Cardiovascular tissue engineering and regeneration based on adipose tissue-derived stem/stromal cells
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CHAPTER 5

Recombinant Collagen-based Microspheres for Soft Tissue Repair

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Abstract

Stem cell therapy to treat cardiovascular disease is in need to improved tissue retention. As a first step, we developed delivery collagen based scaffolds i.e. microspheres (MS) to adhere stem cells. Recombinant collagen peptide-based (RCP-RGD and RCP-DGR) microspheres (MS) was used to generate two types of MS (average size 50μm) via chemical crosslinking hexamethylene diisocyanate (HMDIC) Human adipose tissue-derived stromal cells (ADSC) were seeded onto the MS and cultured overnight. Stem cell-loaded or bare MS were injected subcutaneously on the back of rats. The injected MS and surrounding tissue were dissected at day 1, 3, 14 and 28 to investigate biological response. Our results showed that HMDIC cross-linked RCP MS supported the adhesion of ADSC as assessed through SEM analyses. The tissue response comprised of influxes of inflammatory cells such as macrophages as well as vascularization. The loading of ADSC on RCP MS caused a reduced influx of macrophages at d14 and at d28 the deposition of collagen was lower in ADSC-loaded MS implants compared to controls. The loading of ADSC onto both types of MS did not affect the vascularization during the tissue response. The newly deposited extracellular matrix was produced mainly by fibroblasts, while myofibroblasts were not detectable in any explant. Degradation of the MS was observed at day 14 and 28. In conclusion, we showed that (ADSC) RCP MS are suitable for tissue repair with an appropriate tissue reaction after subcutaneous implantation.
Introduction

Following damage to any tissue, repair mechanisms are activated as part of the normal biological response. However, after severe damage such as acute myocardial infarction (aMI), the body lacks adequate repair capacity [1,2]. In this case, assistance with therapies that augment tissue repair (TR) are required. The main approach to treating large damage or defects is closing or filling the gaps that arise as the consequence of the tissue loss [3]. Scaffolds are one of the suitable candidates, which can be used either as a delivery tool or filler or both [4,5]. Collagen is a major component of the natural extracellular matrix (ECM) [2] and is well-suited for tissue engineering and regenerative medicine-based therapeutic approaches. In contrast to natural collagen, recombinant collagen peptide (RCP) can be genetically modified to introduce e.g. moieties, such as RGD, that augment cell binding. The production of RCP is possible to free of animal products that might compromise the biological safety of the final product. For this purpose, large-scale fermentation of recombinant yeasts in a large volume (multiple cubic meters) bioreactor is used to produce in a consistent, efficient and safe manner. The course and phenotype of the foreign body response (FBR), which occurs after implantation of virtually all biomaterials, is difficult to predict. Ideally, the FBR augments the natural wound healing, while assuring the complete degradation of the implanted biomaterial simultaneously with the resolution of the wound healing response. After implantation of a biomaterial, an inflammatory activation sets off, similar to physiological wound healing, that comprises of a tightly regulated series of cellular fluxes. Firstly, influxes of granulocytes set the stage for attraction and maturation of macrophages that remodel the implanted biomaterial in interaction with attracted (myo) fibroblasts that deposit new extracellular matrix. During the FBR, angiogenesis warrants cellular fluxes, while implanted biomaterials are often encapsulated by a fibrous capsule.

The use of RCP-based scaffolds is hampered by a fast biodegradation rate and low mechanical strength which limits its use in vivo [6-9]. Cross-linking is an efficient way to modify the biodegradability and physical properties of scaffolds [10-12] Amon stem cells, mesenchymal stem cells (MSC) derived from adipose tissue (ADSC) are promising cells for tissue engineering and repair due to their availability, and differentiation capacity to various lineages. Beyond their differentiation capacity, however, ADSC have been shown to suppress inflammatory and fibrotic responses; both of which are associated with the FBR. Furthermore, ADSC promotes angiogenesis, another important part of the FBR. Much of the action of ADSC is attributed to their secretion of trophic factors [13]. To date, the influence of ADSC or their secreted components e.g. absorbed by biomaterials, on the FBR has not been studied. We argued that ADSC could both aggravate and modulate the FBR to implanted biomaterials. In this study, we produced RCP-based microspheres (MS) and describe the adhesion of ADSC on these MS. In addition, we investigated biodegradability and biological responses against bare or ADSC loaded RCP MS (RCP-RGD and RCP-DGR) at day 1, 3, 14 and 28 after subcutaneous implantation in rats.
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Materials and Methods

Materials
Rough-surface RCP microspheres were prepared via water-in-oil emulsification using calcium carbonate as a porogen, as described previously [1,14]. Recombinant collagen peptide (RCP), containing genetically added RGD sequences (RCP-RGD), was produced by a fermentation processes using genetically modified yeast as described elsewhere [1,14]. A scrambled version RCP, which RGD sequence was replaced by DGR (RCP-DGR) was prepared as a control. Covalent crosslinking of RCP was done 1 v/v % high concentration of hexamethylene diisocyanate (HMDIC) in ethanol overnight. The resultant MS were size-fractionated by sieving through a series of sieves to yield MS with an average diameter of 50μm ± 25μm.

Adipose derived stem cell isolation and culture
Adipose tissue derived stromal cells (ADSC) were isolated from human subcutaneous fat (adipose tissue) that was acquired through liposuction (Bergman Clinics, The Netherlands). ADSC were isolated as previously described [1,15]. Briefly, collected adipose tissue was extensively washed with PBS to remove red blood cells, then tissue was enzymatically digested with 0.1% Collagenase A, (Roche Diagnostic, Mannheim, Germany) 1:1 in PBS, containing 1% bovine serum albumin (BSA; Sigma-Aldrich, Boston, MA) shaking at 37ºC for 1h. Digested tissue was filtered and washed with 1% PBS/BSA. Collected cells were suspended and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Hemel Hempstead, UK), 1% L-glutamine (Lonza Biowhittaker, Verviers, Belgium), and 1% penicillin/ streptomycin (P/S) (Gibco, Invitrogen, Carlsbad, CA) at 37ºC in a humidified incubator with 5% CO2. Medium was refreshed three times per week until cells had reached 80-90% confluency, then they were passaged and used at passage 3-6. The use of adipose tissue as source of ADSC was approved by the local Ethics Committee of the University Medical Centre Groningen, given the fact that it was considered anonymized waste material. All anonymous donations the clients had written informed consent as part of the surgical admission procedure.

ADSC loading onto microspheres
12.5mg of RCP-RGD and RCP-DGR microspheres were mixed with 1x10^6 ADSC and incubated in DMEM containing 10% FBS, 1% L-glutamine, and 1% P/S at 37ºC for 16-18h. Ultra-Low Cluster plates (Corning, NY) were used for cell-loading onto biomaterials to prevent the cells to adhere to the tissue culture plate.

Scanning electron microscopy
ADSC-loaded MS were collected and washed three times with PBS and fixed in 3.7% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After 48 h, samples were washed twice with PBS and incubated with 3.7% PFA for 30 min. Then, PFA was removed and samples were dehydrated in graded alcohol dilution series. Next, 1ml of hexamethyldisilazane was added and samples were incubated for 10 min. Finally, hexamethyldisilazane was removed and samples were dried overnight in a fume hood.
Animal and injection procedure

All animal experiments were approved by the Local Animal Care and Use Committee of the University of Groningen and performed according to governmental and international guidelines on animal experiments. 8-week-old male Brown Norway rats (Harlan, Horst, the Netherlands) were used in this experiment. Animals were anesthetized with 5% isoflurane (induction) followed by 2% isoflurane (maintenance) inhalation in combination with a 2:1 mixture of O₂. The back of the animals was shaved and disinfected, and then either 1x10⁶ ADSC per 12.5 mg MS or bare MS in 200μl of saline were injected in 6 spots on the back of the animals.

Explantation

The injected MS and surrounding tissues were dissected at day 1, 3, 14 and 28 (n=12 per time point) post-implantation. The dissected tissue was snap frozen (n=3 per time point), fixed in 4% paraformaldehyde (PFA) (n=3 per time point) and fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for histological analysis (n=3 per time point). Afterwards, the animals were sacrificed by cervical dislocation.

Light microscopy

The glutaraldehyde-fixed samples were used for plastic embedding. Collected samples were dehydrated in graded alcohol dilution series and were embedded in Technovit 7100 (Heraeus Kluzer, Wehrheim, Germany) according to manufacturer’s protocol. Sections were cut (2 μm) and stained with Toluidin Blue (Fluka Chemie, Buchs, Switzerland) and analyzed by light microscopy.

Histological analysis

PFA fixed samples were embedded in paraffin and sections of 5 μm were cut. Hematoxilin and eosin staining was performed as described previously to investigate the histological overview of the explants at different time points. Masson’s trichrome staining, which stains collagen blue and cells red, was performed on sections according to a standard protocol. High-resolution images were exported using NDPI > OME-TIFF software [16]. The amount of blue staining, representing ECM after Masson’s trichrome staining was determined.

Immunohistochemistry & Immunofluorescent staining

Sections were deparaffinized as described previously and incubated in 0.1 M Tris/HCl buffer (pH 9.0) at 80oC overnight. The next day, samples were incubated at room temperature (RT) for 30 min and then washed three times with PBS. This was followed by 0.1 % H2O2 treatment (15 min) to remove endogenous peroxidase activity. Afterwards, sections were washed three times with PBS and incubated with 10% serum in PBS from the species that produced secondary antibodies. Subsequently, sections were incubated with primary antibodies: anti-collagen IV antibody (Abcam, Cambridge, UK), PDGFRβ antibody (Santa Cruz, CA, USA), anti-smooth muscle actin antibody (Dako Heverlee, Belgium) or anti-CD 68 antibody (Bio-Rad Puchheim, Germany). For immunohistochemistry bound antibodies were detected with peroxidase-conjugated rabbit-anti-mouse or rabbit-anti-goat IgG (Dako, Glostrup, Denmark). Staining was performed with 3, 3’-Diaminobenzidine (DAB). Stained samples were
scanned by Nano-Zoomer digital slide scanner (Hamamatsu, Japan) and quantified either with Aperio ImageScope software (Leica, the Netherlands). Macrophage infiltration, after IHC for CD68, was quantified in FIJI using the color-threshold plugin. The number of positive pixels in between MS were corrected for the surface area where MS were present and used for further analysis. In case of Immunofluorescent staining, bound antibodies were detected with Alexa Fluor 555- or 647-conjugated secondary antibodies (Invitrogen) diluted 1:500 in PBS containing 10% normal rat serum (NHS) and 4',6-diamidino-2-phenylindole (DAPI) 1μg/ml in PBS. After extensive washing steps, samples were mounted in Citifluor (Agar Scientific, UK) and images were taken with a Leica inverted microscope.

**Statistical analysis**
All data are presented as the mean values with standard error of the mean (SEM) and analyzed by GraphPad Prism 5 (Graph Pad Software, La Jolla, CA). Statistical analyses were performed using Student’s t-tests. Values of p<0.05 were considered significant.

**Results**

**MS support adhesion of ADSC**
Previously we reported [14] that HMDIC cross-linking of RCP has no negative influence on the survival and adhesion of ADSC in vitro. HMDIC cross-linked RCP MS supported adhesion of ADSC as assessed by SEM, which showed ADSC spread on the MS, yet ADSC also formed intercellular connections between MS. No signs of cell damage and blebbing, were observed (Fig. 1 A, B, C and D H).
Figure 1. Adhesion of ADSC. SEM micrographs show the adhesion of ADSC on different materials after 16 h culture. HMDIC cross-linked RCP MS supports ADSC adhesion (A, B). HMDIC cross-linked RCP-DGR MS has a lower adhesion capacity in comparison to RCP MS (C, D).

Both preloading of MS with ADSC and integrin binding motifs affect MS degradation and turnover
Irrespective of cell-loading or modification with integrin-binding motifs (RGD or DGR), implanted MS remained largely intact on the first and three days post-implantation (Suppl. Fig. 1). Fourteen and 28 days post-implantation irrespective of integrin binding motifs or ADSC loading MS were largely degraded, however still detectable. The implantation of bare MS (RCP-RGD and RCP-DGR), irrespective of integrin-binding motifs, showed the gradual increase of deposited extracellular matrix (Fig. 2A, blue staining). In contrast, ADSC-(pre)loaded MS, irrespective of integrin-binding motifs (RCP-RGD; p=0.015 and RCP-DGR p=0.015), had a significantly lower deposition of extracellular matrix at 28d post-implantation (Fig. 2B). The newly formed extracellular matrix was deposited between the implanted MS.
Figure 2. Immunochemical analyses of extracellular matrix deposition. Masson’s trichrome staining was performed on paraffin sections at different time points of explantation. The results showed no differences in the collagen deposition between the groups at day 1, 3 and 14. At day 28, collagen bundles had deposited (light blue strands) between the MSs in ingrown areas of the explants. At this time point, less collagen deposition was observed in ADSC-loaded MS. MS are indicated with arrows.
Macrophage infiltration is suppressed by ADSC-loaded MS

One day after implantation, irrespective of the type of material or loading with ADSC, a infiltration of macrophages was observed in the surroundings of the implanted MS (Fig. 3A). Three days after implantation a modest influx of macrophages was observed predominantly in the outer rim of the implanted MS (Fig. 3A). The influx was throughout the implants at d14 post-implantation, and had a significantly lower influx in ADSC-loaded MS in comparison to bare MS (RCP-RGD; p= 0.046 and RCP-DGR p=0.04) (Fig. 3b). However, one month after implantation, the influx of macrophages did not differ between bare and ADSC-loaded MS (Fig. 3A, B). At day 14 and 28, foreign body giant cells (FBGCs) has been formed around the MS (asterisk).
Immunochemical detection of macrophages and giant cells. The areas containing microspheres were quantified by morphometric analysis (Aperio Image scope, Leica Microsystems B.V. the Netherlands). Already at day 1 and 3, macrophages were present mostly in the surrounding tissue, however, macrophage infiltration increased in time. The macrophage influx was throughout the implants at d14 post-implantation, and had a significantly lower influx in ADSC-loaded MS in comparison to bare MS. At day 14 and 28, foreign body giant cells (FBGCs) were also observed (asterisk). Data are presented as mean with SEM, as calculated using unpaired student’s t-test assuming equal variances. Values of p<0.05 were considered significant. MS are indicated with arrows.
Preloading of MS with ADSC nor RCP MS with integrin-binding motifs does not affect neovascularization

At day 1 and 3 vessels were present ubiquitously in the surrounding tissue of the implanted MS with no apparent differences between types of material or ADSC-loading. The density of new vessels increased in time inside the implants and reached a maximum at day 28 in both bare and ADSC-loaded MS. (Fig. 4).

Figure 4. Immunochemical detection of newly formed vessels. The areas containing the microspheres were quantified by morphometric analysis (Aperio Image scope, Leica Microsystems B.V. the Netherlands). At day 1 and 3, vessels were detected only in the outer area of the implants. At day 14, new vessels were detected in ingrowth tissue. No differences were observed within the experimental groups. MS are indicated with arrows.

Fibroblasts but not myofibroblasts deposit ECM after implantation of MS

The stromal tissue that is formed between the MS after implantation comprised of blood vessels (see previous section), deposited extracellular matrix (previous sections) and fibroblast. Fibroblasts and myofibroblasts generally are responsible for ECM deposition during the FBR. The commonly used marker for myofibroblasts, αSMA, is also expressed by vascular smooth muscle cells. myofibroblasts were not detected after implantation of MS (Fig. 5) neither around nor inside the implants, while the staining pattern matched the
vascularization pattern (Fig. 4) in particular of the large(r) vessels. These observations were independent of time of implantation, both bare materials or with ADSC-loading. Although no specific marker for fibroblasts exists, the receptor for platelet-derived growth factor (PDGFRβ) has been used as fibroblasts marker (besides vascular smooth muscle cells). This showed that fibroblasts were present during all time points and in all materials. PDGFRβ showed similar pattern either with ADSC or without (Fig. 6).

**Figure 5.** Myofibroblast activation. Immunohistochemical staining for α-SMA was performed to detect myofibroblasts. There was no detection of myofibroblast in samples. α-SMA stained the vasculature, which overlapped with Col-IV staining. MS were indicated with arrows.
Figure 6. Immunochemical detection of fibroblasts. A staining for PDFGRβ was performed to detect fibroblasts at day 14 and 28. Fibroblasts were present inside the implants. MS are indicated with arrows.
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Discussion

The purpose of this study was to investigate the biological response against either bare or ADSC loaded RCP MS (RCP-RGD and RCP-DGR). It shows that injectable RCP-MS are biocompatible and start to degrade within 28 days. Macrophages and fibroblasts are the main players in the foreign body response, which involved in biodegradation, ECM deposition and capsule formation. It has been shown that inflammatory response depends on the size, shape and surface area of the biomaterials [17]. For instance, if implanted materials are smaller than 25µm, these will be cleared by macrophages via phagocytosis. However, if the MS are bigger to phagocytose, macrophages will form foreign body giant cells. Fibroblasts contribute to ECM formation to repair the tissue. But, they might differentiate to myofibroblasts, which is involved in formation of fibrotic tissue after e.g. organ damage such as myocardial infarction [17]. Myofibroblasts in the foreign body reaction are primarily investigated in capsules that surround implants such as silicone implants, pacemakers to mention a few, and also in biomaterial implants where these contribute to biomaterials dysfunction [19].

In this study, we describe the FBR against RCP-MS in which integrin-binding motifs were introduced. We aimed to improve stem cell adhesion and function by RGD motifs. Our previously published results showed beneficial effect of HMDIC cross-linked RGD MS on ADSC function in in vitro set up. In addition, motifs that mediate integrin bindingsuch as RGD, also affect inflammatory responses and macrophage behavior. For instance, these motifs are involved in fusion of macrophages to FBGC, but also for phagocytosis. [17,20,21]. Macrophage infiltration was slightly higher in RGD-containing MS in comparison to DGR MS. In addition, no differences were observed in ADSC-loaded MS in comparison to bare MS at day 1, 3 and 28. Interestingly, at day 14 less macrophages were observed in ADSC loaded MS group in comparison to bare MS. As mentioned previously, RGD mortifies play a role in FBR, however, in the current study no differences were observed in MS either with or without RGD motifs. In this study we applied human ADSC in wild type rats, which means a crossing of the species barrier. Many studies involved xenograft application of MSC, which they tried mostly human derived Mesenchymal stem cells (MSC) in other species. Around 93.6% of the studies were able to show engraftment and function of MSC across different species. In contrast, in 6.4% of the studies there was no effect of MSC or failure of MSC function [22]. In the current study, no adverse tissue responses were observed in human ADSC loaded MS in respect to inflammatory response. Recruited fibroblasts to the implants may differentiate towards a myofibroblasts, which are generally considered as the main cell type during fibrosis excreting high amounts of ECM and pro-fibrotic factors. α-SMA does not only expressed by myofibroblast but also by the smooth-muscle cells in the arteries. αSMA staining indicates blood vessels that match well with the pattern seen by staining for collagen type IV as a marker of newly formed vessels. We detected the collagen type IV staining in the surrounding tissue at all time points a common phenomenon in the FBR. Macrophages regulate pro-fibrotic processes and angiogenesis, upon activation macrophages can produce, limited amounts of, ECM components, such as collagen, during wound healing [23,24]. Macrophage and FBGC adhesion on biomaterials can modulate ECM remodeling either by degrading or producing ECM components and as a results affect biomaterial function [17,23,24].
addition, macrophages are important to recruit the fibroblast to the site, which also contribute in ECM production and consequently influence the biomaterials function. [19] Interestingly, all types of implanted MS caused the influx of fibroblasts that did not differentiate to myofibroblasts. The fibroblasts were identified by virtue of these expressing the PDFGRβ receptor, while expression of αSMA expression was observed solely around blood vessels. Yet, extensive ECM was deposited between the MS. Although macrophages can deposit ECM, we surmise that the present fibroblasts, being professional connective tissue cells, were responsible for the newly deposited ECM. The fibroblasts were most likely instructed by the macrophages. ECM deposition was examined by Masson’s Trichrome staining, which revealed that collagen bundles had deposited (light blue strands) between the MS in ingrown areas of the explants at day 14 and 28. Less collagen deposition was observed in ADSC loaded with MS. ADSC and their secreted factors have been reported to be anti-fibrotic [25,26]. Our preceding in vitro findings demonstrated that the adhesion and function of ADSC is affected by the type of cross-linkers used during fabrication of RCP-MS. In this study we showed less collagen deposition in ADSC loaded MS at day 28, and less macrophage infiltration in ADSC loaded MS at day 14. However, no differences were observed in MS degradation and new vessels density. Summarizing, our findings demonstrated that RCP microspheres proved to be biocompatible, biodegradable and appear suitable candidates for tissue repair.

**Conclusion**

HMDIC cross-linked RCP based MS showed an appropriate tissue reaction after subcutaneous implantation in rats. At present, we are examining the application of the HMDIC cross-linked MS as a delivery vehicle for stem cells in pig myocardial infarction model. This study indicates that RCP based MS loaded with ADSC are promising candidates for guiding tissue repair by means of drug and/or stem cell delivery because they showed lower ECM deposition, no fibrotic formation and less inflammation in the used FBR model.
References


Suppl. Fig. 1. Histological overview of the foreign body reaction to implanted RCP MS. Hematoxylin and eosin staining shows MS degradation in time. At day 1 and 3 MS remained largely intact. However at day 14 and 28 were mostly degraded. MS were indicated with arrows.

Suppl. Fig. 2. Macrophages and fibroblasts are govern the FBR. Both macrophages (ED-1, green) as well as fibroblasts (PDGFRβ, red) are present between and around the MS both at days 14 and 28 post-implantation. Nuclei stain blue (DAPI). Size bar indicates 100 µm.