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Effect of direct RGD incorporation in PLLA nanofibers on growth and osteogenic differentiation of human mesenchymal stem cells

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Abstract The aim of this study was to functionalize synthetic poly-(L-lactic) (PLLA) nanofibers by direct incorporation of cRGD, in order to promote adhesion, growth and osteogenic differentiation of human mesenchymal stem cells (hMSC) in vitro. The cRGD was incorporated into PLLA nanofibers either by emulsion [PLLA-cRGD (d)] or suspension [PLLA-cRGD (s)]. Matrices were seeded with hMSC and cultivated over a period of 28 days under growth conditions and analyzed during the course. Although the mode of incorporation resulted in different distributions of the RGD peptide, it had no impact on the fiber characteristics when compared to corresponding unblended PLLA control fibers. However, hMSC showed better adherence on PLLA-cRGD (d). Nevertheless, this advantage was not reflected during the course of cultivation. Furthermore, the PLLA-cRGD (s) fibers mediated the osteogenic potential of collagen (determined as the expression and deposition of collagen and osteocalcin) to some extent. Further studies are needed in order to optimize the RGD distribution and concentration.

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1 Introduction

Over the last decade, polymer nanofibers have been in demand for a broad range of applications. The number of investigations concentrating on scaffolds for tissue engineering prepared by electrospinning is impressive [1, 2]. The great advantage of these fibers is that they can be produced from a broad spectrum of biocompatible and biodegradable polymers [2, 3]. This allows an optimization of the properties of the polymer, depending on the particular area of application. Furthermore, these fibers mimic the extra cellular matrix to some extent [4, 5] and therefore, represent an ideal three dimensional scaffold for cell growth and differentiation. With respect to bone tissue engineering, several groups have demonstrated that nanostructured materials are suitable to culture osteoblast-like cells or mesenchymal stem cells in vitro [6–12].

We reported earlier that nanofibers made from poly (L-lactic acid) (PLLA) did not interfere with the growth and differentiation of human mesenchymal stem cells (hMSC) [13]. Although these cells grew well on PLLA nanofibers, there was an initial down regulation of the expression of genes associated with the osteoblast differentiation [14]. In order to overcome this, several strategies capable of triggering osteogenesis should be included [15]. Here, besides the bioactive incorporation of BMP-2, the functionalization of PLLA nanofibers using collagen I is promising.

Collagen I (COLI) interacts with the integrin receptor and its interaction is sufficient to induce osteogenic differentiation of hMSC, even in the absence of exogenous soluble stimuli [16, 17]. This attribute is maintained in collagen nanofibers [8, 14], as well as in blend nanofibers containing varying ratios of collagen or gelatin [18, 19]. Since the biologic action of the collagen can be reduced to

an RGD motive (R: arginine; G: glycine; D: aspartic acid) interacting with the integrin family ($\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha IIb\beta 3$, $\alpha 1\beta 5$) [20], numerous materials have been RGD functionalized for medical applications [21].

With respect to bone healing, in vitro studies showed evidence that RGD covered surfaces resulted in an increase of both osteoblast cell number and differentiation [22–24]. Therefore, the functionalization of PLLA nanofiber with RGD peptides is of interest. As well as self-assembling peptide-amphiphile molecules containing RGD sequences [6], nanofibers from block co-polymers poly(ethylene glycol)-block-poly(D,L-lactic) [25], poly(ethylene glycol)-block-poly(epsilon-caprolactone) [26] and poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol)-NH2 [27] were constructed and showed promising results in vitro. Nevertheless, all these approaches imply a modification of the polymer backbone, which might compromise the nanofiber properties. Hence we designed this study, with the aim of incorporating an RGD motive into the PLLA nanofibers directly. The feasibility of this approach is based on two characteristics of PLLA electrospinning. First, the poor protein release properties of the polymer [28, 29] and second, the accumulation of hydrophilic compounds on the fiber surface during the electrospinning process [30]. Taking for granted that incorporation has no impact on the PLLA fiber configuration, the large surface and the porous structure of PLLA nanofibers is thought to facilitate contact between RGD and the corresponding integrins.

2 Materials and methods

2.1 Construction of nanofibers and characterization

The preparation of PLLA nanofibers by electrospinning has already been reported in detail earlier [14, 31]. In order to incorporate cyclic Arg-Gly-Asp (Cyclo(-RGDfK) Ana-Spec, Inc. San Jose, USA) into the nanofibers, two different strategies were performed. First, 1 mg solid cRGD was added to 1 ml of a 4% PLLA dichloromethane solution and dispersed in an ultrasonic bath over a period of 30 min (PLLA-cRGD (s)). In a second approach, cRGD was dissolved in water at a concentration of 1.0 mg/ml. Then, 0.1 ml of this solution was added to 0.9 ml of the 4% PLLA dichloromethane solution and mixed extensively using a vortex mixer over a period of 5 min (PLLA-cRGD (d)). Spinning process was performed at a flow rate of 0.12 ml/h with an applied voltage of 20 kV and a distance of 15 cm.

In some experiments cRGD was replaced by a fluorescein isothiocyanate (FITC)-labeled RGD, in order to analyze the distribution within the nanofibers.

2.2 Contact angles

Static contact angles of water were measured using the sessile drop method with a G10 Drop Shape Analysis System (Krüss, Hamburg, Germany) and calculated using Data Physics SCA20 Contact Angle Analyzer Software.

2.3 Scanning electron microscopy

For scanning electron microscopy (SEM), samples were sputter-coated with platinum and examined in a SEM (JSM-7500F, JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 2–5 kV in the SEI mode.

2.4 Human mesenchymal stem cell isolation, characterization and culture

Human mesenchymal stem cells were obtained from consenting patients with the approval of the institutional review board. The patients had no evidence of other bone or auto-immune diseases. The routinely removed bone was obtained from the proximal femur, while preparing the implant bed. Mesenchymal stem cells were isolated and cultured according to the preparation of Pittenger et al. [32], with minor modifications as described by Brendel et al. [33]. Further treatment was done as described in [14].

2.5 Gene expression analysis

RNA extraction, cDNA synthesis and quantitative polymerase chain reaction (PCR) analysis were performed as described earlier [14]. Cycle temperatures and incubation times for human collagen I (COLI), osteocalcin (OC), and 18s rRNA were previously described [14, 34, 35]. Purity of the single PCR products was verified by melting point analysis.

2.6 Immunofluorescence microscopy

Immunofluorescence analysis was carried out as described earlier [14]. Samples were fixed in acetone/methanol, washed, and exposed to blocking buffer (1% donkey serum albumin in PBS) at room temperature. Then cells were incubated with primary antibodies against COLI (Abcam, Ab6308, Cambridge, United Kingdom), OC (Acris, BP710, Hiddenhausen, Germany), and Ki-67 (Darko, Hamburg, Germany). Visualization was done, using cy-2- or cy-3-conjugated secondary antibody (Dianova, Hamburg, Germany) at room temperature (1 h) and stained with 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was performed using a Leica DM5000. Microphotographs of at least three different areas were made. The intensity and area of fluorescence was determined using

Quips analysis software. The total cell count of DAPI stained nuclei was obtained. The proliferation index was calculated as a ratio of Ki-67 positive versus total cells.

2.7 Statistics

All values were expressed as mean ± standard error of at least 3 different patients and compared using students' *t*-test or ANOVA with Bonferroni as a post hoc test. Values of $P < 0.05$ were considered to be significant.

3 Results

3.1 Characterization of cRGD incorporated PLLA nanofibers

The PLLA fibers within this study exhibited a relatively smooth surface, with a diameter of approximately 1024 ± 693 nm and a contact angle of 124.3° ± 17.6° (Fig. 1a, d). When cRGD was incorporated by dispersion (PLLA-cRGD (s)) the diameter of the fibers was reduced to 883 ± 450 nm (Fig. 1c). The contact angle amounted to 126.7° ± 15.2° (Fig. 1d). Fluorescence microscopy using FITC labelled RGD provided evidence that the incorporated RGD accumulated in focal spots (Fig. 1f). When cRGD was incorporated into the fibers as an emulsion (PLLA-cRGD (d)) the fiber diameter turned out to be 792 ± 735 nm and the contact angle was 133.5° ± 9.8° (Fig. 1b, d). Here (FITC) labelled RGD was distributed ubiquitously in the PLLA fibre (Fig. 1e).

3.2 Adhesion, growth and proliferation of hMSC cultured on nanofibers

In order to describe the biological effects of the incorporation of cRGD into PLLA nanofibers, we first analyzed the adhesion of hMSC on the desired scaffolds. Cells were seeded at a density of approximately 12500 cells/cover slip on the scaffolds and allowed to adhere over a period of 4 h. As shown in Fig. 2a, during this time, significantly more cells adhered on PLLA-cRGD (d) scaffolds compared to PLLA nanofibers or PLLA-cRGD (s) nanofibers. Furthermore, on the PLLA-cRGD (d) nanofibers there was a slightly increased proliferation rate (Fig. 2b). Nevertheless, this effect was not significant at all. Although we observed a great inter-patient variability, no difference in the relative cell densities after an incubation of 22 days of cultivation could be observed (Fig. 2c).

3.3 Matrix formation

In order to examine the influence of cRGD incorporation on hMSC differentiation and matrix formation, we compared the gene expression as well as the deposition of COLI and OC of cells grown on PLLA with cells grown on PLLA-cRGD (s) and PLLA-cRGD (d), respectively. As shown in Fig. 3, the incubation of hMSC on PLLA-cRGD (s) but not on PLLA-cRGD (d) resulted in an increase of gene expression of matrix proteins COLI (Fig. 3a) as well as OC (Fig. 3b). This increase was reflected in the deposition of COLI as well as OC (Fig. 3c).

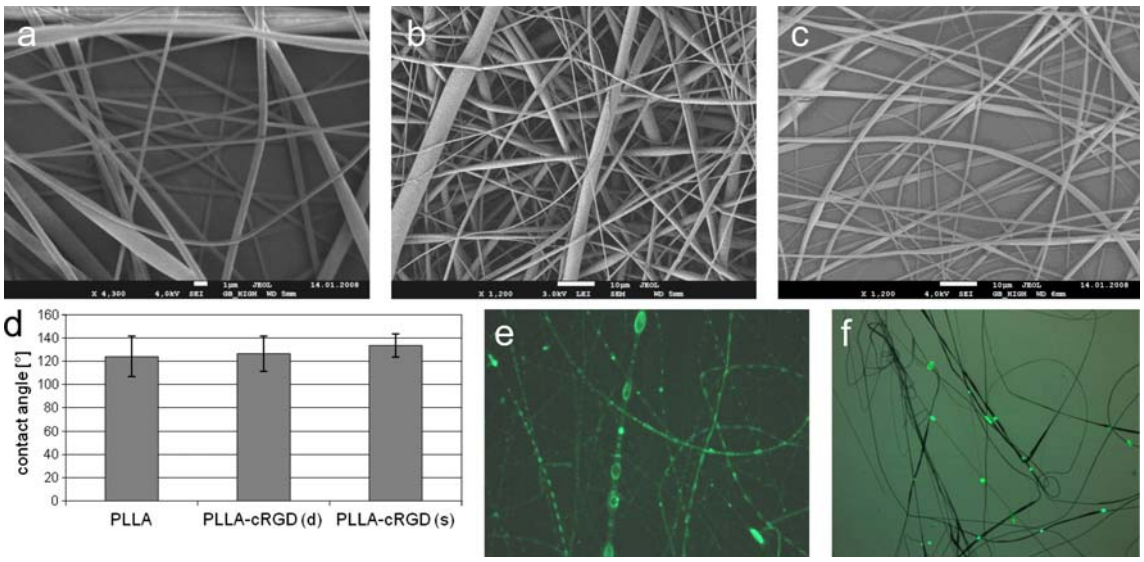

Fig. 1 Fiber characterization. SEM analysis of PLLA (**a**) and PLLA incorporated nanofibers either by emulsion PLLA-cRGD (s) (**b**) or dispersion PLLA-cRGD (d) (**c**). Wettability of nanofibers as described by the contact angle of water (**d**). Fluorescent microscopy of PLLA-cRGD (d) (**e**) and PLLA-cRGD (s) (**f**)

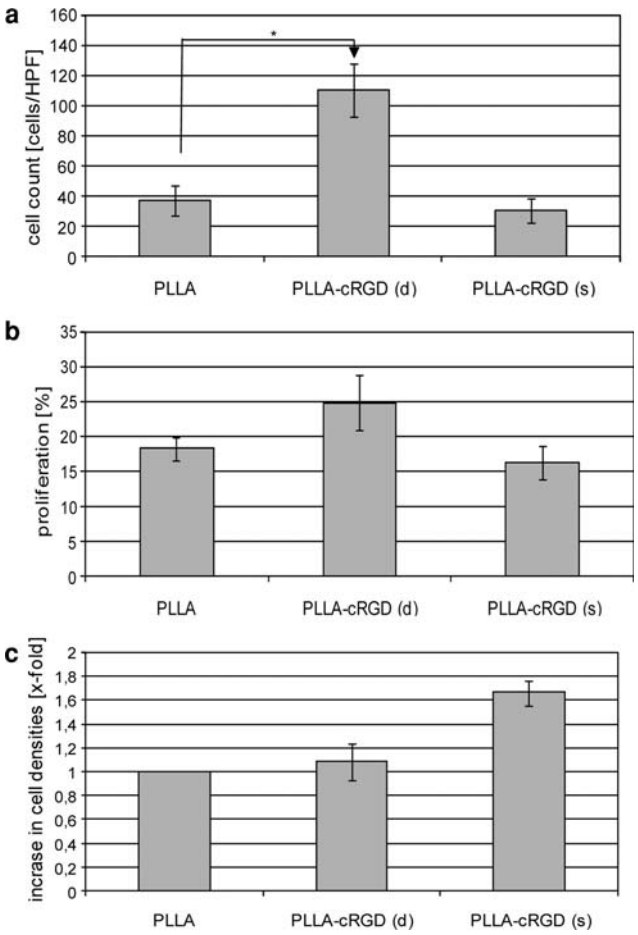

Fig. 2 Influence on adhesion, proliferation and cell density of cRGD incorporation into nanofibers. Cells were seeded on PLLA and cRGD containing nanofibers. After 4 h unattached cells were removed in order to determine the adhesion (**a**). Proliferation was determined by Ki67 staining after 4 days of cultivation (**b**) and total cell count was done at the end of culture (**c**)

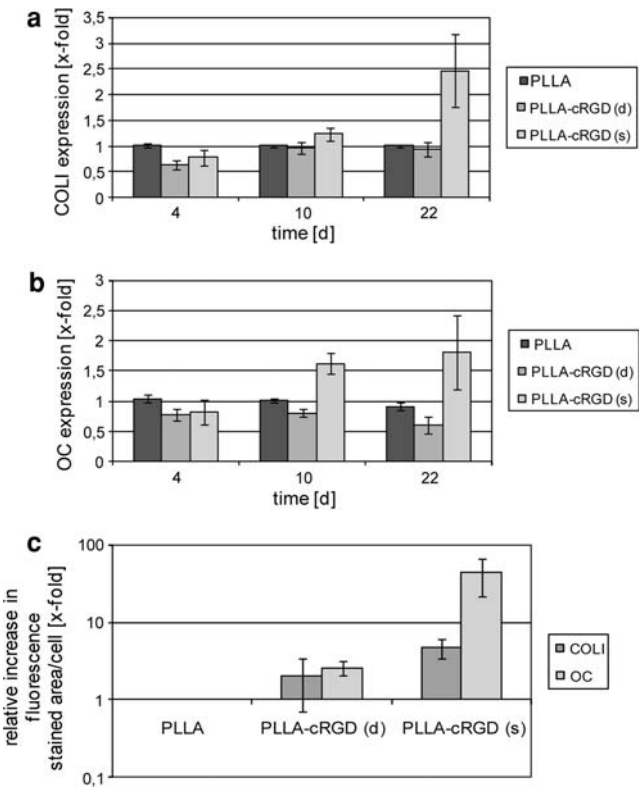

Fig. 3 Influence on deposition and gene expression of matrix proteins of cRGD incorporation into nanofibers. Time course of gene expression of COLI (**a**) and OC (**b**) of hMSC on PLLA nanofibers and PLLA nanofibers with incorporated cRGD, by dispersion (s) or emulsion (d). Relative quantification of COLI and OC deposition of hMSC, cultured under growth conditions on PLLA nanofibers and PLLA nanofibers with incorporated cRGD either by dispersion (s) or emulsion (d) over a period of 22 days (**c**)

4 Discussion

Scaffolds made of PLLA nanofibers represent an appropriate matrix for osteoblast growth and osteogenic differentiation of hMSC in principle [13, 31, 36]. However, we reported earlier that during differentiation of hMSC the initial gene expression of osteoblast marker genes decreased. In order to overcome this disadvantage, a functionalization of PLLA nanofibers using bioactive motives of collagen, the main component of bone, might be a suitable tool. Indeed it has been shown that collagen nanofibers support growth and differentiation of hMSC [8, 14] even in the absence of osteogenic stimuli. Therefore the fibers mediate, at least in part, the osteoinductive potential of collagen [16, 17]. In addition, PLLA nanofibers can be improved by either coating [18] or blending [19] with collagen or gelatin.

Within this study we elucidated that an incorporation of cRGD-peptides might be a suitable tool towards the functionalization of PLLA fibers without modifying the polymer backbone. Therefore two approaches of incorporating bioactive cRGD, shown to interact with the integrin receptor $\alpha v\beta 3$, were tested.

Compared to earlier studies, the PLLA fibers used in this study showed a reduced surface roughness and increased diameter [14], which may be explained by the extremely reduced humidity during the spinning process [37]. A wide range of fiber diameters were shown, which can limit the observation caused by the incorporation of cRGD on nanofibers. However a direct comparison to the PLLA-cRGD (s) and PLLA-cRGD (d) fibers indicates that neither diameter nor wettability were seriously influenced by the incorporation of cRGD.

Therefore, the increased adhesion of hMSC observed on PLLA-cRGD (d) fibers cannot be explained by differences in fiber morphology.

It is more likely that the regular distribution of the RGD (as demonstrated by fluorescence microscopy) facilitates a

RGD-integrin interaction, resulting in an increased adhesion. This conclusion is supported by the finding that this phenomenon did not occur when cRGD was incorporated by emulsion (PLLA-cRGD (s)) where the distribution of the RGD was focal and restricted to distinct areas. Similar findings, with respect to the coating of inorganic carriers with RGD, have been reported [22–24]. However, the increased adhesion of hMSC observed on PLLA-cRGD (d) fibers had no influence on the development of hMSC. Focusing on the matrix formation using either fluorescence microscopy or gene expression analysis, no differences to the PLLA fiber alone were observed.

Unlike the blend fibers described earlier, this kind of fiber did not mediate the osteogenic potential of collagen. On the other hand the PLLA-cRGD (s) fibers mediated the osteogenic potential of collagen (determined as the expression and deposition of collagen and osteocalcin) to some extent. Although the increase in gene expression was much lower, the time course was similar to that observed when stem cells were cultured on collagen nanofibers [14]. The reason for this is unclear and might be ascribed to either a loss of biologic activity of cRGD during dispersion in ultrasonic bath or, more likely to, a lack of distribution of the cRGD within the nanofibers. Here, further studies are needed in order to optimize incorporation and distribution of cRGD.

5 Conclusion

Taken as a whole, this study demonstrates that the direct incorporation of cRGD into PLLA nanofibers has some effect on hMSC growth or differentiation. This effect depends on the mode of incubation and the resulting distribution of the cRGD, respectively. Further studies are needed in order to optimize the concentrations used and to optimize the distribution of the cRGD.

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