 α was added in DM at 200 ng/mL, myotubes were slightly shorter and less well formed (Fig. 6(A)). For cells grown on film@EDC70 in the absence of SDF-1 α , cells differentiated well, as expected (Fig. 6(B)). In the presence of matrix-bound SDF-1 α , troponin T was still highly expressed whatever the adsorbed amount of SDF-1 α . However, we noted that higher amounts of matrix-bound SDF-1 α lead to Troponin-T expressing cells that often contained one nucleus (Fig. 6(B), right panel). When the surface concentration of SDF-1 α was higher than 1.1 $\mu\text{g}/\text{cm}^2$, cells began to aggregate and form “nodules” (Fig. SI5) and even detached after a few days of culture. Quantitative analysis of the cell fusion index (i.e. myotubes having at least two nuclei) revealed that matrix-bound SDF-1 α impaired the fusion process by decreasing the fusion index, although soluble SDF-1 α did not, either on TCPS or on film@EDC70 (Fig. 6(C)). However, the analysis of Troponin T (Fig. 6(D)) and myogenin (Fig. SI6) by Western blot revealed that matrix-bound SDF-1 α increased the expression of these proteins as compared to soluble SDF-1 α (Fig. 6(D)). Indeed, their expression increased as a function of the time (at Day 3

and Day 5 during differentiation) and was higher in the presence of both soluble and matrix-bound SDF-1 α . To note, matrix-bound SDF-1 α was more efficient than soluble SDF-1 α in promoting this expression.

All together, these data indicate that matrix-bound SDF-1 α enhances myogenic differentiation but impairs the fusion process of myotubes resulting in small myotubes containing often a few nuclei.

4. Discussion

SDF-1 α is an important chemokine, which plays a key role in tissue development and regeneration. SDF-1 α is the only recognized ligand of the chemokine receptor CXCR4. The SDF-1 α /CXCR4 interaction has an important role in regenerative processes during tissue remodeling including cell migration, proliferation, and differentiation [48]. Mice, deficient in CXCR4, exhibited severe defects of secondary limb myogenesis [24]. Furthermore, SDF-1 α /CXCR4 signaling was shown to be important in the development of migrating muscle progenitors [9]. Indeed, it was previously shown that SDF-1 α is secreted in muscle tissue and that the CXCR4 receptor is present in cell lines derived from satellite cells such as C2C12 cells [49]. Our results show a moderate CXCR4 expression in C2C12 cells (Fig. 3(A)) and a negligible expression of the other possible SDF-1 α receptor, CXCR7 (Fig. SI3). In addition soluble SDF-1 α induced myoblast migration in a dose-response manner (Fig. 3(B)). Our results are in agreement with a value between the CXCR4 receptor and SDF-1 α of ~200 ng/mL [50].

For the regeneration of tissues *in situ* by means of biomaterials, SDF-1 α is a very interesting candidate, whose chemoattractive properties have already been shown on a large variety of cell types, including mesenchymal stem cells [19], hematopoietic cells and muscle precursor cells [51]. Both synthetic poly(lactic-co-glycolic acid) (PLGA) [19] and natural 3D biomaterials, such as silk-collagen or alginate patches [52] or microspheres [20] have already been used to deliver SDF-1 α locally. Natural materials are interesting since they are more hydrated and can exhibit natural interactions with the chemokine [53]. Indeed, SDF-1 α was found to have an affinity with HA-based hydrogels [23]. In this study, we used ~1 μ m thick films made of PLL and HA as a reservoir for SDF-1 α to deliver it at the basal side of the myoblast cells in a “matrix-bound” manner.

Interestingly, the amount of SDF-1 α in the films could be tuned over a large range from 100 ng/cm² to 6 μ g/cm² by varying the initial SDF-1 α concentration in solution, the loading pH as well as the crosslinking degree of the film. There was an SDF-1 α burst release mostly occurring during the first hours, after which a steady state was reached without further detectable loss of SDF-1 α from the film (Fig. 1(A) and Fig. SI2). The SDF-1 α final adsorbed amounts were remarkably higher for SDF-1 α initially solubilized in a solution at pH 3 as compared to pH 7.4 (Fig. 1(A')). Also, there was a linear increase of the loaded amount as a function of the initial concentration of SDF-1 α in solution (Fig. 1(B')). The loading of SDF-1 α in the (PLL/HA)₁₂ films is reminiscent of the very efficient loading of the osteoinductive growth factor bone morphogenetic protein 2 (BMP-2) in the same type of films [34]. The BMP-2 amount, loaded in such films, could also be controlled by varying the

deposition conditions and the film thickness. Of note, both BMP-2 and SDF-1 α are basic proteins (pI 8.5 for BMP-2 as compared to 9.8 for SDF-1 α) and are therefore protonated at the applied pHs. SDF-1 α is smaller with an MW of 8 kDa as compared to 13 kDa for BMP-2, but both chemokines are active in their dimeric state [54]. These chemokines are known to interact with sulfated polysaccharides, such as heparan sulfate [14], but interestingly, both also interact with hyaluronan [34,23], which may explain their affinity with HA-containing gels [23] and films [34].

All together, our results suggest that the trapping of SDF-1 α in the (PLL/HA) films is a combination of electrostatic interactions, H-bond interaction, natural affinity for HA and nanoporosity of the film.

We noted that SDF-1 α had a significant effect on cell spreading for cells deposited on films with a low crosslinking degree. Whereas myoblast cells were mostly round on film@EDC30 without SDF-1 α , they were spread, elongated and exhibited multiple protrusions on film@EDC30 with matrix-bound SDF-1 α (Fig. 4(A)). This effect was especially visible for the film@EDC30 as compared to the film@EDC70, onto which cells spread even in the absence of SDF-1 α (Fig. 4(B)) [42]. Interestingly, our results with SDF-1 α are very similar to those obtained with matrix-bound BMP-2, which also increased cell adhesion and spreading [55]. Avigdor and coworkers [48] previously observed that SDF-1 α delivery in solution to hematopoietic progenitor cells plated on HA-coated substrates *in vitro* had a drastic effect on the cell cytoskeleton [48]. These authors showed that SDF-1 α in solution induced the rearrangement of the actin cytoskeleton in hematopoietic cells adhered via CD44 on HA coatings. This suggests that there is a crosstalk between CD44 and CXCR4 receptors. In our case, the increase in spreading observed on matrix-bound SDF-1 α delivered from film@EDC30 might also involve a molecular interaction between CXCR4 and adhesion-related cell surface receptors. This crosstalk might allow the cells to overcome an adhesion threshold that, on more crosslinked (i.e. stiffer films), is achieved by other mechanisms such as stiffness sensing.

A similar picture emerges when the effect of SDF-1 α on cell migration speed is analyzed (Fig. 5). SDF-1 α is already known to be a key chemokine in the migration of mouse muscle progenitor cell [56] and muscle satellite cells [49], which respond to soluble gradients of SDF-1 α . Here, when SDF-1 α was presented in a matrix-bound manner, its effect on cell migration was more prominent on soft film@EDC30 than on stiffer films. Migration speed also depended on the amount of matrix-bound SDF-1 α presented to the cells in a graded manner (Fig. 5(C)) and was specific for CXCR4 (Fig. SI4).

Of note, Hesselgesser et al. [57], reported $163,521 \pm 35,875$ binding sites per cell for SDF-1 α in Jurkat cells (our positive control for CXCR4 receptor expression), which is estimated to correspond to ~ 100 binding sites per μm^2 of cell surface area. The lowest mbSDF-1 concentration used here for cell migration was 135 ng/cm^2 , which corresponds to $\sim 10^5$ SDF-1 molecules per μm^2 . This should be theoretically enough to saturate the endogenous SDF-1 α receptors in C2C12 cells, as these cells were proved to have less receptors than Jurkat cells (see Kd values obtained from Fig. SI1 and Fig. 3). When

delivered in solution (soluble SDF-1), a high concentration of SDF-1 α was used (200 ng/mL) in order to saturate the receptors.

C2C12 myoblast cells normally differentiate into myotubes in low serum-containing medium (DM) on TCPS [46]. It is well known that cell fate is highly controlled by a fine-tune combination of physical, chemical and biological parameters [58]. In this respect, our PEM-based film approach has the advantage to combine, in a tuneable fashion, multiple parameters that influence cell fate. In our previous study on C2C12 cell differentiation on (PLL/HA) films crosslinked to different extents, we showed that films@EDC30 were not suitable for myoblast differentiation, whereas cells differentiated efficiently on films@EDC70 [36]. *In vivo*, SDF-1 α is recognized as an important chemokine for the regeneration of skeletal muscle by impacting on the mobilization of CXCR4 positive cells [51]. So far, effects of SDF-1 α on myogenesis have only been studied by delivering SDF-1 α in solution to myoblasts, C2C12 being the most acknowledged *in vitro* cellular model [6,26,27,44]. CXCR4 is recognized as being an important receptor for regulation of myoblast migration and fusion [26,6,27], but *in vitro* results are somewhat contradicting depending on the study design. Pavlath and coworkers [6] showed *in vitro* that soluble SDF-1 α regulated migration of both proliferating and terminally differentiated muscle cells, and was necessary for proper fusion of muscle cells [6]. Furthermore, Mechionna et al. [27] showed that 24 h treatment by soluble SDF-1 α at 100 ng/mL led to myosin heavy chain accumulation and induction of myotube formation in both C2C12 myoblasts and mouse satellite cells [27]. Moreover, inhibition either by specific inhibitors or RNA interference of CXCR4 signaling blocked myogenic differentiation of C2C12 cells [27]. Very recently, Chen and coworkers [47] showed that CXCR4 knockdown did not affect the differentiation index but decreased the fusion index and average myonuclei number. In contrary to these positive effects of SDF-1 α on myoblast differentiation and fusion, Engle and coworkers [26,25] showed that soluble SDF-1 α at 10 ng/mL to 100 ng/mL almost fully inhibited the differentiation of both C2C12 and primary mouse myoblasts, decreasing MyoD, myogenin and myosin heavy chain expression.

Our results using soluble SDF-1 α at 200 ng/mL showed that C2C12 differentiation and fusion is globally similar to the control condition (TCPS or film@EDC70 without SDF-1 α), with a slight increase in the expression of myogenic markers (Fig. 6). Matrix-bound SDF-1 α increased the expression of myogenic markers as compared to the condition without matrix-bound SDF-1 α (Fig. 6(D)) but the fusion index decreased in a dose-dependent manner with the SDF-1 α adsorbed amount (Fig. 6(C)). Thus, without impacting terminal cell differentiation, SDF-1 α had an effect on the fusion process.

Different experimental conditions could account for the discrepancy of the observed effects of SDF-1 α , including the different modes of presentation (soluble versus matrix-bound presentation) and the amount of SDF-1 α provided to the cells and their state. A hypothesis is that matrix-bound SDF-1 α might induce the expression of soluble factors regulating fusion. The SDF-1 α loaded PEM films hold potential to further elucidate the signaling events underlying SDF-1 α mediated cell response.

5. Conclusion

In this study, we showed that (PLL/HA) films are able to efficiently trap SDF-1 α and deliver it locally to cells in a matrix-bound manner. SDF-1 α local delivery had several effects on myoblast cytoskeletal organization, migration as well as differentiation. Matrix-bound SDF-1 α notably increased cell spreading for cells grown on the low-crosslinked films (film@EDC30) where cells were otherwise poorly spread in the absence of SDF-1 α . In addition, cell migration was strikingly enhanced on both film@EDC30 and EDC70 in the presence of matrix-bound SDF-1 α , the CXCR4 receptor being involved in this process. Here again, cells were more sensitive to matrix-bound SDF-1 α presented from film@EDC30. The expression of cell differentiation markers was promoted by matrix-bound SDF-1 α but cell fusion decreased in a dose-dependent manner with the adsorbed amount of SDF-1 α . All together, these findings suggest that the delivery of SDF-1 α in a matrix-bound fashion has potent effects on myogenesis and that presentation by a biomaterial may potentialize the effects of the chemokine. Our future studies will aim to further understand the signaling pathways triggered by matrix-bound SDF-1 α when delivered from a biomaterial surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMP	bone morphogenetic protein
EDC	1-ethyl-3-(3-dimethylamino-propyl) carbodiimide
HA	hyaluronan
PDMS	poly-dimethylsiloxane
PEM	polyelectrolyte multilayer film
PLGA	poly(lactic glycolic acid)
PLL	poly(L-lysine)
SDF-1α	stromal derived factor-1 α

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Fig. 1. Initial release and loaded amount of SDF-1α in (PLL/HA)₁₂ films cross-linked@EDC30 and EDC70

(A) Percentage of SDF-1α released during washes in the Hepes-NaCl buffer as a function of time (inset: zoom on time 0–50 min); (B) amount of SDF-1α loaded as a function of the pH of the SDF-1α solution added in contact with the film. The initial SDF-1α concentration was 5 μg/mL. (C) Percentage of SDF-1α released as a function of the time for increasing SDF-1α initial concentrations (inset: zoom on time 0–50 min) and (D) final amount of SDF-1α loaded as a function of the initial SDF-1α concentration in solution in contact with the films (for EDC30 or EDC70).

Fig. 2. Atomic force microscopy images ($5 \mu\text{m} \times 5 \mu\text{m}$, z scale 60 nm) of $(\text{PLL-HA})_{12}$ films crosslinked@EDC30 (A) or EDC70 (B), before loading with SDF-1 α (A and B) and after loading with SDF-1 α (A' and B'). SDF-1 α was loaded at an initial concentration of 20 $\mu\text{g/mL}$ at pH 3. The control images (A and B) have been obtained after dipping of the films in the 1 mM HCl solution and thorough rinsing with Hepes-NaCl buffer (corresponding to the plateau value of Fig. 1(A) and (C)).

Fig. 3. SDF-1 α promotes the migration of C2C12 cells via CXCR4 receptors

(A) CXCR4 expression in C2C12 cells measured by FACS. Cells were incubated with an anti-mouse CXCR4 antibody (uncolored peak) or an isotype control antibody (gray peak). (B) Chemotaxis of C2C12 cells induced by SDF-1 α added in solution at different concentrations tested in a Transwell assay. The control (cell migration towards DM without SDF-1 α) was arbitrarily set to 100%. Values are the means $\pm$ S.D of three different samples from a typical experiment. Experiments were performed in triplicate.

Fig. 4. Effect of matrix-bound SDF-1 α on cytoskeleton organization and cell spreading
 Cell morphology was observed 24 h after plating, by staining their actin cytoskeleton. C2C12 cells were grown on the different films in the absence (no SDF-1 α) or in the presence of matrix-bound SDF-1 α (SDF-1 α initial solution concentration of 20 mg/mL): (A) film@EDC30 and (B) film@EDC70 Upper row: Fluorescence images, 20 $\pm$ obj; lower row: Confocal images, 63 $\pm$ obj, rhodamine phalloidin for actin filament (red) DAPI (blue) for nucleus. (A', B') Quantitative analysis of the cell spreading area and circularity using a Box plot representation (lower 5% limit, median and upper 95% limit, mean value is represented by an open square and ($\times$) represents the 1 and 99% respectively). At least 200 cells were counted for each condition. * $p < 0.05$.

Fig. 5. Matrix-bound SDF-1 α potentiates C2C12 cell migration

(A) Phase-contrast images and representative migratory trajectories of C2C12 cells on film@EDC30 loaded with increasing amounts of SDF-1 α from 0 to 2600 ng/cm² (corresponding to 2.5, 10 and 100 μ g/mL initial loading SDF-1 α concentrations). (B) Migration speed as a function of the adsorbed amount of SDF-1 α and corresponding fit (black lines). At least 20 cells were analyzed for each condition. * $p < 0.05$. (C) Migration speed at different positions along the matrix bound SDF-1 α gradient. The corresponding fluorescence intensity profile of the SDF-1 α gradient on the (PLL/HA)₁₂ film is also shown (black squares).

Fig. 6. Myoblast differentiation using soluble and matrix-bound SDF-1α

C2C12 cells grown on TCPS were differentiated in the presence of soluble SDF-1α for 5 days. For the study of matrix-bound SDF-1α, C2C12 cells were cultured on film@EDC70 loaded with increasing amounts of SDF-1α. (A) Phase-contrast images and immunostaining of troponin T for C2C12 cells on TCPS with and without SDF-1α in culture medium and (B) on films with SDF-1α added in solution at 200 ng/mL or with matrix-bound SDF-1α (adsorbed amounts of 0–470 ng/cm² corresponding to 0, 2.5, and 5 mg/mL initial loading SDF-1α concentrations). (C) Quantitative analysis of myoblast fusion index for the different conditions. (D) Expression of troponin T assessed by immunoblot analysis as a function of time over 5 days in DM. Cells were either grown on TCPS in the presence of soluble SDF-1α, or on film@EDC70 loaded with 470 ng/cm² of SDF-1α. Corresponding quantitative analysis fold increase expressed in % for the expression of Troponin T in the presence of SDF-1α (ratio of Troponin T over tubulin) as compared to its expression in the absence of SDF-1α.