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Original Article

Paraffin processing of stented arteries using a postfixation dissolution of metallic and polymeric stents

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ABSTRACT

Studying the morphology of the arterial response to endovascular stent implantation requires embedding the explanted stented artery in rigid materials such as poly(methyl methacrylate) to enable sectioning through both the in situ stent and the arterial wall, thus maintaining the proper anatomic relationships. This is a laborious, time-consuming process. Moreover, the technical quality of stained plastic sections is typically suboptimal and, in some cases, precludes immunohistochemical analysis. Here we describe a novel technique for dissolution of metallic and plastic stents that is compatible with subsequent embedding of "destented" arteries in paraffin, fine sectioning, major staining protocols, and immunohistochemistry.

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1. Introduction

With more than 1 million devices deployed yearly worldwide [1], endovascular stents are by far the most widely used type of artificial implants in medical practice. Evolution of stent designs, especially pertaining to those that combine mechanical support with local drug elution from stent surface, has led to a rapid expansion of indications for coronary and peripheral artery stenting and to ever decreasing rates of complications [2]. However, two clinical issues, in-stent restenosis (ISR) [3] and late stent thrombosis (LST) [4], present a formidable problem even with the state-of-art, third-generation drug-eluting stents. While currently below 10% and 1%, respectively, ISR and LST carry significant medical and economic burden due to the voluminous use of stenting [5]. High-quality histological analysis allowing identification of specific cell types associated with stent struts, their physiological status, and secretome is an essential tool for studying normal physiological healing response and the pathological reaction of the vessel wall to stent implantation. An inherent technical problem associated with histologic processing of stented arteries is the presence of metallic or polymeric struts encased in the neointima of long-term implants. These elements are harder than surrounding vascular tissue, thus precluding successful sectioning of stented arteries embedded in paraffin. To enable sectioning, the stent-bearing arteries are embedded into a rigid media, such as poly(methyl methacrylate). Sectioning is carried out using either a precision saw with subsequent polishing [6,7] or a heavy-duty microtome equipped with a tungsten carbide knife [7]. This is a low-yield, laborious process requiring costly equipment and technical expertise. Moreover, the sections are often poor quality because the dragging momentum of the microtome results in strut displacement and tissue tears [7]. Additionally, immunohistochemical or immunofluorescence evaluation of sections is often challenging mostly due to excessive heat generated during embedding and sectioning [6]. Exposing arteries to harsh organic solvents to remove the polymeric imbedding media may also play a role in the impeded epitope binding by antibodies that readily work on paraffin-embedded arterial tissue.

Recently, electrochemical dissolution of stents within the formalin-fixed arteries was reported by two groups [8,9]. This method allows paraffin embedding of partially destented arteries, thus facilitating subsequent sectioning and staining. Despite demonstrated proof of principal, it is difficult to envision routine use of electrochemical methods for stent dissolution since they are extremely laborious and destroy the proximal and distal ends of the stented segment. Moreover,

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since electric current stops when the circuit is broken, dissolution of stent struts is never complete, leaving islands of metallic wires that impede sectioning.

No prior study has investigated a direct chemical dissolution of metallic or polymeric stents. Metal alloys used for stent manufacture (316L stainless steel, cobalt-chromium-tungsten-nickel alloy L605, cobalt-platinum alloy, nitinol) are corrosion resistant. These materials withstand treatment with strong acids due to a rapid formation of a surface layer consisting of metal oxides, which, being insoluble in acid, protects the underlying bulk of material from acidic dissolution. Interestingly, addition of relatively weak hydrofluoric acid (pK_a=3.17) to nitric acid (pK_a=−1.3) enables dissolution of stainless steel because of the high solubility of metal oxides in hydrofluoric acid [10]. Polyesters used for the fabrication of biodegradable stents and scaffolds, such as poly-L-lactide (PLLA), are readily soluble in nonpolar organic solvents, dichloromethane and chloroform.

Here we report a novel method that provides complete stent dissolution in explanted, formalin-fixed stented arteries with a fully formed neointima. We show that this "destenting" protocol enables paraffin embedding, preserves the morphology of the arterial tissue, and is fully compatible with virtually all standard histological staining protocols and immunohistochemistry unlike conventional techniques using rigid imbedding media.

2. Materials and methods

2.1. Dissolution kinetics

Multilink 316L stainless steel stents (n=3; Laserage, Waukegan, IL), nitinol self-expanding stents removed from Smart Control Vascular Stent systems (n=3; Cordis, Hialeah, FL) and 1 mm×0.1 mm×8 mm coupons of L605 cobalt-chromium-tungsten-nickel alloy (n=3) cut from L605 foil (Goodfellow, Coraopolis, PA) were cleaned with isopropanol and chloroform and dried at 70°C. The weight of each sample was recorded. The samples were then individually exposed to 20 ml of a 1:1 (v/v) mixture of 3 N nitric acid and 12.8 N hydrofluoric acid at 28°C with mild shaking. After 5, 30, 90, and 360 min of incubation, the samples were removed from the acid solution, washed in water, wiped to dryness, weighed, and placed back in the acid. The relative decrease of the samples weight was plotted over time to reflect dissolution kinetics. The dissolution of PLLA fibers (PL-32, 565 kDa, 65 μm diameter; Purac, Netherlands) in chloroform was similarly studied and presented.

2.2. Preparation of stents

Biodegradable stents were manufactured in-house (University of Texas, Dallas, TX) from PLLA resin, PL-32 (565 kDa, Purac, Netherlands). PLLA was melt-extruded and drawn to a final diameter of 0.065±0.01 mm [11]. The drawn fibers were then wound on a specially created jig to make the double opposed coiled stent. Axial reinforcing rods, made of the same drawn PLLA fiber, were inserted into the coil and fixed at the fiber overlaps. Stent crimping on the balloons of the PTA catheters (NuMed, Hopkinton, NY) was performed using a manual crimp tool (Model RJ25, Blockwise Engineering, Chicago, IL) to a final external diameter of 1.02 mm. The mounted stents were sterilized in 70% ethanol prior to use. Multilink stents (304 stainless steel grade; Laserage) were cleaned with isopropanol and chloroform and sterilized in 70% ethanol prior to mounting on PTA catheters (NuMed).

2.3. Animal experiments

Carotid stent angioplasty procedures were carried out in 400–450 g male Sprague–Dawley rats (n=10). After performing carotid balloon denudation with 3 passages of a 2F Fogarty catheter (Edwards LifeSciences, Irvine, CA), the stents (n=5 for each stent type) were deployed with PTA catheters (1.5 mm nominal diameter; NuMed) at 16 atm in the mid-segments of the left common carotid arteries as described in our previous publications [12–14]. The animals were euthanized 14 days after stent deployment. Bromodeoxyuridine (40 mg/kg) was injected intraperitoneally 6 h before sacrifice. Harvested stented arterial sections were flushed with heparinized saline and fixed in 10% buffered formalin for a minimum of 48 h. All animal studies were approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee and conducted in accordance with principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

2.4. "Destenting" procedures

2.4.1. Stainless steel stents

Formalin-fixed arteries incorporating metallic stents were washed in running tap water for 2 h, rinsed in distilled water, and individually treated with 20 ml of a 1:1 (v/v) mixture of 3 N nitric acid and 12.8 N hydrofluoric acid at 28°C with mild shaking for 5 h. Completeness of stent dissolution was confirmed by microscopy using a dissecting microscope. The destented arteries were then washed in running tap water for 30 min, postfixed in 10% neutral buffered formalin for additional 24 h, and routinely processed for paraffin embedding and sectioning.

We also tested feasibility of stent dissolution within archival plastic-embedded arteries to allow paraffin reembedding. The uncut edge fragments of poly(methyl methacrylate)-embedded rabbit iliac arteries bearing stainless steel stents were deplastified with 2-methoxyethyl acetate (Aldrich) and hydrated through graded ethanol sequence. Strut dissolution was carried out in HNO3/HF formulation as described above. Destented segments were then refixed for 24 h in formalin and routinely embedded in paraffin.

2.4.2. PLLA stents

The formalin-fixed arteries incorporating PLLA stents were washed in running tap water for 2 h and dehydrated in graded ethanol and xylene. After the second xylene change, PLLA stent-deployed arteries were exposed to chloroform at 28°C with mild shaking for 20 min. Completeness of stent dissolution was confirmed microscopically as above. The destented arteries were then transferred to 100% xylene and routinely processed for paraffin embedding and sectioning.

2.5. Immunohistochemistry and special stains

The slides were deparaffinized and boiled in a pressure cooker with antigen retrieval buffer (H-3300; Vector Labs, Burlingame, CA) for 3–5 min. The sections were then blocked with 10% horse serum for 20 min, followed by application of either a primary antibody or nonimmune IgG at room temperature for 1 h. After PBS washing, the sections were consecutively treated with the secondary biotinylated anti-mouse (BA-2001, Vector Labs) or anti-rabbit (BA-1100, Vector Labs) antibodies (1:100–1:200 dilution; 45 min), Vectastain Elite ABC reagent (PK-6100, Vector Labs), and peroxidase substrate (ImmPact DAB; SK-4105, Vector Labs). Finally, the sections were counterstained with Gill 3 hematoxylin (5 and 40 s for the PLLA destented and stainless steel destented samples, respectively) and mounted. Nonimmune special stains, Verhoeff-van Gieson (VVG) elastic stain, and Masson's trichrome staining were carried out according to the instructions provided by the manufacturers of the respective kits (HT25A; Sigma-Aldrich, St. Louis, MO and 25088; Polysciences, Warrington, PA). Digital images were captured under magnification ×40–200 with a Nikon Eclipse 80i microscope.

3. Results

3.1. Dissolution kinetics of nonimplanted stents

Optimization experiments (not reported here) have established the optimal molar ratio of 3–3.4 between hydrofluoric and nitric acid for dissolution of 316L grade stainless steel and investigated the stainless

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