A new source for cardiovascular tissue engineering: human bone marrow stromal cells☆

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Abstract

Objective: Vascular-derived cells represent an established cell source for tissue engineering of cardiovascular constructs. Previously, cell isolation was performed by harvesting of vascular structures prior to scaffold seeding. Marrow stromal cells (MSC) demonstrate the ability to differentiate into multiple mesenchymal cell lineages and would offer an alternative cell source for tissue engineering involving a less invasive harvesting technique. We studied the feasibility of using MSC as an alternative cell source for cardiovascular tissue engineering. **Methods**: Human MSC were isolated from bone marrow and expanded in culture. Subsequently MSC were seeded on bioabsorbable polymers and grown in vitro. Cultivated cells and seeded polymers were studied for cell characterization and tissue formation including extracellular matrix production. Applied methods comprised flow cytometry, histology, immunohistochemistry, transmission (TEM) and scanning electron microscopy (SEM), and biochemical assays. **Results**: Isolated MSC demonstrated fibroblast-like morphology. Phenotype analysis revealed positive signals for alpha-smooth muscle actin and vimentin. Histology and SEM of seeded polymers showed layered tissue formation. TEM demonstrated formation of extracellular matrix with deposition of collagen fibrils. Matrix protein analysis showed production of collagen I and III. In comparison to vascular-derived cell constructs quantitative analysis demonstrated comparable amounts of extracellular matrix proteins in the tissue engineered constructs. **Conclusions**: Isolated MSC demonstrated myofibroblast-like characteristics. Tissue formation on bioabsorbable scaffolds was feasible with extracellular matrix production comparable to vascular-cell derived tissue engineered constructs. It appears that MSC represent a promising cell source for cardiovascular tissue engineering. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Tissue engineering; Cardiovascular; Marrow stromal cells; Polymer scaffold

1. Introduction

Currently valve replacement is an effective treatment using mechanical or biological protheses [1]. However, there are certain limitations regarding long-term benefit. Mechanical valves are associated with a substantial risk of thromboembolism while biological valves lack the ability to repair, grow, or remodel resulting in structural dysfunction [2,3].

Tissue engineering offers a promising approach to overcome these limitations by trying to create viable valve structures with a thromboresistant surface and a living interstitium with repair and remodeling capabilities [4]. Several groups reported the feasibility of creating autologous living cardiovascular structures by seeding cells on synthetic polymer, collagen or xenogenic scaffolds [5–8]. Previously our group demonstrated the feasibility of tissue engineering living heart valve constructs by seeding bioabsorbable polymer scaffolds [9]. However, all of these approaches are based on vascular-derived cell sources with certain shortcomings. Harvesting of the cells prior to scaffold seeding necessitates the sacrifice of an intact vessel. Furthermore, vascular-derived cells demonstrate different characteristics compared to valve interstitial cells, qualities which may be vital to the development and function of a tissue engineered heart valve [10]. Due to these limitations we investigated the feasibility of applying marrow stromal cells (MSC) as an alternative cell source for tissue engineering. The usage of MSC may offer several advantages by: (i) showing characteristics of multipotent progenitor cells which are able to differentiate into a variety of mesenchymal cell types; (ii) easy collection and isolation methods avoiding the sacrificing of intact cardiovascular structures;

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and (iii) demonstrating immunological unique characteristics allowing persistence in an allogenic setting [11–13].

2. Materials and methods

2.1. Cell isolation

MSC were isolated from human bone marrow by washing in Dulbecco's phosphate buffered saline (DPBS, Gibco) for 10 min at 1500 rpm. The cells were recovered after centrifugation and resuspended in DPBS. A low-density cell fraction of bone marrow was obtained by centrifugation of the cell suspension over a Ficoll step gradient (density 1.077 g/ml) (Ficoll-Histopaque 1077, Sigma) at 1500 rpm for 10 min. The nucleated cells were collected from the interface, diluted with two volumes of DPBS and centrifuged at 1500 rpm for 10 min. Following, the cells were resuspended, counted and plated at 200 000 cells/cm^2.

2.2. Cell cultivation

The isolated cell fraction was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin (Gibco), and streptomycin (Gibco) in tissue flasks (Corning, Inc.) and left to adhere at 37°C for 4–5 h. The non-adherent cells floated off, while mesenchymal cells adhered, spread, and grew. Medium was replaced at 24 and 72 h and every 6 days following. Daily growth progress was monitored by phase-contrast microscopy. The cells were serially passaged and expanded in a humidified incubator at 37°C with 5% CO$_2$. Sufficient cell numbers for cell seeding on bioabsorbable polymer scaffolds were obtained after 21–28 days.

2.3. Bioabsorbable polymer scaffolds

Non-woven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany Int.) was coated with poly-4-hydroxybutyrate (P4HB, MW: 1×10^6, PHA 4400, TEPHA Inc., Cambridge, MA). From the PGA/P4HB composite scaffold material strips (20×15 mm) were cut and cold gas sterilized with ethylene oxide.

2.4. Cell seeding and in vitro culture of polymer constructs

MSC were seeded onto the polymer scaffolds ($n = 6$) with an approximate cell density between $4.5–5.5 \times 10^6$ per cm^2 and cultured in nutrient medium (DMEM, Gibco) for 14 days in a humidified incubator (37°C, 5% CO$_2$).

2.5. Analysis of MSC cultures

2.5.1. Flow cytometry (FACS)
A single cell suspension of MSC was prepared for FACS. $0.5–1 \times 10^6$ cells in 100 μl phosphate buffered saline (PBS) plus BSA were incubated with saturating concentrations of monoclonal antibodies CD 31-FITC (Sigma, St. Louis), LDL-Dil (Biomedical Technologies Inc, Stoughton, MA), CD 14-FITC (Beckon Dickinson, San Jose, CA). For intracellular staining, cells were permeabilized with ethanol for 30 min and incubated with monoclonal antibodies against ASMA (Sigma, St. Louis) and vimentin (NeoMarkers, Fremont). Following washing, staining with a secondary FITC-conjugated IgG goat-anti-mouse antibody (Chemicon, Temecula, CA) was performed for 30 min. Forward and side scatter gates were set to exclude debris and 10 000 gated events were counted per sample. Corresponding isotype and positive controls were performed for each antibody. Cells were analyzed with the flow cytometer FACS-Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data analysis was performed with the CELL QUEST software program (Becton Dickinson Immunocytometry Systems, San Jose, CA). Expression levels were calculated as mean fluorescence intensity ratio (MFIR) defined as mean fluorescence intensity of the studied antibodies divided by mean fluorescence intensity of corresponding isotype controls.

2.5.2. Histology and immunohistochemistry
Isolated MSC were cultivated onto glass coverslips in nutrient medium (DMEM, Gibco). After 2–3 days cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. Cells were examined histologically by hematoxylin & eosin (H & E) and Trichrome-masson stain. Immunohistochemistry was performed by incubation with monoclonal mouse antibodies for ASMA (Sigma, St. Louis), vimentin (NeoMarkers, Fremont), desmin (NeoMarkers, Fremont), collagen I–IV (Oncogen, Boston), and elastin (Sigma, St. Louis). Incubation with a secondary biotin-labeled goat-anti-mouse IgG antibody (Sima, St. Louis) was performed and the signal was developed with the avidin-peroxidase system (ABC kit, Vector Lab, Burlingame CA). Prior to intracellular staining, permeabilization of the cells was performed by incubation with 0.1% Triton (Sigma, St. Louis) for 10 min.

2.6. Analysis of MSC-seeded polymer constructs

2.6.1. Histology and immunohistochemistry
After 2 weeks in culture, sections of MSC seeded polymer scaffold strips were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Paraffin sections were cut at 5-μm thickness and studied by H & E and Trichrome-masson stain. Immunohistochemistry was performed as described above by incubation with monoclonal mouse antibodies for ASMA, vimentin, desmin, collagen I–IV and elastin.

2.6.2. Scanning and transmission electron microscopy
Additional samples of MSC-seeded polymer strips were fixed in 2% glutaraldehyde (Sigma, St. Louis) and dehydrated in acetone followed by drying in carbon dioxide for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

2.6.3. Biochemical assays

Cellular and extracellular components of the MSC-seeded constructs were analyzed by biochemical assays and compared to vascular-derived cell (VC) seeded constructs. VC seeded constructs were identically generated as MSC-seeded constructs by using jugular venous fibroblasts. Total DNA was isolated and purified by sequential organic extractions with phenol and phenol/chloroform/isoamyl alcohol and quantitated by spectrophotometry. For determination of total collagen content, the tissue was completely acid-digested and total 5-hydroxyproline was measured. Total proteoglycan/glycosaminoglycan (GAG) and elastin content were quantitated with a BLYSCAN™ and FASTIN™ assay (Biocolor, Belfast, Ireland) after tissue extraction [14]. Data of biochemical testing was statistically analyzed by SPSS 8.0 Software and expressed as mean ± standard error of the mean. An unpaired t-test (Student t-test) was performed, considering a P-value <0.05 as statistically significant. Six samples of each group were studied per test.

3. Results

3.1. Cell morphology

Isolated cells appeared small and round with a tendency to grow in clusters. Non-adherent cells were removed by medium change at 24 h and every 4 days thereafter. Elongated cells with fibroblast-like morphology appeared after 72 h and reached confluence after 10–14 days.

3.2. Flow activated cell scanning

Table 1 shows the results of FACS analysis for MSC. Flow cytometry characterization of MSC demonstrates no significant difference in expression of ASMA (MFIR 3.66) and vimentin (MFIR 12.59) compared to vascular-derived myofibroblasts. No positive signal was detected for CD 14 (MFIR 1.13), CD 31 (MFIR 1.1), and LDL (MFIR 1.94) among the isolated cell population (Table 1).

3.3. Histology and immunophenotyping of MSC

H & E and Trichrome-masson staining of fixed cells demonstrated the deposition of extracellular matrix throughout the cell culture. Immunohistochemistry showed the expression of ASMA and vimentin by MSC. The deposition

Table 1
MFIR of FACS analysis

Antigen	MSC	Positive controls
Vimentin	12.59	15.67 (myofibroblasts)
ASMA	3.66	3.53 (myofibroblasts)
CD 14	1.13	4.6 (PBMC)
LDL	1.94	7.19 (endothelial cells)
CD 31	1.1	55.58 (endothelial cells)

of collagen I and III was detected by positive staining. In contrast, no signal was observed following antibody staining for desmin, collagen II, IV, and elastin.

3.4. Histology and immunohistochemistry of MSC-seeded polymer constructs

H & E and Trichrome-masson staining of the MSC-seeded polymer constructs showed a layered tissue formation and a dense upper layer with deposition of extracellular matrix proteins. Irregular cellular ingrowth was observed into less cellularized deeper parts of the polymer strips (Figs. 1A,B). Immunohistochemistry of MSC-seeded constructs showed positive staining for ASMA and vimentin (Figs. 1C,D). Extracellular matrix analysis demonstrated the deposition of collagen I and III (Figs. 1E,F). No positive staining was studied for desmin, collagen II, IV, and elastin.

3.5. Transmission and scanning microscopy

TEM of sections of MSC polymer constructs showed secretionally active fibroblasts with deposition of collagen fibrils (Fig. 2A). SEM revealed loose tissue formation and demonstrated the degradation of the polymer scaffold by multiple hydrolytic breakages and fragmentation of the polymer fibers (Fig. 2B).

3.6. Biochemical assays

Results of the biochemical assays are shown in Fig. 3. DNA content of the MSC constructs (2.7 µg/mg dry tissue ±0.6) was significantly superior ($P < 0.01$) compared to VC constructs (0.9 µg/mg dry tissue ±0.5). Biochemical assays detected a hydroxyproline content of MSC constructs of 3.1 µg/mg dry tissue ±0.4 which was comparable to VC constructs (2.8 µg/mg dry tissue ±0.5). The quantitative analysis of the glycosaminoglycan amount revealed 4.9 µg/mg dry tissue ±0.7 not significantly different ($P > 0.05$) to VC constructs (5.8 µg/mg dry tissue ±0.6). Elastin was not detectable in MSC- and VC-constructs.

4. Discussion

Presently, vascular-derived cells represent an established cell source for seeding of tissue engineered cardiovascular constructs. By using venous or arterial vascular cells, functional tissue engineered patches, vascular grafts, and heart valves were generated [5,7,15]. In prior in-vitro and in-vivo studies Hoerstrup et al. demonstrated the feasibility of tissue engineering a complete and living autologous heart valve by using myofibroblasts of carotid artery origin [9]. Following 5 months implantation in sheep the tissue engineered valves functioned satisfactorily in-vivo while tissue formation gradually evolved to resemble native pulmonary valve morphology and histology. However, there is evidence that native heart valves consist of a mixed population of
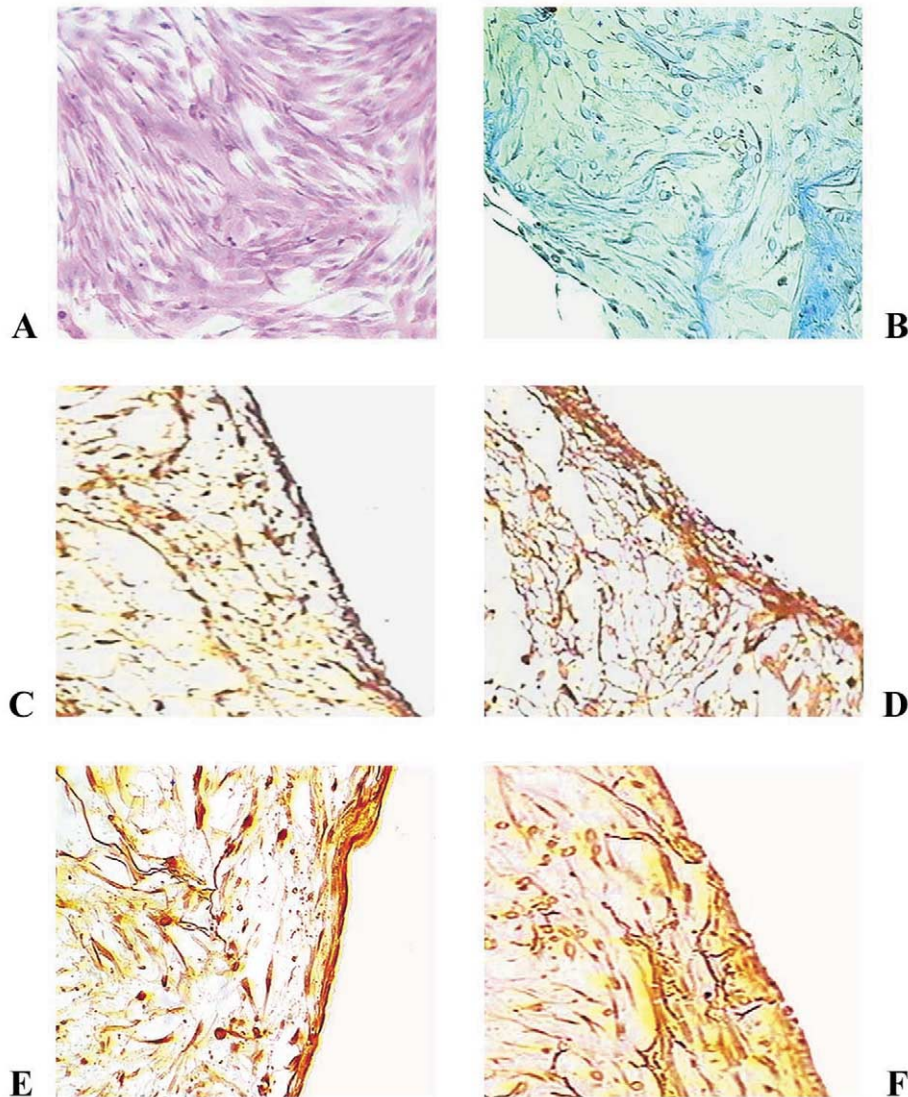
Fig. 1. Histology and Immunohistochemistry of MSC culture and polymer constructs. H & E (A); and Trichrome-masson (B) staining show cells with a fibroblast morphology and layered tissue formation with deposition of extracellular matrix proteins. MSC-seeded polymer constructs show a irregular cellular growth into less cellularized deeper layers. Immunohistochemical staining demonstrated the expression of ASMA (C); and vimentin (D). Extracellular matrix analysis demonstrated the deposition of collagen I (E); and III (F).

interstitial cells (IC) which show specific and unique characteristics different to vascular-derived fibroblasts [16,17]. Recently, Taylor et al. demonstrated the feasibility of using IC for tissue engineering [8]. A possible clinical application of creating a tissue engineered valve with IC is limited by using non-autologous IC.

Given these problems it appears that the appropriate cell source for tissue engineering of heart valves is still unclear. In the present study we evaluated the feasibility of using an alternative cell source for tissue engineering heart valves – mesenchymal stromal cells.

Isolation of MSC was easy to perform avoiding the sacrificing of an intact vascular structure. Isolated cells appeared small, round, and elongated with a fibroblast-like morphology. After 72 h cells appeared to grow in a colony-forming pattern. Identical morphological characteristics and growth pattern are reported for mesenchymal precursor cells by other studies using a similar isolation procedure [18,19]. Analysis of the cell population showed characteristics of a myofibroblast-like differentiation. MSC expressed ASMA, vimentin and the deposition of collagen I and III was observed. MSC did not stain positive for the monoclonal antibody against desmin, a muscle cell marker. A similar staining pattern is reported for valve IC by Taylor et al. [8]. FACS analysis did not detect any CD 14, CD 31, or LDL positive cells, indicating the absence of myeloid and endothelial cell differentiation of MSC. Furthermore, we did not observe any positive staining for collagen II, implying the absence of an osteoblastoid differentiation of the isolated cells.

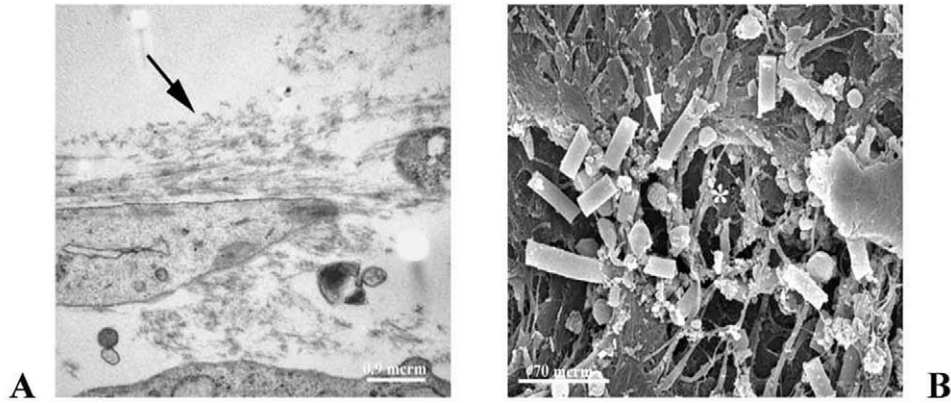The formation of tissue by MSC showed identical results

Fig. 2. Transmission and scanning electron microscopy. (A) TEM of sections of MSC polymer constructs shows cell elements typical of viable, secretionally active myofibroblasts with deposition of collagen fibrils (arrow). (B) SEM revealed loose tissue formation with good cell-polymer-attachments (arrow) and demonstrated the degradation of the polymer scaffold by multiple hydrolytic breakages and fragmentation of the polymer fibers (*).

compared to vascular-derived cells seeded on polymer constructs after 14 days in culture. We observed a layered tissue structure formation with a dense upper layer. Overall, morphology and ultra-structural analysis showed good cell-polymer adhesion and growth of MSC into deeper parts of the polymer strips. Furthermore, the gradual biodegradation of the scaffold and the replacement by viable tissue was observed. Results of biochemical assays demonstrated no significantly different amount of extracellular matrix proteins of MSC polymer constructs compared to VC seeded constructs.

In previous studies it was demonstrated that exposure of tissue engineered heart valve constructs to a pulsatile flow significantly improved tissue development and mechanical properties [7,20]. A homogenous, dense tissue developed resembling native valve morphology. In addition, biochem-ical analysis of the tissue engineered heart valves showed comparable values to native tissue. Given these results we anticipate that exposure of MSC-seeded constructs to a biomimetic pulsatile flow system will demonstrate improved tissue formation and differentiation. We also believe that future animal experiments are required to evaluate the remodelling capacity, the growth potential, and long-term function of MSC-seeded tissue engineered constructs under physiologic conditions.

In conclusion, this study demonstrates the feasibility of using MSC as a new alternative cell source for tissue engineering. Cell isolation was easy to perform without the need to sacrificing intact vascular structures. MSC showed characteristics of myofibroblast-like differentiation with neo-tissue formation comparable to tissue engineered constructs based on vascular-derived cells.
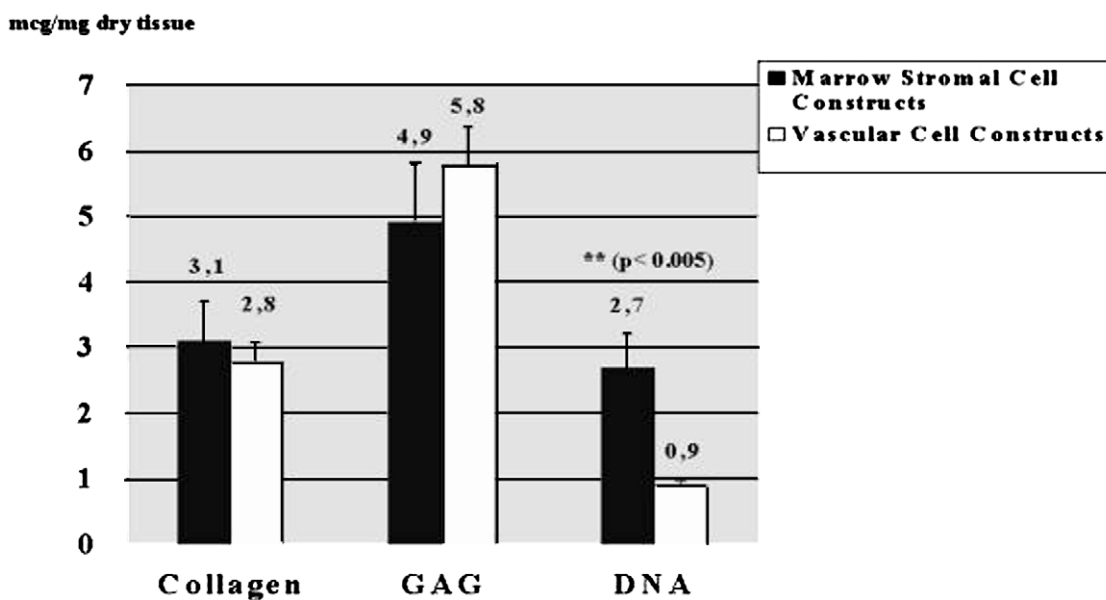
mcg/mg dry tissue

Fig. 3. Biochemical assays.

Acknowledgements

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Appendix A. Conference discussion

Mr S. Stoica (*Cambridge, UK*): A very elegant concept. I thought the histology slides were most convincing. Did you use a negative and positive control for your monoclonal antibodies?

Dr Kadner: Certainly, we were performing both negative and positive controls.

Dr G. Gerosa (*Padova, Italy*): I congratulate you. You did very excellent work. I would like to ask you, the myofibroblasts that you identified with your immunohistochemistry were fetal type or adult type?

Dr Kadner: The bone marrow stromal cells were adult type, and the vascular-derived cells came from saphenous vein.

Dr A. Haverich (*Hannover, Germany*): I have a more general question, and that is that many other researchers in other fields use the same sort of cells and they are making liver and they are making pancreas, so they are using pluripotent neural stem cells and cellularized tissues out of that. What do you think will be the necessary environment for these cells when used for cardiovascular applications?

Dr Kadner: I would have to speculate about this. It appears that initially these cells take spontaneously a fibroblast-like lineage. However, other groups have shown that by using such cell medium supplements as dexamethasone, insulin or transforming growth factor beta 3 that you can induce chondrocytic, osteocytic or adipocytic lineages. What would be very interesting for cardiovascular tissue engineering is the differentiation of these cells in a endothelial lineage.

Dr B. Messmer (*Aachen, Germany*): I think one of the most important things is the expression of collagen. Can you explain why you have an inhomogenous expression of collagen 1 and 2, which you have shown; you didn't show 3 and 4? This is very inhomogenous, and this may be a disadvantage.

Dr Kadner: I agree with you, and I think the reason is that these constructs were cultured only for a very brief period, for 7 days under steady conditions. In this context I would like to refer to a talk my colleague, Simon Hoerstrup, is giving tomorrow. He will present our data for using these cells for creating tissue engineered heart valve constructs, which were cultured under dynamic conditions for an extended period of time.