A New Method for the Joint Visualization of Vascular Structures and Connective Tissues: Corrosion Casting and 1 N NaOH Maceration

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KEY WORDS corrosion casting technique; 1 N NaOH method; scanning electron microscope

ABSTRACT Corrosion casting combined with scanning electron microscopy (SEM) has been widely used to study the morphofunctional aspects of microcirculation in many organs. In this study, we present an optimization of the corrosion casting (CC) technique associating it with NaOH 1 N maceration method to obtain a clear visualization of the relationships existing between the microvascular architecture of an organ and its extracellular matrix. Briefly, experiments were performed macerating the tissue previously injected with a low viscosity acrylic resin in 1 N NaOH and then observing it at SEM. In this study, we present an application of this technique to better evaluate the extracellular components of the vascular wall in medium-sized and capillary vessels both in skin and in kidney. The results obtained yielded clear images of the three-dimensional layout of medium-sized and capillary vessels in comparison with the extracellular environment. Furthermore, detailed information was obtained on the three-dimensional layout of fibers constituting the walls of venules, arterioles, and capillaries. In addition, the tubular collagenic structures surrounding the excretory tubules of the kidney and the dermal glands of the skin were depicted and their relationships with their vascular supply described in detail. *Microsc. Res. Tech. 69:919–923, 2006.*

INTRODUCTION

The corrosion casting technique has been widely used to obtain a detailed three-dimensional picture of the vascular architecture in many organs, both under physiological and pathological conditions. Combined with scanning electron microscopy (SEM), corrosion casting yields detailed information on capillary morphology at structural and ultrastructural level. The spatial layout of capillaries is readily displayed in three dimensions and the whole vascular architecture of an organ can be visualized (Castenholz, 1989; Hodde and Nowell, 1980; Lametschwandtner et al., 1990; Murakami, 1971; Weiger et al., 1986).

To produce a three-dimensional image, both the cellular and the interstitial collagenic components must be removed using strong alkalis and/or acids to digest all the organic tissues around the vessels. This process destroys important information regarding the close relationships existing between vessels and the surrounding organic tissue.

For a long time, semicorroded specimens obtained by stopping the digestion process at different times appeared to be the only method available to study these three-dimensional relationships. To date, no protocols have been devised to standardize a useful technique to readily interpret and reproduce these data. Only few reports described semicorrosion casting methods to visualize elastic fibers and pericytes using a low KOH, NaOH concentration of corrosion solutions (Goncalves, 1995; Song et al., 1992; Song 1994). Other authors used ultrasound-treated and plastoid-injected specimens to be able to visualize the periendothelial cells in arterioles and capillaries of rat brain (Castenholz, 1983a) or fractured the injected specimens with a particular method that allowed a detailed visualization of outer surface morphology of blood vessels (Castenholz, 1983b).

We devised a modification of corrosion casting by associating it with 1 N NaOH maceration method (Ohtani et al., 1988) for the study of the three-dimensional layout and morphology of interstitial collagen tissue. Here, we present the application of this new technique using as a model kidney and skin of wistar rats.

MATERIALS AND METHODS

Thirty male Wistar rats weighting an average of 325 g were used. The rats were deeply anesthetized with a subcutaneous injection of 2 mL ketamine and then killed by high dose of anesthetic, according to the guidelines for use and care of laboratory animals, approved by the ethical veterinary service of our university. Soon after death, the abdominal cavity was opened. A 24G cannule was then inserted into the abdominal aorta and the vena cava punctured to allow reflux blood outflow. We then manually perfused the kidneys with 10 mL of heparinized solution (4%) to pre-

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Received 8 February 2006; accepted 7 July 2006

DOI 10.1002/jemt.20366

Published online 18 August 2006 in Wiley InterScience (www.interscience.wiley.com).

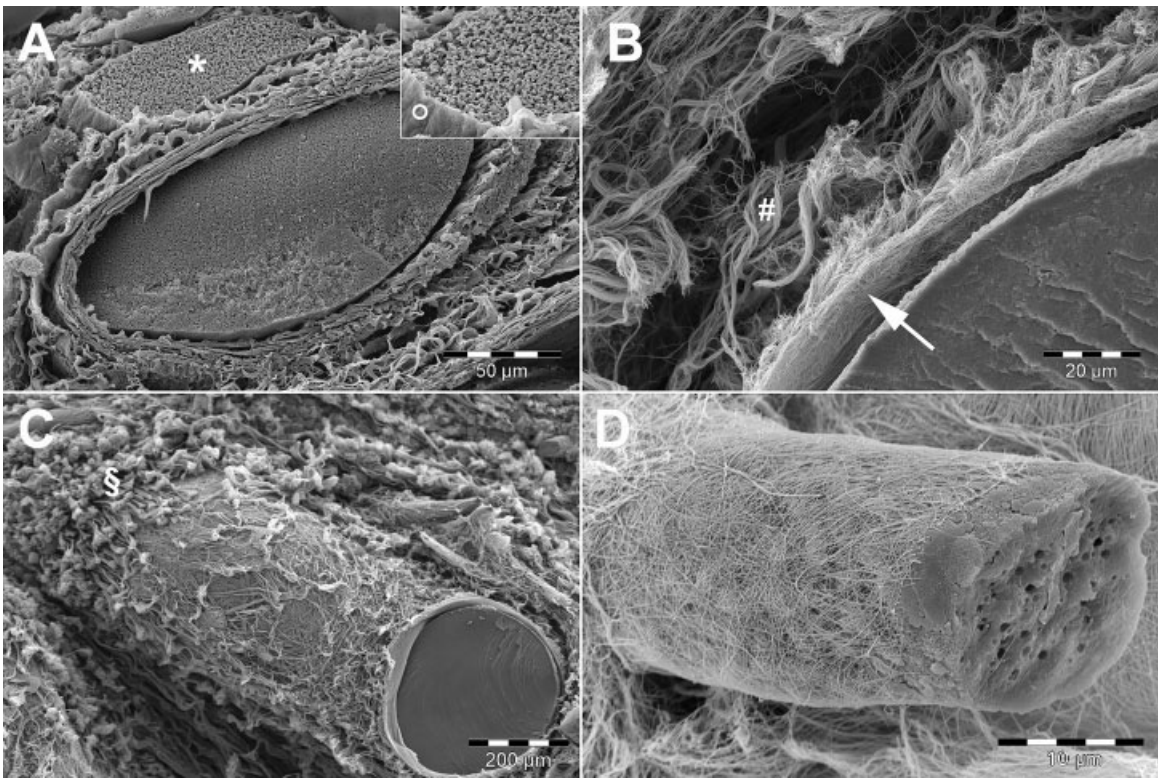

Fig. 1. Skin. (A) A venule filled with the resin in transverse section, with its collagenic wall made up of multiple layers, next to a nerve (*). Inset: High magnification of the nerve: it is possible also to observe the collagenic structure of the perinevrium wall (○). Note the ovoidal section of the cast, typical of the venule and the orientation of collagen fibers constituting its wall. (B) High magnification of the collagenic wall of an artery; note the subendothelial layer (arrow) facing the cast (transversally sected) and made up of a thin layer of interlaced collagen fibers. The free space included between the cast and this collagenic layer is usually occupied, in noncorroded specimens, by the endothelial cells. This layer represents their basement membrane. Note the collagen bundles of the intermedial layer that do not follow any particular direction (#) and the external one. (C) The two layers collagenic wall of an arteriole (roundish transverse section); note the tangentially sectioned collagen fibers oriented parallel to the major axis of the vessels and belonging to the external layer (§) and the randomly placed collagen fibers of the internal layer. (D) The thin collagenic layer of a capillary. Note the thin fibers variously oriented enveloping the cast.

vent blood clotting. We proceeded fixing the vessels with 25 mL of Karnovsky solution to prevent the resin leaking and to reduce the modifications to endothelial cells occurring during the injection phase.

In the meantime, we mixed 20 mL of an acrylic low-viscosity resin (Mercox, SPI Supplies; viscosity of 50 cp) with 5 mL of methyl-methacrylate (Jeol) and 5 g of catalyzer (benzoyl-peroxide; SPI Supplies). The solution obtained was then injected at 1 mL/min flow rate through the cannule until we observed reflux from the vena cava and the injection pressure gradually increased.

After partial polymerization of the resin, the kidneys and a small specimen of the dorsal skin of the animals were surgically explanted and immersed in a warm saline solution (60°C) to complete the hardening process. The specimens were then immersed in formaldehyde (4%) for 6 days and kept in cold storage.

Six kidneys were then cut in 1 mm slices with a razor blade under stereomicroscopic control, embedded in Cryoembed medium and reduced to 0.4 mm using a Leica HM560 cryostat (Leica Microsystems AG, Wetzlar, Germany). The slices obtained underwent the digestion process in 1 N NaOH for at least 5 days. The resulting specimens were rinsed in distilled water, dehydrated in ascending grades of ethanol, and critical-point dried in CO_2. The specimens were then mounted on adhesive films applied on standard aluminum stubs, gold coated in an Emitech K550 sputter coater (Emitech Products, Houston, TX), and then observed under a Philips XL-30 SEM-FEG microscope (FEI, Eindhoven, the Netherlands) at 10 keV.

RESULTS

Skin

Many arteries and veins, readily distinguished observing both their transverse sections and the collagenic architecture of their perivascular wall, were seen in the dermal layer of the skin.

Medium-sized arteries characterized by a roundish transverse section, and veins with an ovoidal one were observed. It was possible to study the fine arrangement of collagen bundles constituting the walls of medium-sized arteries and veins from the intimal layer to the adventitial one. It was also possible to observe the clear-cut relationship between the vessels and the nerves, represented by a dense group of fibers, all oriented parallel to one another along the vessel's major axis (Fig. 1A). We observed each of the multiple collagenic

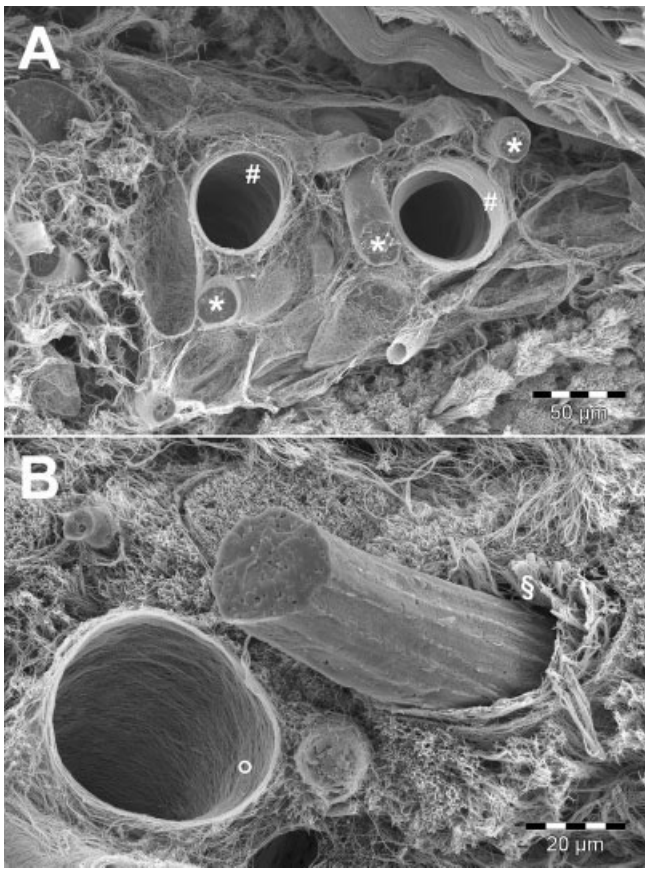

Fig. 2. Dermal layer of the skin. (A) The vascular and collagenic structure of a sweat gland. Note the high density of capillaries filled with the resin and transversally sected (*), surrounding empty tubules with a well-organized collagenic wall (#). (B) High magnification of the collagenic wall of the tubule (○ characterized by a single collagenic sheet and that of an arteriole (§) characterized by longitudinally oriented collagen fibers along the major axis of the vessel (its cast, partially extruded, is characterized by longitudinal endothelial imprints). Note the space visible between the cast and collagenic layer usually occupied by endothelial cells in noncorroded tissue specimens. It is also possible to observe the collagen of interstitial space around the wall of the arteriole and the tubule in which two other capillaries, filled with the resin and transversally sected, are visible.

layers surrounding the vascular cast. The closest to the cast was the intima, made up of interlaced collagen bundles regularly arranged to form a dense subendothelial collagenic net, most of them directed along the major axis of the vessel (Fig. 1A). Just below this layer some thicker collagen bundles were seen following any direction and forming a more disorganized wide net of collagen. The external layer of these medium-sized vessels, in direct contact with the dermal collagen, seemed to be formed by thick collagen bundles directed along the major axis of the vessel (Fig. 1B).

The arterioles were characterized by a roundish transverse section and a collagenic wall made up of two main layers. The first (the intima) was formed by a dense sheet of collagen fibers, variously arranged in the two dimensions of the wall. The second was composed of thick collagen bundles oriented along the major axis of the vessels, as readily observed in a transverse section (Fig. 1C). The capillaries were characterized by a roundish transverse section and a collagenic wall made up of a single irregular net of thin collagen fibers enveloping the vascular cast (Fig. 1D).

The dermal sheet of the skin contained many tubular structures mostly belonging to sweat and sebaceous glands, and was characterized by groups of empty tubular structures (adenomer tubules), surrounded by a capillary three-dimensional net (Fig. 2B).

The tubule wall was formed by a single collagenic layer corresponding to the basal lamina of the ductal cells. The radius of the tubules varies from 20 to 25 μm. The walls of the capillaries and arterioles of the glands had no specific characteristics in the collagenic architecture of their wall that revealed to be similar to that of the other capillaries and arterioles sited in the dermal layer (Fig. 2B).

Kidney

The kidney can be divided into a cortical and a medullary portion. The cortical part was characterized by a high density of glomerular structures made up of convoluted and interlaced capillaries well visible in a transverse section. It was also possible to observe the afferent or efferent arteriole entering or exiting the glomerulus and its thin collagen capsule (Bowman capsule). It was also readily apparent that the intercapillary spaces are filled by variously displaced collagen fibers (Fig. 3A).

The excretory tubules in the medullary area were easy to distinguish from the surrounding capillaries. They consisted of empty tubular structures made up of a collagenic layer characterized by a circular arrangement of collagen fibers. Each tubule was surrounded by the cast of its own capillary network, made up of arteries and veins well distinguished from one to another, on observing their transverse sections (Figs. 3B and 3C).

DISCUSSION

The results obtained demonstrate the utility of this technique in visualizing the very close relationship existing between the vascular and collagenic architecture of an organ.

We applied this technique to the skin and the kidney of wistar rats to focus on their microvascular architecture and relate it to its collagenic component (De Takats, 1932; Edwards, 1960; Hundeiker, 1971; Kawabe et al., 1985; Kikuta, 1989; Ditrich, 1990; Sangiorgi et al., 2004a,b; Sangiorgi et al., 2005). The technique proved to be a sharp method to give a precise three-dimensional topographic collocation of vessels, giving the vascular casts a precise "grid" for the interpretation of their position in space. We could also distinguish the tubular collagenic empty structures, belonging mostly to tubular glands and excretory tubules, from their vessels filled with the resin.

Moreover, the cast itself gives important mechanical support to the stromal interstitial component most of all in those organs and structures lacking a strong connective architecture thereby enhancing the study of their three-dimensional collagenic architecture.

It is worth to note that, using our technique, it was also possible to evaluate the exact diameters of the vessels without any distortion due to the dehydrating processes and to estimate the true vascular volume of

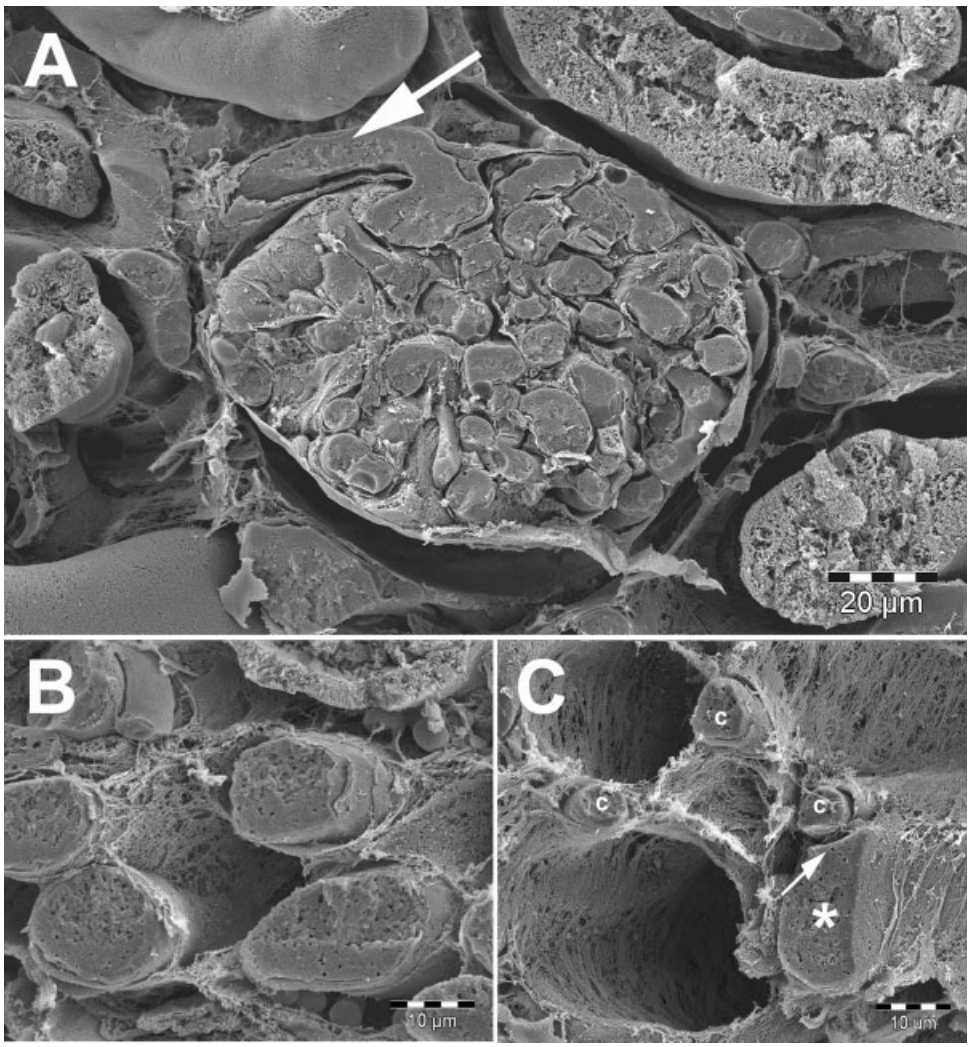

Fig. 3. The kidney. (A) Cortical region: note the afferent or efferent arteriole entering or exiting the glomerular body (arrow), the capillary structure visible as roundish sectioned casts, the collagenic capsule surrounding the glomerulus and containing intercapillary collagen fibers. (B) Medullary region: high magnification of an empty tubule surrounded by four capillaries filled with the resin and transversally sected. Note its collagenic structure made up of a thin layer of circular collagen fibers constituting the basal membrane of tubular cells digested away. (C) High magnification of two tubules divided by collagenic septi in which small capillaries can be seen (c). We can also observe a venule (*) next to the tubule characterized by an irregular transverse section and the imprint of a capillary (arrow).

an organ and the exact proportion between its vascular and interstitial components.

The cast allows a fine visualization of the venules that often collapse soon after organ explantation because of the diminishing pressures and because they lack a strong elastic and muscular wall. For this reason, the cast offers the possibility to observe the explanted organ in its real physiologic appearance and volume as when filled with blood.

In addition, filling the vessels with resin improved the correct visualization of the collagenic structure of the vessel walls, maintaining the true physiological disposition of its fibers that often collapse, retract, or fail and do not maintain their shape using traditional NaOH 1 N maceration.

In some tangential sections, the mechanical support given by the cast allowed us to visualize one by one the layers constituting the vessel walls, a condition hard to reproduce without this technique.

These conditions can be achieved only if a correct and physiological pressure of injection is maintained to prevent any distortion or ballooning of vessels filled with the resin. Moreover, it is very important to make a correct mixture of the resin to prevent any shrinkage when processing the specimens for SEM analysis, mostly manipulating them with dissectors or forceps.

The cons of this technique is that, upon performing NaOH 1 N maceration, we loose every cell belonging to the vascular wall. For this reason, we can only imagine the localization of endothelial cells, fibroblast, and smooth muscular cells. However, this is the price we have to pay to better visualize the relationships between the collagenic fibers and casted vessels.

This problem could be solved by joining these results with those obtained by already described two techniques (Castenholz, 1983a). The first one consists in the use of ultrasounds to treat injected specimens, while the second is based on fracturing the injected specimens and on the consequent observation by SEM. Although these methods allow a detailed visualization of both smooth muscular perivascular cells and pericytes, describing their position in the space after SEM observation, on the other hand, it is not possible to obtain any detailed information regarding the collagenic components of each layers of the vascular wall. Moreover, the adventitial layer is always loosed after the ultrasound treatment.

The same effect is obtained using enzymatic treatment as described by Evan et al. (1996).

For these reasons, our technique fits well to the study of those tissues affected by a pathologic process, where the relationship between the vessels and the collagenic component along with its morphological structure underlies a pathophysiological mechanism (e.g., intimal thickening in high hypertensive vascular disease, diabetic vascular wall degeneration, connective diseases, and tumoral vascular and interstitial remodelling).

In conclusion, corrosion casting can be successfully improved by combining it with the 1 N NaOH maceration method both in the study of normal tissue (mostly if characterized by a small collagenic component) and pathological tissue (Ribel–Madsen et al., 2005) to be better oriented and to understand the relationship existing between the vascular architecture and the collagenic component of an organ.

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