Establishment of a neovascular bed in a collagen-impregnated polyurethane sponge

M. H. Lipsky and P. Lamberton
Department of Medicine and Division of Endocrinology, Rhode Island Hospital, Providence, Rhode Island

A technique for promoting vascularization of a polyurethane sponge is demonstrated in the present study. Collagen-impregnated polyurethane sponges (Hypol, 2002) Foamable Hydrophilic Prepolymer (FHP) were implanted in the femoral fossa of rats for 1 day to 6 weeks. The ligated femoral artery/vein was pulled through the sponges to facilitate more complete neovascularization. Light-microscopic evaluation of the implanted sponges revealed that significant vascularization had occurred by the seventh day of implantation, and was maximal by the fourth to sixth week. Sponges containing collagen had a more thorough vascularization process than sponges without collagen, perhaps due to a more uniform pore size as demonstrated by scanning EM. Time course studies suggested that the artery/vein pull-through enhanced the development of the neovascularization process in the center of the sponges. We conclude that significant vascular tissue in-growth can be developed in polyurethane sponges and that both collagen and centrally placed blood vessels help promote the vascularization process. Potential applications could extend to a variety of bioartificial systems including endocrine or hepatic transplantation, soft-tissue prosthetic materials, bone grafts, or drug delivery systems. Further studies would be useful in providing additional information on the factors promoting neovascularization, and on the potential applications of this methodology using the present or similar biomaterials.

INTRODUCTION

Vascularized biomaterials have a number of potential applications including bioartificial endocrine organs, soft tissue prostheses, or vascularized drug delivery systems. Previous studies have suggested that porous biomaterials can provide a framework for the in-growth of vascularized connective tissue.[1,2] The purpose of this investigation was to determine if significant in-growth of vascular tissue could be promoted into a polyurethane hydrophilic flexible sponge. This biomaterial, which has an intercommunicating open-pore structure, has been previously shown to be of value in immobilizing microbial cells.[3] The polymer was chosen for the present studies primarily because of ease of sponge formation, the ability to control pore/sponge characteristics, the ability to incorporate a variety of additives into the sponge during formation, and polyurethane's reported biocompatibility.

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MATERIALS AND METHODS

Animals and materials

Female Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Hypol, 2002 Foamable Hydrophilic Prepolymer (FHP) was a gift of W. R. Grace and Co. (Lexington, MA). Microfil was purchased from Canton BioMedical Products, Inc. (Boulder, CO). Collagen (Vitrogen 100) was purchased from Collagen Corporation (Palo Alto, CA). Rat fibrinogen was purchased from Sigma (St. Louis, MO), and sodium heparin (1000 units/mL) was obtained from Invenex Lab. Inc. (Chagrin Falls, OH).

Formation of sponges

Upon mixing the hydrophilic polyurethane liquid (Hypol, 2002) with an equal volume of water and Vitrogen 100 (50% v/v), or water alone. Foaming after mixing takes place rapidly, even without a catalyst. Sponge formation is complete within 3–6 min, and demolding times range from 5 to 10 min. Corning polypropylene centrifuge tubes (50 mL) were used as molds for sponge formation. The cylindrical sponge was removed from the formation chamber and sliced cross-wise into pieces of about 1–2 cm^3.

Techniques of vascularization

The polyurethane sponges, sterilized by ethylene oxide, were washed in sterile normal saline and then soaked in a sterile solution of heparin/fibrinogen in saline (100 units sodium heparin + 1 mg rat fibrinogen + 10 mL normal saline).

Female Sprague-Dawley rats weighing 150–200 g were used to study the vascularization process. Following IP injection of ketamine anesthesia (20 mg/100 g bwt IP) the animals were given IM injections of procaine penicillin (10,000 units/100 g bwt) and gentamicin (0.5 mg/100 g bwt). The femoral/thigh regions were shaved and prepped with Betadine, then the femoral vein and artery were exposed through a small skin incision. A 1.5-cm segment of artery and vein was isolated, ligated at the distal end, and cut. A small mosquito clamp was then inserted into the center of a sponge. The silk sutures on the distal ends of the femoral vein and artery were pulled through the sponge, and the ligated vessels were anchored in the center of the sponge by tying the vessel ligatures to the distal end of the sponge. The sponge was then placed into the femoral fossa, and the subcutaneous tissue and skin were sutured with 4.0 chromic gut.

Light microscopy and scanning EM

Sponges containing collagen were compared by scanning EM to sponges without collagen to assess the role of collagen in modifying the surface characteristics of the sponges. The scanning was performed on an Amray 1000 scanning electron microscope operating at 20 KV. Photographs were taken at a magnification of 35×. To assess the degree of vascularization, sponges were removed from animals after varying time periods, placed into formalin, and then embedded in plastic for light microscopy. After sectioning (5 μm), the sponge sections were stained with hematoxylin and eosin.

Microfil study (silicone rubber injection compounds)

To further study the microvasculature inside the implanted sponges, a microfil experiment was performed. Three rats, in which sponges had been implanted 6 weeks earlier, were anesthetized with ketamine and perfused with paraformaldehyde. The circulatory system was then flushed with heparinized saline and microfil was perfused for 5 min throughout the circulatory system via a cannula placed in the left ventricle. After the microfil perfusion was terminated, the animals were placed overnight in a refrigerator at 4°C. The femoral fossa was opened the next day and the sponges were removed and placed into formalin. The sponges were then embedded in Glycol-methacrylate, sectioned, and examined by light microscopy for the presence of blue microfil within the microvasculature in the sponges.

Time course study

A time course study was done to determine the rate of the vascularization process. Sponges were removed from four animals at 1 day, 3 days, 7 days, 2 weeks, and 6 weeks after surgery. The sponges were embedded and sectioned for light microscopy and compared for degree of vascular tissue development.

Controls

Polyurethane sponges were modified and implanted as follows: 1) with no artery/vein pull-through, three animals; 2) without collagen impregnated in the polyurethane, three animals; 3) with no heparin or fibrinogen, three animals; 4) with artery and vein pull-through without ligation, three animals (artery and vein placed intact into a slit in the side of the sponge). Cellulose acetate sponges were also studied to provide a comparison with the polyurethane material.

RESULTS

Figure 1 shows the time course of the vascularization process. As can be seen, after 24 h there was essentially no tissue in-growth. By day 3, however, a thin layer of connective tissue was present along the outside surface of the sponge. By day 7, 1–2 mm of tissue in-growth had occurred. The 2-week specimens had considerable vascular tissue, as seen in Figure 1. Well-formed capillaries containing red blood cells (RBCs) were present over the outer margins of the sponges, penetrating 4–6 mm toward the center. By 4 to 6 weeks, nearly the entire sponge was filled with highly vascularized connective tissue. This is illustrated in Figure 2, where multiple RBC-containing vessels are evident. Maximum vascularization of the sponge occurred by 4 to 6 weeks.

Controls

To investigate the role of the artery/vein pull-through, we placed sponges into the femoral fossa but did not pull vessels through the center of the sponges. In these controls, four animals, there was incomplete vascularization of the central regions of the sponges, even by 6 weeks. Figure 3 suggests that the central vascularization spreading outward from an artery/vein pull-through enhanced the growth of vascular connective tissue. Sponges containing nonligated femoral vessels also developed excellent central vascularization. Thus, the presence of the femoral vessels, even when nonligated, was useful in enhancing more thorough sponge vascularization.

In addition, we varied sponge composition, to determine the effect of each component. Sponges containing collagen had more extensive vascular tissue in-growth than sponges without collagen. The comparison of sponges with and without heparin/fibrinogen did not, however, reveal a demonstrable difference in the vascularization process. A comparison of vascularization in the polyurethane material with that occurring in cellulose acetate sponges revealed a more complete vascular-tissue in-growth in the former. Finally, the degree of tissue reaction (in terms of number of giant cells seen per high-powered field) was considerably more pronounced in the cellulose acetate than in the polyurethane sponges, despite similar preparation techniques. Table I summarizes the degree of vascularization and tissue reaction in the various sponge preparations.

Scanning EM

In order to study the role of the collagen further we carried out scanning EM on two polyurethane sponges containing collagen and on two made without collagen. Figure 4, showing scanning micrographs at 35×, illustrates the more uniform sponge pore size and more reticulated cell structure found in the collagen-treated polyurethane material.

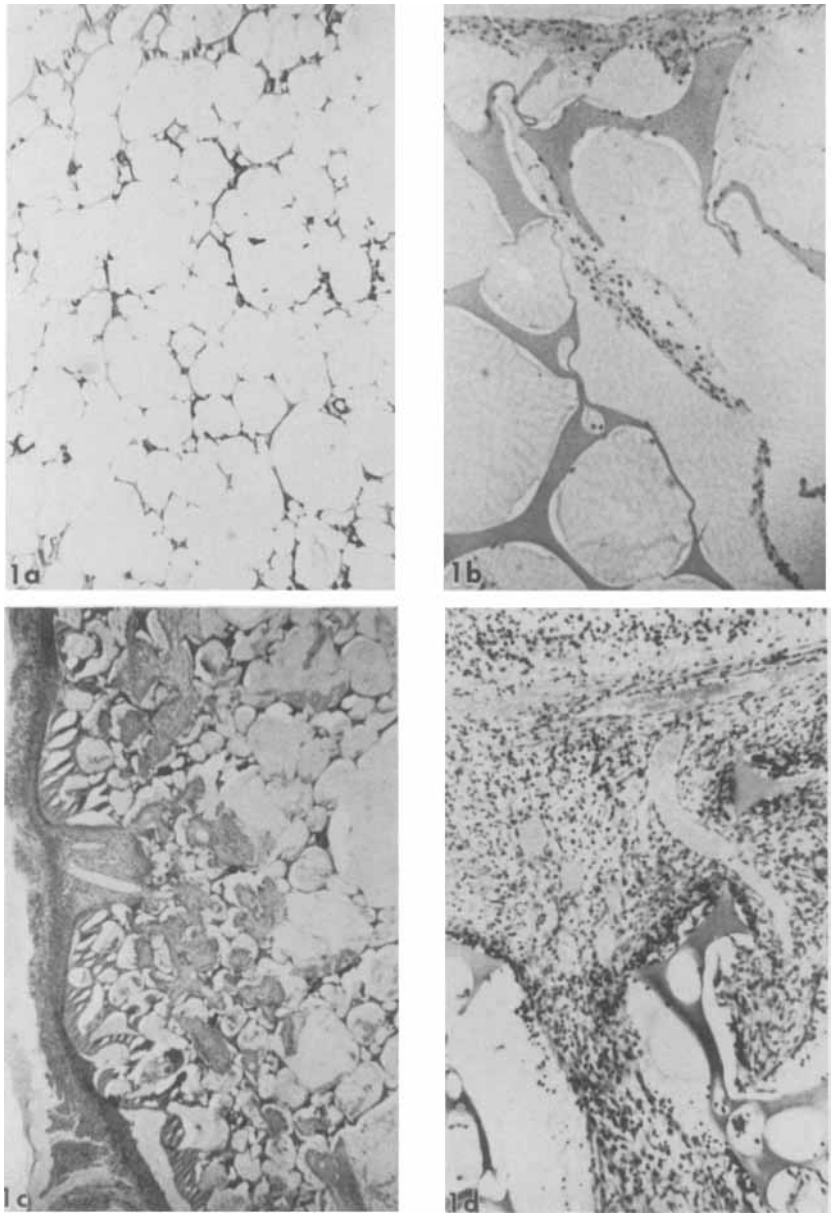

Figure 1. Time course of vascular tissue in-growth into polyurethane sponges over time. Figures 1(a), (b), (c), and (d) are of sponges removed 1, 3, 7, and 14 days, respectively, after implantation. (Original magnifications: [(b), (d), ×100]; [(a), (c) ×25].

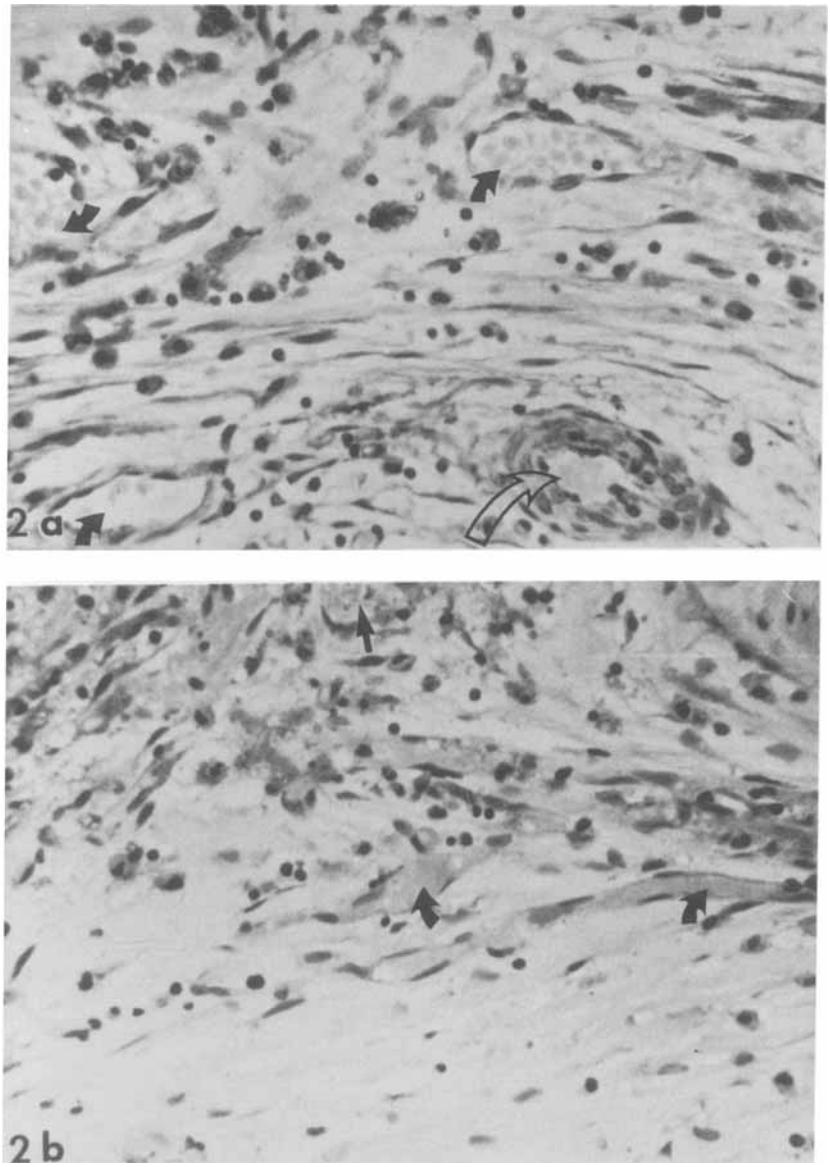

Figure 2. High-power views (original magnification, ×400) of representative areas of polyurethane sponges implanted for 6 weeks. Note multiple capillaries containing red blood cells (RBCs).

Microfil study

Figure 5 shows a sponge taken from an animal perfused through the left ventricle with microfil. Examination of the sponge by light microscopy revealed multiple large and small vessels containing blue microfil particles, providing evidence of the vascular continuity of the vascularized sponges.

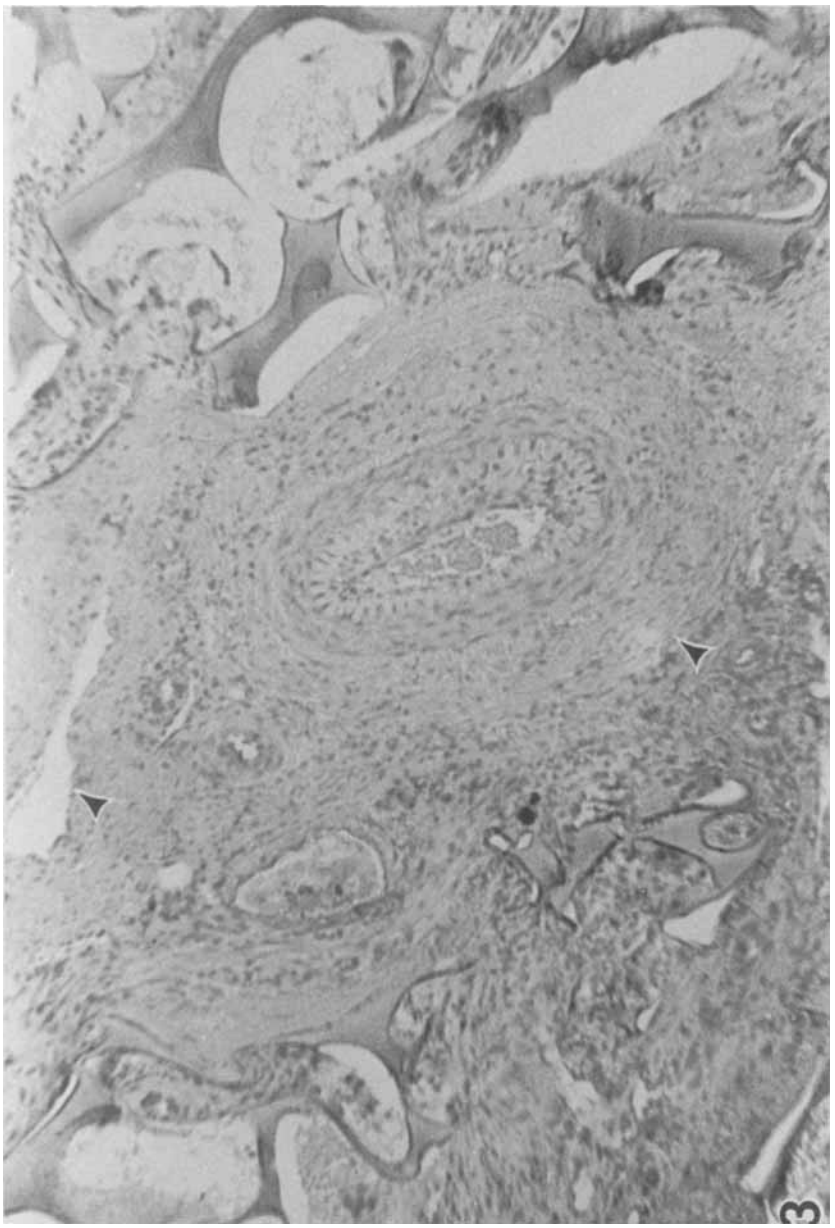

Figure 3. Light micrograph (original magnification, ×100) of a polyurethane sponge removed after 4 weeks. In the center of the figure is the femoral artery (arrow) and in the upper part of the figure is the femoral vein (arrow).

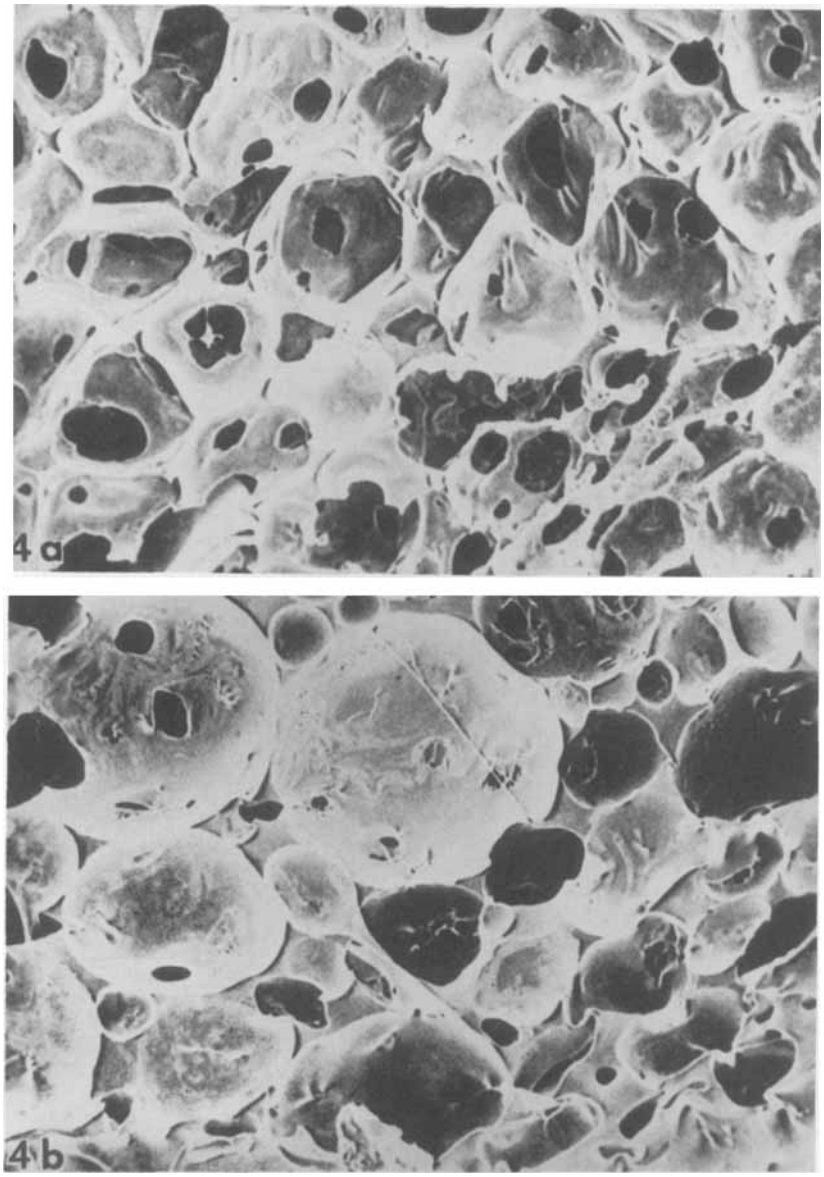

Figure 4. Scanning EM (×35) of polyurethane isocyanate sponges. Shown in (a) is a sponge containing collagen while (b) shows a sponge without collagen.

DISCUSSION

The present investigation demonstrates that a porous polyurethane biomaterial can be vascularized within a relatively short period of time: 4 to 6 weeks for a 1–2 cm^3 size sponge. Previous investigators have examined several factors promoting tissue in-growth into various porous biomaterials and have shown that pore size is an important determinant of the degree of

TABLE I
Degree of Sponge Vascularization and Tissue Reaction

Sponge	Vascularization[a]	Degree of Tissue Reaction[b]
Day 1 Hypol	0	0
Day 3 Hypol	+1	+1
Day 7 Hypol	+2	+1
Day 14 Hypol	+3	+2
Week 6 Hypol	+4	+2
Hypol with artery	+4	+2
Hypol with collagen (No Heparin Fibrinogen)	+4	+2
Hypol with heparin/fibrinogen (No Collagen)	+2	+2
Cellulose acetate	+2	+4

[a] Scale 0–4; +4 represented maximal vascularization seen.
[b] +4 represented maximal tissue reaction as assessed by degree of giant cell formation, fibroblasts, etc.

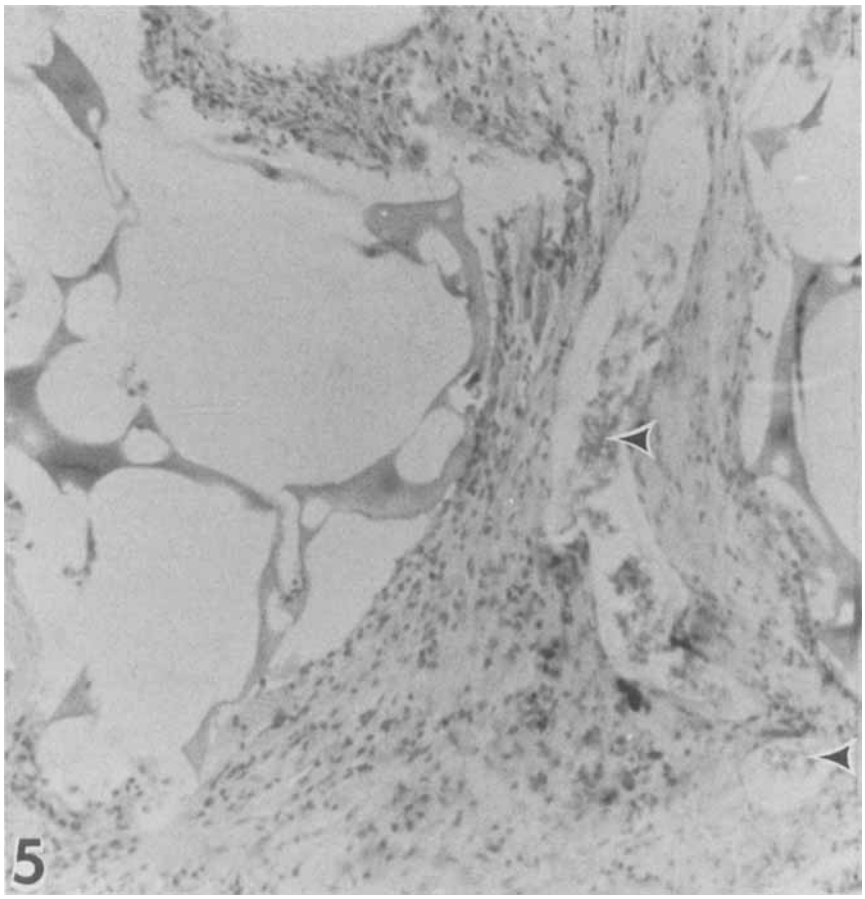

Figure 5. Light micrograph (original magnification, ×100) of a polyurethane sponge removed from a microfil-injected animal. The arrows point to blue microfil particles noted within larger and smaller vessels.

tissue and vascular in-growth.[1,2] The current biomaterial, which can be formed into sponges with varying degrees of porosity and texture, seems well suited for a number of potential biomedical applications. Christie et al. have recently documented the utility of studying polyurethane surface characteristics with scanning EM.[4] The present investigation demonstrates, by scanning EM, that the addition of collagen to the polyurethane resulted in a more consistent pore size.

The cellular changes occurring within the sponges represent a process similar to wound healing[5] in that leukocytes and minimal connective tissue were evident during the first several days. During the following weeks, significant vascularity developed through the sponge pores interspersed between various elements of connective tissue. Folkman[6,7] has suggested that neovascularization can be stimulated by several factors including heparin, fibrinogen, and the presence of a collagen-coated surface: This provided the rationale for the methods we used in the current study to examine techniques to promote maximal vascularity in the biomaterial. No benefit was shown, in this study, from the presence of heparin and fibrinogen. Our results do suggest collagen may aid in sponge vascularization, but it is not clear whether this is due to biochemical interactions or to the more-uniform pore size of collagen-containing sponges.

The role of existing blood vessels in providing the endothelial cells from which new capillaries and larger vessels are eventually formed has also been discussed by Folkman.[7] We have demonstrated the value of providing a source of endothelial cells, in the form of the femoral artery and vein. It is unlikely, however, that the endothelial cells lining the femoral artery or vein are the cells from which new vessels were formed.[7] Rather, it is likely that endothelial cells from small vessels attached to the femoral vascular bundle were the source of the new capillaries seen in the areas of the sponge surrounding the femoral artery/vein pull-through.

Previous investigators have characterized the cells at the surface of an implanted material to evaluate the biocompatibility of the implant.[8,9] Behling and Spector noted that, at 100–118 weeks, approximately 50% of the surface area of polysulfone implants and polyethylene implants was covered by macrophages; about 20% of the surface area of these implants was also covered by giant cells.[8] The present study was shorter, which prevented a direct comparison of the degree of reactivity. However, when examined by light microscopy at 4–6 weeks, the polyurethane sponges exhibited less reactivity than did the cellulose acetate sponges. That is, the polyurethane sponges showed less surface coverage by macrophages and giant cells. We believe that the present polymer, despite displaying some reactivity at 4–6 weeks, is still a useful material for the further study of the vascularization process and potential vascularized bioartificial applications.

Because the purpose of our study was to promote biomaterial vascularization, we focused on methods to increase vascular development. Recent evidence suggests that macrophages may enhance factors promoting angiogenesis[10,11] as a component of the inflammatory process. In this regard, some

degree of tissue reactivity may be beneficial, as suggested by Hall et al.[12] Of interest is our observation that despite a maximal degree of tissue reactivity in the cellulose acetate sponges, there was less vascularization than seen in the Hypol sponges where the degree of tissue reactivity was less. Clearly there are a number of potential factors enhancing vascularization and in the Hypol sponges the polyurethane material appears superior overall to cellulose acetate in promoting neovascularization. At this time, the reasons for this difference are unclear. However, additional studies would be useful to further define the methods which would allow maximum angiogenesis, while minimizing the bioreactivity of the sponge in terms of the inflammatory response.

There are several potential applications of this vascularization process. The vascularized sponge model was originally developed in our laboratory for the purpose of providing a vascular bed in which to implant endocrine tissue. In addition, bioartificial skin, bone,[13] or vascular tissue[14] could incorporate vascularized polyurethane materials in their design. Vascularized biomaterials might also be applied to tracheal prostheses,[15,16] dental grafts, or vascularized drug-delivery systems in which the drug is incorporated into the polyurethane material[17] with the vascular supply facilitating drug delivery outside of the sponge. Similarly, microencapsulated endocrine cells[18] or hepatic cells attached to immunoprotected microcarriers[19] could potentially be injected into a vascularized open-pore sponge.

CONCLUSION

The present study documents the methods for vascularizing a polyurethane biomaterial by connective tissue in-growth. The artery/vein pull-through technique appears to speed vascularization in the center of the sponge. The inclusion of collagen also improves vascularization, possibly because it promotes a more uniform pore size. Additional studies are needed to clarify the role played by collagen-impregnation in the promotion of vascularization, and the potential role(s) of angiogenesis factors, such as heparin, as well as to explore methods for minimizing tissue reactivity while still allowing vascularization to proceed. Potential applications involving bioartificial organs and drug delivery systems deserve study.

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