Immunohistochemical Characterization of Neotissues and Tissue Reactions to Septal Defect–Occlusion Devices

Rudi Foth, MD; Thomas Quentin, PhD; Ina Michel-Behnke, MD; Manfred Vogt, MD; Thomas Kriebel, MD; Anne Kreischer; Wolfgang Ruschewski, MD; Thomas Paul, MD; Matthias Sigler, MD

Background—We sought to evaluate tissue reactions within and at the surface of devices for interventional therapy of septal defects and to identify antigen characteristics of neotissues.

Methods and Results—Atrial or ventricular septal defect–occlusion devices (Amplatzer, n=7; Cardioseal/Starflex, n=3) were processed using a uniform protocol after surgical removal from humans (implantation time, 5 days to 4 years). Devices were fixed in formalin and embedded in methylmethacrylate. Serial sections were obtained by sectioning with a diamond cutter and grinding, thus saving the metal/tissue interface for histologic evaluation. Immunohistochemical staining was performed using conventional protocols. Superficial endothelial cells stained positive for von Willebrand factor. Within the newly formed tissues, fibroblast-like cells were identified with a time-dependent expression of smooth muscle cell maturation markers (smooth muscle actin, smooth muscle myosin, h-caldesmon, and desmin) beside extracellular matrix components. Neovascularization of the newly formed tissues was demonstrated with the typical immunohistochemical pattern of capillaries and small vessels. Inflammatory cells could be identified as macrophages (CD68+) and both T-type and B-type lymphocytes (CD3+, CD79+).

Conclusions—This is the first presentation of results from serial immunohistochemical staining of a collection of explanted human septal-occlusion devices. A time-dependent maturation pattern of the fibroblast-like cells in the neotissues around the implants could be described. Neoendothelialization was seen in all specimens with implantation times of 10 weeks or more. The time course of neoendothelialization, as seen in our study, further supports the clinical practice of anticoagulant or antiplatelet therapy for 6 months after implantation. This time interval should be sufficient to prevent thromboembolic events due to thrombus formation at the foreign surface of cardiovascular implants. (Circ Cardiovasc Intervent. 2009;2:90-96.)

Key Words: heart septal defects ■ immunohistochemistry ■ catheterization ■ occlusion ■ pathology

Clinical interest and information in literature on transcatheter closure of septal defects are generally focused on feasibility, safety, and long-term clinical outcome. Because the implants remain in the human heart for lifetime, biocompatibility has additionally emerged into the focus of interest. Hitherto, there are few data on histopathology of explanted occlusion devices for atrial and ventricular septal defects. Concerning immunohistochemical characterization of tissue reactions, data are scarce. Just a single “clinical vignette” presenting a figure of an immunohistochemically labeled specimen has been published so far.

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The purpose of this work was to characterize cellular and extracellular matrix (ECM) components that are formed within and around the devices after implantation and to illuminate the previously unknown way of ingrowth.

Methods

Devices

The Amplatzer septal defect occluder (AGA Medical Corporation, Golden Valley, Minn) is a self-expanding device made from nitinol wire mesh. It has a double disc shape and consists of two retention occlusion devices for atrial and ventricular septal defects. Concerning immunohistochemical characterization of tissue reactions, data are scarce. Just a single “clinical vignette” presenting a figure of an immunohistochemically labeled specimen has been published so far. This is the first presentation of results from serial immunohistochemical staining of a collection of explanted human septal-occlusion devices. A time-dependent maturation pattern of the fibroblast-like cells in the neotissues around the implants could be described. Neoendothelialization was seen in all specimens with implantation times of 10 weeks or more. The time course of neoendothelialization, as seen in our study, further supports the clinical practice of anticoagulant or antiplatelet therapy for 6 months after implantation. This time interval should be sufficient to prevent thromboembolic events due to thrombus formation at the foreign surface of cardiovascular implants. (Circ Cardiovasc Intervent. 2009;2:90-96.)

Key Words: heart septal defects ■ immunohistochemistry ■ catheterization ■ occlusion ■ pathology
Table 1. List of Specimens for Histopathologic Workup

<table>
<thead>
<tr>
<th>Patient</th>
<th>Implant</th>
<th>Implantation Time</th>
<th>Indication for Explantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amplatzer ASD</td>
<td>5 days</td>
<td>Malpositioning</td>
</tr>
<tr>
<td>2</td>
<td>Amplatzer VSD</td>
<td>10 weeks</td>
<td>Valve incompetence</td>
</tr>
<tr>
<td>3</td>
<td>Amplatzer VSD</td>
<td>7 months</td>
<td>Malpositioning</td>
</tr>
<tr>
<td>4</td>
<td>Amplatzer VSD</td>
<td>10 months</td>
<td>Malpositioning</td>
</tr>
<tr>
<td>5</td>
<td>Amplatzer ASD</td>
<td>12 months</td>
<td>Malpositioning</td>
</tr>
<tr>
<td>6</td>
<td>Amplatzer ASD</td>
<td>15 months</td>
<td>Residual shunt</td>
</tr>
<tr>
<td>7</td>
<td>Amplatzer ASD</td>
<td>24 months</td>
<td>Residual shunt</td>
</tr>
<tr>
<td>8</td>
<td>Cardioseal ASD</td>
<td>25 months</td>
<td>Residual shunt</td>
</tr>
<tr>
<td>9</td>
<td>Cardioseal ASD</td>
<td>36 months</td>
<td>Atypical configuration of the device</td>
</tr>
<tr>
<td>10</td>
<td>Starflex ASD</td>
<td>48 months</td>
<td>Recurrent neurology/apoplexy</td>
</tr>
</tbody>
</table>

Human specimens (n=10). ASD indicates atrial septal defect; VSD, ventricular septal defect.

Human Explants

Ten human tissue specimens were collected during corrective surgery at 5 centers and sent to us for routine histopathologic workup. The time intervals between implantation and explantation of septal defect–occlusion devices ranged from 5 days to 48 months. Indications for device explantations are summarized in Table 1.

Tissue Preparation

Immediately after explantation, the tissue block containing the implant was dissected free with a minimum of surrounding tissue. After briefly flushing with saline, the specimens were fixed in formalin (buffered 4%).

Embedding, Sectioning, and Histology

To obtain immunostaining of resin-embedded specimen, the sections were mounted on glass slides using silicon glue and underwent deplastification, as described previously. Staining with Richardson blue, Movat pentachrome, or Picrosirius red was performed according to standard protocols. For Richardson blue staining of resin-embedded specimen, the sections were mounted on plastic slides and stained without deplastification. For Movat pentachrome and Picrosirius red staining, the resin-embedded specimens were mounted on glass slides and deplastificated as for immunostainings.

Before embedding, macroscopic evaluation and documentation was accomplished. After fixation, the tissue block with the device was embedded in resin methylmethacrylate (Technovit 9100, Kulzer & Co, Wehrheim, Germany). After hardening, the resin blocks were subsequently sectioned in slices of 0.8 mm using a diamond band saw (300 CP, Exakt GmbH, Norderstedt, Germany). These slices were grinded down to 5 to 30 μm with a horizontal rotatory grinder and polisher (400 CS, Exakt GmbH).

Immunohistochemistry

Details of the staining protocols are shown in Table 2. Umbilical cord sections served as positive controls for immunostaining of smooth muscle markers and human tonsil sections for immune cells, respectively. Negative controls were processed without the antigen-specific antibody.

Nomenclature

To clarify the assignment of findings to different regions in the specimen, the following terms were used, as shown in Figure 1:

Table 2. Pretreatment and Dilutions of Antibodies for Immunohistochemical Labeling

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse antihuman desmin, clone D9 (Progen Biotechnik GmbH)</td>
<td>1:50</td>
<td>Basic buffer, pH 9 (Target Retrieval Solution, High pH, Dako), 20 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Mouse antihuman smooth muscle myosin heavy chain, clone ID6 (Chemico International)</td>
<td>Paraffine 1:100 Resin 1:500</td>
<td>Citrate buffer, pH 6 (Dako REAL Target Retrieval Solution), 40 minutes in a steamer</td>
<td>EnVision amplification system</td>
</tr>
<tr>
<td>Mouse antivimentin, clone V9 (Dako)</td>
<td>1:100</td>
<td>Citrate buffer, pH 6, 40 minutes in a steamer</td>
<td>EnVision amplification system</td>
</tr>
<tr>
<td>Mouse antihuman smooth muscle actin, clone 1A4 (Dako)</td>
<td>1:50</td>
<td>Citrate buffer, pH 6, 40 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Polyclonal rabbit antihuman von Willebrand factor (Dako)</td>
<td>1:400</td>
<td>Citrate buffer, pH 6, 40 minutes in a steamer</td>
<td>Swine antirabbit immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Mouse antihuman caldesmon, clone h-CD (Dako)</td>
<td>Paraffine 1:100 Resin 1:400</td>
<td>Proteinase K (Dako, diluted 1:500 in 50 mmol/L TBS, pH 7.6) at 37°C for 5 minutes, followed by citrate buffer, pH 6 for 40 minutes in a steamer</td>
<td>EnVision amplification system</td>
</tr>
<tr>
<td>Polyclonal rabbit antihuman CD3 (Dako)</td>
<td>1:100</td>
<td>Citrate buffer pH 6 (Dako), 40 minutes in a steamer</td>
<td>Polyclonal rabbit antigoat immunoglobulin/horseradishperoxidase (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Monoclonal mouse antihuman CD79a/cy, clone HMS57 (Dako)</td>
<td>1:100</td>
<td>Tris/EDTA, pH 9, 20 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Monoclonal mouse antihuman CD68, clone PG-M1 (Dako)</td>
<td>1:200</td>
<td>Tris/EDTA, pH 9, 20 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Monoclonal mouse anticollagen III (Acris)</td>
<td>1:500</td>
<td>Citrate buffer, pH 6, 40 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
<tr>
<td>Monoclonal mouse antifibrin (No. 0.350) (American Diagnostica Inc)</td>
<td>1:500</td>
<td>Citrate buffer, pH 6, 40 minutes in a steamer</td>
<td>Rabbit antimouse immunoglobulin antibody (Dako), diluted 1:100</td>
</tr>
</tbody>
</table>
1. “Neoendothelium” for the superficial cell layer if present;  
2. “Pseudointima” for the tissue between the neoendothelium and the implant; and  
3. “Neotissue” within the implant for tissue inside fabric and metal wires of the implant.

The term pseudointima was used according to previous descriptions of this type of tissue that is being formed intracardially after device implantation.1,2

**Statement of Responsibility**

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

Presentation of results is focused on immunohistochemical labeling and results of Movat pentachrome and Picrosirius red staining. Results of macroscopic evaluation and standard histology were, in part, presented and discussed previously by our group.1

**Neoendothelium**

Macroscopically, the Amplatz atrial septal defect occluder with an implantation time of only 5 days (patient 1) was covered with a thin layer of dull grayish material (Figure 2A). By means of immunostaining, this superficial material could be identified as fibrin with included blood cells (Figure 2B). All other devices with implantation times of ≥10 weeks were covered by a shining layer of whitish tissue of variable thickness macroscopically (Figure 2C). Endothelialization was complete, except for some prominent parts of the occluder of patient 2. Von Willebrand factor stained positive in all these specimen (Figure 2D). Results of immunostainings are summarized in Table 3.

**Fibrin Deposition**

Formation of condensed fibrin with included blood cells around the foreign material was the principle finding in the specimen, with an implantation time of 5 days (Figure 3A). In the superficial portion of this fibrin deposit, single longitudinally oriented cells were seen. These cells stained positive for vimentin (Figure 3B), but not for other muscle cell markers. Residual islets of fibrin deposits were observed in single specimen with implantation times of up to 15 months (Figure 3C). These fibrin residues were solely seen in the central portion of the implants. In all other specimen with implantation times of ≥24 months, there was no evidence of thrombotic material within the implants.

**Pseudointima**

Except for the implant with an implantation time of 5 days, all specimen with an implantation time ≥10 weeks had a well-definable pseudointima with an organized cellular arrangement. The cells of the pseudointima showed a predominantly longitudinal orientation (parallel to the neoendothelium; Figure 1B) and stained positive for vimentin in all specimen (Figure 4A). In addition, staining was positive for the muscle cell marker smooth muscle actin and smooth muscle myosin (Figure 4B and 4C). h-caldesmon as a marker of mature smooth muscle cells was detected in a single specimen with an implantation time of 15 months (Figure 4D). Desmin stained positive in 2 specimens with implantation times of 10 months and 48 months.

By means of Movat pentachrome stain, proteoglycans were identified as the main component of ECM within the pseudointima. In addition, collagen and few elastic fibers were seen. Immunohistochemically, antibodies against collagen III stained positive with a homogeneous distribution pattern.

Few capillaries and small vessels could be detected within the pseudointima. The cells lining the lumen of the capillaries stained positive with antibodies against von Wille-
brand factor and vimentin (Figure 5A). In addition, cells in the wall of the small vessels showed positive staining for smooth muscle actin, smooth muscle myosin, and h-caldesmon even in specimen with implantation times of only 10 weeks (Figure 5B).

**Neotissue Within the Implant**

The neotissue within the implant can be clearly distinguished from the formerly described pseudointima (1) by its localization, (2) by the configuration of cellular and extracellular components, and (3) by its rich vascularization opposed to the sporadic appearance of capillaries within the pseudointima. It morphologically consisted of areolar connective tissue with mostly irregular oriented cells (Figure 1A). These cells stained positive with antibodies against vimentin and CD68 (Figure 7A). In almost all antibodies against collagen III (Figure 6C). Proteoglycans were identified as the main constituent of this part of the specimen besides collagen and few elastic fibers (Figure 6D). Composition of ECM components was not different in the parts adjacent to Dacron fibers and metal parts of the implants.

**Inflammation**

Foreign body giant cells were seen usually in local relation to Dacron fibers. These cells stained positive with antibodies against vimentin and CD68 (Figure 7A).

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**Table 3. Staining Patterns of Primary Antibodies**

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<tbody>
<tr>
<td>Surface</td>
<td>vWF</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pseudointima</td>
<td>Vimentin</td>
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<td>+</td>
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<td></td>
<td>SMA</td>
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<td>h-cald</td>
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<tr>
<td>Tissue within the implant</td>
<td>Vimentin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>SMM</td>
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<tr>
<td></td>
<td>h-cald</td>
<td>–</td>
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</tbody>
</table>

ASD indicates atrial septal defect; VSD, ventricular septal defect; vWF, von Willebrand factor; SMA, smooth muscle actin; SMM, smooth muscle myosin; h-cald, h-caldesmon; –, negative; +, positive; ++, strong positive.

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**Figure 3.** Micrographs show representative immunohistochemical staining of fibrin deposits (positive immunohistochemical staining, brown; metal wires, black). A, Fibrin staining of a specimen with an implantation time of 5 days (patient 1). B, Micrograph of vimentin staining in the same patient. C, Micrograph of fibrin staining in patient 6 (implantation time 15 months). DF indicates Dacron fibers.

**Figure 4.** Micrographs show representative immunohistochemical staining of cellular components of the pseudointima with typical longitudinal orientation of cells and few capillarization (positive immunohistochemical staining, brown). A, Micrograph of vimentin staining of a specimen with an implantation time of 48 months (patient 10). B, Micrograph of smooth muscle actin staining of a specimen with an implantation time of 12 months (patient 5). C, Micrograph of smooth muscle myosin staining of a specimen with an implantation time of 12 months (patient 5). D, Micrograph of h-caldesmon staining of a specimen with an implantation time of 15 months (patient 6). DF indicates Dacron fibers.
explants, inflammatory infiltrates with lymphocytes were observed within the implant. In 2 of the implants, marker for T cell and B cell was used for further differentiation. In both specimen, lymphocytes stained positive with antibodies against the T-cell marker CD3 and with the B-cell marker CD79 (Figure 7B and 7C).

The clinically most important aspects of the healing response (neoendothelialization and cellular organization of initially formed fibrin deposits) after implantation of a septal defect–occlusion device are summarized in Table 4.

**Discussion**

To the best of our knowledge, this is the first study that characterizes tissue reactions in a series of human explanted septal defect–occlusion devices by means of immunohistochemistry. Two of the currently and most commonly used septal occluders, the Amplatzer and Cardioseal/Starflex devices, were evaluated in our study.

Except for 1 case report, no immunohistochemical stainings of explanted septal defect–occlusion devices have been published so far. This may be for the most part because of the technical challenge to perform immunohistochemistry in hard resin-embedded specimen. The need for deplastification of the grinded sections to uncover antigens aggravates the complexity of specimen processing. In addition, establishment of primary antibodies in resin-embedded specimen requires far more time and effort as compared to paraffin wax-embedded specimen.

Neoendothelialization of septal defect–occlusion devices after interventional application is of major clinical relevance because superficial thrombus formation is a possible source for embolism and subsequent organ damage. For prevention, most centers give antiplatelet or anticoagulant therapy for 6 months until neoendothelialization is expected to be completed.

In animal studies and human explants, endothelial cells were observed as soon as 30 days after implantation. In previous publications, these characterizations were accomplished solely by standard stain or scanning electron microscopy. In this study, we were able to identify endothelial cells by means of immunohistochemical labeling for the first time. Antibodies against von Willebrand factor and vimentin stained positive superficial cells in all specimen with implantation times.

<table>
<thead>
<tr>
<th>Table 4. Healing Response Over Time</th>
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</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
</tr>
<tr>
<td>Surface</td>
</tr>
<tr>
<td>Pseudointima</td>
</tr>
<tr>
<td>Tissue within the implant</td>
</tr>
</tbody>
</table>
thrombotic material has been demonstrated to be transformed to connective tissue consisting of mainly ECM and embedded cellular components, which morphologically resemble fibroblast-like cells in septal defect–occlusion devices and occlusion devices for other applications.\(^1\) The results of our present study give some insight into the time course of this process, as cellular organization of fibrin deposits was advanced after 10 weeks already, but completed after 24 months only (Table 4).

To further characterize the “fibroblast-like” cells within the newly formed tissue, we used a panel of muscular maturation marker. The cells seen in the pseudointima and within the implant concordantly stained positive for the mesenchymal marker, vimentin, and the early muscular differentiation marker, smooth muscle actin and smooth muscle myosin, resembling a myofibroblast-like phenotype.\(^{11}\) h-caldesmon is part of the contractile apparatus of muscle cells and is regarded as a marker for mature and highly differentiated muscle cells.\(^{12,13}\) In our series, it only stained positive in a single specimen with implantation times of 15 months. Likewise, desmin as a marker for intermediate filaments is expressed in highly differentiated muscle cells.\(^{12,14}\) The late occurrence of desmin and h-caldesmon in our specimen can be taken as signs for the slow progression of cell maturation after device implantation. Concerning immunostaining, there were no differences between cells in the pseudointima (longitudinally orientated) and cells within the implant (irregularly orientated).

Cells in close local relation to metal struts stained positive for vimentin, smooth muscle actin, and smooth muscle myosin independently of the implantation time. Thus, we found no evidence for a possible toxic reaction to metal ions, because release of nickel ions from metal wires of septal defect–occlusion devices is a well-described phenomenon.\(^{15,16}\)

In analyzing the newly formed tissues around occlusion devices, the specimen with the shortest implantation time of only 5 days is of special interest. Besides the typical pattern of thrombus with fibrin septations and included blood cells, occurrence of some vimentin positive fibroblast-like cells could be demonstrated. These fibroblast-like cells could be fibrocytes. Fibrocytes are circulating cells, which migrate into wounds and adopt a smooth muscle-like phenotype (spindle-shape morphology, positive staining for vimentin, and smooth muscle actin). Varcoe et al\(^{17}\) demonstrated that these cells are involved in intima formation. It can be assumed that these cells are involved in the organization of the thrombotic material and the formation of the pseudo-intima, as seen in our collection of specimen.

With regard to ECM components, we found a similar areolar-like pattern in central parts of the specimen, as described by Kreutzer et al\(^2\) with mainly proteoglycans, besides collagen and few elastic fibers. Multinucleated foreign body cells have constantly been observed in local relation to Dacon fibers of cardiovascular implants, such as the Amplatzer or the Cardioseal/Starflex device.\(^2,4\) Foreign body giant cells in the specimen of our study stained positive with antibodies against CD68 identifying these cells as macrophages, which are considered as being part of the innate immune response.

In addition to the foreign body reaction, mild lymphocytic infiltrations have been described within the newly formed tissue after implantation of septal defect–occlusion devices.\(^1,2\) In the present study, we were able to further differentiate this lymphocytic reaction for the first time, because we could demonstrate a lymphocytic reaction of a mixed type (CD3 and CD 79 positive). Thus, we have evidence of some kind of adaptive cellular response in addition to the reaction of the innate immune system locally related to the implants.

No difference in pattern or time course of immunohistochemistry-based findings was observed comparing Amplatzer and Cardioseal/Starflex occlusion devices, different sites of implantation (atrial septal defect versus ventricular septal defect), or indication for explantation. This is in accordance with the results of comparative analysis of standard staining of the 2 devices, as it has been reported earlier by our group.\(^1\)

In summary, this study demonstrates the value of immunohistochemical staining for characterization of tissues within and around septal defect–occlusion devices after interventional implantation. We can for the first time describe antigen patterns of cells in the neotissues. Fibroblast-like cells were identified with a slowly progressing maturation pattern over time as demonstrated by immunohistochemically labeling with a variety of smooth muscle cell markers. Superficial cells could be identified as endothelial cells. The time course of neoendothelialization, as seen in our study, further supports the clinical practice of anticoagulant or antiplatelet therapy for 6 months after implantation. This time interval should be sufficient to prevent thromboembolic events due to thrombus formation at the foreign material surface of cardiovascular implants.

**Acknowledgments**

We thank Andrea Poppe and Karin Baer for technical assistance.

**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

More than 100 000 septal defect–occlusion devices have been implanted as yet. Much experience has been gained with the implantation procedure during the past years, making it a safe and effective procedure nowadays. In contrast, little is known on the healing response after implantation. To our knowledge, this study is the first systematical evaluation of tissue reactions using immunostainings in a series of 10 septal defect–occlusion devices, which were explanted from humans for malpositioning, residual shunting, or other reasons. Superficial cells could be identified as endothelial cells. The time course of neointimalization, as seen in our study, further supports the clinical practice of anticoagulant or antiplatelet therapy for 6 months after implantation. This time interval should be sufficient to prevent thromboembolic events due to thrombus formation at the foreign material surface of cardiovascular implants. As seen by immunohistochemical staining, the process of complete cellular organization of the initially formed fibrin deposits takes longer than previously reported. Even in a device which had been implanted for 15 months, residual islets of condensed fibrin were seen. The cellular components of the neotissue were demonstrated to mainly consist of fibroblast-like cells, which showed a slowly progressing maturation pattern over time.
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