CHEMICALLY MODIFIED COLLAGENOUS MATERIALS WITH HEPARIN SLOW RELEASE APPLIED TO A VASCULAR GRAFT AND AN ANTIADHESIVE MEMBRANE

Yasuhiro Noishiki, 1) Teruo Miyata, Masayasu Furuse, 2) and Yoshihisa Yamane, 3)
1) Division of Surg., Dept. of Rehabilitation Medicine, Medical School, Okayama University, Misasa, Tottori, 682-02, Japan. 2) Koken Bioscience Institute, 2-11-21, Nakane, Meguro-ku, Tokyo, 152, Japan. 3) Small Animal Clinical Research Center, 142 Yatsuya, Kurayoshi, Tottori, 682, Japan.

INTRODUCTION
New technologies to treat collagenous materials were introduced. One is a cross-linking method and another is a heparinization method. Both technologies were applied to develop a vascular graft and an antiadhesive membrane.

In general, biological materials have an affinity to the host tissue. To adapt the biological materials for medical use, one needs to perform some modification, except when the tissues are used autologously. Until now, only glutaraldehyde (GA) has been used for this purpose. GA cross-linking makes the materials less biodegradable, insoluble, and less antigenic, but GA treatment completely changes the native properties of the materials. To overcome these difficulties, a new cross-linking method was introduced 1). The cross-linking reagent was Polyepoxy Compounds (PC) named "Denacoil" produced by Nagase Chemical Co. Ltd. in Himeji, Japan.

Another technology is a heparinization method 2,3). A basic protein, protamine, was used to increase heparin binding to collagenous materials. The protamine was impregnated and cross-linked covalently by cross-linking reagent such as GA and PC. The collagen-protamine complex was interacted with a heparin solution to ionically bind heparin to the material. The amount and the distribution of the bound heparin can be controlled by changing the protamine to the material.

I. Vascular graft experiment

MATERIALS AND METHODS

Preparation of the graft
1. Fresh carotid artery with ID 2.5 to 3.0 mm was obtained from a dog. It was soaked in distilled water for 1 hour and sonicated at 28 kc for 20 seconds in order to cause cell destruction. Cell debris was removed by washing with distilled water. In this way, a natural tissue tube composed of collagen and elastic laminae was obtained.
2. A 2% protamine sulfate solution at pH 5.9 was poured into the natural tissue tube graft lumen, and the graft was inflated with air pressure of 80 to 100 mmHg for 30 minutes to force the protamine into the graft wall.
3. The graft inflated with air pressure was treated with a 5% PC
solution in 50% ethanol and 0.1M Na2CO3 at pH 10.0 for 5 hours to cross-link the tissue and covalently immobilize protamine impregnated into the wall. The graft was then washed with distilled water.

4. The graft was soaked in a 1% heparin solution at pH 7.0 for 5 hours at 45°C and repeatedly washed with distilled water.

5. The graft was then preserved and sterilized in a 70% ethanol solution.

6. Before implantation, the graft was soaked and washed in saline solution several times to remove ethanol completely.

The control graft

For the control experiment, unheparinized grafts treated with either GA and PC were prepared, and used for mechanical property measurement and animal study. ε-NH2 groups of collagen molecules in each graft reacted with the reagent was analyzed by TNBS method 4).

Mechanical properties of the developed graft

1. Strength. Fresh canine carotid arteries, GA cross-linked, PC cross-linked and heparinized (new graft) were used. Cylindrical specimens were fixed longitudinally and tensile strength measurements were performed. The elongation and breaking tensile strength were measured in each specimen.

2. Stiffness and elastic behavior. Each cylindrical shaped specimens was placed in an evaluation system developed by Hayashi 5). The relationship between the interluminal pressure and the external radius of each specimen was plotted as the logarithm of pressure ratio versus distension ratio.

Carotid artery replacement.

Fifty-six mongrel dogs weighing 8 to 12 kg were used for the experiment. About 6 cm of both carotid arteries were harvested and a 6 cm long by 2.5 to 3.0 mm internal diameter segment of heparinized graft was implanted end to end. Five hundreds mg of penicillin was given but no anticoagulants were used at any time. Eighty heparinized grafts were implanted, as well as additional 16 GA treated control grafts. The experimental animals had free access to standard dog food and water. They were sacrificed electively at approximate time intervals. The implantation periods ranged from one hours to 429 days.

Determination of heparin concentration in the graft

Heparin concentration in the whole graft wall was measured with the method of D.Lagnoff et all 6).

RESULTS

Mechanical properties of the graft

Using Hayashi's method, the vascular compliance of each specimen at 90 mmHg was calculated. The strength and elongation of each specimens was also measured. These results are shown in Table 1. The reaction rate of the ε-NH2 groups of each specimens is also shown.
Table 1. MECHANICAL PROPERTIES OF DOG CAROTID ARTERIES

<table>
<thead>
<tr>
<th>Properties</th>
<th>Tensile strength (g/mm²)</th>
<th>Elongation rate (%)</th>
<th>Stiffness parameter &quot;B&quot;</th>
<th>Compliance at 90mmHg (x10⁻³mmHg⁻¹)</th>
<th>Reaction rate of ε-NH₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated Arteries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native Artery</td>
<td>207±27</td>
<td>116±15</td>
<td>16.8</td>
<td>2.33</td>
<td>0</td>
</tr>
<tr>
<td>GA crosslinked A.</td>
<td>175±39</td>
<td>57±12</td>
<td>38.1</td>
<td>0.80</td>
<td>41±6</td>
</tr>
<tr>
<td>PC crosslinked A.</td>
<td>199±97</td>
<td>127±11</td>
<td>21.1</td>
<td>1.70</td>
<td>53±7</td>
</tr>
<tr>
<td>PC, Protamine crosslinked A.</td>
<td>215±82</td>
<td>84±37</td>
<td>25.4</td>
<td>1.40</td>
<td>45±8</td>
</tr>
</tbody>
</table>

Implantation of the grafts
The heparinized grafts were white, pliable, and more elastic than the yellowish control. The inner surface of both grafts were shiny and smooth, but the heparinized grafts were easier to suture and match with the host arterial wall. There was no blood leakage through the grafts wall on implantation, and no kinking occurred even when the graft was bent.

Removal of the grafts
At the time of the graft removal, 77 grafts were patent and 3 were occluded. In one dog, sacrificed at 172 days after implantation, a graft implanted in the right carotid artery was patent, but the graft implanted on the left was occluded. The occluded graft was soft and white, but anastomotic lines were hard. In another dog, sacrificed at 11 days, the cervical wound was infected and the grafts implanted in both carotid arteries were occluded. As these were the only graft occluded, the patency rate of the heparinized graft was 96%. All the patent grafts were still soft and pliable as native artery. Within 100 days after implantation, the inner surface were completely free from the thrombus deposition. The surface were as shiny, white, smooth and glistening as that of the host arterial intima. In the case of those grafts that remained in place for more than 100 days, slightly yellowish or whitish and semi-transparent small spots were sporadically observed on the surface. The control grafts were occluded within one week after implantation, and they were very hard and dark brown in color.

Microscopical observations
Microscopic observations confirmed that there was neither thrombus nor fibrin deposition on any graft. The luminal surface was composed of the internal elastic membrane, with no endothelial cells on the surface. Early on, there was no foreign body reaction such as giant cell infiltration on the outer surface of the graft. A small number of plasma cells were noticed in short period of time after implantation. At more than 30 days, some macrophages were noticed in the inter elastic laminal spaces near the luminal surface. After more than 6 months, smooth-muscle-like cells infiltrated these spaces.
from the adventitia side. In the walls of long-term grafts, cells filled the spaces completely. In 75% of the spaces near the luminal surface, these elongated cells were arranged circumferentially, as in 25% of spaces near the adventitia, these cells were oriented longitudinally. The inner surface was covered with endothelial cells, which imprinted on the surface of elastic lamina. The structure of the graft following long-term implantation closely resembled that of the native arterial wall. There was no foreign body reaction in the long term specimens, and no degenerative changes such as hyalinization, calcification, or arteriosclerosis.

Concentration of the heparin in the graft

Before and after the implantation, the total amount of heparin in the graft was measured. The results indicated that the amount before implantation was about 7.0u/cm², but in specimens in place for more than 80 days, there was no heparin in the graft wall.

II. Antiadhesive membrane experiment

MATERIALS AND METHODS

Preparation of the membrane

1. A thin membrane of human amnion was obtained aseptically from a labor room and was dipped into distilled water for 1 hour, and sonicated at 28 kc for 20 seconds in order to cause cell destruction. Cell debris was then removed by washing with distilled water.

2. The membrane was lyophilized.

3. It was dipped into 1% solution of protamine sulfate.

4. The protamine impregnated membrane was treated with 2% PC of 50% ethanol solution for 5 hours to cross-link the protamine to collagen and washed with distilled water.

5. The membrane was soaked into 1% heparin solution for 5 hours at 45°C and repeatedly washed with distilled water.

Implantation of the membrane

Nine adult mongrel dogs weighing 6-12 kg were used, five of them for the heparinized experiment and the other 4 of them for the control. The peritoneal cavity was opened via a midline skin incision under general anesthesia. Two 5X5 cm² areas of the serosa with the thin layer of the longitudinal muscle were peeled off from the large intestine in each animal. The naked areas were covered with the heparinized membrane, which was fixed on the surface of the intestine using 5-0 tevdeck. Antibiotics were used at the surgery, but no antiadhesive drugs were used. The peritoneal cavity was closed with a 2-0 silk suture. As the control experiment, no membrane was used on the naked area. All the animals were sacrificed at a period of 2 to 130 days after the surgery. All the specimens excised were submitted to macroscopic and light and scanning electron microscopic observations.
RESULTS

The heparinized membrane showed an excellent antiadhesive effect in every part of all 5 dogs (10 parts). Only a tiny adhesion of omentum to the suture on the edge of the membrane was noticed in two parts. After 2 days, oozing blood stayed under the membrane, and no blood coagulation nor adhesion was noticed. After 9 days, the oozing blood was already absorbed. The naked area was covered with the membrane, which prevented from adhesion completely. In the long-term experiment, the wound healed completely. The surface was shiny and smooth. No scar formation was observed on the area. The intestine could be inflated naturally with an internal air pressure. The light microscopical observation revealed that the surface was covered with a layer of serosal cells. The heparinized membrane was already absorbed. However, in the control experiment, the greater omentum, mesoterium, uterus or urine bladder were adhered in 5 of 8 areas. Three areas were free from the adhesion. The intestine had a scar on each adhered area and could not be inflated naturally with inner air pressure.

DISCUSSION

GA has been used for the treatment of biological materials, however, adverse effects of GA treatment have been reported. GA cross-linking makes the materials less biodegradable, insoluble, and less antigenic, but the materials become less flexible and less affinity with cells. In this report, we used new cross-linking reagent, PC. A remarkable difference between materials treated with GA and PC is their color; GA treatment makes the materials yellow, but PC makes them white. There are also remarkable difference in their softness and elasticity. One of the characteristics of PC treatment is the hydrophilic property imparted to the material which is important for affinity with host tissue, and makes the graft more non thrombogenic. The strength of the materials treated with PC is also important because despite its softness, it has sufficient strength to withstand mechanical stress.

Reconstruction of an arterial wall with the vascular graft was most successful with smooth-muscle-like cells infiltrating the graft wall 6). We observed the healing process of the implanted vascular graft and learned that the smooth muscle cells infiltrated the neointima of the graft and were arranged parallel to the direction of the tensile stress placed upon the graft wall. If the tensile stress is not present, smooth muscle cells seldom appear. The appearance of such cells in the graft treated with PC suggested that there is enough compliance of the graft to induce the migration of these cells. In the case of GA treated grafts, the environmental condition in the graft wall is considered to be insufficient for the infiltration of the smooth-muscle-like cells. The grafts cross-linked with GA become yellowish and lose their pliable and elastic characteristics. By contrast, PC cross-linked grafts maintain their natural vessel compliance and are stronger than the
original vessels, thus providing excellent sutureability and compliance match. Furthermore, the PC cross-linked grafts are hydrophilic because of hydroxyls in the molecular structure, while the GA cross-linked grafts are hydrophobic. In this respect, the PC cross-linked graft would have superior antithrombogenic characteristics because the hydrophilicity give the material antithrombogenicity. Therefore, in terms of antithrombogenicity, as well as compliance match, PC cross-linked grafts are superior to GA cross-linked grafts when applied as a small caliber vascular substitute 8).

In the case of the antiadhesive membrane, native mesothelial cells covered the amnion membrane after the release of heparin, because, the PC cross-linked collagen membrane provided a good supporting framework for cellular infiltration.

We already clarified that the deposition of fibrin on the intestinal surface leads to the formation of cellulosifibrous adhesive tissue in the early stage of intestinal inflammation and adhesion 9). Therefore, heparin which inhibits the precipitation of fibrin could be effective in preventing adhesion. In this study, an effective antiadhesive membrane with slow releasing heparin was demonstrated. After the release of heparin, the mesothelial cells covering the surface contribute to the prevention of adhesion naturally and permanently 10).

In the case of the vascular graft, heparin is slowly released following implantation. As the heparin gradually absorbed, the graft becomes naturally antithrombogenic because endothelial cells advantageously cover the graft surface. Consequently, the graft can remain permanently antithrombogenic by endothelialization. Animal experiments reveal that this method produced stable antithrombogenicity in small caliber artery grafts.

From these two experiments, these new technologies were recognized as useful methods for the development of new artificial organs using collagenous materials.

REFERENCES