NANOFIBER SCAFFOLDS FOR LOCAL DELIVERY OF STEM CELLS AND IMMUNOSUPPRESSIVE DRUGS FOR THERAPEUTIC PURPOSES

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Abstract

Cell-based therapy of local tissue injuries or damages requires application of stem cells and inhibition of harmful inflammatory reaction which could impede the healing process. To increase the effectiveness of this therapy, a local administration of drugs can avoid their side effects associated with a systemic treatment. A local therapy requires suitable carriers, which can transfer the cells and drugs to the site of injury. As a promising carriers turned out nanofiber scaffolds prepared by electrospinning technology from various types of polymers. The main advantage of this technology is a possibility to define properties of nanofiber scaffolds, optimal for the growth and transfer of stem cells, and which could incorporate various types of immunosuppressive drugs. Here we describe the formation and use of nanofiber scaffolds prepared by needleless electrospinning technology from poly (L-lactic acid) (PLA) which are loaded with immunosuppressive drug Cyclosporine A (CsA). We show that CsA-loaded nanofibers effectively and selectively inhibit proliferation of activated T cells and suppress the production of T cell cytokines *in vitro*. Simultaneously, these nanofiber scaffolds enable growth of mesenchymal stem cells (MSCs) and thus can serve as stem cell carriers. Moreover, using an experimental mouse model of skin transplantation, we showed that covering skin allografts with MSC-seeded and CsA-loaded nanofibers significantly inhibited the local production of pro-inflammatory cytokines IL-2, IL-17 and IFN-γ, and supported healing. Thus, nanofiber scaffolds seeded with stem cells and loaded with CsA can serve as carriers of cells and drugs for a local cell therapy and for simultaneous effective immunosuppression.

Keywords: Nanofiber scaffold, immunosuppressive drug CsA, cytokines, stem cells

1. INTRODUCTION

Nanofibers prepared by the electrospinning technology has been shown as promising carriers for targeted drug delivery or as scaffolds for cell transfer in regenerative medicine. So far, various molecules have been incorporated into nanofiber scaffolds or were bound on their surface [1,2]. To modulate the kinetic of drug release from nanofibers, different kinds of polymers or composite nanofiber structures, including nanofibers, nanoparticles or self-assembling proteins, have been tested [3,4]. These materials have proved out as perspective carriers for number of molecules, including antibiotics, vitamins, growth factors and chemotherapeutics [5].

Nanofiber scaffolds, which represent an optimal 3D matrix [6,7], have been successfully used also for cell-based therapy. If genetically different cells are used for cell-based therapy, a local immunosuppression, compared to a systemic suppression of immunity, would be profitable for the protection of these cells from immunological attack. The use of biocompatible nanofibers which could serve as cell carriers and simultaneously would release selective immunosuppressive drugs will be of main priority.

To approach this task, we prepared and tested nanofibers which were fabricated by original needleless electrospinning technology from a biocompatible polymer poly (L-lactid acid) (PLA). Nanofibers prepared from the PLA can be seeded with different cell types and can serve as carrier for cell-based therapy. We have recently shown that nanofibers can be successfully used as a scaffold for stem cell transfer to treat ocular

surface injuries and limbal stem cell deficiencies [7]. Here we show that PLA nanofibers can be simultaneously loaded with immunosuppressive drug Cyclosporine A (CsA) and seeded with different types of cells. These constructs can be used for cell-based therapy and for a simultaneous local suppression of harmful T cell-mediated immune reactions.

2. METHODS

2.1. Nanofiber preparation

A modified needleless Nanospider™ technology [8] was used for the preparation of PLA nanofibers. This technology flexibly enables the formation of fibers tens of nanometers to tens of micrometers in diameter. The nanofibrous samples used during this study were prepared at a basic weight of 5, 10 or 25 g/m^2 and nanofibers had diameter size ranging from 290 - 539 nm. The morphology of CsA-free and CsA-loaded PLA nanofibers and their nanofibrous architecture was analysed using scanning electron microscopy (SEM).

2.2. T cell proliferation assay and assessment of immunosuppression *in vitro*

The single spleen cell suspension (0.5 x 10^6 cells/ml) from BALB/c mice was cultured in a volume of 200 µl of the complete RPMI 1640 medium unstimulated or stimulated with 1.0 µg/ml of Concanavalin A (Con A). To determine cell proliferation, ^{3}H-thymidine (1 µCi/well) was added to each well for the last 6 h of a 72-h incubation period. The ^{3}H-thymidine incorporation into cells was measured using a scintillation counter.

To characterize immunosuppressive potential of CsA-loaded nanofibers, nanofiber samples of variable size (ranging from 1 mm^2 to 25 mm^2), various basic weight (5, 10 or 25 g/m^2) and different concentrations of CsA (0, 1, 2.5, 5 or 10 wt.%) were added into wells with Con A-activated spleen cells. CsA at concentrations ranging from 1 pg/ml - 100 µg/ml was added to the cultures of Con A-stimulated cells as a positive control.

2.3. Determination of kinetics of CsA release from nanofibers *in vitro*

Samples of nanofibers (size 5 x 5 mm, basic weight 10 g/m^2, concentration of CsA 10 wt.%) were soaked into wells with 250 µl of RPMI 1640 medium with 10% FCS at the laboratory temperature. The nanofibers were repeatedly transferred after 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h into new wells containing fresh medium. The supernatants from the individual wells after each transfer were harvested, stored at -20°C and tested for the inhibition of Con A-induced T-cell proliferation and IL-2 production.

2.5. Proliferation and cell viability of nonlymphoid cells

Mouse limbal stem cells (LSC) and mesenchymal stem cells (MSC) were prepared from BALB/c mouse tissues as we have described [9]. Mouse embryonal fibroblast cell line 3T3 was obtained from the American Cell Culture Collection. LSC, MSC or 3T3 cells were cultured at a concentration 25 x 10^3 cells/ml in a volume of 200 µl of a complete RPMI 1640 medium alone or in the presence of CsA-free or CsA-loaded nanofibers. Proliferation of cells was determined by ^{3}H-thymidine incorporation.

To determine the cell growth on nanofibers, nanofiber scaffolds were fixed into CellCrownTM^{24} inserts. The inserts with nanofibers were placed into 24-well tissue culture plates and seeded with 10^5 cells in a volume of 700 µl of a complete RPMI 1640 medium. The plates were incubated for 24 h and cell viability and metabolic activity was determined by the Cell proliferation reagent WST-1 assay [7].

2.6. Production and detection of cytokines and nitric oxide (NO)

Spleen cells from BALB/c mice (0.5 x 10^6 cells/ml) were stimulated with Con A (1.0 µg/ml) in a volume of 800 µl of the culture medium in the absence or presence of nanofiber samples. The concentrations of cytokines

IL-2, IFN-γ and IL-17 in the supernatants were assessed by an enzyme-linked immunosorbent assay (ELISA) following the instructions of the manufacturers (R & D Systems).

For production of IL-12 and NO, mouse peritoneal macrophages (1 x 10^6 cells/ml) were stimulated with lipopolysaccharide (LPS, 1.0 μg/ml) and IFN-γ (5 ng/ml) in the absence or presence of nanofibers. The concentrations of IL-12 in the supernatants were measured by ELISA (R & D Systems). The levels of NO in the supernatants were determined after 48 h using the Griess reaction [10].

2.7. Mouse model of skin grafting

Skin grafts from B6 donors were transplanted in BALB/c recipients according to the technique of Billingham et al. [11]. The grafts (size 8 x 8 mm) were covered with CsA-free or CsA-loaded nanofibers. The grafts were removed on day 7 after transplantation and the grafted tissue was used for real-time PCR analysis to detect expression of genes for pro-inflammatory molecules, or the graft explants were cultured *in vitro* for 48 h [12] and the concentrations of IL-17 in the supernatants were assessed by ELISA.

To determine the kinetic of CsA release *in vivo*, the CsA-loaded nanofibers were removed 1, 2, 4 or 8 days after transplantation and the content of CsA retained in nanofibers was determined. The immunosuppressive potential (suppression of T cell proliferation, inhibition of IL-2 production) of the collected nanofibers was compared with that of the original CsA-loaded nanofibers or with a pure CsA standard, and the percentage of CsA remaining in nanofibers was calculated.

2.8. Characterization of inflammatory reaction by real-time PCR

The expression of genes for pro-inflammatory cytokines IL-2 and IFN-γ in cells from control or nanofiber-covered skin allografts was determined by a real-time PCR. The details of RNA isolation, transcription and PCR parameters we have described [7, 9].

3. RESULTS

3.1. Immunosuppressive properties of CsA-loaded nanofibers

The addition of CsA-loaded PLA nanofibers, but not nanofibers from PLA without CsA, into wells with Con A-stimulated spleen cells strongly inhibited T cell proliferation (**Figure 1A**). CsA-loaded nanofibers also inhibited in a dose-dependent manner the production of IL-2 (**Figure 1B**), IL-17 (**Figure 1C**) and IFN-γ (**Figure 1D**) by Con A-stimulated spleen cells. The inhibition depended on the concentration of CsA in PLA (**Figures 1A-D**) and on the basic weight of the nanofibers (data not shown). On the contrary, production of IL-12 (**Figure 1E**) or NO (**Figure 1F**) by LPS/IFN-γ stimulated macrophages was not inhibited in the presence of CsA-loaded nanofibers.

3.2. The kinetics of the drug release *in vitro* and *in vivo*

To study the kinetics of CsA release from CsA-loaded nanofibers, the samples of nanofibers were soaked into 250 μl of a complete RPMI 1640 medium and at the indicated time intervals the nanofibers were transferred into the same volume of fresh medium. The presence of released pharmacologically active CsA in the culture media obtained at individual time intervals was assessed by their ability to inhibit T cell proliferation and IL-2 production. A significant amount of CsA was detected in the samples harvested at the intervals within the first 12 h of incubation, but the release of CsA continued consequently for at least 96 hours (data not shown).

To evaluate the release behavior of CsA in vivo, CsA-loaded nanofibers covering skin allografts were collected 1, 2, 4 and 8 days after grafting and the presence of CsA in the nanofibers was tested for its ability to inhibit Con A-induced T cell proliferation and IL-2 production. The results showed that nanofibers collected at all

tested days still retained a sufficient amount of CsA to effectively inhibited T cell proliferation or IL-2 production. A quantitative determination of CsA content in nanofibers collected at the individual time points showed, in comparison with the amount of CsA in original nanofibers at the time 0, that about 50% of CsA was retained in nanofibers on day 2 and about 35% of the original CsA content remained in nanofibers on day 8 after grafting (data not shown).

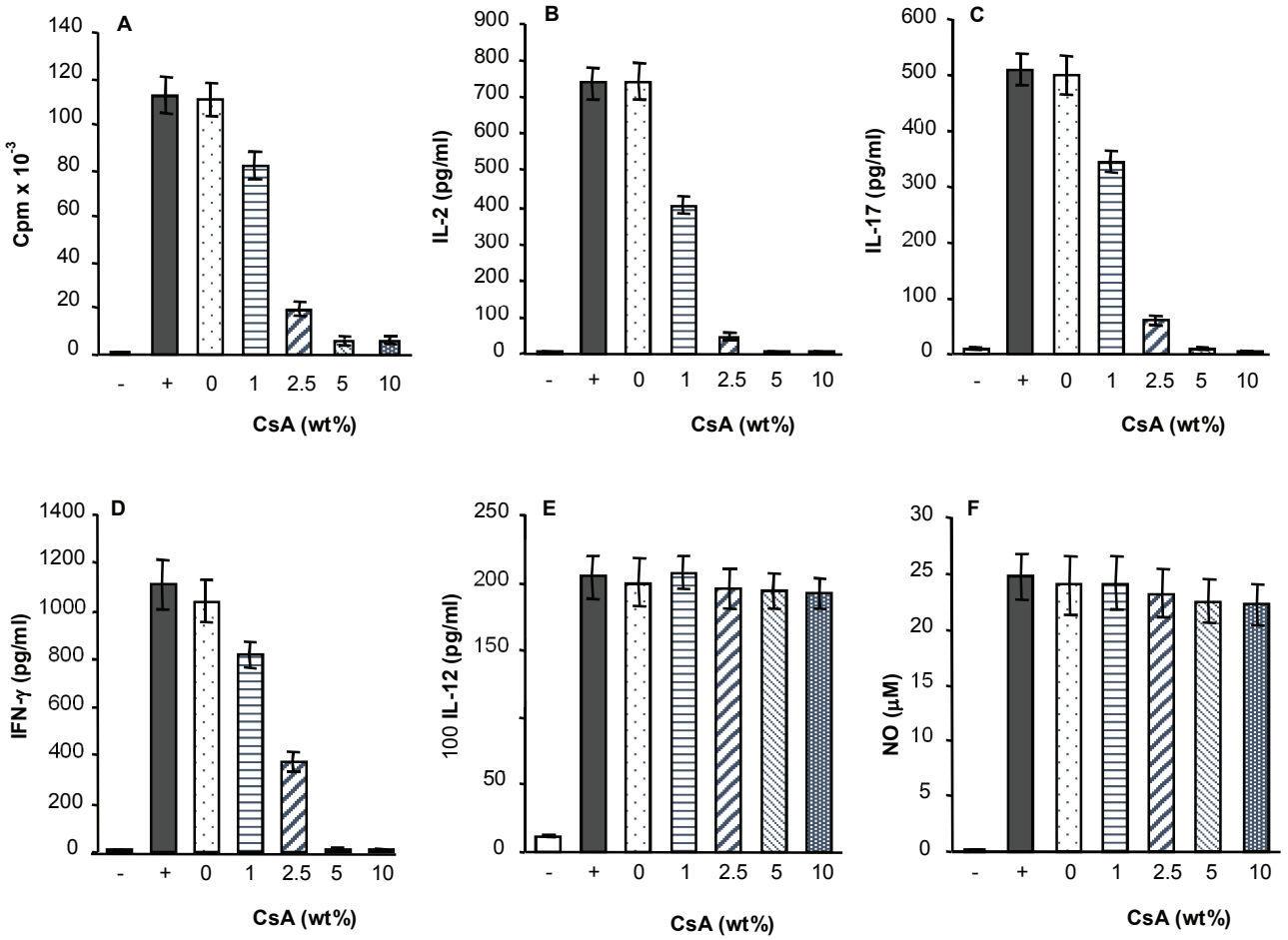

Figure 1 Selective inhibition of T cell proliferation and cytokine production by CsA-loaded PLA nanofibers. Mouse spleen cells were cultured unstimulated (-), stimulated with Con A (+) or were stimulated with Con A in the presence of nanofibers (size 2 x 2 mm, basic weight 10 g/m^2) with indicated content (wt%) of CsA. Cell proliferation (A) and production of IL-2 (B), IL-17 (C) and IFN-γ (D) were determined. The production of IL-12 (E) and NO (F) by macrophages stimulated with LPS/IFN-γ in the presence of nanofibers with the indicated content of CsA was also assessed

3.3. The growth of cells on CsA-loaded PLA nanofibers

Three types of cells, LSC, MSC and 3T3 fibroblasts, were cultured in plastic tissue culture plates in the presence of CsA-loaded PLA nanofibers. It was observed that the cell growth in the presence of CsA-loaded nanofibers was comparable as in the presence of nanofibers prepared from PLA only or in wells without nanofiber samples (data not shown). In addition, the cells were seeded on plastic of 24-well tissue culture plate and on PLA nanofibers without CsA or on CsA-loaded nanofibers. Cell proliferation was determined after a 24-h incubation period by the WST-1 assay. All cell types grew on CsA-containing nanofibers comparably as on nanofibers without CsA (data not shown).

3.4. Suppression of a local inflammatory reaction *in vivo*

Rejection of skin allograft induces a local inflammatory reaction which can be detected by the expression of genes for pro-inflammatory cytokines IL-2, IL-17 and IFN-γ. Covering of the inflammatory sides with CsA-loaded nanofibers significantly inhibited the expression of genes for IL-2 and IFN-γ and attenuated production of IL-17 in the cultured explants of skin allografts which had been covered by CsA-loaded nanofibers (data not shown).

4. DISCUSSION

Transplantation of organs or a transfer of genetically different cells requires an extensive systemic immunosuppression to inhibit immune response to antigens on the grafted cells. The systemic immunosuppression is regularly associated with the harmful side effects which prevent the use of high and effective doses of immunosuppressive drugs. To avoid this problem, the local delivery of drugs offers a promising treatment. Nanotechnologies provide a perspective tool for preparing an optimal carrier for targeted drug delivery. Here we showed that CsA, a potent inhibitor of T cell functions, can be effectively loaded into PLA nanofibers and used for a local immunosuppression to treat inflammatory and transplantation reactions.

CsA represents one of the widely used immunosuppressive drugs, but its use is limited by the side effects, as are nephrotoxicity, gingivitis or hirsutism, regularly observed at higher doses of this drug. At lower concentrations, CsA selectively inhibits T cell functions without apparent effect on other cell populations. Therefore, we prepared nanofibers with various basic weights and with different content (1 - 10 wt. %) of CsA. The addition of these CsA-loaded nanofibers into cultures of spleen cells stimulated with Con A inhibited T cell proliferation and suppressed production of T cell cytokines. The production of IL-12 or NO by activated macrophages or the growth of LSC, MSC and 3T3 fibroblasts was not inhibited in the presence of CsA-loaded nanofibers. These results indicate that incorporation of CsA into PLA nanofiber scaffold do not impair the immunosuppressive properties of CsA. The studies of kinetic of CsA release in aqueous solutions revealed that the majority of CsA was released into culture medium from nanofibers within the first few hours, but a significant amount of CsA was retained in nanofibers and was gradually released for more than 96 h. The sustained release of CsA for few days is different from the pattern of a release of water-soluble small molecular weight substances which are rapidly released from nanofibers within few minutes or hours [13]. The drug release profile *in vivo* may be quite different from that observed in aqueous solutions *in vitro*. Indeed, we observed that CsA-loaded nanofibers retain a substantial amount of the drug for more than 8 days when they are placed on skin allograft *in vivo*.

Although the potential use of drug-loaded nanofibers has been extensively discussed, there are only few studies proving the effectiveness of drug-loaded nanofibers in biological systems *in vitro* [14, 15] and *in vivo* [16,17]. Our data presented here show that CsA-loaded nanofibers fabricated from PLA polymer effectively and selectively inhibit proliferation of activated T cells and suppress production of T cell cytokines *in vitro*. Moreover, using an experimental model of skin transplantation in mice we showed that covering of the inflammatory sides with CsA-loaded nanofibers significantly inhibited a local production of pro-inflammatory cytokines IL-2, IL-17 and IFN-γ.

In addition to their ability to serve as drug carriers, CsA-loaded nanofibers can be used as scaffolds for the growth and transfer of cells. We showed that LSC, MSC and 3T3 fibroblasts grow on CsA-loaded nanofibers comparably as on nanofibers prepared from a CsA-free PLA. Since nanofibers prepared from PLA polymer have comparable mechanical properties as nanofibers from polyamide, that we recently described and used for LSC and MSC transfer to treat ocular surface injuries [7], CsA-loaded and simultaneously cell-seeded PLA nanofibers appear to be a suitable scaffold also for cell transfer in cell-based therapy and regenerative medicine.

5. CONCLUSION

In conclusion, we showed that immunosuppressive drug CsA can be directly loaded into electrospun nanofibers without any loss of its pharmacological activity. Such CsA-loaded nanofibers can be used as drug carriers for a local suppression of inflammatory reactions and as scaffolds for cell transfer in tissue repair and regeneration. These observations suggest that the CsA-loaded PLA nanofibers may be highly perspective for simultaneous cell-based therapy and a local suppression of harmful T cell-mediated immune reactions.

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