The effect of type I collagen on osteochondrogenic differentiation in adipose-derived stromal cells in vivo

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Background
The objective of this study was to investigate whether type I collagen would elicit in vivo bone formation of passaged rat adipose-derived adult stromal cells (ADASC) placed extraskeletally.
Methods
After expansion for 1–4 passages (P), cells were incubated in osteogenic medium containing dexamethasone, ascorbic acid and β-glycerol phosphate for 2–4 weeks. Undifferentiated cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) and von Kossa staining as well as by gene expression of ALP, osteopontin (OP), osteonectin (ON), osteocalcin (OC), collagen I (colI), collagen II (colII), bone sialoprotein (BSP), periostin (Postn), runx2, osterix (Osx), sox9, msx1 and msx2. Diffusion chambers were filled with 1×106 cells mixed with or without type I collagen gel and implanted subcutaneously into rats. Controls included chambers exposed to (1) undifferentiated cells (with or without collagen, (2) collagen without cells and (3) empty chambers (n=5 per group).
Results
Four weeks after implantation, in vivo bone and cartilage formation was demonstrated in implants containing 4-week osteo-induced P1 and P4 cells wrapped in the collagen gel, as confirmed by Goldner's trichrome and Alcian blue staining, respectively. Newly formed bone stained positive for type I collagen. Control implants had no bone or cartilage and were primarily filled with fibrous tissue at that time interval.
Discussion
Recent studies have demonstrated that ADASC offer great promise for cell-based therapies because of their ability to differentiate toward bone, cartilage and fat. However, the influence of different matrices on the in vivo osteogenic capability of ADASC is not fully understood. These findings suggest that type I collagen may support the survival and expression of osteogenic and chondrogenic phenotypes in passaged rat ADASC in vivo.

Keywords
adipose-derived adult stromal cell, bone/cartilage formation, chondroblast, osteoblast, osteochondrogenic differentiation, type I collagen.

Introduction

The repair of massive segmental bone defects and non-healing fractures remains a challenging problem in orthopedic surgery. Although conventional autologous and allograft bone grafting are currently available for treatment of these orthopedic disorders, early expectations of these methods have yet to be realized [1-4]. The utilization of tissue engineering in the repair of bone and cartilage has shown great promise, particularly in disunion fractures where endochondral ossification occurs during wound healing [5]. Therefore, of the potential targets for tissue engineering and regenerative medicine, skeletal defects are perhaps most likely to meet with clinical success in the near future [6-8]. In combination with appropriate biomaterials and growth factors, autologous and syngeneic bone marrow (BM)-derived stem cells have been shown to enhance bone repair significantly in animal fracture models [9,10] and differentiate into various human tissue types in vitro and in vivo [11,12].

More recently, a second large stromal compartment found in adipose tissue has received attention and is believed to contain multipotent cells [13-16]. Although it remains to be determined whether adipose-derived adult stromal cells (ADASC) meet the definition of stem cells, they are multipotential, available in large numbers and easily accessible and attach and proliferate rapidly in culture, making them an attractive cell source for tissue engineering [17-20]. Moreover, ADASC demonstrate a substantial in vitro bone and cartilage formation capacity, equal to that of BM but much easier to culture [21-25]. The in vivo osteogenic capability of ADASC placed extraskeletally has been described [26-28] as well as their ability to heal critical-size skeletal defects [6,29-31]. However, the influence of different osteo-inductive matrices on the in vivo osteogenic differentiation of ADASC remains poorly defined [2,4,7,31]. This study demonstrates that passaged rat (r) ADASC mixed with type I collagen gel are capable of forming bone-like and cartilage tissue in vivo when implanted with diffusion chambers. These findings support further the potential application of these cells for tissue regeneration.

Methods

Cell isolation and culture

The inguinal fat pads were harvested from adult male Wistar rats and washed extensively with phosphate-buffered saline (PBS). They were then excised, finely minced and incubated in 100-mm2 tissue culture plates (NuncTM) containing antibiotic media: Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 3% antibiotic-antimycotic (all from Sigma, St Louis, MO, USA) for 1 h. Afterward, the tissue was rinsed three times in PBS for 5 min, followed by digestion with 0.075% type I collagenase (Gibco-BRL, Life Technologies, Grand Island, NY, USA) and gentle shaking for 40 min at 37°C in a 50-cc centrifuge tube. Next, enzyme activity was neutralized with DMEM containing 10% FBS and centrifuged at 1500 r.p.m. for 10 min to obtain a cell pellet. The pellet was resuspended in lysis buffer (0.16 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 10 min at room temperature to remove contaminating erythrocytes. The remaining cells were collected by centrifugation, as detailed above, filtered through a 100-mm nylon mesh (Cell Strainer, Becton Dickinson and Co., Franklin Lakes, NJ, USA) to remove cellular debris and resuspended with DMEM containing 10% FBS, 100 mL/mL penicillin- streptomycin and 2 mm l-glutamine (Sigma). After cell counting using trypan blue, the cells were

plated at a concentration of 1.5 $X10^6$ cells/75-cm^2 tissue culture flask and maintained in control medium at 37°C in a humidified atmosphere and 5% CO_2.

Osteogenic differentiation

After primary culture in the control medium (DMEM+ 10% FBS) and expansion to 1-4 passages (P), the rADASC were trypsinized and replated onto 75-cm^2 flasks at a density of 2 $X10^4/cm^2$. The cells were incubated in the control medium for a day to adhere to the flasks and the medium was then replaced with osteogenic medium (OM): DMEM supplemented with 10% FBS, 10^{-8} M dexamethasone (DEX), 50 mM ascorbic acid-2-phosphate (ASC-2-P) and 10 mM b-glycerol phosphate (b-GP) (all from Sigma). The medium was changed every 3 days.

Osteogenic differentiation was assessed by alkaline phosphatase (ALP) and von Kossa staining 2 and 4 weeks after initial induction, respectively. Expression of osteogenic cells specific to osteopontin (OP) and osteocalcin (OC) was confirmed by reverse transcriptase-polymerase cell reaction (RT-PCR) 2 and 4 weeks after initial osteogenic induction.

ALP detection

rADASC were seeded at 10^5 cells/well in 6-well plates in triplicate and incubated for 24 h in maintenance medium. Cells were then fed either maintenance medium or OM for 14 or 28 days. The medium was changed every 3-4 days. Cells were rinsed with PBS, fixed in 10% phosphate-buffered neutral formalin for 10 min and then stained for ALP using a staining buffer including a 1% volume of a 50 mg/mL solution of naphtol ASBI phosphate dissolved in dimethyl sulfoxide and 1 mg/mL Fast Red TR salt (Sigma) in 0.05 mol Tris-HCl, pH 9, at 37°C for 30 min. After counterstaining in hematoxylin, cells were rinsed with tap water and examined by light microscopy. Positive ALP activity, which is indicative of osteoblastic differentiation, was detected as a red stain.

Von Kossa staining

To determine the potential to mineralize extracellular matrix, rADASC were plated in 6-well plates at 105 cells/ well in triplicate and incubated for 24 h in maintenance medium. The cells were then fed either maintenance medium or OM for 14 or 28 days and the medium changed every 3-4 days. The plates were rinsed with PBS and fixed in 4% paraformaldehyde for 15 min. They were then incubated in 5% silver nitrate solution (w/v) for 10 min in the dark, washed thoroughly with distilled water, and exposed to bright light for 1 h. The cells were fixed with sodium thiosulfate for 5 min, rinsed with distilled water, and then observed with an inverted microscope. Images were recorded digitally with a Nikon camera and secretion of calcified extracellular matrix was observed as black nodules with von Kossa staining.

RT-PCR determination

rADASC were maintained in control medium or induced toward the osteogenic lineage for 2 and 4 weeks. Total cellular RNA was isolated using Real Total RNA Spin Plus (Real, Spain) and 1 mg of total RNA was used as a template for RT into cDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). cDNA was amplified by PCR with oligonucleotide primer sets as outlined in Table 1, and all primer sequences

were determined through established GenBank sequences. DNA amplifications were performed with an initial denaturation at 95°C for 8 min, followed by 28 cycles of denaturation at 95°C for 30 s, annealing at 608C for 30 s and extension at 72°C for 1 min. The final cycle included 7 min for extension at 72°C. PCR products from each sample were analyzed by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide. Triplicate PCR reactions were amplified using the designed rat primer Aldolase A (ALD) as a control for assessing PCR efficiency.

Flow cytometry analysis

Flow cytometry was used to characterize ADASC obtained from Wistar rats and grown for 1 and 4 passages under control and experimental conditions (4 weeks after induction of ostegenesis). Cultured cells were trypsinized, spun and washed in flow cytometry buffer consisting of 10 mM HEPES (Gibco), 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mg/mL bovine serum albumin (BSA; Sigma) in Leibovitz's L-15 medium (Gibco). Cell aliquots (1 $X10^6$ cells) were incubated for 30 min in flow cytometry buffer containing a fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) against CD34 (R&D Systems) and CD166 (AbD Serotec) followed by phycoerythrin (PE)-conjugated anti-CD29 and allophycocyanin (APC)-conjugated anti-CD45 and anti-ALP (R&D Systems). As an isotype control, FITC-, PE- or APC- coupled non-specific mouse IgG (Sigma) was substituted for the primary antibody (Ab). For STRO-1 staining, the cells were incubated for 30 min with Ab against STRO-1 (mouse IgM; R&D Systems). The cells were then incubated with a secondary Ab (PE-conjugated anti-mouse IgM; AbD Serotec) for 30 min. As an isotype control for STRO-1, control anti-mouse IgM (Sigma) was substituted. To exclude dead cells, 7-aminoactinomycin D (7-AAD; BD Pharmigen, San Diego, CA, USA) was added to each tube. Live cells were analyzed using a MoFloTM odular flow cytometer (DakoCytomation, Denmark) and Summit software (DakoCytomation). The isotype Ab control samples obtained from each individual cell population were used to set the dot-plot intercepts used for the analysis.

In vivo implantation

For the in vivo new bone formation assay, the cultured P1 and P4 rADASC were trypsinized and suspended in 150 mL DMEM mixed with 0.85 mg/mL rat type I collagen gel [32] (BD Biosciences, Bedford, MA, USA) at a concentration of 1 $X10^6$ cells/150 mL. These cell suspensions were then loaded into diffusion chambers (0.45 mm pore size; Millipore, Bedford, MA, USA). The rats were randomly divided into three groups, which received chambers loaded with either (1) P1 and P4 undifferentiated cells mixed with or without collagen, (2) P1 and P4 cells osteo-induced for 2 weeks with or without collagen or (3) P1 and P4 cells osteo-induced for 4 weeks with or without collagen. Controls also included chambers exposed to collagen without cells and empty chambers. The chambers (n =5 per group) were subcutaneously implanted into the dorsal side of 3-month-old female rats in pockets formed by blunt dissection. Animals were killed at 4 weeks after cell implantation and the newly formed tissues in the chambers were analyzed for osteogenesis histologically and immunohistochemically. The removed chambers were fixed in 10% phosphate-buffered neutral formalin or Bouin's fluid, dehydrated, embedded in paraffin and sectioned at 7

mm. Serial sections were either stained with Picrosirius- hematoxylin (PSH), Alcian blue, Toluidine blue and Goldner's trichrome for histologic analyzes or used for immunohistochemical staining for type I collagen.

Immunofluorescence

To detect bone matrix formation in the diffusion chambers, 7-mm sections were assessed immunohistochemically for the presence of type I collagen. Briefly, deparaffinized sections were rehydrated and then washed in PBS three times for 5 min. They were incubated with rabbit polyclonal anti-collagen type I Ab (Calbiochem-Novabiochem Co., USA) at a 1:60 dilution in buffer consisting of Tris-PBS, pH 7.8, 0.5% Triton X-100, 1% BSA and 5% sheep serum (Sigma) overnight at 4°C. Sections were washed extensively with PBS and incubated for 1 h at room temperature in buffer containing a Cy3-conjugated goat anti-rabbit IgG (Sigma) diluted 1:400. They were then washed with PBS and mounted in PVA-DABCO (Sigma).

Results

Osteogenic differentiation in vitro

The inguinal fat pads from each rat yielded approximately 8 X106 nucleated cells. When plated, the rat osteochondrogenic progenitor cells appeared as a heterogeneous population of fibroblast-like cells. Within 1-4 passages after initial plating of the primary culture, rADASC appeared as a monolayer of large, flat cells. As the cells approached confluence, they acquired a spindle-shaped or fibroblast-like appearance. Phase-contrast microscopy demonstrated the spindle-shaped morphology of control rADASC at day 7 (Figure 1A), while cells grown with OM, containing DEX, had become polygonal, more numerous and begun to form nodular aggregates (Figure 1B). Islands of extracellular matrix were secreted from these cells 7 days after osteogenic induction. By day 14, rADASC maintained in control medium were only slightly positive after ALP staining (Figure 1C) but cells grown with OM had formed nodular aggregates that were strongly stained for endogenous ALP activity (Figure 1D). Moreover, the cells cultured in control medium remained von Kossa negative by day 28 (Figure 1E) whereas von Kossa staining revealed mineralized nodular structures formed within 4 weeks in rADASC cultured in OM (Figure 1F).

Expression of osteoblast-specific genes

To confirm the osteoblastic differentiation potential of these rADASC, we used RT-PCR to probe osteoblast- specific gene expression in 14- and 28-day cell cultures supplemented with DEX, ASC-2-P and b-GP (Figure 2). As the results obtained from P1 and P4 cells were similar, only data concerning P4 ADASC are presented. Although the osteogenic induction conditions used in this study were specific for the bone lineage and did not result in the expression of collagen II (colII), consistent with cartilage differentiation, expression of sox9 (specific to chondro- genic differentiation) was noted at the 2- and 4-week time points in the differentiated ADAS cells. Expression levels of runx2 and osterix (Osx), transcription factors essential for bone formation and osteoblastic differentiation, were observed in osteo-induced ADASC at days 14 and 28. Furthermore, for all three of these genes (sox9, runx2 and Osx), the expression was not restricted to osteogenic induction, as basal expression was seen in ADASC maintained

in control medium (Figure 2).
Additional osteoblast-specific genes were investigated, including ALP, OP, bone sialoprotein (BSP), osteonectin (ON), OC, collagen I (colI), periostin (Postn) and the homeodomain proteins msx1 and msx2 (Figure 2). ALP, an early indicator of osteoblastic differentiation, was demonstrated in the controls and differentiated ADASC at day 14; however, the gene expression of the induced cells was higher than that of the controls. As shown in Figure 4, expression of the bone matrix proteins OP, ON and colI was observed with consistent expression levels in both differentiated and control ADASC. Expression of the bone-specific gene OC was also detected in ADAS cells after 2 and 4 weeks of induction. In addition to these matrix proteins, ADASC also expressed transforming growth factor (TGF)-b1 and Postn, both before and after induction. A qualitative increase in TGF-ß1 expression level was observed in induced ADASC at day 28 compared with differentiation at day 14 and non-induced controls. A low level of expression of BSP was observed at day 28 only in osteo-induced ADASC. Finally, both osteo-induced and control ADASC expressed msx1, a gene involved in osteoblast differentiation, whereas msx2 was not detected at any time point in either differentiated or control ADASC.

Epitope profile of the rADASC
We used flow cytometry to evaluate the expression of CD45, CD34, CD29, CD166, STRO-1 and ALP cell-surface antigens (Ag) on control and 4-week induced ADASC (after P1 and P4) obtained from Wistar rats. The rADASC from this strain were negative for CD45 (a hematopoietic cell-surface marker) and ALP (Figure 3). rADASC were positive for CD166, CD29, CD34 [mesenchymal stromal cell (MSC) and progenitor cell markers] and STRO-1 (an osteoprogenitor precursor marker) at the time of flow cytometry analysis (Figure 3). The marker profiles of control rADASC at P1 and P4 were similar and low (c. 1-7%) whereas they were much higher (c. 15-57%) in osteo-induced cells (Figure 4). The flow cytometry analysis also revealed that the osteo-induced rADASC marker profile changed during expansion. At P1, the rate of positivity for CD34, STRO-1 and CD29 (b1 integrin) in the osteo-induced cells was 39%, 45% and 57%, respectively, compared with 20% at P 4 (Figure 4A). The frequency of CD166 (ALCAM) positivity in the osteo-induced P1 and P4 rADASC was approximately 31% and 15%, respectively. Therefore, after P4 (Figure 4B), the osteo-induced cells showed decreased marker expression (an average decrease of roughly 55%) com- pared with the cells at P1.

Osteochondrogenic differentiation in vivo Histologic analysis of the implants showed that the osteo- induced rADASC differentiated toward chondrogenic and osteogenic lineages in vivo (Figure 5). Four weeks after implantation, in vivo bone and cartilage formation was demonstrated in implants containing both P1 (Figure 5A) and P4 (Figure 5B) rADASC that had been osteo-induced for 4 weeks and mixed with type I collagen gel, as confirmed by Goldner's trichrome (Figure 5C) and Alcian blue (Figure 5D) staining, respectively. Abundant cartilaginous matrices together with chondrocyte-like cells were remarkable in these implants (Figure 5D, E). Immunostaining of collagen type I also supported the histologic findings that the implanted collagen-wrapped osteo-induced rADASC continued to survive and participate in the generation of ectopic

bone-like tissue for up to 4 weeks (Figure 5F). On the other hand, no evidence of new bone or cartilage formation was found following implantation of 2-week osteo-induced rADASC with (Figure 5G) or without (data not shown) collagen. Similarly, the implants containing 4-week osteo-induced rADASC without collagen (Figure 5H), and the control implants containing undifferentiated cells mixed with (Figure 5I) or without (data not shown) collagen had no bone or cartilage and were primarily filled with fibrous tissues at that time interval.

Discussion

In this study, rat ADAS cells isolated from inguinal fat pads were cultured, osteo-induced and subsequently implanted into rats (the in vivo bone formation assay). We have demonstrated that the abundant and easily obtained adipose tissue could be an alternative stem cell source for bone repair because the osteo-induced ADAS cells can differentiate into osteoblasts in vitro and induce new bone and cartilage tissue formation in vivo when implanted with type I collagen gel. These cells had a rapid population-doubling rate, generating enough cells for implantation in a short-term culture, and thus avoided the prolonged culture that is expensive and easily contaminated. More- over, we were able to control reproducibly the differentiation of these rat adipose-derived stromal cells down the osteogenic lineage. The induction of osteogenesis was seen in all rADASC cell cultures placed in media supplemented with DEX, ASC-2-P and b-GP. This was supported by the osteoblastic cell structure, increased endogenous ALP activity and formation of islands of mineralized extra- cellular matrix containing calcium hydroxyapatite.

Consistent with previous results observed in ADASC [16,24,25] and supportive of osteogenic differentiation, the expression of the majority of genes examined by RT-PCR (runx2, Osx, msx1, ALP, TGF-ß1, OC, ON, OP, colI and Postn) was confirmed in both control and induced ADASC populations. Expression of the key transcription factors runx2, Osx and msx1 that bind to the promoters of several osteogenic genes in rADASC indicates their osteogenic commitment [34-37]. Although the expression of ON and OP and increased expression of ALP is strongly suggestive of osteogenesis, these genes are not considered to be specific markers for differentiation [25]. The RT-PCR results demonstrated that the osteo-induction of ADASC resulted in expression of OC, an extracellular matrix protein that is often used as a marker of the mature osteoblastic phenotype [25,37], after 14 and 28 days in culture. It is possible that this pattern observed in rADASC may be the result of the differentiation of multiple osteoprogenitor subpopulations with distinct temporal and developmental profiles. In addition to these matrix proteins, ADASC also expressed TGF-ß1, a growth factor that has dramatic effects on the recruitment of osteoblast precursors [38,39] and Postn, a protein with increased expression by TGF-b and the capacity to induce osteoblast attachment and spreading [39], both before and after induction. A qualitative increase in TGF-ß1 expression level was observed in induced ADASC at day 28 compared with differentiation at day 14 and non-induced controls.

A limitation of the present study is that we did not assay the corresponding control ADASC gene expression at days 14 and 28, as it was confined to a single time point (day 0). Although studies have documented no changes in control mouse ADASC gene expression at different time points (days 0, 14 and 28) [24], more studies concerning the

gene expression of control rADASC throughout the differentiation period will reveal if there are changes in gene expression relative to day 0 controls. Furthermore, we only selected representative transcription factors and marker genes in this study. Further study of gene expression profiling of ADASC differentiation will deepen the understanding of gene expression pattern during differentiation.

Previous studies have performed characterization of human and mouse ADASC using the expression of cell- specific proteins and CD markers [16,25,40-43]; however, no study has addressed rat marker profiles. Our observations of rADASC under control and inductive conditions revealed changes in their expression of CD166, CD34 and CD29, surface proteins used to identify MSC and progenitor cells [40-43], and STRO-1, an osteogenic precursor marker [44,45]. Our analysis of rADASC at P1 and P4 indicated that marker profiles were much higher (at least 2.5 times that of the controls) in differentiated cells. The results also showed that there was a striking increase (from 1% to 57%) in the frequency of positivity for CD29 (b1 integrin) in P1 ADASC after 4 weeks of induction. It is interesting to note that integrins are the central transducers of extracellular matrix signals that regulate the process of osteoblast commitment and differentiation. In fact, V2b1 integrin is highly expressed on osteoblast-like cells and it is one of the predominant adhesion receptors used by osteoblast-like cells to adhere to the collagen matrix [37].

The flow cytometry analysis also revealed that the osteo-induced rADASC marker profile changed during expansion. rADASC at P4 showed an average decrease of roughly 55% in marker expression compared with the cells at P1. We are investigating whether these changes in the marker expression profile reflect the different proliferation rates of stem cells and differentiated cells. We ruled out the possibility of contamination of the rADASC population with hematopoietic progenitors caused by disruption of the blood supply during isolation, as flow cytometry revealed no CD45-positive cells in the rADASC population. Moreover, the lack of CD45 on these cells is consistent with the localization of ADASC to a non- hematopoietic tissue. The lack of ALP on 4-week induced ADASC at the time of flow cytometry supported the histochemical and RT-PCR observations, where an increase of endogenous ALP activity was observed after 2 weeks of culture in osteogenic media. In support of this, time-course studies on BMSC have shown that ALP peaks early, correlating with matrix mineralization, and is down-regulated during terminal differentiation into osteocytes [46]. However, differing from the flow cytometry data, a constitutive level of ALP on control rADASC was observed in both histochemical and RT-PCR analyzes.

In the present study, the frequency of STRO-1 expression in the osteo-induced ADASC was 45% at P1, whereas only 4% was detected in control cells. STRO-1 was originally reported to identify osteogenic precursor cells isolated from BM [45] and has been shown to have some promise for playing a role in immunophenotyping of MSC [44]. However, STRO-1 Ag is progressively lost in culture [47] and our observations are consistent with this. The expression of STRO-1 by induced rADASC at P4 was only 20%. Additionally, in some reported studies, BMSC had negative [48] or only dimly positive [49] expression of STRO-1. With regard to MSC derived from locations other than the BM, Gronthos et al. [50] reported that STRO-1 Ag was not detected in human ADASC, although Zuk et al. [25] reported that the rate of expression of STRO-1 in these cells was 31%. These diverse findings on MSC phenotype indicate a lack of

consensus concerning the value of STRO-1 as a single marker [40].

In contrast to findings in previously reported studies, in which elevated levels of osteogenic markers were prominent in the less passaged rat BMSC cells (P0 through P2 cells) [51,52], the expression of osteogenic markers in our DEX-treated ADASC cultures did not decrease with passing. Different from rBMSC, rADASC retained their potential for differentiation from P1 through to P4. According to these authors, rBMSC continuously cultured in the presence of DEX initially showed high ALP expression and abundant mineralization, but no ALP activity and calcification were found in cells passaged three times.

Although in vivo bone formation by implantation of a limited number of passaged ADASC has previously been demonstrated [7,30,43], detailed documentation of several passaged ADASC with regard to in vivo osteogenic potential has not been delineated. Similar to the in vitro experiment, the in vivo osteogenic potential to form chondro-osseous tissues was demonstrated in both P1 and P4 osteo-induced ADASC. This could be an attractive advantage of ADASC over BMSC when contemplating clinical strategies, because the culture conditions permit the rapid expansion of cells while retaining their potential for differentiation with passaging. Ishikawa et al. [51] reported that the in vivo osteogenic potential of BMSC tended to decrease with passages. According to these authors, the failure of in vivo osteogenesis in implanted P3 cells was a dilemma for autologous cell therapy for bone regeneration. Less passaged cells can be applicable for use in cell therapy for the repair of bone defects. For clinical feasibility, however, large cell numbers with osteogenic potential will be required.

Bone-like and cartilage tissue formation could be constituted by transplanted osteo-induced ADASC as the host cells did not penetrate into the diffusion chambers. Although the implanted cells showed expression of osteogenic markers, cartilage formation associated with positive Alcian blue and Toluidine blue staining was characteristic, in addition to intramembranous ossification adjacent to the membrane. The cartilage tissue, bordering on the bone nodules with no evidence of vascularization, seemed to be suggestive of the so-called transchondroid ossification that was observed specifically in distraction osteogenesis [51]. The exact reason for in vivo chondro-genesis is still unknown, but ADASC with chondrogenic potential may be included in the implanted cells or the differentiated cells may be influenced by the

microenvironment within the chamber, leading to the unique chondro-osseous tissue formation [51]. Interestingly, control and induced rADASC showed expression of the transcription factor sox9, specific to chondrogenic differentiation [53,54], as detected by conventional RT- PCR. Better understanding of the molecular mechanisms directing the differentiation of ADASC will eventually allow us to manipulate ADASC properly both in vitro and in vivo for bone and cartilage repair [17,20,41,55]. Like other investigators [7,22,27,30], we found that the salient characteristic of these cells is that their bone-forming ability needs the presence of an osteogenic inducer, i.e. type I collagen. Similarly, our experiments demonstrate that fat pad adipocytes required prior induction in an osteogenic medium before being able to form bone.

Lastly, methods need to be developed to more rapidly isolate and enrich fat-derived inducible osteoprogenitor cells for clinical use [13,18,19,56,57]. From a clinical standpoint, the ubiquitous adipose tissue is abundant, expendable and easily harvested. Furthermore, compared with BM biopsy, liposuction or fat biopsy is more readily performed, safer and more reliable [15]. It has been demonstrated that a large number of multipotent cells can easily be obtained by human lipo-aspiration and induced to differentiate into osteoblasts [5,7,25,26]. There is also significant experimental potential for the use of these osteochondrogenic progenitor cells in animal models of bone and cartilage formation [5-7,26,28]. For example, the ease of isolation of the inguinal fat pads makes rADASC ideal for experimental models of autologous tissue engineering. Moreover, the efficacy of various osteo- inductive scaffolds such as type I collagen [7,58] and the osteo-inductive capability of various growth factors can be studied by using these cells [4,7].

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. Oligonucleotide primer sequences for RT-PCR

Gene	Primer sequence (forward, reverse)
Transforming growth factor-b1 (TGF-ß1)	5‘-CTGCTGCTTTCTCCCTCAAC-3‘
	5‘-GACTGGCGAGCCTTATTTG-3‘
Alkaline phosphatase (ALP)	5‘-CGCCTATCAGCTAATGCACA-3‘
	5’-AGGGAAGGGTCAGTCAGGTT-3’
Osteopontin (OP)	5’-GAGGAGAAGGCGCATTACAG-3’
	5’-AAACGTCTGCTTGTGTGCTG-3’
Osteonectin (ON)	5’-AAACATGGCAAGGTGTGTGA-3’
	5’-GGTGACCAGGACGTTTTTG ‘-3’
Osteocalcin (OC)	5’-GAGGGCAGTAAGGTGGTGAA-3’
	5’-AGGGTCGAGTCCTGGAGAGT-3’
Type I collagen (colI)	5’-CTGCTGGAGAACCTGGAAAG-3’
	5’-GGAAACCTCTCTCGCCTCTT-3’
Type II collagen (colII)	5’-AAGGGTGATCGTGGTGAGAC-3’
	5’-AGGGCCAGAAGTACCCTGAT-3’
Bone sialoprotein (BSP)	5’-AAAGAGCAGCACGGTTGAGT-3’
	5’-AGACCGTAGCACCATTCCAC-3’
Periostin (Postn)	5’-AACCAAGGACCTGAAACACG-3’
	5’-GCCACTTTGTCTCCCATGAT-3’
Runt-related transcription factor 2 (runx2)	5’-GCCGGGAATGATGAGAACTA-3’
	5’-GAGGCGGTCAGAGAACAAAC-3’
Osterix (Osx)	5’-GCTGCCTACTTACCCGTCTG-3’
	5’-TGTGAATGGGCTTCTTCCTC-3’
Sex-determining region Y-box 9 (sox9)	5’-CTGAAGAAGGAGAGCGAGGA-3’
	5’-TGTAATCGGGGTGGTCTTTC-3’
Homeo box, msh-like 1 (msx1)	5’-TCCTCAAGCTGCCAGAAGAT-3’
	5’-TTCACCTGGGTCTCGGTAAG-3’
Msh homeo box homolog 2 (msx2)	5’-TCCGCCAGAAACAGTACCTC-3’
	5’-CTTGCAAGGGGGAGTTGATA-3’
Aldolase A (ALD)	5’-TGTACCCATTGTGGAGCCTGA-3’
	5’-CAGACAACACTGCACACACGA-3’

All primer sequences were determined with established GenBank sequences.
ALD, a glycolitic enzyme that is widely distributed in almost all tissues and cells, was used as control.

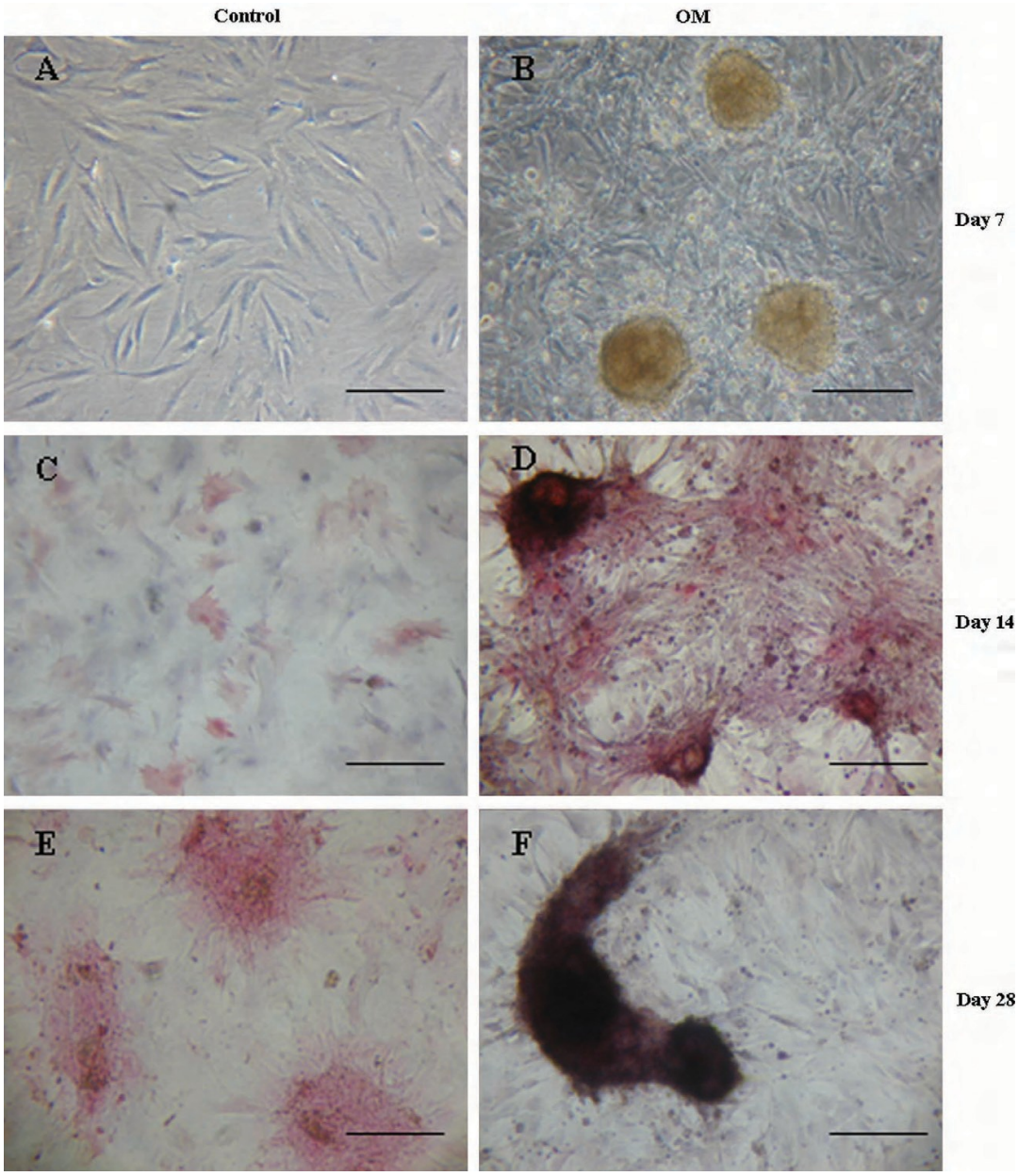

Figure 1. Effect of OM on cell morphology, ALP expression and mineral deposition in rADASC cultures. Except for (A) and (B), all specimens were stained by ALP and von Kossa histochemical techniques as described in the Methods. Phase-contrast microscopy demonstrated the spindle- shaped morphology of control rADASC (A) at day 7, while cells grown with OM (B) had become polygonal, more numerous, and begun to form nodular aggregates. By day 14, rADASC maintained in control medium (C) were only slightly positive after ALP staining, but cells grown with OM (D) had formed nodular aggregates that were strongly stained for ALP and began to mineralize their matrix (shown as black granular deposits). The cells cultured in control medium remained von Kossa negative by day 28 (E), whereas von Kossa staining revealed calcified extracellular matrix (black) in rADAS cells cultured for 4 weeks in OM (F). Bars: 150 mm.

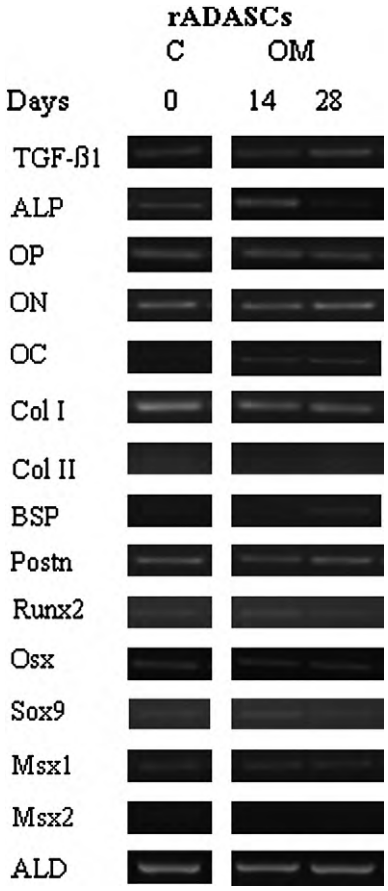

Figure 2. Osteo-induced rADASC express several osteogenic genes. rADASC were cultured in OM or control medium (C) for 2 and 4 weeks and analyzed by RT-PCR for the indicated genes. Expression of ALD was assessed as an internal control.

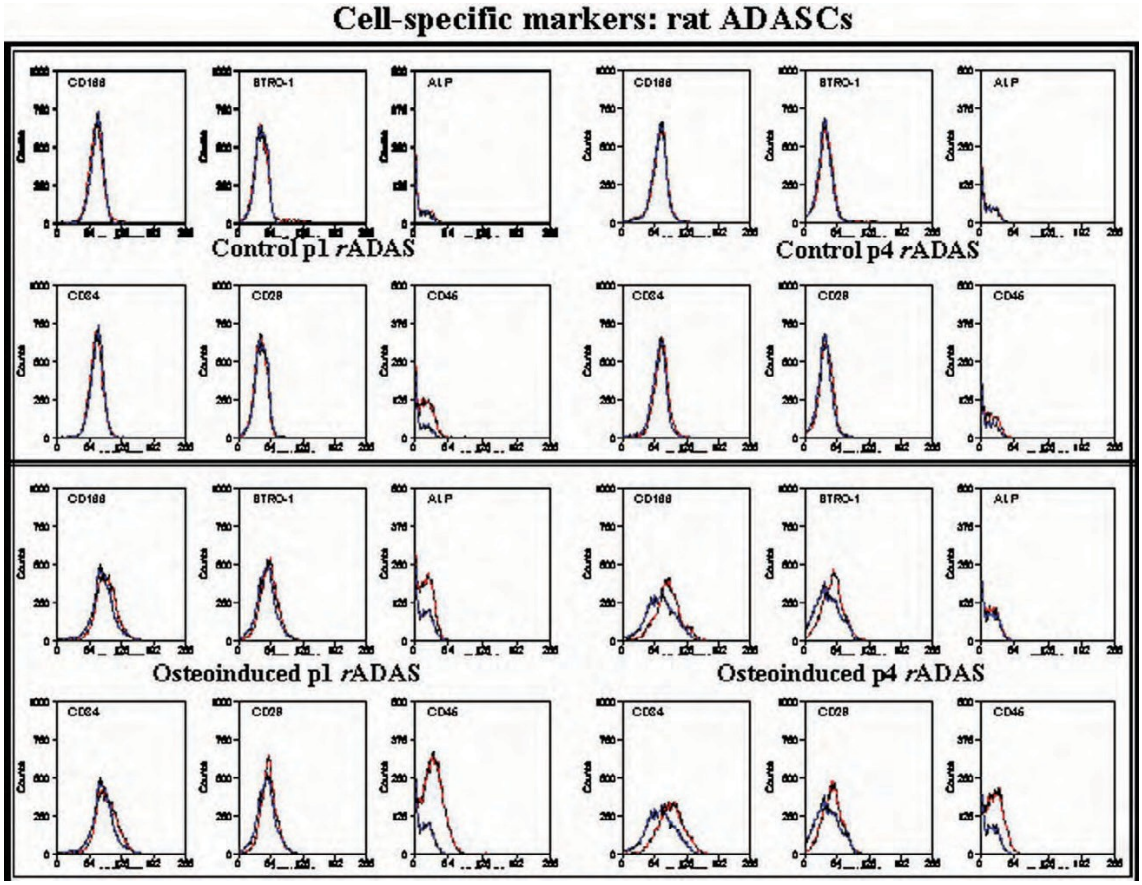

Figure 3. Expression of cell-surface proteins on rADASC, determined by flow cytometry. The cell composition of representative control and osteo- induced rADASC samples at P1 (left) and P4 (right) was determined by incubating the samples with the following MAb: CD166, STRO-1, ALP, CD34, CD29 and CD45 (red). Cells stained with a fluorochrome-conjugated non-specific IgG were examined as a control (blue). rADASC were negative for CD45 and ALP but positive for CD166, CD29, CD34 and STRO-1. The marker profiles of control rADASC at P1 (above, left) and P4 (above, right) were similar and low. After osteo-induction, P1 (below, left) and P4 (below, right) rADASC showed increased marker expression (at least 2.5 times that of the controls). Flow cytometry analysis also revealed that the osteo-induced rADASC marker profile changed during expansion. After P4, the cells showed decreased marker expression (an average decrease of roughly 56%) compared with the cells at P1.

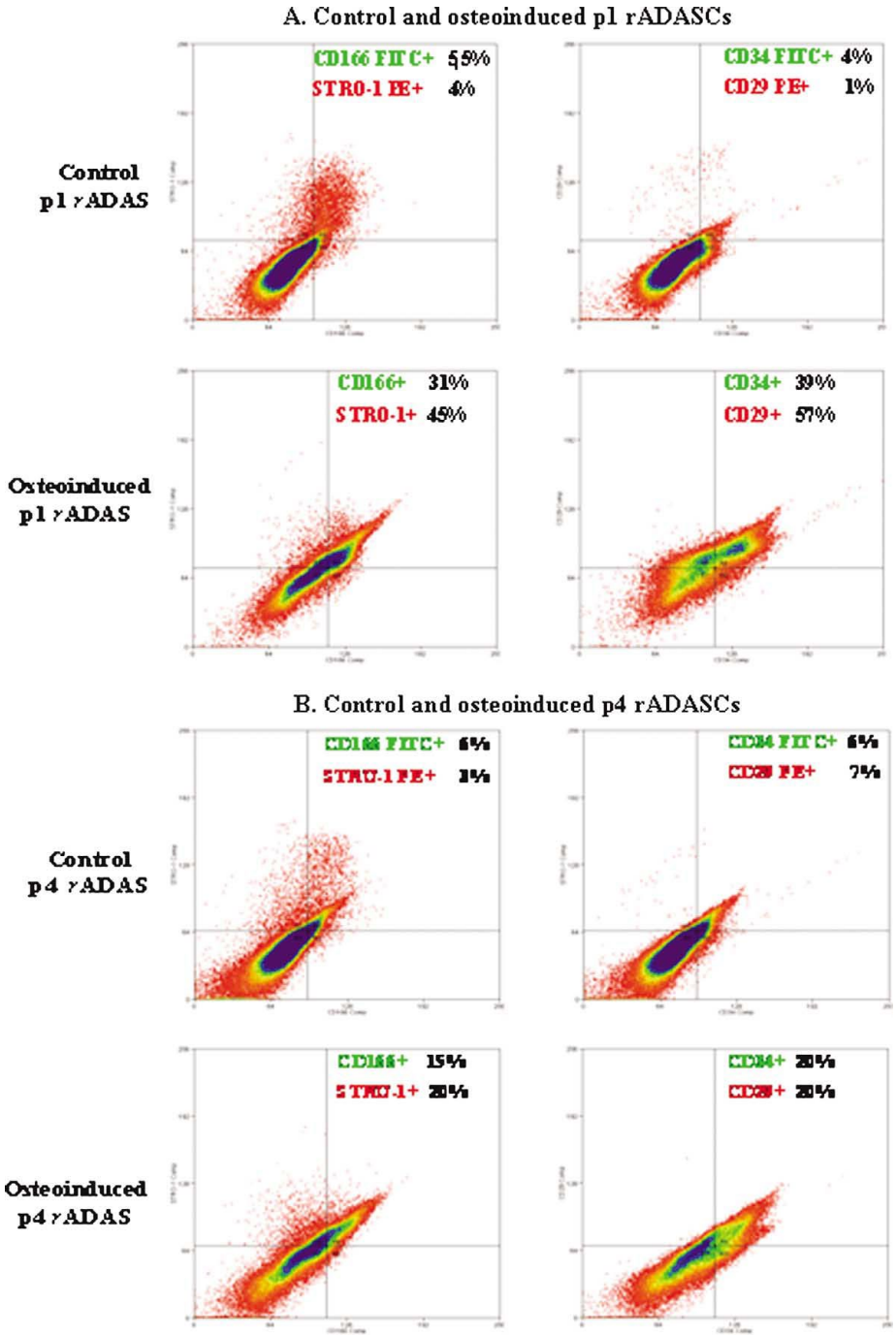

Figure 4. Flow cytometry analysis of CD166 and STRO-1 (left) and CD34 and CD29 (right) on control and osteo-induced rADASC at P1 (A) and P4 (B). Flow cytometry data were collected and the mean number of positive events of each cell-specific marker was expressed as a percentage of total rADASC number. Marker expression was low (1-7%) on control cells whereas it was significantly higher (15-57%) on osteo-induced cells. At P1 (A), 31% and 45% of the osteo-induced cells were CD166 and STRO-1 positive whereas 39% and 57% were CD34 and CD29 positive, respectively, compared with 15-20% of them at P4 (B).

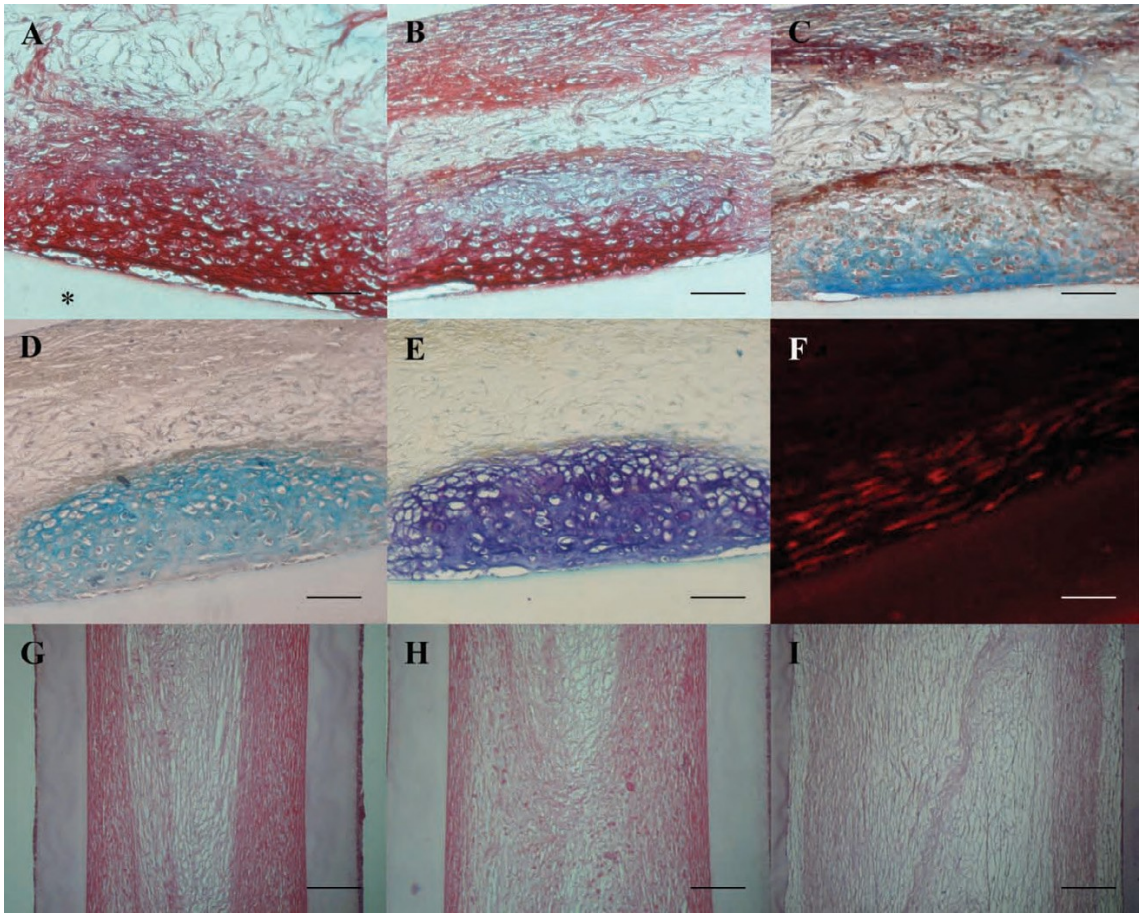

Figure 5. Histologic sections of the diffusion chambers that were filled with rADASC at 4 weeks after implantation. Chambers containing collagen- wrapped P1 (A) and P4 (B) rADASC osteo-induced for 4 weeks revealed nodules of cartilage and bone-like tissue inside the membrane (*). The bone-like areas were positively stained for PSH (red) (A, B) and Goldner's trichrome (blue) (C), indicative of accumulation of type I collagen, as confirmed by positive immunostaining for collagen type I (F). In vivo chondrogenesis by rADASC was determined by positive Alcian blue (D) and Toluidine blue (E) staining, which indicated the presence of highly sulfated proteoglycans that are characteristic of cartilaginous matrix. No evidence of new bone or cartilage formation was found following implantation of collagen-wrapped rADASC induced for 2 weeks (G). Implants containing 4-week induced cells without collagen (H) or undifferentiated rADASC with collagen (I) were partially filled with loose fibrous connective tissue containing collagen fibrils adjacent to the membranes (PSH staining). Bars: 200 mm.