
Original Article

Experimental Biliary Reconstruction with an Artificial Bile Duct Using *in situ* Tissue Engineering Technique

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The development of vitally functional biliary substitutes has long been awaited. However, despite numerous approaches for replacement of biliary defects with a variety of materials, an “artificial bile duct” has not yet been realized clinically. Recently, tissue engineering has become a promising technique for regenerating defective organs. In the present study, we attempted to regenerate bile duct tissue using *in situ* tissue engineering. We prepared experimental scaffolds composed of collagen sponge (CS) as a base material and performed biliary reconstruction using these artificial bile ducts in canine models. We replaced circumferential biliary defects with our prosthesis in 7 dogs. In group A, we used prostheses consisting entirely of CS with biliary stenting for 2 weeks (2 dogs). In group B, the biliary stents were left in place for 12 weeks (2 dogs). In group C, we used prostheses consisting of a CS reinforced with a polypropylene (PP) mesh framework with biliary stenting for 2 weeks (3 dogs). In group A, stenosis of the prostheses progressed and the dogs became jaundiced within 2 months. In group B, regeneration of biliary epithelium was not seen, although the prostheses maintained the patency of the duct due to long-term stenting. In group C, a reconstructed bile duct composed of newly formed biliary epithelium and connective tissue successfully replaced the defects and maintained the patency without long-term stenting. Thus, our artificial bile duct consisting of CS and a PP mesh framework shows potential for regeneration of bile duct tissue.

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Key words

artificial bile duct, tissue engineering, regeneration, collagen scaffold, polypropylene mesh

Introduction

A wide variety of experimental materials have been utilized in the past for bridging gaps in the common bile duct in order to establish bile flow between the liver and the intestinal tract¹⁻⁸⁾, but there has been little opportunity to adapt these grafts to the field of clinical surgery. On the other hand, progress in surgical techniques for bilioenteric anastomosis has outdistanced development of an artificial bile duct. Currently, biliary-enteric bypass is the most reliable surgical method for treating diseases of the bile duct system. However, loss of papillary function and the reflux of intestinal contents into the biliary tree following the use of this procedure often cause postoperative complications such as cholestasis, cholangitis, or increased risk of cholangiocarcinoma⁹⁾. If a clinically effective biliary substitute could be obtained, it would enable sphincter-preserving biliary reconstruction and prevent subsequent complications, providing bile drainage through an intact sphincter of Oddi.

Recently, tissue engineering has become a promising treatment strategy for malfunctioning or lost organs. One of the most important factors for successful tissue engineering is provision of a scaffold suitable for tissue regeneration. Collagen is a suitable material for tissue scaffold because of it promotes tissue self-repair through cell migration, attachment, and proliferation. However, a prosthesis constructed of collagen sponge (CS) alone lacks mechanical strength. From this viewpoint, use of composite material seems to be a promising approach in terms of mechanical strength. Indeed mechanically reinforced collagen scaffold have been applied for artificial esophagus^{10,11)} or trachea¹²⁻¹⁴⁾. In them, the artificial trachea has already been applied clinically since 2002¹⁵⁾.

In the present study, we attempted to fabricate an artificial bile duct composed of CS with or without a reinforcing inner stent or mesh framework, and used these prostheses to replace biliary defects in a canine model. Our aim was to analyze the optimal design of a CS scaffold as an artificial bile duct for regeneration of biliary ductal tissue *in vivo*.

Materials and Methods

1)Artificial bile duct

We prepared two types of artificial bile duct: a prosthesis consisting entirely of CS (Fig.1A) and a prosthesis consisting of CS reinforced with a mesh framework (Fig.4A).

2)Prosthesis consisting of CS

The CS was prepared as described previously¹¹⁾. Briefly, the collagen was extracted from porcine skin by enzymatic treatment, and was composed of type I (70% to 80%) and type III

(20% to 30%) atelocollagen. This purified collagen was dissolved in hydrochloric acid with a pH of 3.0, so that its concentration became 3.0%. An inner tube 3 mm in diameter and 25 mm in length was inserted in the center with its axis parallel to the outer tube, which had an inner diameter of 10 mm and a length of 25 mm, to create a cylindrical space. The homogenized collagen solution was poured into this space between the inner and outer tubes. This material was frozen once at -20°C and then freeze-dried for 24 h to produce a porous structure, followed by dehydrothermal treatment at 140°C for 24 h in a vacuum to induce cross-linkage of the collagen molecules.

3)Prosthesis consisting of CS reinforced with a mesh framework

The CS reinforced with a mesh framework was prepared with the method described previously¹²⁾. Briefly, the mesh framework consisted of a cylinder formed by surgical PP mesh (Bard Mesh: CR Bard, Crawley, UK), reinforced with a continuous PP spiral. The mesh cylinder was 6 mm in inner diameter and 25 mm in length. The PP spiral, with a diameter of 0.8 mm, was attached to the external surface of the mesh cylinder and fixed with 6-0 Prolene sutures (Ethicon, Somerville, NJ). The mesh cylinder was exposed to corona discharge at 9 kV for 15 min to render the polymer surface hydrophilic. The collagen solution was poured into the space between the inner and outer tubes in which the mesh framework was placed. This material was then freeze-dried and cross-linked as described above. In this process the CS sealed both the inner and outer surfaces of the mesh framework.

The prosthesis was sterilized with ethylene oxide gas before implantation.

4)Animal experiment

Seven beagle dogs, weighing 11.0 to 13.0 kg, were anesthetized with an intramuscular injection of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (3 mg/kg) and intubated

Table 1 Biliary reconstruction using prostheses

	Dog No.	Prosthesis	Duration of stent (weeks)	Survival time (weeks)	Stenosis	Epithelization
Group A	1	Entirely CS	2	6	+	-
	2	Entirely CS	2	8	+	-
Group B	3	Entirely CS	12	12	-	-
	4	Entirely CS	12	12	-	-
Group C	5	CS and PP	2	12	-	+
	6	CS and PP	2	12	-	+
	7	CS and PP	2	12	-	+

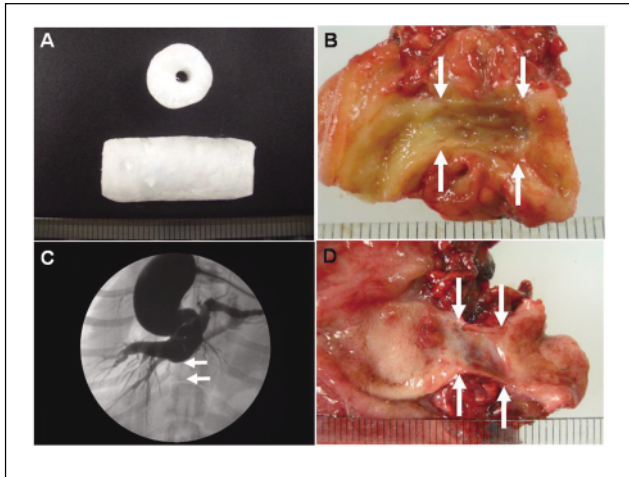


Fig.1
 A: The artificial bile duct consisting entirely of CS. B and C: The reconstructed bile duct (B) and cholangiogram (C) in group A. Occlusion of the prosthesis is noted at the implant site. D: The reconstructed bile duct in group B. Arrows, anastomosis.

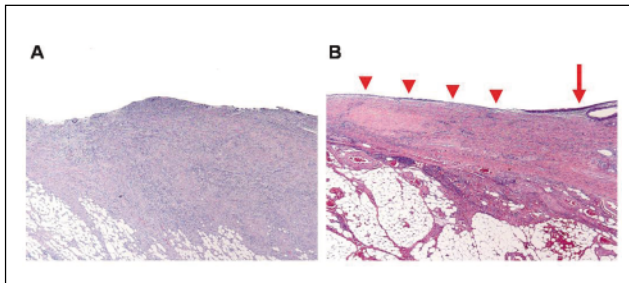


Fig.3
 Microscopic findings in group A (A) and group B (B). A: The reconstructed bile duct is occupied by only inflammatory granulation tissue. B: Biliary neopithelium is observed only near the anastomoses (arrow), while the middle of the prosthesis lacks an epithelial lining (arrowhead). (Hematoxylin and eosin stain; original magnification in both, x40.)

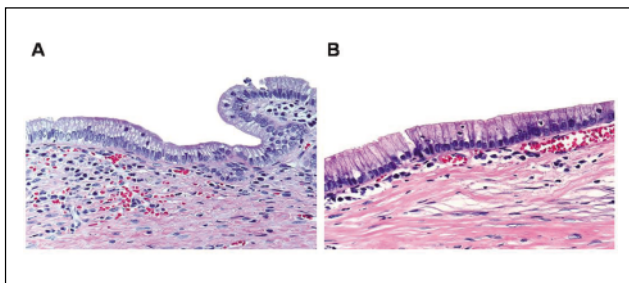


Fig.5
 Microscopic findings of the native common bile duct (A) and the reconstructed bile duct in group C (B). The regenerated epithelial layer is similar to that of the native common bile duct. (Hematoxylin and eosin stain; original magnification in both, x100.)

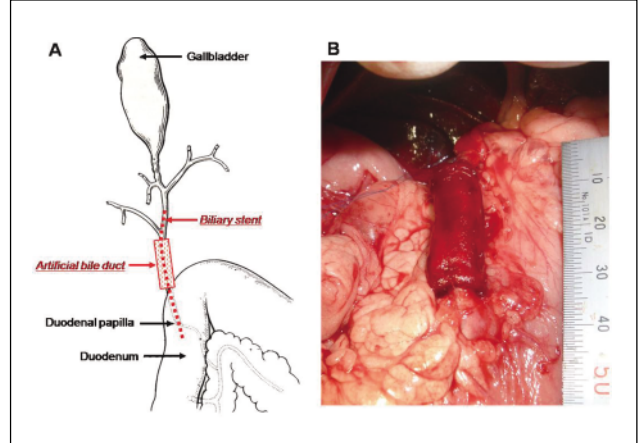


Fig.2
 A: Scheme of biliary reconstruction with a prosthesis and biliary stenting in a canine model. The common bile duct was replaced with an artificial bile duct by end-to-end anastomosis. (Remarked from the original figure 7-46 (page 295) in “Miller’s anatomy of the dog”, by Dr. Howard E. Evans & George C. Christensen, published from W.B. Saunders Company, Philadelphia, USA, 1979.) B: Intraoperative findings after replacement with the artificial bile duct.

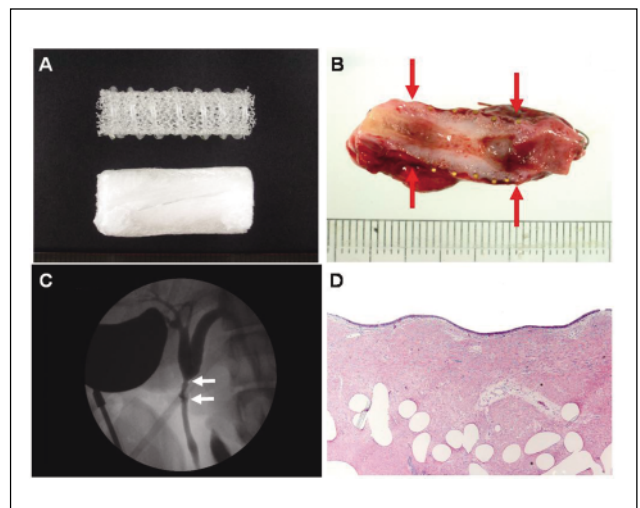


Fig.4
 A: The artificial bile duct consisting of CS reinforced with a mesh framework. B and C: The reconstructed bile duct (B) and cholangiogram (C) in group C. The prosthetic lumen remains patent, and little stenosis is evident. D: Microscopic findings in group C. The luminal surface is covered completely with columnar epithelium. (Hematoxylin and eosin stain; original magnification, x40.) Arrows, anastomosis.

endotracheally. Anesthesia was maintained under mechanical ventilation using sevoflurane (1%) and nitrous oxide (50%). The common bile duct was approached through a midline abdominal incision, and a 20-mm segment of the common bile duct was resected circumferentially. Seven dogs were separated into three groups (Table 1). In groups A (2 dogs) and B (2 dogs), a biliary prosthesis consisting of CS alone was used. The artificial bile duct was invaginated outside the proximal cut ends of the native bile duct and anastomosed by interrupted suturing with 6-0 Vicryl (Ethicon). After the proximal anastomosis had been completed, a PTCO tube (8 Fr, Create Medic, Yokohama, Japan) was inserted into the common bile duct through the prosthesis lumen and fixed with a 6-0 Prolene suture (Ethicon). This PTCO tube was positioned to extend from the common bile duct to the duodenum through the papilla as a biliary stent, in order to protect the prosthesis from bile and prevent biliary leakage until the prosthesis had been covered by host tissue (Fig.2). The distal stump of the bile duct was invaginated into the other end of the prosthesis and anastomosed in the same manner. After both anastomoses had been completed, the replaced area was wrapped with greater omentum. The gallbladder was left intact. Finally the abdominal incision was closed in layers. In group C (3 dogs), a biliary prosthesis consisting of CS reinforced with a mesh framework was used employing the same operative procedure. An antibiotic (cefazolin sodium, 50 mg/kg/day) was administered intramuscularly for 5 days.

In groups A and C, a second laparotomy was performed under general anesthesia 2 weeks after the first operation. The biliary stent in each dog was removed by cutting the sutures anchoring it, with access through a small incision in the duodenum. The duodenal incision then was closed with 3-0 Vicryl (Ethicon) using interrupted sutures. The abdominal incision was then closed in the same manner. In group B, the biliary stent was not removed, and was left in the prosthetic lumen during the experiment.

Dogs were euthanatized 12 weeks after prosthesis implantation after cholangiography had been performed via the gallbladder in each dog. The artificial bile duct was resected en bloc, including both the distal and proximal sites of the native bile duct, and prepared for histologic examination. All experiments were conducted in accordance with the Rules and Regulations of the Committee for Animal Research of Kyoto Prefectural University of Medicine, Japan.

Results

All dogs survived the first and second operations, and no op-

erative death occurred in any animal. At autopsy, all of the artificial bile duct was seen to have become incorporated into the native bile duct with varying degrees of adhesion to surrounding tissues, especially the greater omentum. The 2 dogs in group A showed decreased appetite, diarrhea, and weight loss after removal of the stent, and gradually became jaundiced within 2 months. These animals were euthanatized, and at autopsy circumferential stenosis of the artificial bile duct was noted. Occlusion of the reconstructed area with severe proximal biliary dilation was recognized on cholangiograms (Fig.1B,C). Microscopic examination of specimens taken from the dogs in group A revealed that the CS of the prosthesis had been replaced with only severe inflammatory granulation tissue comprising predominantly macrophages, fibroblasts, and polymorphonuclear cells. No regeneration of the biliary epithelium was evident (Fig.3A).

The animals in groups B and C appeared clinically well and survived without serious events until their scheduled sacrifice at 12 weeks. In group B, the silicone tube was retained in the prosthetic lumen as a biliary stent for 12 weeks. Cholangiograms revealed free passage of contrast medium into the duodenum through the drainage tube. Macroscopically, the reconstructed area was covered with host tissue surrounding the silicone tube (Fig.1D). Microscopically, the reconstructed bile duct wall was composed of granulation tissue with an inflammatory appearance. The luminal surface mostly lacked an epithelial lining, although biliary neoeepithelium was present in the lumen at the edges of the prosthesis (Fig.3B).

In group C, the artificial bile duct consisting of CS reinforced with a mesh framework maintained the patency of the graft after the removal of the inner stent at 2 weeks and provided satisfactory bile drainage throughout the study period even at 12 weeks after implantation. The animals in group C had normal cholangiograms and no narrowing of the reconstructed bile duct (Fig.4C). It appeared that stricture formation with no biliary stenting, like that observed in group A, was prevented by supporting the tissue with the mesh framework of the prosthesis. Macroscopically, the prosthesis was covered entirely with host tissue and the prosthesis mesh was not exposed to the biliary lumen (Fig.4B). Importantly, bile crystals did not form within the reconstructed bile duct. Microscopic examination indicated that the reconstructed bile duct was composed of a layer of fibrous collagen bundles including small vessels that had covered the mesh framework of the prosthesis. The inflammatory response was minimal. Furthermore, biliary neoeepithelium extending over the connective tissue layer was present on the internal surface throughout the reconstructed bile duct (Fig.4D). This regenerated epithelial

layer was composed of a monolayer of cubic columnar epithelial cells, similar to that found in the native bile duct (Fig.5A,B).

Discussion

The main components required for tissue engineering are cells, growth factors and a scaffold. Among these three elements, the scaffold is central to the concept of *in situ* tissue engineering because this technique utilizes autologous cells and growth factors derived from the body *in vivo*. Once an adequate scaffold is applied to a tissue defect as an appropriate “field” for tissue regeneration, it utilizes the natural healing potential of the body, which subsequently achieves natural regeneration of tissues and organs *in vivo*. If this technique works well, it can provide a more realistic and clinically acceptable approach than *in vitro* tissue regeneration. In the present study, we investigated the specific design of an artificial bile duct used as a scaffold for bile duct tissue regeneration using *in situ* tissue engineering.

We used CS as a base material for this artificial bile duct because of its high biocompatibility and optimal porosity. This CS is a biodegradable material which has been shown to be an effective scaffold and promotes the regeneration of various tissues^{11,16,17}. Once autologous cells derived from tissues surrounding the area of a tissue defect infiltrate an implanted scaffold, they themselves can produce the intact extracellular matrix (ECM) that accelerates their proliferation and differentiation, resulting in natural tissue regeneration. In groups A and B, we used an artificial bile duct constructed entirely of CS.

On the other hand, although the collagen scaffold can facilitate cell attachment and tissue infiltration, it is permeable to bile and cannot prevent bile from leaking through it. In our animal experiments, therefore, a bile drainage tube was needed as a biliary stent to protect the prosthesis from bile and to prevent bile leakage, especially in the initial stage after implantation. Therefore, we placed the biliary stent that extended from the common bile duct to the duodenum because it was technically easy to remove the stent through a small incision on the duodenum by the second laparotomy. The stents of the dogs in group A were removed 2 weeks after the operation when CS was completely replaced with autologous tissue that had infiltrated from the area surrounding the graft. However, constriction of the reconstructed bile duct gradually progressed after stent removal, resulting in subsequent biliary stricture within 2 months.

By contrast, in group B, the biliary stents had been left in the prosthetic lumen for 12 weeks in order to avoid luminal constriction and scar formation. This long-term stenting made the prosthesis resistant to compression, and the stenosis that was

observed in the group A dogs did not occur. Although microscopic examination revealed that the reconstructed bile duct was occupied by granulation tissue with an inflammatory appearance, no high-order structure appeared. We considered that at least two factors may have been responsible for these unsatisfactory results. First, the long-term stenting would have resulted in prolonged mechanical stimulation. The friction between the lumen of the reconstructed bile duct and the inner stent might not only have evoked chronic inflammation of the tissue that had infiltrated into the CS but also delayed the regeneration of the biliary epithelial layer. Second, the function of the duodenal papilla may have been inhibited during the period of stent placement, and the resulting long-term interference with the sphincter mechanism might have created a potential risk of back-flow of duodenal contents into the biliary tree. The findings in group B revealed that long-term stenting offered no benefit for biliary epithelization, and that the duration of biliary stent retention should be a minimum.

Based on the results obtained in groups A and B, we speculated that a key factor contributing to the success of biliary reconstruction using an artificial bile duct was the specific design of the prosthesis, maintaining a tubular structure for bile duct tissue regeneration *in vivo*. In order to function successfully, an artificial bile duct should itself have sufficient mechanical strength to support the regenerated tissue, thus maintaining a three-dimensional configuration until tissue remodeling has been accomplished, without long-term stenting. In group C, therefore, we designed a new type of artificial bile duct consisting of CS reinforced with a PP mesh.

PP meshes are commonly used in patients with hernia and have high mechanical strength to provide reinforcement for the infiltrated tissue¹⁸. The mesh structure of our prosthesis enabled the regenerated tissue induced by the CS to maintain a tubular structure, supporting the tissue from outside of the prosthesis, while not inhibiting the regeneration of biliary epithelium on the luminal surface. As we expected, the artificial bile duct in group C maintained its patency even after removal of the stent and provided satisfactory bile drainage for 12 weeks. The biliary stricture that was observed in the dogs of group A was not evident on the cholangiograms in group C, and microscopic examination showed that the reconstructed bile duct was similar to the native bile duct structurally and morphologically. Fibrous collagen bundles, including small vessels, had covered the prosthesis and a biliary neopithelial layer was present over the entire luminal surface. Minimal tissue reaction was observed around the PP elements of the mesh.

Our present findings suggest that *in vivo* tissue regeneration of the bile duct can occur if an appropriate environment serving as an artificial bile duct is provided at a site that requires repair. *In situ* tissue engineering of the common bile duct can be potentially compromised due to the presence of bile at the site of prosthesis placement, and this may be associated with complications, such as leakage, stasis, or infection. In fact, our prosthesis was exposed to bile juice after removal of the inner stent. Bile salts are known to be potentially cytotoxic, due to their potent detergent properties, causing cellular membrane injury^{19,20}. Previous experiments in pigs have provided evidence that bile salts can seriously exacerbate injury of the biliary epithelium²¹. Despite these disadvantageous conditions, however, the biliary epithelium was able to regenerate on the luminal surface of the prosthesis.

In general, PP mesh is a stiff material. Therefore, the reconstructed bile duct containing the PP mesh was different from the native bile duct in terms of tissue flexibility. Although the PP mesh was beneficial for providing good tissue strength and maintaining the patency of the reconstructed bile duct, it also contributed to fibrosis. Eventually, the connective tissue layer in the reconstructed bile duct containing the PP mesh became much thicker than that of the native bile duct. Reduced flexibility and increased stiffness caused by the implanted PP mesh may therefore lead to a reduction in function. Further long-term assessment seems necessary to exclude the possibility of long-term complications attributable to the rigidity of the reconstructed bile duct. Also, to overcome the problem of tissue rigidity, a more flexible material could be promising for use as a mesh framework.

There is also room for improvement of the inner structure of our artificial bile duct. We employed biliary stenting in order to prevent bile leakage. If the luminal surface would be impermeable to bile, no stenting of the prosthetic lumen would be required, even in the early stage of implantation. Coating the prosthetic lumen might be promising for another improvement.

In conclusion, we have confirmed that our artificial bile duct consisting of CS and PP mesh provides adequate tissue proliferation as a template for tissue remodeling. This composite material has both sufficient biocompatibility and mechanical properties and provides an appropriate three-dimensional structure at the implanted site for bile duct tissue regeneration *in vivo* without the need for long-term biliary stenting. Our prosthesis has potential for regeneration of biliary defects through the concept of *in situ* tissue engineering if the flexibility of the reconstructed tissue can be further improved.

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