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Accumulation of fibronectin in the heart after myocardial infarction: a putative stimulator of adhesion and proliferation of adipose-derived stem cells

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Abstract Stem cell therapy is a promising treatment after myocardial infarction (MI). A major problem in stem cell therapy, however, is that only a small proportion of stem cells applied to the heart can survive and differentiate into cardiomyocytes. We hypothesized that fibronectin in the heart after MI might positively affect stem cell adhesion and proliferation at the site of injury. Therefore, we investigated the kinetics of attachment and proliferation of adipose-tissue-derived stem cells (ASC) on fibronectin and analysed the time frame and localization of fibronectin accumulation in the human heart after MI. ASCs were seeded onto fibronectin-coated and uncoated culture wells. The numbers of adhering ASC were quantified after various incubation periods (5–30 min) by using DNA quantification

assays. The proliferation of ASC was quantified after culturing ASC for various periods (0–9 days) by using DNA assays. Fibronectin accumulation after MI was quantified by immunohistochemical staining of heart sections from 35 patients, after different infarction periods (0–14 days old). We found that ASC attachment and proliferation on fibronectin-coated culture wells was significantly higher than on uncoated wells. Fibronectin deposition was significantly increased from 12 h to 14 days post-infarction, both in the infarction area and in the border-zone, compared with the uninfarcted heart. Our results suggest that a positive effect of fibronectin on stem cells in the heart can only be achieved when stem cell therapy is applied at least 12 h after MI, when the accumulation of fibronectin occurs in the infarcted heart.

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Introduction

Cardiovascular diseases are a leading cause of morbidity and mortality in the western world (Wang and Sjoquist 2006), with heart failure attributable to myocardial infarction (MI) being the most common aetiology (Lu et al. 2004). Heart failure partly is caused by the loss of cardiomyocytes and the limited capacity of spontaneous regeneration of the heart. This results in the replacement of injured myocardium by scar tissue, which adversely affects cardiac function (Smits et al. 2005; Wang and Sjoquist 2006).

To reduce heart failure after MI, current therapeutic strategies focus on the restoration of the blood flow to the myocardium and on reducing cardiac remodelling by using drug therapy (inhibitors of angiotensin-converting enzyme

and beta blockers; Fraser et al. 2004). Although these strategies significantly reduce mortality (Khalil et al. 2001; Lange and Hillis 2002), they do not replace the lost cardiomyocytes.

A promising tool to restore contractile function after myocardial infarction is stem cell therapy. Stem cells not only restore neovascularization, but also replace lost cardiomyocytes, since they have the capacity to differentiate into cardiomyocytes (Orlic et al. 2001; Strauer et al. 2002; Wang and Sjoquist 2006). However, to restore contractile function of the heart effectively after MI, sufficient stem cells need to attach to the infarcted area and subsequently proliferate and differentiate into cardiomyocytes, thereby replacing a substantial fraction of the jeopardized cardiomyocytes. Unfortunately, recent studies suggest that only a small proportion of stem cells applied to the heart invade the infarcted area and finally differentiate into cardiomyocytes. Furthermore, most of the applied stem cells die within the first week after transplantation (Wang and Sjoquist 2006; Zhang et al. 2001).

This high stem cell death rate is probably caused by the inferior environment that is found in the heart after myocardial infarction and into which the cells are transplanted; this poor environment is caused by the presence of inflammatory mediators and ischemia (Azarnoush et al. 2005; Wang and Sjoquist 2006; Zhang et al. 2001). Therefore, stem cell therapy has to be applied at that moment after infarction when the environment is most favourable for stem cell adhesion and cardiomyocyte formation. However, to the best of our knowledge, this ideal time frame has yet not been determined (Mishra 2005). In most animal studies, investigators apply stem cells during the same operational procedure as the infarct induction, whereas recent studies have shown that later time points might be more favourable for the survival of the stem cells (Bermejo et al. 2006; Lu et al. 2004; Wang and Sjoquist 2006).

Homing, growth and differentiation of stem cells after myocardial infarction is known to depend on several environmental factors, including the presence of adhesion factors at the site of injury (Chastain et al. 2006; Lu et al. 2004; Malek et al. 2006; Salaszyk et al. 2004; Wang and Sjoquist 2006). Fibronectin has been demonstrated to enhance the attachment of bone-marrow-derived stem cells *in vitro*, compared with several other extracellular matrix (ECM) molecules (Cool and Nurcombe 2005; Ogura et al. 2004). Fibronectin is a large molecular weight glycoprotein, present at low levels in the ECM of the healthy heart (Heng et al. 2004; Willems et al. 1996). In addition to the influence of fibronectin on cell attachment, the presence of fibronectin can also affect stem cell behaviour such as migration and proliferation, both *in vitro* and *in vivo* (Tate et al. 2002; van der Loo et al. 1998; Voermans et al. 1999;

Wijelath et al. 2004). However, the effect of fibronectin on adipose-tissue-derived stem cells (ASC) has as yet not been investigated. Therefore, we have examined whether fibronectin stimulates the attachment of ASC and have compared this with the attachment of ASC to other ECM proteins that are normally expressed in the heart and that are upregulated after acute MI (AMI), namely collagen type 1 and laminin (Deten et al. 2001; Morishita et al. 1996). We have also investigated the effect of fibronectin on the proliferation of ASC *in vitro*.

In vivo, fibronectin is strongly upregulated in the heart after MI (Froen and Larsen 1995; Knowlton et al. 1992; Willems et al. 1996). We have hypothesized that fibronectin serves as an attractant for ASCs, thereby improving stem cell homing, attachment and proliferation. Therefore, we have studied the kinetics and the localization of fibronectin accumulation in the human heart after MI in detail, in heart sections from 35 patients, representing various infarction periods.

Materials and methods

Adipose tissue donors

Human subcutaneous adipose tissue samples were obtained as waste material after elective surgery and donated upon informed consent of the patients from various clinics in Amsterdam, The Netherlands. Adipose tissue was harvested from the abdomen or hip and thigh region by using either resection or tumescent liposuction. Five donors (age-range: 27–49 years) were included in this study.

Isolation of the stromal vascular fraction of adipose tissue

Adipose tissue was stored in sterile phosphate-buffered saline (PBS) at 4°C and processed within 24 h after surgery as described previously (Oedayrajsingh-Varma et al. 2006). In brief, resected material was minced by using a surgical scalp before processing. After extensive washes with PBS, the ECM was enzymatically digested with 0.1% collagenase A (Roche Diagnostics, Mannheim, Germany) in PBS containing 1% bovine serum albumin (BSA; Roche Diagnostics) under intermittent shaking for 45 min at 37°C. Then the material was washed with Dulbecco's modified Eagle's medium (DMEM-glucose; BioWhittaker, Cambrex, Verviers, Belgium) containing 10% fetal bovine serum (FBS) and centrifuged for 10 min at 600g. The cell pellet was resuspended in PBS and passed through a 200- μ m mesh (Braun/Beldico, Marche-en-Famenne, Belgium) to obtain a single cell suspension. To remove contaminating erythrocytes, the cells were subjected to Ficoll density centrifugation (lymphoprep, $\rho=1.077$ g/ml, osmolarity: 280 ± 15 mOsm; Axis-Shield, Oslo, Norway). The cell-containing interface

was harvested and washed with DMEM containing 10% FBS. Cells were frozen under “controlled rate” conditions and stored in liquid nitrogen until needed in experiments.

Cell culture

Cells from the stromal vascular fraction were seeded at 1×10^5 cells/cm² and cultured for several passages in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Invitrogen, Calif., USA; normal culture medium), in a humidified atmosphere of 5% CO₂ at 37°C. Media were changed twice a week. When reaching 80%–90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Gibco, Invitrogen) for 5 min at 37°C and replated.

Attachment of ASC

To investigate attachment of ASC to different coatings, culture-expanded cells (passages 3–6; p3–6) were seeded in a 96-well culture plate at a density of 25,000 cells per well. Wells were either uncoated or coated with fibronectin (5 or 0.5 µg/cm²; Roche Diagnostics), laminin (0.12 µg/cm²; Roche Diagnostics) or collagen (2.5 µg/cm², rat tail collagen type I; BD Biosciences, Bedford, Mass., USA). Cells were then allowed to attach undisturbed in a humidified incubator (5% CO₂, 37°C) for different incubation times (5 min, 10 min, 30 min), after which unattached cells were removed by washing with PBS. To standardize the conditions, all the conditions at the same timepoint were added simultaneously to one microplate. The number of attached cells was quantified by assaying for DNA with a CyQUANT Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer’s protocol.

To achieve an *in vitro* situation more comparable to the *in vivo* situation after AMI, attachment of ASC to cardiac muscle cells and to fibronectin-coated cardiac muscle cells was investigated. Cardiomyoblasts (H9c2, p18; ATCC, Manassas, Va., USA) were seeded into 24-well culture dishes, at a density of 10,000 cells per well, and when wells were 70% confluent, cells were either coated with fibronectin (0.5 or 5.0 µg/cm²) or remained uncoated. Then culture-expanded cells (p3–p7) from four donors were seeded into the culture dishes, at a density of 40,000 cells per well. Cells were allowed to attach undisturbed in a humidified incubator (5% CO₂, 37°C) for various incubation times (5 min, 10 min, 30 min), after which the unattached cells were removed by washing with PBS. The cells were subsequently trypsinized, washed, centrifuged at 600g for 5 min, resuspended in 50 µl PBS and incubated with anti-CD90 phycoerythrin-labelled antibody (1:20; BD Biosciences Pharmingen, San Diego, Calif., USA) for 30 min on ice. The percentage of CD90-positive cells (ASC)

was quantified by using fluorescence-activated cell sorting (FACS) analysis (FACScaliber, Becton Dickinson, San Jose, Calif., USA).

Proliferation of ASC

To assess ASC proliferation on fibronectin-coated culture dishes, culture-expanded cells (p3–p6) from three donors were plated into 6-well culture plates, at a density of 25,000 cells per well, either coated with 5 µg/cm² fibronectin or uncoated, in normal culture medium. After a 1-day incubation in a humidified incubator (5% CO₂, 37°C), unattached cells were removed by washing with PBS. Cells were then allowed to proliferate for various incubation times (0 days, 3 days, 6 days, 9 days). Cells were washed with PBS to remove dead cells. The amount of living cells removed by this washing was negligible (<0.5%). The number of attached cells was quantified by assaying for DNA with a CyQUANT Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer’s protocol.

Myocardial tissue from infarction

Myocardial tissue from 27 autopsied patients who had died from AMI and from eight autopsied patients with no cardiac lesions of any kind was obtained from the Department of Pathology for autopsy, with approval of the ethics committee of the VU Medical Centre, Amsterdam. The use of left-over material after the pathological examination is completed, is part of the standard patient contract in our hospital. Heart tissue was sampled as soon as possible, within 24 h of death.

Characterization of infarction phase

Recent AMI was defined by using macroscopic (lactate dehydrogenase decolouration) and microscopic criteria to estimate infarct duration and the viability of cardiomyocytes in all myocardial tissue specimens (paraffin-wax-embedded sections stained with haematoxylin and eosin). Clinical data with respect to the duration of the infarction corresponded to the time intervals of the various morphological phases of infarction. Jeopardized myocardium was characterized by the intensity of eosinophilic staining of involved myofibres, cell condensation, loss of nuclei and cross striation and was visualized by complement (C3d) staining. We defined heart tissue sections without microscopic changes but with macroscopic lactate dehydrogenase decolourization as a phase 1 infarct (3– to 12-h-old AMI), infiltration of polymorphonuclear leucocytes as a phase 2 infarct (12-h to 5-day-old AMI) and infiltration of lymphocytes and macrophages and fibrosis as a phase 3

Table 1 Characterization of the different infarct phases used in the immunohistochemical study (*LDH* lactate dehydrogenase)

Infarction phase	Number of patients	Infarct duration	Definition
0	8	No infarction	Does not apply
1	11	3–12 h	LDH decolourization, without extravascular neutrophilic granulocytes.
2	9	12 h–5 days	Extravascular neutrophilic granulocytes.
3	7	5–14 days	Granulation tissue formation

infarct (5– to 15-day-old AMI; Krijnen et al. 2003). Table 1 shows the distribution of the various infarct phases among the different patients.

Immunohistochemistry

Myocardial tissue was fixed with 4% buffered formaldehyde and embedded in paraffin. Paraffin sections (3–4 μm) were mounted on glass slides, dried, deparaffinized, rehydrated, and then used for immunohistochemistry. Sections were stained for fibronectin. Serial sections were stained for complement factor C3d to define the microscopical infarction area (Krijnen et al. 2003). All antibodies used were from DakoCytomation (Glostrup, Denmark).

For C3d staining, antigen retrieval was performed by boiling sections in 10 mM sodium citrate buffer, pH 6.0, for 10 min in a microwave oven. Sections were pre-incubated with normal swine serum (1:10; DakoCytomation) for 10 min at room temperature, followed by an incubation with rabbit antibodies against human C3d (1:1,000) for 1 h at room temperature. The sections were then washed with PBS and incubated with biotin-conjugated swine-anti-rabbit antibodies (1:300) for 30 min, washed in PBS and incubated with streptavidin-biotin complex/horseradish peroxidase (1:200) for 1 h. Staining was visualized by using 3,3'-diaminobenzidine (DAB; 0.1 mg/ml, 0.02% H_2O_2).

For fibronectin staining, antigen retrieval was performed by incubating the sections with 0.1% pepsine-HCl for 30 min at 37°C. Sections were incubated with rabbit antibodies against human fibronectin (1:18,000) for 1 h at room temperature, followed by incubation with 100 μl envision (ready for use kit, anti-Mouse IgG and anti-Rabbit IgG) for 30 min at room temperature. Staining was visualized with envision DAB (DakoCytomation). Finally, the sections were counterstained with haematoxylin, dehydrated and cover-slipped. Control sections were incubated with PBS instead of the primary antibody.

All slides were judged and scored for infarct age and anatomical localization of fibronectin and C3d, as visualized

by immunohistochemical staining. The border-zone of the infarct was defined as the area surrounding the microscopic infarction area, as characterized by C3d staining. Since only the fibronectin on the plasma membrane and in the ECM are available for ASC attachment, fibronectin deposition was scored separately for the ECM, plasma membrane and intracellular components (nucleus and cytoplasm). The extent of the fibronectin deposits was determined by quantifying the mean surface area occupied by cardiomyocytes positive for fibronectin and expressed as a percentage of the total surface area of the sections in the infarcted region, border-zone and non-infarcted areas. Areas were scored as negative (0), 1%–5% of the cells positive (1), 6%–25% positive (2), 26%–50% positive (3), 51%–75% positive (4) or 76%–100% positive (5). Since positivity of fibronectin in the ECM is difficult to quantify, fibronectin staining in ECM was related to its intensity score as follows: negative (0), minor positive (1), intermediate positive (2) or strongly positive (3).

Statistics

Statistics were performed with the SPSS statistics program (Windows version 9.0) and Graphpad Prism (version 4). Attachment and proliferation data were analysed by using Repeated Measures. Fibronectin accumulation was analysed by the Kruskal Wallis and the Dunn's Multiple Comparison Test. A *P*-value of less than 0.05 was considered to be significant.

Results

Attachment and proliferation of ASCs on fibronectin

To investigate the attachment of ASC to various ECM molecules, the percentage of ASCs adhering to laminin, fibronectin, collagen-1 and uncoated culture wells was analysed. We monitored the number of ASCs in the culture wells and compared this with the numbers of cells in uncoated culture wells. As shown in Fig. 1, after 5 and 10 min of incubation, significantly higher numbers of ASC were detected on fibronectin-coated culture wells as compared with the uncoated culture wells ($P < 0.05$). This effect was dose-dependent, since a lower concentration of fibronectin (0.5 $\mu\text{g}/\text{cm}^2$ instead of 5.0 $\mu\text{g}/\text{cm}^2$) did not increase ASC attachment. Laminin and collagen-1 did not significantly affect ASC attachment, although collagen-1 showed a trend towards increased attachment of ASC. After 30 min, a plateau level seemed to be reached for all conditions. This suggested that ASC adhered more rapidly to fibronectin than to laminin, collagen or uncoated culture wells.

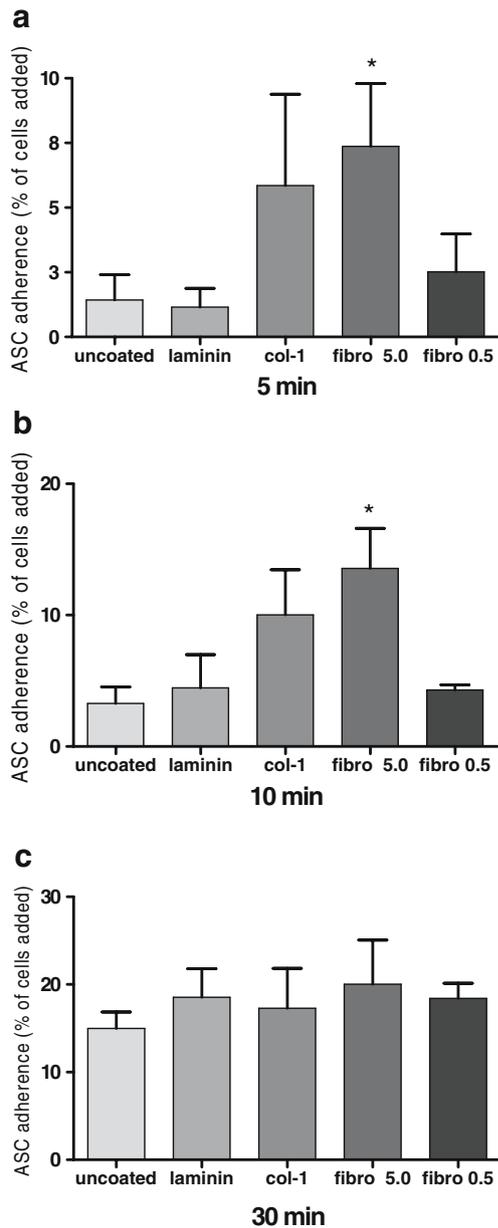


Fig. 1 ASC adherence to laminin-coated, collagen-1 (*col-1*)-coated and fibronectin (*fibro*)-coated (5 and 0.5 $\mu\text{g}/\text{cm}^2$) and uncoated culture wells monitored with time. Numbers of adhered ASCs were quantified after 5 (a), 10 (b) and 30 (c) min of attachment. Results are expressed as the percentage of applied cells. Error bars represent means \pm SEM ($n=4$, $*P<0.05$, Repeated measures)

To investigate the kinetics of ASC attachment in a setting more comparable to the *in vivo* situation after AMI, the percentage of ASCs adhering to fibronectin-coated and uncoated cardiac muscle cells was monitored with time. Numbers of adhered ASCs were quantified after 5, 10 and 30 min of attachment and are shown as the percentage of total cells applied in Fig. 2. ASC rapidly adhered to the fibronectin-coated cardiac muscle cells. This rapid attachment was most prominent after 5 min of incubation, when

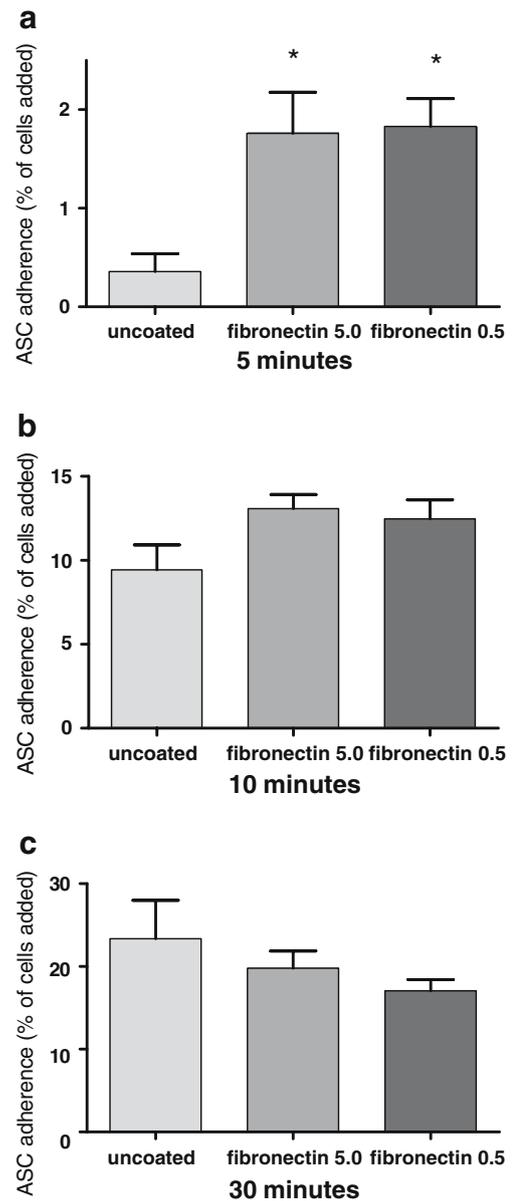


Fig. 2 Kinetics of ASC adherence to fibronectin-coated (5 and 0.5 $\mu\text{g}/\text{cm}^2$) and uncoated cardiac muscle cells monitored with time. Numbers of adhered ASCs were quantified after 5 (a), 10 (b) and 30 (c) min of attachment. Results are expressed as the percentage of applied cells. Error bars represent means \pm SEM ($n=4$, $*P<0.05$, Repeated measures)

5 times more ASC attached to the fibronectin-coated cells than to the uncoated cells ($P<0.05$). This effect was independent of the dose of fibronectin, since the ASC also attached rapidly to the cardiac muscle cells coated with a lower concentration of fibronectin (0.5 $\mu\text{g}/\text{cm}^2$ instead of 5.0 $\mu\text{g}/\text{cm}^2$). On the uncoated cardiac muscle cells, however, attachment was limited when compared with the fibronectin-coated cells and increased only gradually the first 10 min. For all conditions, a plateau level was reached after 30 min of attachment. These findings suggested that

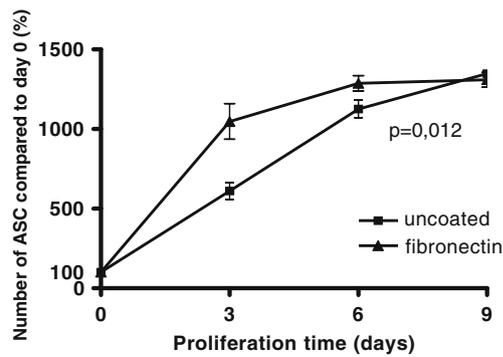


Fig. 3 Kinetics of ASC proliferation on fibronectin-coated ($5 \mu\text{g}/\text{cm}^2$) and uncoated culture wells. Numbers of adhered ASCs were quantified after 0, 3, 6 and 9 days of culture by using the CyQUANT cell proliferation assay. Results are expressed as percentage of cells, compared to day 0, both for the fibronectin-coated and uncoated wells. Error bars represent means \pm SEM. ($n=3$, $p=0.012$, Repeated measures)

ASC attached more rapidly on fibronectin-coated cardiac muscle cells than to uncoated cardiac muscle cells.

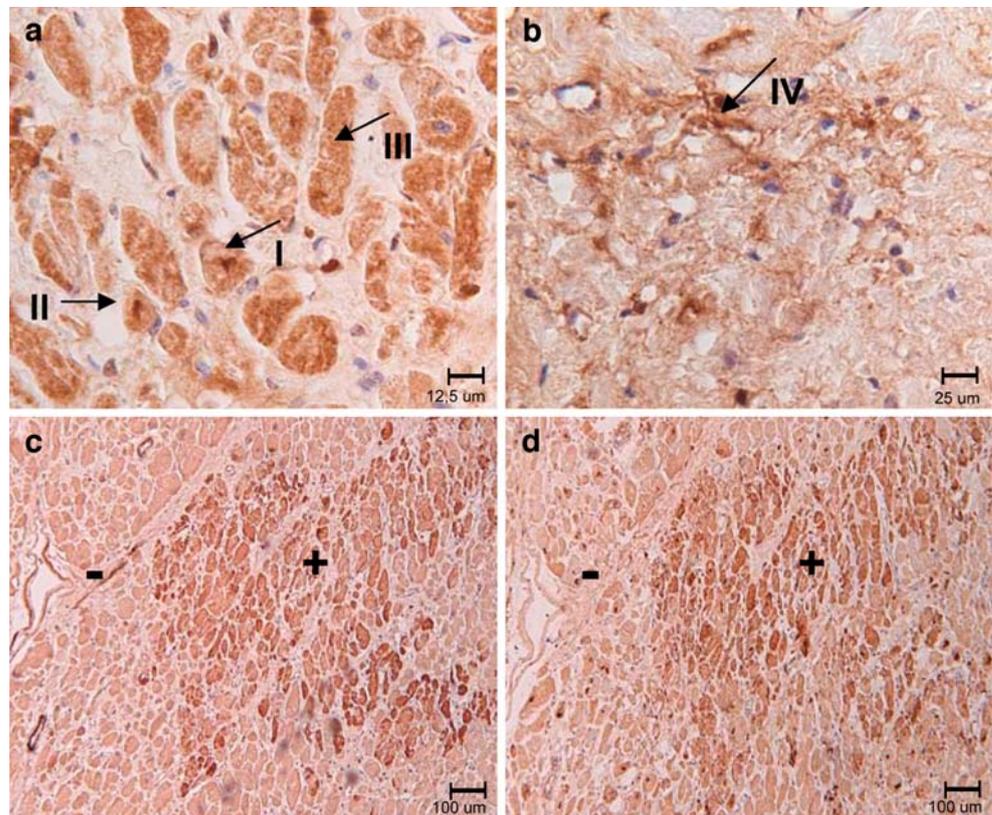
To study the proliferation of ASCs on fibronectin, ASCs were seeded in fibronectin-coated culture wells. Unattached

cells were washed away after 24 h (day 0). We monitored the number of ASCs in the culture wells with time (days 0, 3, 6, 9) and compared this with the numbers of cells in uncoated culture wells. Significantly higher numbers of ASC were detected on fibronectin-coated culture wells compared with the uncoated culture wells during the 9-day culture period ($P=0.012$, Repeated measures, $n=3$; Fig. 3).

Fibronectin accumulation after infarction

Fibronectin accumulation after myocardial infarction was investigated in heart sections from 35 patients, representing various infarction periods (no infarction, infarction of 3–12 h old, 12 h–5 days old, and 5–14 days old). To define jeopardized cardiomyocytes and thus the microscopical infarction area, we stained serial sections for complement factor C3d (Fig. 4c). Fibronectin was found not only in cardiomyocytes (plasma membrane, cytoplasm and nucleus; Fig. 4a), but also in the ECM (Fig. 4b). Fibronectin colocalized with complement factor C3d but fibronectin deposition was also found in the border-zone adjacent to the infarction area (complement-negative). To analyse

Fig. 4 Localization of fibronectin in myocardial infarction of the human heart. Fibronectin accumulation after myocardial infarction was investigated in heart sections representing different infarction periods. **a** High power view of fibronectin deposition in infarct phase 2. Fibronectin depositions were found on the plasma membrane (arrow I), in the nucleus (arrow II) and in the cytoplasm (arrow III) of jeopardized cardiomyocytes. **b** Fibronectin depositions in infarct phase 3 in the extracellular matrix (ECM; arrow IV). **c** C3d deposition in infarct phase 2 (+ jeopardized cardiomyocytes, i.e. complement-positive cells, – complement-negative cells). **d** Fibronectin colocalization within C3d-positive areas in a serial section from the same patient as in **c**, in infarct phase 2. As a control, AMI sections were incubated with PBS instead of mAb and gave no positive staining (not shown)



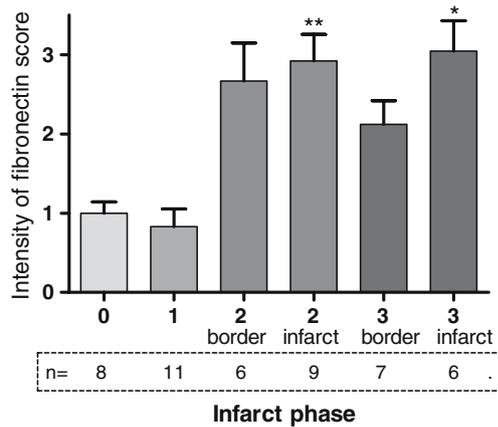


Fig. 5 Fibronectin deposition in the ECM of the human heart after myocardial infarction. Intensity of the fibronectin deposition in the ECM was scored and compared with that of the non-infarcted heart (stadium 0). Areas were scored negative (0), minor positive (1), intermediate positive (2) or strongly positive (3). Error bars represent means±SEM (* $P<0.05$, ** $P<0.01$)

fibronectin depositions in the human heart after myocardial infarction, we scored all sections for the extent and the anatomical localization of fibronectin depositions. Notably, in AMI phase 1, the border-zone and infarcted area could not be analysed as such with the slides from the macroscopical infarction area, since it was not possible to identify a microscopical infarction area clearly at this early phase, as complement was then negative. As shown, the intensity of fibronectin depositions in the ECM (Fig. 5) was significantly increased at the site of myocardial infarction of AMI phases 2 ($P<0.01$) and 3 ($P<0.05$) compared with the uninfarcted heart. The extent of fibronectin deposition was also increased in the border-zone of phases 2 and 3; however, this increase was not statistically significant. In phase 1 infarction and in the uninfarcted areas, no increase in ECM fibronectin intensity score was found, when compared with uninfarcted hearts (not shown).

We subsequently scored fibronectin positivity in the cardiomyocytes. The extent of fibronectin deposition was significantly increased in the infarcted area of AMI phase 2, compared with controls, at the plasma membrane ($P<0.05$), in the cytoplasm ($P<0.001$) and in the nucleus ($P<0.05$). Fibronectin depositions were increased not only in the infarcted area, but also in the border-zone of phase 2; however, this increase was only statistically significant in the nucleus when compared with the non-infarcted heart. In AMI phase 3, no cardiomyocytes in the infarction area could be analysed since this area no longer contains cardiomyocytes in this stadium, but only granulation tissue. The extent of fibronectin deposition was still significantly

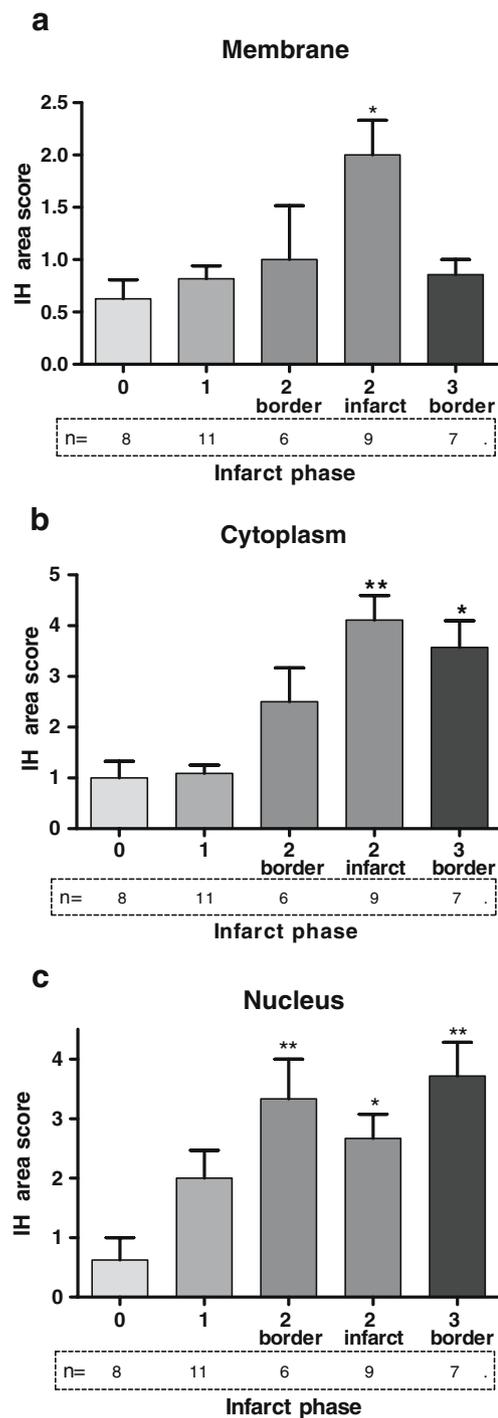


Fig. 6 Fibronectin deposition in cardiomyocytes in the human heart after myocardial infarction (IH immunohistochemical). **a** Area of fibronectin deposition in the plasma membrane. **b** Area of fibronectin deposition in the cytoplasm. **c** Area of fibronectin deposition in the nucleus. Areas are scored from 0 for no fibronectin-positive cells up until 5 for 75%–100% fibronectin-positive cells. Error bars represent means±SE (* $P<0.05$, ** $P<0.01$) (Phase 0: $n=8$, phase 1: $n=11$, phase 2 border: $n=6$, phase 2 infarct: $n=9$, phase 3 border: $n=7$)

increased in the border-zone of AMI phase 3 in the nucleus and cytoplasm but was not positive on the plasma membrane (Fig. 6a). Neither in AMI phase 1 (Fig. 6) nor in non-infarcted areas (not shown) was an increase in fibronectin positivity found in the cardiomyocytes when compared with controls (Fig. 6).

Discussion

We hypothesized that accumulation of fibronectin in the heart after MI might improve the fate of ASC after transplantation in the heart. Therefore, we have studied the effect of this protein on the attachment and proliferation of ASC in vitro. In addition, we have investigated the time course and the localization of the accumulation of fibronectin after myocardial infarction in the human infarcted heart. We have found that ASC attachment and proliferation on fibronectin-coated plates is significantly increased compared with that on uncoated plates (early attachment: $P < 0.05$, proliferation: $P = 0.009$, Repeated measures, $n = 4$) and that attachment of ASC to cardiac muscle cells is significantly increased when cells are coated with fibronectin ($0.5 \mu\text{g}/\text{cm}^2$ and $5.0 \mu\text{g}/\text{cm}^2$), after 5 min of attachment. In the ECM of the human heart, fibronectin depositions are significantly increased at AMI phase 2 and phase 3 in the infarction area, whereas fibronectin deposition in the cytoplasm, at the plasma membrane and in the nucleus of cardiomyocytes is significantly increased at AMI phase 2, but not in AMI phase 3, except for depositions in the nucleus.

When culture wells are coated with fibronectin, we have found an increased attachment of ASC when compared with uncoated plates, even though ASC are mesenchymal stem cells that are defined by their strong attachment to plastic culture wells (Wagner et al. 2005). We have also found that neither laminin nor collagen-1 significantly increases the attachment of ASC, unlike fibronectin, which does cause an increase. These findings are comparable with studies of Ogura et al. (2004) and Cool and Nurcombe (2005), in which an increased attachment of human bone-marrow-derived MSC to fibronectin-coated culture plates has been found when compared with albumin-coated or other ECM-coated culture plates. Ogura et al. (2004) have also reported that attachment to fibronectin is most prominent within the first 20 min of incubation. We now have shown that fibronectin also improves the attachment of ASC.

In an experiment mimicking the in vivo situation after a myocardial infarction, we have demonstrated that fibronectin also increases the attachment of ASC to cardiac muscle cells, even when a relatively low concentration of fibronectin is used. These results are in agreement with our

hypothesis that fibronectin depositions will also increase stem cell attachment in vivo, independent of fibronectin concentration, underlining the importance of fibronectin in stem cell transplantation.

In this study, we have additionally demonstrated a significant increase in the proliferation of ASC cultured in fibronectin-coated wells, compared with proliferation in uncoated culture wells ($P = 0.012$). This effect is most prominent during the first 5 days of culture; thereafter, the cells grow confluent and the effect diminishes. Cool and Nurcombe (2005) have found no significant effect of fibronectin on proliferation, although a trend is visible; this might be caused by the timepoint of their study, since they have only measured the difference in cell numbers at day 7, or could be related to the source of stem cells.

Since the presence of fibronectin promotes ASC attachment and proliferation in vitro in our study, we have hypothesized that the presence of fibronectin in the heart after myocardial infarction might positively affect stem cell adhesion and proliferation at the site of injury. As such, stem cell therapy would be most efficient when fibronectin depositions are high. We have found an increase in the extent of fibronectin depositions in cardiomyocytes during AMI phase 2, whereas fibronectin deposition in the ECM is significantly increased in the infarction area during AMI phase 2 and phase 3, when compared with the uninfarcted heart.

These results are comparable with the findings of several animal studies, in which increased fibronectin mRNA expression has been demonstrated, together with increased fibronectin deposition in the infarcted area as early as the first day after infarct induction (Carlyle et al. 1997; Froen and Larsen 1995; Knowlton et al. 1992; Shekhonin et al. 1990; Ulrich et al. 1997). Moreover, several studies have shown increased deposition of fibronectin in the infarction area after MI in human (Hu et al. 1996; Ishikawa et al. 2000, 2003; Ortmann et al. 2000; Willems et al. 1996). However, none of these studies have scored the fibronectin depositions as accurately as we have during the different infarction phases, i.e. by using complement to define the microscopic infarction area, by differentiating between infarction area and border-zone and by separately scoring the ECM and the various anatomical locations in the cells.

We have studied the various anatomical locations of fibronectin depositions separately, since theoretically only fibronectin on the plasma membrane and in the ECM should play a direct role in stem cell attachment. Although fibronectin has been detected on the ECM and the plasma membrane of cardiomyocytes, depositions in the cytoplasm and the nuclei are more widespread than on the plasma membrane. These strong depositions in the nucleus and cytoplasm have also been reported by Ishikawa et al. (2000);

however, they have only described these nuclear depositions and not scored them.

Notably, we have found that the extent of fibronectin depositions in the heart is only increased in the infarcted area and the border-zone, and not in the non-infarcted areas. This is an important observation, since stem cell attachment and differentiation is only desirable in the infarcted area and the area surrounding it for stem cell therapy. In accordance with this finding, Tran et al. (2006) have indeed shown that rat bone marrow MSC are retained better in the infarcted area, when compared with the intact myocardium.

Several other studies have also investigated the time frame after MI with respect to when best to apply stem cell therapy, both in animal models and human stem cell transplantation studies, by investigating molecular events and stem cell retention at the site of infarction (Bermejo et al. 2006; Lu et al. 2004; Ma et al. 2005). These studies suggest that stem cell therapy should neither be applied during the acute inflammation phase (within 1 day after infarction), nor after a period longer than 2 weeks when scar tissue has been formed. In agreement with this suggestion, our results indicate that a putative effect of fibronectin on stem cell therapy can only be achieved when stem cell therapy is applied more than 12 h but within 14 days after infarction.

In conclusion, we have shown, for the first time, that fibronectin improves ASC attachment and proliferation. To achieve a positive effect of fibronectin on stem cell therapy after AMI in the heart, this therapy should be applied when fibronectin depositions in the heart are high. We have accurately scored fibronectin deposition in the human heart after AMI and found that the fibronectin intensity score significantly increases from 12 h after MI in both the infarction area and the border-zone.

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