Experimental ASD closure using autologous cell-seeded interventional closure devices

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Abstract

Objective: Atrial septal defect (ASD) occluders are permanent implants in paediatric cardiology which serve as mechanical shields until complete overgrowth and incorporation of the occluding device by autologous tissue has occurred. Thereafter, the foreign body material making up the device is dispensable and bears potential long-term adverse effects. Rapid, firm and complete incorporation into the atrial septal wall should be a prerequisite for biodegradable devices. In this study, the feasibility of using autologous cell-seeded devices was investigated by (a) testing the influence of a collagen coating on cellular stress resistance in vitro and (b) comparing the short-term effects between cell-seeded, collagen-coated and acellular ASD occluders in vivo. Methods: Native and collagen-coated Dacon fabrics and Starflex-devices were pre-seeded with autologous fibroblasts (skin biopsy) and evaluated using various mechanical stress tests. In a sheep model interventionally created ASDs were closed using either autologous pre-seeded or conventional (acellular) Starflex-devices. Results: ASD closure devices were successfully pre-seeded with autologous cells. The incubation period needed, the cellular density achieved and the mechanical stability of the cytolayer after mechanical stressing (implantation) were improved by applying a collagen matrix on the fabric. Compared to the thin layer of ingrown tissue seen on conventional occluders after 30 days in vivo, a thicker layer of organising, newly formed granulation tissue on pre-seeded collagen-coated devices embedded not only the Dacron fabric, but also completely covered the spring arms of the device underneath a layer of neo-endothelium. Conclusion: Autologous cell pre-seeding of interventional closure devices is feasible since the cells survive the mechanical stress encountered during implantation. Rapid, firm and complete ingrowth of occluder devices into a thicker layer of young fibrous granulation tissue can be achieved, but an increased thrombogenicity currently limits the in vivo application. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

During recent years the interventional closure of atrial septal defects (ASDs) has become an attractive alternative to surgical therapy, obviating the need for a thoracotomy (scar) and cardiopulmonary bypass in up to 70% of patients [1]. Various devices for interventional occlusion of these defects have been developed [2–12]. Despite considerable difference of the implant designs, all devices consist of a metal framework and a synthetic fabric. After implantation of the devices, ingrowth of tissue from the defect periphery and endothelialisation occurs. Studies in experimental animals have characterised the histological features and time course for the device ingrowth up to 2 years after implantation [2,4,8,9]. Incomplete and delayed incorporation of devices into the surrounding atrial septal tissue result in a potential for residual defects, infections, thromboembolism, friction lesions or fractures of the framework. A matter of concern is that these devices are implanted into children with a life expectancy of up to 70 years or more. The potential long-term consequences

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related to the implantation of foreign body material into the growing and moving (beating) heart of a young child for his or her future adult life remain uncertain. However, the device is no longer necessary once the defect is closed and covered by a complete and firm layer of ingrown, endogenous “repair” tissue. Moreover, the foreign material making up the device, contrary to “biological implants”, lacks the ability to grow along with the hearts of young recipients and to undergo a remodelling according to the bodies needs and thus may be potentially harmful.

For a number of tissues (e.g., skin, cartilage, bone) there are now in-vitro cultured cell-, tissue- and matrix-constructs under clinical evaluation [13–15]. The perspective of a new generation of “biological devices” which act temporarily and disappear as soon as autologous tissue has taken over its stabilising function could therefore be a promising approach for future cardiovascular devices [16–24]. A prerequisite for any resorbable or “biodegradable” septal occluder as a temporary mechanical device is therefore, that rapid, firm and complete overgrowth by endogenous tissue occurs which subsequently takes over the permanent occluding function.

The aim of this study was to evaluate whether this prerequisite could be achieved using devices pre-seeded with autologous cells. In addressing this question, the work was divided into two sub-studies: The interventional therapy of atrial septal defects requires the use of “compressible” devices which can be delivered through small size sheaths or catheters. Thus mechanical stress resistance tests of cell-seeded devices and fabrics were performed, comparing the pre-seeding of collagen-coated devices with a cell-seeded non-coated control. The second objective was to compare the in situ, biological short-term characteristics of such pre-seeded occcluders with conventional acellular devices after implantation into the atrial septal wall.

2. Methods

2.1. Cell isolation and culture, pre-seeding of devices and viability test

Human fibroblasts were isolated from foreskin tissue obtained from circumcision operations. Fibroblasts of young sheep (35–40 kg) were derived from small skin biopsies (~0.25 cm²) taken from the groin of the individual animals during the ASD creation procedure (see below). The tissue was cut into cubes of 2–3 mm³, placed into a T-75 culture flask (Becton Dickinson, NJ, USA) and incubated in DMEM medium containing antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml; Gibco Life Technologies, Karlsruhe, Germany) and 10% fetal calf serum (FCS, Gibco) at 37°C in a humidified atmosphere of 95% air–5% CO₂. Medium was changed twice a week. Outgrowth of cells was observed starting at day 3 of culture. Confluent cultures were subcultured using trypsin. Only first and second passage cells were used in the experiments described. Cell-seeded fabrics and devices were cultured under the same conditions. One occluder device used in the in animal studies was pre-seeded with keratinocytes and fibroblasts. This additional experiment served to further discriminate morphologically between pre-seeded cells and ingrown tissue from the defect edge. The autologous keratinocytes were also derived from a skin biopsy using standard techniques [15,25–27].

Cellular viability was assessed using a commercially available fluorescence test (Live/dead viability/cytotoxicity assay, Molecular Probes). This test discriminates between vital and damaged or apoptotic cells by measuring intracellular esterase activity (calcein) and plasma membrane integrity (ethidium homodimer) as described elsewhere [28–31]. The cellular viability index expressed in percentage was calculated as the ratio of vital cells to total cell number at the endpoint of each experiment.

2.2. Stress resistance after simulated implantation of pre-seeded devices in vitro

Two groups of STARFlex® devices (Nitinol Medical Technologies, Boston, MA, USA) were used with either native fabric or fabric precoated with a thin layer of collagen (Vitrogen 100, bovine type I collagen; Collagen, Palo Alto, CA, USA). The chilled collagen solution (4°C) was diluted with phosphate-buffered saline and neutralised with 0.1 M NaOH to a final concentration of 2 mg/ml and a pH of 7.4. Gelation (fibrillogenesis) of suffused devices occurred at 37°C overnight. The collagen sealed the mesh network of the fabric and adhered well to the surface. Both, native and collagen-coated devices were pre-seeded on both surfaces with human fibroblasts (4±0.8×10⁶ cells) for 4 days. To simulate device implantation in vitro, pre-seeded Starflex devices were taken from the culture medium, loaded into the delivery system [32,33] and flushed with saline. Thereafter, the delivery catheter was advanced through a long sheath, the distal umbrella opened in a culture medium filled basin, (re-) positioning procedures were simulated and then the proximal umbrella opened and the device released into the medium. The total device manipulation time took on average 6 min. Immediately after the simulated implantation and after 4, 8 and 12 days of continued culture the fabric was carefully removed from both sides of the devices for determination of cellular viability (n=4 per group).

2.3. Mechanical stressing of cell-seeded fabrics by applying compressive force

Cell-seeded fabrics were exposed to compressive force to confirm and extend the above stress resistance tests. The
dose–response relationship and time–course of cell damage was established by application of defined mechanical force. Native and collagen-coated Dacron fabrics identical to those used for Starflex devices (knitted polyester, 41 wales/in. thickness 0.006 in. size 1 × 1 cm) were seeded with human fibroblasts (1 in. = 2.54 cm). Mechanical force was applied to subconfluent cell-seeded Dacron fabrics which had been cultured for 5 days (n = 4 per group and treatment). A square of fabric was placed onto the inner surface of a plastic cylinder (inner diameter 10 mm, wall thickness 2.5 mm) with the cytotlayer pointing towards the lumen. The cylinder was then immersed in a basin filled with cell culture medium and a deflated 12 mm balloon angioplasty catheter (Opta, Cordis Europe, Roden, The Netherlands) was advanced into the inner lumen of the cylinder carefully avoiding any abrasive contact with the cytolayer and placed centrally over the fabric. The balloon catheter was inflated to a pressure of 1 to 6 atm for least one arm of the device was in contact with the cytolayer and placed centrally over the fabric. Delivery catheter and sheath were then withdrawn from the cylinder and the pre-seeded fabric removed for determination of cellular viability. Cellular viability was assessed microscopically as described above (fluorescent live/dead assay) and expressed as a percentage of living cells per cm² of fabric. The experiment was repeated twice with similar results.

2.4. Animal model/defect creation

ASDs were created in young sheep (35 kg, n = 13) by transseptal puncture and subsequent balloon dilatation of the interatrial septum [2] under fluoroscopic control. Anaesthesia was introduced by an intravenous bolus infusion of 1.5 mg/kg propofol and 4 mg/kg carprofen. Animals underwent endotracheal intubation and were mechanically ventilated with isoflurane and oxygen–room air. ECG, heart rate, respiratory rate and end-tidal CO₂ were monitored throughout the procedure. After puncture of the right femoral vein a transseptal needle (Cook, West Lafayette, IN, USA) was advanced through a 7F Mullins long sheath (Medtronic AVE Ireland, Galway, Ireland) to gain access to the left atrium via a transseptal puncture. A defect within the interatrial septum was created by balloon dilatation of the interatrial septum using balloon diameters of 4, 8 and 12 mm (Opta, Cordis Europe) followed by a 20 mm balloon catheter (Balt, Montmorency, France) using a guidewire inserted into the left atrium or a pulmonary vein. Heparin (4,000 IE after transseptal puncture) and antibiotics (fluclxacillin 1.5 g at the end of the procedure) were administered intravenously. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The investigations were approved by the local governmental animal ethics committee.

2.5. Device implantation/defect closure

After allowing for healing of the ASD edges (2–3 weeks), animals were divided randomly into two groups. Defect closure was performed using either native (=acelular, without collagen coating) or autologous cell-seeded, collagen-coated Starflex devices [32,33]. Animals were anaesthetised as described above. Prior to defect occlusion balloon sizing of the defect was performed (Arrow, Reading, PA, USA) and heparin (4,000 IE i.v.) was administered. A 23 mm Starflex device was then loaded into the delivery system and advanced percutaneously through a 10F long sheath (Cook) via the femoral vein and across the ASD into the left atrium. The distal umbrella was opened by advancing the delivery catheter through the sheath. Delivery catheter and sheath were then gently retracted together until fluoroscopy showed that at least one arm of the device was in contact with the interatrial septum. When the position appeared acceptable, the right atrial umbrella was deployed by retraction of the sheath with the device/delivery catheter held in place. After release of the device, sheath and delivery catheter were pulled back and a contrast angiogram was performed to test for significant residual shunting. Before device implantation antibiotics were given (fluclxacillin. 1.5 g i.v.). Animals received no anticoagulation during the follow-up period.

2.6. Device explantation/macrosopic and histological examination

Animals from both groups were sacrificed 4 days (native/acelular group n = 2, collagen-coated and pre-seeded group n = 2), 12 days (collagen-coated and pre-seeded group n = 3) and 30 days (native/acelular group n = 3, collagen-coated and pre-seeded group n = 3) after defect closure. Prior to sacrifice device position was confirmed by contrast injection via the superior caval vein into the right atrium under fluoroscopy control and anaesthesia. Each animal received an intravenous dose of heparin (400 units/kg body weight) to prevent post mortem thrombus formation on the device, followed by a lethal injection of pentobarbital (800 mg according to recommendations by the local animal ethics committee). Complete autopsy was performed by two veterinary pathologists. Specimens from brain, liver, spleen, kidney, gut, salivary glands, intestine and lungs were taken and fixed in 5% buffered formalin for 48 h for histopathological examination. The heart and adjacent vessels were inspected for the gross appearance of the implanted device and then fixed in alcoholic formaldehyde [95% ethanol–formaldehyde (40% gas, w/w) (2:1, v/v)] for 48 h. Representative sections from the right and left atrial wall including the device were taken. The tissue samples were embedded in paraffin wax and stained as follows: general stain and evaluation of cellularity – hematoxylin and
eosin; fibrous tissue – azan stain; fibrin – Ladewig-stain; iron-ions – Turnbull blue stain; neutral mucopolysaccharides, glycoproteins, basement membranes – periodic acid Schiff-reaction (PAS); mesenchymal tissue – vimentin staining with immunhistochemical detection using a murine monoclonal antibody (Dako, Hamburg, Germany, No. M0725); endothelial cells – anti-factor VIII antibody (BioGenex); collagen distribution – Picrosirius red stain; elastic fibres – elastic van Gieson; proliferation assessment – immunhistochemical staining using a Ki67 antibody (Ki67:MiB1, murine monoclonal antibody; Dianova, Hamburg, Germany, No. Dia 505) and cytokeratin – immunohistochemical staining using a CK murine monoclonal pan-cytokeratin antibody (Dako, No M0717). All gross and microscopic specimens were reviewed by two experienced veterinarian pathologists (PW. and M.B.).

2.7. Statistics

Data are given as mean±standard deviation (S.D.). In each analysis, the distribution mode was evaluated by the Kolmogorov–Smirnov test. For comparison of two normally distributed groups, two-tailed, unpaired Student’s t-test was used. p<0.05 was considered statistically significant.

3. Results

3.1. Stress resistance after simulated implantation of pre-seeded devices in vitro

When native Dacron fabrics identical to those used in Starlex devices were seeded with fibroblasts, the cells adhered to the meshwork (Fig. 1a) from where they continued to proliferate when grown in vitro. It took up to 10 days in culture until a continuous cellular layer filled all the spaces between the woven Dacron meshwork (comparable to Fig. 1b and h).

After a simulated implantation, fluorescent viability staining revealed extensive damage to the cytolayer. In addition to a significant surface area of the device being denuded of cells, 61±3% of the remaining cells had lost their viability (red fluorescent staining, Fig. 1c and e). After 5 days of continued culture the extent of cell-free area increased further and almost all vital cells vanished from the device thereafter (Fig. 1g).

In devices impregnated with a matrix of collagen, a higher density of cells was reached immediately after the seeding procedure (Fig. 1b) leading to a confluent cellular layer after only 3–4 days of culture. When these devices were exposed to mechanical stress by simulated implantation, the majority of cells (65±3%) remained on the device and maintained their viability (green fluorescent staining, Fig. 1d). In small areas of the device cells were sheared off and some of the remaining cells on the device lost their viability. However, cells that survived the device implantation stress continued to proliferate in subsequent culture. Fig. 1f shows a subconfluent layer after 4 days and a confluent layer of fibroblasts after 12 days (Fig. 1h) of continuous post-stress culture. In the latter group, cellular density resembled that of a control group not exposed to mechanical implantation stress.

3.2. Cellular viability after mechanical stressing of cell-seeded fabrics by applying compressive force

In order to determine the resistance of fibroblasts seeded onto the Dacron fabrics of native and collagen-coated devices more systematically, cellular viability was studied after exposure to a defined pressure load for variable periods of time. Dose–response curves (Fig. 2) show the decrease of the cellular viability index (defined as percentage of surviving cells) with both increasing pressure (Fig. 2a) and prolonged exposure time (Fig. 2b). The cellular viability index declined from 93±2% (control group) to 72±4% on native fabric when exposed to a pressure of 6 bar for a duration of 10 s. A more marked decrease in cellular viability index to 56±3% was observed on native fabric in case a given pressure (4 bar) was applied for a prolonged period of time (5 min). The respective values were significantly higher on fabrics coated with collagen: 80±3% of pre-seeded cells remained viable after exposure to a compressive force of 6 bar for 10 s (p<0.05 versus non-coated fabrics) and 72±4% of cells survived a pressure of 4 bar for 5 min (p<0.001 versus non-coated fabrics).

3.3. Short-term ingrowth of cell-seeded devices in vivo

The interventionally created ASDs measured between 11 and 14 mm in diameter as determined by balloon sizing immediately before device implantation. Collagen-coated and pre-seeded as well as native Starflex devices were successfully implanted in all animals. The measured device to defect ratio varied from 1.6 to 2.1, with no significant statistic difference between the two groups. Collagen-coating and pre-seeding of devices did not lead to any problem during the loading or deployment of the device, although slightly more force was needed during initial retraction of the device into the loading pod.

All animals survived the defect creation and closure procedures. Postoperatively, there were no premature deaths and no signs of thrombo-embolism, acute infection or other adverse effects were observed in either the cell-seeded or the native control group during follow-up. No signs of systemic or pulmonary venous stenosis were observed in the angiography prior to sacrifice of the animals.

Collagen-impregnated Starflex devices pre-seeded with autologous fibroblasts were followed in parallel to native
Fig. 1. Fluorescence microscopy of vital (green) and damaged or apoptotic fibroblasts (red stain) on a pre-seeded atrial septal defect occluder (Starflex): viable cells after seeding of a native device and 1 day of culture in vitro (a). A higher cellular density is achieved on a fabric pre-coated with collagen after the same amount of time (b). Pre-seeded native fabric immediately after application of mechanical stress by simulated device implantation (c, seen in detail in panel e). The remaining cells expired under continued culture (g). The pre-seeded, collagen-coated fabric immediately after application of mechanical stress by simulated device implantation (d). A significantly increased mechanical stability and density of cells in the pre-cultured cytolayer is observed. Subsequently, after 4 (f) and 12 days (h) of continued culture a confluent cytolayer is re-established. Original magnification ×40 (c, d, h), ×100 (a, b, e–g).
Fig. 2. Cellular viability (% of vital cells) after defined application of compressive force on native Dacron fabrics pre-seeded with human fibroblasts (circles, full lines) and on collagen-coated Dacron fabrics pre-seeded with human fibroblasts (squares, dashed line) as described in Methods. Dose–response curves demonstrate the decrease of cellular viability with enforced exposure to stress for a duration of 10 s (a) and after prolonged exposure time under a pressure of 4 bar (b). The cellular viability after exposure to mechanical stress is better preserved on collagen-coated fabrics. Data are mean±S.D. of four parallel cultures per group and treatment. Statistics (pertain to each point beyond control) by t-test. *, p<0.05; **, p<0.001.

Starflex devices after implantation for ASD closure for up to 30 days in vivo. On gross inspection all devices were macroscopically intact. There were no metal arm fractures in either group and no adverse effects from the centring spring. No compromise of blood flow, pulmonary or systemic veins nor heart valves was observed in any of the animals.

After 4 days in vivo the fabrics of both native and cellular pre-seeded devices adhered to the adjacent atrial wall in most parts of the circumference and could not be lifted freely. In the native device group the fabric was incompletely coated with a thin layer of shiny-reddish material with a fine granular surface. All metal of the spring arms and part of the fabric remained uncovered. Histologically the fragile shiny-reddish material found on the surface of native devices consisted of condensed plasma proteins, leukocytes and erythrocytes. The thickness of material found on the device varied from virtually nil to 0.3 mm. The deposits were covered in some areas by a monolayer of "neo”-endothelium. However, due to section artefacts caused by the loose consistence of the material, the full extent of endothelial coverage could not be determined reliably. At the defect edge activation of endothelial cells, single fibroblastoid cells migrating into the plasmatic components on the device periphery and mild predominantly histiocytic infiltration were observed (Fig. 3a).

By contrast, the collagen-coated, pre-seeded devices exhibited a surface completely embedded in shiny-reddish material. No part of the Dacron fabric nor the metal arm and joints were visible on gross inspection after the same time in vivo. Collagen-coated, cell-seeded devices were cushion-like embedded into a markedly thicker layer (up to 1.4 mm) of plasma proteins, partially degenerated lymphocytes and numerous erythrocytes. The atrial surface was incompletely endothelialized (Fig. 3b). Especially near the central part of the device there was no clear evidence of a continuous endothelial layer. In immediate spatial relation to the Dacron fabric numerous fibroblastoid cells were found in contact with each other and formed a three-dimensional network with the fabric inserted. Some focal areas showed detritus of expired cells. A mild, predominantly histiocytic infiltration containing some macrophages, as seen in the native group, was observed. Some collagen-coated, cell-seeded areas contained young granulation tissue rich of fibro-angioblastic structures. This group showed a significantly higher degree of endothelial cell and local connective tissue cell activation at the atrial septal defect edge. At the periphery, fibroblastoid cells were found in the plasmatic components covering the fabric. The device centre was connected with the interatrial septum by structures of young granulation tissue and thrombotic material. No difference in histology was observed between the right and left atrial umbrella.

Collagen-coated, pre-seeded devices sectioned after 12 days in vivo were in close contact with the interatrial septum. On average, seven of the eight corners of the device were firmly attached to the atrial tissue and could not be lifted with moderate force. All parts of the device, including the metal (spring arms and joint coils), fabric,
corners and edges were incorporated into a greyish tissue of approximately 0.1 cm thickness. This tissue was in continuity with the adjacent endocardium. While the surface of the device periphery was smooth and shining like the surrounding atrial surface, central parts of the device were covered with slightly elevated greyish–yellow tissue which had a glassy, fine granular surface due to numerous tiny, partially coalescing red spots. In the corresponding histology of these cell-seeded devices the periphery of the surface was completely covered, but the central surface remained incompletely covered by endothelial cells after 12 days in vivo. The periphery of the fabric was firmly attached to the adjacent endocardium by fibroblastic granulation tissue with a low amount of newly formed fibres (Azan-stain positive; elastica-stain negative. Data for special staining results not shown). Picrosirius red-stain showed low amounts of type III collagen fibres within the immature granulation tissue. The Dacron fibres were surrounded by a mild granulomatous inflammatory reaction consisting of macrophages (partially hemosiderin-laden, as indicated by Turnbull blue-staining), multinucleated giant cells and lymphocytes. In some areas focally circumscribed necrosis/cellular debris was found close to the Dacron fibres. Central parts of the fabric were attached to the endocardium by immature granulation tissue, condensed plasma protein, erythrocytes, white blood cells, a mild granulo-histiocytic infiltration and a fine, proliferating meshwork of fibroblasts. Vimentin staining showed numerous positive fibroblastic, histiocytic and endothelial cells (capillaries) within deeper parts of the device, indicating well vascularized granulation tissue.

After 30 days in vivo, native Starlex devices were well healed into the defect. The device edges were firmly sealed with the adjacent tissue. The fabric itself was covered with a thin layer of grey–white tissue with a smooth surface. Although most parts of the metal arms, central pin and joints remained visible, all metal parts were covered by a fine glistening layer (Fig. 4a). At the microscopic level, the
Fig. 5. The histology of a collagen-coated, pre-seeded occluder device after 30 days in vivo demonstrates complete endothelial coverage (■) of the ingrown devices in continuity with the adjacent atrial tissue. The mark (□) indicates the boundary of the former defect edge, with native atrial tissue to the left and young repair tissue on top of the occluder device to the right. The dashed line (--) runs along the level of the device fabric. The histology corresponds to the gross appearance of the device in Fig. 4b. Vimentin staining. Original magnification ×125.

The surface of the device was totally covered with a continuous layer of endothelial cells (anti-factor VIII antibody positive). The endocardium at the device margin was slightly thickened compared to the layer on the more central parts of the fabric. A moderate amount of granulation tissue was found to proliferate from the defect rim into central parts of the device. In the latter, the fabric meshwork was penetrated by young immature granulation tissue with a marked amount of ground substance (periodic acid Schiff-reaction positive) and few small blood vessels. In general, the layer of newly formed fibrous granulation tissue on the device appeared slightly thicker on the right atrial aspect than on the left (~0.2 mm vs. ~0.3 to 0.4 mm).

Focally, a mild to moderate granulomatous inflammatory

Fig. 6. Vital, proliferating autologous keratinocytes on the fabric of a device explanted after 30 days in vivo. The collagen-coated occluder was pre-seeded with host fibroblasts and keratinocytes as described in Methods. (a) Overview showing the level of the Dacron fabric (--) and neoeendothelial surface (■) (H/E staining). (b) Detail of keratinocyte tissue. The cells display differentiation towards a non-cornifying, stratified squamous epithelium (cytokeratin staining). Original magnification ×125.
reaction was found surrounding the Dacron. Macrophages, giant cells, few lymphocytes and hemosiderin loaded cells were observed in these areas.

Compared to this native, uncoated control, collagen-coated, cell-seeded devices were found to be embedded in a two- to threefold thicker layer of smooth, greyish–white fibrous tissue. This tissue was in firm continuity with the neighbouring atrial tissue and extended slightly into the adjacent subendocardium (Fig. 4b). No metal parts of the ingrown device were in contact with the surface, but completely buried in the tissue. Histologically, vimentin staining showed that a continuous layer of endothelial cells extended from the atrial tissue onto the device. Fibroblastic granulation tissue was found underneath the “neo”-endothelium arranged parallel to the surface (Fig. 5). This tissue was poorly vascularised in the periphery with a moderate fibre content (azan stain). It bordered on centrally located, more loosely arranged well vascularised granulation tissue with a lower amount of fibres. Type I and III collagenous fibres were found in the granulation tissue by picrosirius red-stain. Elastic-stains showed some very fine fibres in the superficial fibroblastoid granulation tissue. In focal areas the Dacron fibres were surrounded by a mild to moderate granulomatous inflammatory reaction with infiltration of predominantly hemosiderin-laden macrophages, multinucleated giant cells and single lymphocytes.

3.4. Keratinocyte pre-seeded device

In order to further test cellular viability after mechanical stressing of the device implantation process and to discriminate between pre-seeded cells and host fibroblasts growing in from the defect edges, one device pre-cultured with autologous fibroblasts and keratinocytes was evaluated. Histology after 30 days in vivo showed vital epithelial cells with some differentiation towards a non-cornifying, stratified squamous epithelium (Fig. 6). These cells exhibited marked proliferating activity as confirmed by immunocytochemistry. Using a Ki67 antibody, marked nuclear staining of basal epitheloid cells was observed (data not shown).

4. Discussion

In order to test the feasibility of cellular pre-seeded septal occluders, devices pre-seeded with human fibroblasts were exposed to the mechanical stress of device implantation by a simulated implantation manoeuvre in vitro. Cell-seeding of interventional atrial septal defect occluder devices proved to be possible since pre-seeded cells survived the mechanical stress of the implantation process, especially when the device fabric was pre-coated by a collagen matrix.

When cells were seeded onto an occluder without any pre-coating (native device) the majority of them appeared to be scrubbed off by the shear stress of the implantation process (Fig. 1c and e). Most of the cells remaining on the device lost viability and underwent necrosis or apoptosis under subsequent culture in vitro. This process is probably favoured by detrimental factors like the release of intracellular enzymes from damaged cell leading to a local shift in pH or the lack of growth promoting paracrine cellular stimulation.

This course was effectively prevented when cells were seeded onto devices pre-coated with a thin layer of collagen matrix. Due to a higher initial cell density on the device it took shorter time in culture (3–4 versus 8–10 days) until a confluent cytolayer on the fabric was achieved. In addition, the increase in cellular stability and viability during the (simulated) implantation process was of superior importance. Only small areas of the cell-seeded collagen coated device fabrics were denuded of cells and 65±3% of the cells remaining on the device maintained their viability, continued to proliferate, and refilled the defects when in vitro culture was continued. Thus, it appears that a collagen matrix support and a critical cellular density is required to prevent the cytolayer from losing its structural stability and reconstructive capabilities after mechanical stressing of pre-seeded devices.

In order to further characterise the extent of cellular damage following defined mechanical stress, native and collagen-coated Dacron fabrics pre-seeded with fibroblasts were exposed to different levels of compression and to a constant pressure force application over various periods of time (Fig. 2). After mechanical compression, cellular viability decreased time and dose dependently. In both experimental subgroups, the viability of cells was significantly better preserved after application of mechanical stress on collagen-coated fabrics than on the non-coated fabrics. However, the effect of the duration of compressive force on cellular viability was much more pronounced in favour of the collagen-coated group (72±4% vital cells versus 56±3% in the native group, p<0.001) than the effect of the degree of force (80±3% vital cells versus 72±4% in the native group, p<0.05). In the experimental setting used, a pressure load of up to 4 bar for 10 s decreased the proportion of viable cells with intact cell membranes to 73±4% on non-coated fabric and 81±3% on collagen-coated fabrics. An additional increase in pressure (up to 6 bar) did not result in a significant further decline of the viability index. It is likely, that beside the compressive force, the shear stress transmitted to the cells during balloon inflation, is an important component of the overall mechanical stress load. This stress form predominates in the low pressure range, while subsequently the merely compressive component preponderates. Shear stress, however, is exerted in the phase of device loading (loading pod, delivery catheter) to the future endocardial surface of the device fabric, but not the luminal surface. Once the device is loaded, the double umbrella design of the device used in this study protects the cells growing on...
its luminal surface because the outer surfaces of both umbrellas are folded to the inside. Thus, these areas are protected from shear stress during the advancing of the delivery catheter through the long delivery sheath and are only exposed to compression.

This experimental study demonstrates for the first time the feasibility of atrial septal defect occlusion with autologous collagen-coated, cell-seeded devices. Fibroblasts isolated from individual skin biopsies survived the mechanical stress of occluder implantation when seeded onto a fabric coated with collagen. Previous histological studies have shown, that ingrowth of fibroblastic tissue onto the device occurs from the defect edges [8]. The origin of fibroblastoid cells detected on the device after 12 days and at later stages (30 days) could not be determined exactly. However, since the ingrowth of host cells starts at the periphery of the device, it is likely that the cells seen earlier (after only 4 days) and more centrally on the device were of autologous, pre-seeded origin.

This assumption is strengthened by an experimental animal in which a device was pre-seeded with autologous (ectopic) cells of different morphology. A device pre-cultured with both, fibroblasts and keratinocytes, showed vital, proliferating and differentiating keratinocytes (stratification layers) on the fabric after 30 days in vivo (Fig. 6).

In the light of a potential clinical application, the observed increase in thrombogenicity of the pre-seeded devices is of major concern. Despite this, the parenchymatous organs of all animals in this study showed grossly and histo-pathologically no hints of thrombo-embolic lesions in either organs. Physically, the animals behaved normally, there were no signs of illness and no premature death during follow-up. Nevertheless, the significantly thicker cushion-like layer of condensed plasma proteins and blood cells seen on the pre-seeded device bear a considerable risk for the individual as long as the material is completely covered by “neo”-endothelium and firmly organised. These devices had been pre-coated with collagen, a well-known thrombogenic substance. It is possible to coat the device with variable concentrations of collagen which may have different effects in vivo [34,35]. The effects thereof have not been tested in the study. Thus, although this study demonstrates the eminent importance of a matrix to increase cellular density and resistance against mechanical stress, it must be mentioned critically that the ideal matrix for this purpose has yet to be determined. In an attempt to develop artificial heart valves especially by means of tissue engineering, numerous biocompatible scaffolds have been evaluated. These included among others polymeric substances [19,22–24,36], fibrin gel [37,38], acellular matrix biomaterials [39–41] and collagen-based scaffolds including various cross-linking methods [42,43]. In this context it seems to be important that none of the animals used in our experimental groups received any anti-coagulant treatment, while this is routinely performed in humans. The control group (native Starflex devices) showed minimal thrombogenicity. It may, however, be necessary for cell-seeded, collagen-coated devices to administer temporarily limited anti-coagulant medication until full “neo”-endothelialisation of the implanted device has occurred (up to 4 weeks). Currently, studies are under way in our lab to test various (biological/biocompatible) substances that protect the pre-seeded cytolyser during implantation while avoiding the thrombogenic potential.

Over the short-term follow-up period of 30 days, pre-seeding of collagen-coated devices with autologous cells lead to rapid, more complete and firmer ingrowth of the occluder underneath a thicker layer of young granulation tissue. Contrary to the native, acellular group, these devices were fully covered by host tissue leaving none of the fabric or metal (spring arms, joint coils) exposed. Such a complete incorporation of the device into stable autologous tissue is a prerequisite for the development of totally biodegradable devices which resolve once this state has been reached.

References


