Quantification of Vascular Ingrowth into Dacron® Velour

TRUDY D. ESTRIDGE
Collagen Corporation
Research and Development
1850 Embarcadero Road
Palo Alto, CA 94303

DALE S. FELDMAN
Biomedical Engineering
University of Alabama at Birmingham
Birmingham, AL 35294

ABSTRACT: Evaluation of vascular ingrowth into Dacron® velour was performed to compare methods and to determine the amount of vascular ingrowth. Microcasting and direct measurement were compared based on their ability to quantify ingrowth, the type of information obtainable, and the time required for data acquisition. Microcasting did not produce results indicative of the actual vascular ingrowth; however, direct measurement of histological slides gave quantifiable data for comparing vascular ingrowth.

KEY WORDS: tissue ingrowth, Dacron® velour, vascular ingrowth quantification.

INTRODUCTION

Porous materials have been used extensively as soft tissue biomaterials in many different applications including fixation or stabilization of various implantable devices, tissue ingrowth to create blood compatible surfaces, scaffolding to help the body repair or regenerate tissue, and prevention of epidermal downgrowth in percutaneous devices [1]. Although porous soft tissue implants are common, there is little information about the design criteria that could lead to optimal tissue ingrowth into these porous materials.

0885-3282/91/02 0157-013 $06.00/0
©1991 Technomic Publishing Co., Inc.

Edwards v. Aortic Innovations
U.S. Patent 11,497,634
Edwards Ex. 1106

The success or failure of porous soft tissue biomaterials is a result of porosity, bulk material properties, surface properties or chemistry, material configuration, implant location, implant function, and animal model used [1]. The most commonly used porous soft tissue biomaterial is Dacron® velour [2]. This material has been used extensively as an artificial vascular graft material and in percutaneous devices. Tissue vascularity has been cited as the key to long-term ingrowth. Therefore, pores of a size sufficient to allow vascular ingrowth are required. Most researchers suggest that 40 μm diameter pores or more are needed for soft tissue ingrowth, while others argue that pores of at least 100 μm are needed [1]. In either case, cells die due to lack of nutrition if removed by more than 100 μm from their blood supply [3]. Therefore, vascular ingrowth is critical to the success of soft tissue porous implants.

Several methods are available to look at the vascular system [4] (see Figure 1). As illustrated, radiopaque substances can be injected into the host and X-rays can be taken. This method, however, is limited by inadequate resolution for visualizing the fine capillary beds in and around implants. X-rays are generally composite images through the whole animal, while techniques do exist to subtract or filter out portions of X-rays, these have been found ineffective in showing capillary beds, again due to the poor resolution.

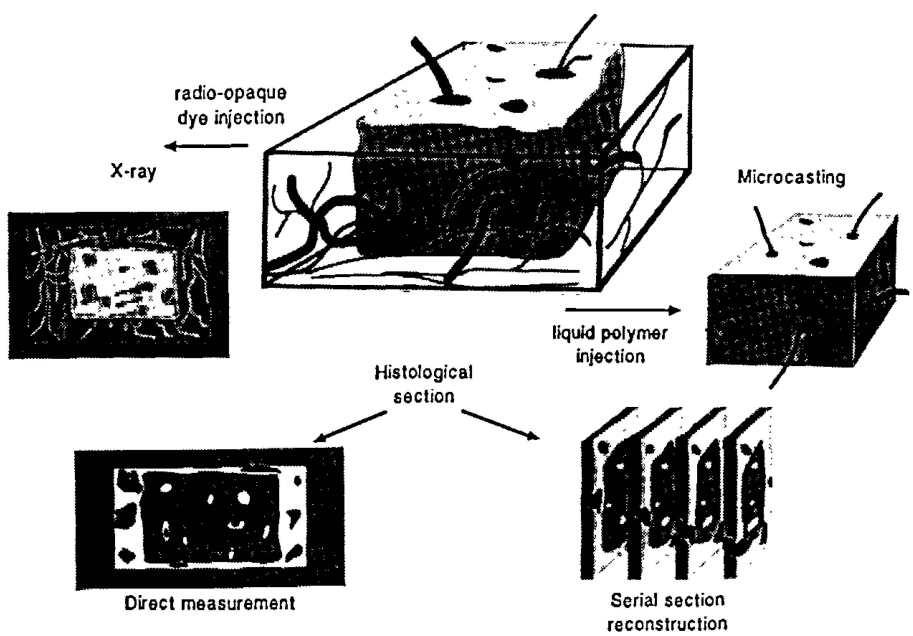

Figure 1. Schematic of vascular visualization techniques.

Histological sections of the implant and the surrounding tissue, shown in Figure 1, can be made and direct measurement of vascularity can be recorded [5]. Direct measurement of structures; however, is cumbersome, time consuming, and therefore not usually done. Serial sections of tissue specimens can be used to reconstruct the three dimensional orientation within the specimen.

Another method called microcasting, also exists. This method has been used for numerous years to study the vascular systems of animals and cadavers [4]. This method involves replacing the animal model's circulatory system with a methylmethacrylate monomer. The monomer is polymerized and the tissue of the specimen is digested away. The three dimensional vascular cast that remains can then be studied under a scanning electron microscope or a stereoscope.

RATIONALE

The absence of an adequate vascularity results in tissue hypoxia and continual necrosis in the area [3,6]. Previous studies of tissue ingrowth in porous biomaterials have used light microscopic studies of the cells and tissue types present. While these studies noted the presence of the blood vessels, the relationship between the degree of revascularization and other wound healing phenomena has not been investigated. The number and size of blood vessels present, as well as their orientation to the implant and surrounding tissue, should be examined. Ingrowth microvasculature studies can be used to compare and evaluate different implant materials, different surface modifications, or different implant configurations. The goal of this study, therefore, was to develop and examine the relative merits of two methods (microcasting and direct measurement) to quantify microvascular ingrowth.

MATERIALS AND METHODS

Material Fabrication

Dacron® velour was obtained from USCI (Cat. No. 001642, USCI, A Division of C.R. Bard, Inc., Billerica, Massachusetts). Dacron® velour is the registered trademark of E.I. du Pont de Nemours, Inc. for polyethylene terephthalate (PET). This material was prepared for implantation by gluing together the knitted sides of two sheets of PET fabric with medical grade Silastic® (Dow Corning MDX 4-4210, Dow Corning Corporation, Midland, Michigan). Therefore, the velour sides provided the tissue interface. The sheets were then cut into specimens of approx-

imately two centimeters by one centimeter. The thickness was approximately three millimeters.

Forty-nine implants were prepared by washing them in a non-oil based soap (Alconox, Alconox, Inc., New York, New York). Implants underwent a fifteen minute wash in an ultrasonic cleaner, a five minute distilled water rinse, a fifteen minute wash with distilled water in the ultrasonic cleaner, and a final five minute distilled water rinse. During these steps, the implants were handled with clean forceps. Implants were then placed in distilled water in a soxhlet extractor for twenty-four hours (to remove low-molecular-weight impurities, including the adhesive reaction by-product acetic acid). After removal from the extractor, the implants were allowed to dry and were placed in sterilizing bags and sterilized by standard steam autoclaving.

Implantation

Two adult (male) dogs weighing approximately 40 lbs. were anesthetized with an intravenous injection of 6–10 ml og 2.5% Surital (sodium-5-allyl-5[1-methlybutyl]-2-thiobarbiturate, Parke-Davis, Division of Warner-Lambert Company, Morris, New Jersey). Each animal was maintained with 0.5–1.5% halothane throughout the surgery. The sites of implantation were shaved and scrubbed in preparation for surgery. Four incisions, two on each side of the spine, were made with a scalpel and subcutaneous pockets were bluntly dissected using a Kelly hemostat. Six independent subcutaneous pockets were made around each incision site. Therefore, there were twenty-four implants per animal for a total of forty-eight implants. The implants remained in place for one month.

Microcasting

At the time of harvesting, one animal was anesthetized with sodium pentobarbital (sodium 5-ethyl-5[1-methylbutyl] barbiturate, Abbott Laboratories, North Chicago, Illinois) and a catheter was placed in the forearm. A slow drip of saline solution was administered, containing two milliliters of physiological saline. With the dog placed in dorsal recumbency, a midline thoracotomy was done to expose and isolate the descending aorta. Once cannulated, the catheter was secured in place with a ligature. The saline solution drip was then changed to the aortic cannula. The inferior vena cava was catheterized in the same manner, and the venous return dripped freely into a bucket. Perfusion with saline was maintained until the venous return was clear (approx-

imately 6 liters). The dog was then given an overdose (10 ml) of sodium pentobarbital.

The microcasting medium (Batson's #17 Anatomical Corrosion Compound and Kit, Polysciences Inc., Warrington, Pennsylvania) was then mixed in a ratio of 40 ml of component A; to 5 ml of component B; to 3 drops of component C; and to 25 ml of dibutyl phthalate prior to injection into the aortic cannula. The blue dye, supplied with the kit, was mixed into the injection medium so perfusion could be monitored visually by noting the color change of the animal's organs. The medium was injected over a period of approximately forty minutes. Due to the modifications made in the microcasting medium, complete polymerization took one and one-half hours, although the medium was "set" in forty-five minutes; therefore, the animal was left undisturbed for approximately two hours and then the implants were retrieved. The medium was modified from the original ratios suggested by the manufacturer to give a less viscous medium with an increased duration of polymerization.

Implant Processing

Twenty-four specimens from animal one were processed, using one of two maceration procedures. Twelve were macerated with potassium hydroxide (30% aqueous solution) and twelve with sodium hydroxide (30% aqueous solution). All twenty-four of these specimens were prepared for SEM by rinsing with distilled water, air drying, and then mounting on aluminum stubs. The specimens were then sputter coated with 200 Å palladium-gold and examined at 5–15 kV in a scanning electron microscope.

Twelve specimens from animal one were processed, using a procedure that was designed to make the surrounding tissue transparent to light, thereby allowing the blood vessels to be visualized. Two versions of this procedure were performed. First, six of the specimens were placed in a graded series of EtOH (30%, 50%, 70%, 90%, 95%, 2 × 100%) for 24 hrs. each, then in methyl salicylate for 24 hrs. Six of the specimens were placed in graded solutions of glycerin/EtOH (50%/50%, 75%/25%, 85%/25%, 100%/0%) for 24 hrs. each.

Thirteen specimens, from dog two, were processed for light microscopy, by fixing in glutaraldehyde/paraformaldehyde. The individual implants were then cut into three pieces and each piece by (i.e., end or middle piece) was labeled. The implant pieces were then embedded in JB-4, sectioned at 5 μm, and stained with hematoxylin and eosin. The remaining specimens, were removed and used for another project.

Data Collection

Specimens viewed with the SEM were evaluated based on the amount of cast present using the grading method in Table 1. The twelve specimens processed with the methods were evaluated with a stereomicroscope. Samples prepared for histology were used to evaluate vascular ingrowth and measure the ability of the casting medium to reach the blood vessels in the ingrowth region.

The specimen thin sections were divided into regions (Figure 2). The first region was the capsule region (C), the thin fibrous capsule around the implant. The next three regions were equal divisions of the area between the capsule edge and the knitted edge (M1, M2, M3). The fifth region consisted of the knitted part of the fabric adjacent to the Silastic® (I). Direct measurement of the number of vessels in each region, number of cast versus non-cast vessels, and average size of vessels in each region were performed.

Data was analyzed using SAS software on an IBM model 30. The analysis of variance was used to compare the blood vessel size, number of blood vessels, and size weighted by number of blood vessels for each piece, region, and the region within each piece. Differences were considered significant when the p value was equal to or less than 0.05.

RESULTS

The six specimens cleared with methyl salicylate and analyzed with the stereomicroscope showed no visible cast. The six specimens cleared

Table 1. Grading scale of microcast specimens.

Grade	Criteria
0	No microcast covering specimen
1	0–10% microcast covering of specimen
2	11–20% microcast covering of specimen
3	21–30% microcast covering of specimen
4	31–40% microcast covering of specimen
5	41–50% microcast covering of specimen
6	51–60% microcast covering of specimen
7	61–70% microcast covering of specimen
8	71–80% microcast covering of specimen
9	81–90% microcast covering of specimen
10	91–100% microcast covering of specimen

Figure 2. Representative sample showing tissue regions.

with glycerin and analyzed with the stereo microscope showed only small amounts of cast present in the capsule region.

The results of the grading of twenty-four specimens analyzed by SEM are summarized in Table 2. Examples of specimens are shown in Figure 3. The use of NaOH was not found to decrease tissue maceration time; and these specimens had to be rinsed extensively to remove a surface residue, which appeared to be salt. The grading of the implants reveals that less than 20% of both sides of the specimens were found to contain cast. The results of grading, shown in Table 2, reveal the mean grade

was 0.6 ± 0.7. This value, however, was found to be significantly different from zero using the standard student's *t*-test.

The results of the specimens graded by light microscopy are summarized in Table 3. The evaluation by direct measurement gives both blood vessel size and distribution throughout the ingrowth region. In addition, it was found that, less than 0.01% of the total number of blood vessels were found to contain cast in these histological sections and of these 100% were located in the capsule region. Examples of the specimens are shown in Figure 4.

The results of the analysis to determine differences in the size of blood vessels are summarized in Table 3. There was no significant difference found when comparing pieces. When comparing the regions within pieces a significant difference was found among the capsule and all other regions. There also was a significant difference in the size of

Table 2. SEM analysis for microcast.

Implant #	Grade
1/1	1
1/2	2
1/3	0
1/4	0
2/1	3
2/2	3
2/3	0
2/4	0
3/1	1
3/2	0
3/3	0
3/4	0
4/1	1
4/2	1
4/3	0
4/4	0
5/1	1
5/2	0
5/3	1
5/4	1
6/1	0
6/2	1
6/3	0
6/4	1
Mean	0.6 ± 0.7

Figure 3. SEM of a representative microcasted specimen. D, Dacron®; C, casted capillary.

blood vessels between the capsule and the other four regions. In addition, significant differences were found between I and regions C, M1, M2; but not M3 ($p = 0.01$); M1 and regions C and I; but not M2 or M3.

The results of the analysis to determine differences in the number of blood vessels among the pieces and regions is also summarized in Table 3. No significant differences were found when comparing pieces or regions within pieces. Significant differences, however, were found between M2 and I and when these two regions were compared to all other regions.

DISCUSSION

The use of either methyl salicylate or glycerin as used in this study was not found to be useful. While both of these compounds are reported to produce tissue that is transparent, in this study, the microcasted vessels could not be identified. Therefore, the use of either of these compounds is not recommended for vascular visualization of subcutaneous tissue.

The two maceration solutions used in this experiment worked equally well. The NaOH solution, however, required additional rinses

in order to remove residue from the implant specimens. Therefore, the use of potassium hydroxide is recommended for maceration, although, care should be taken when using this solution, because long-term contact with Dacron® velour can cause breakdown of the material.

The scant number of vessels with cast suggest that there were problems with the microcasting technique. The problem appears to be due to insufficient clearing of the vascular system with saline. Therefore, using larger volumes of perfusion or flushing saline is indicated for any future microcasting. The viscosity of the microcasting medium was changing throughout injection as the polymerization of the material proceeded. This caused problems trying to achieve continuous steady flow of the microcasting medium. This indicates further modification of the microcasting medium to increase the time before polymerization proceeds into a stage in which the viscosity becomes too unworkable. The small percentage of microcasted vessels in histological sections showed that the casting did not succeed in replicating the capillaries in the tissue ingrowth.

The statistical analysis of the microcasting data shows that the coefficient of variation is 0.23 for the 24 implants. In this study approx-

Table 3. Results of analysis of directly measured specimens.

		Mean		
Piece	Region	Size (μm)	Size Wt. by Number	Number
End (E)	All	23.7 ± 8.0	25.1 ± 18	4.9 ± 4.4
Middle (M)	All	26.0 ± 9.7	25.8 ± 18	4.6 ± 5.1
E	Capsule (C)	26.1	25.3	5.0
E	Middle 1 (M1)	24.6	26.2	3.9
E	Middle 2 (M2)	25.2	26.4	8.1
E	Middle 3 (M3)	21.6	24.1	5.1
E	Inner (I)	20.0	20.5	2.6
M	C	33.2	31.3	6.0
M	M1	23.7	25.1	4.6
M	M2	25.4	25.5	7.3
M	M3	24.8	21.1	3.7
M	I	18.1	17.5	1.4
All	C	29.5 ± 10	28.5 ± 21	5.5 ± 5.3
	M1	24.2 ± 8.1	25.6 ± 16	4.3 ± 3.5
	M2	25.3 ± 6.4	26.0 ± 18	7.7 ± 6.0
	M3	23.1 ± 9.4	22.9 ± 17	4.4 ± 3.8
	I	19.3 ± 6.7	19.5 ± 9.6	1.9 ± 2.3

Figure 4. Light micrograph of a representative specimen. D, Dacron®; C, capillary; M, macrophage; F, fibroblast.

imately a 39% difference in the means would be needed to be significant. In order to get a coefficient of variation of 0.05, about 516 additional implants would have been needed.

The statistical analysis of the data gathered by direct measurement shows the potential value of this method in determining ingrowth vascularity. No differences were found between pieces; but in general, the largest number and sizes of vessels were found in the capsule region, while smaller numbers and smaller sizes were found in the M1, M2, M3, and I regions.

The methods utilized in this study showed few consistent differences between individual regions. For example, adjacent regions (i.e., C and M1, M1 and M2, M2 and M3, and M3 and I) were not found to be consistently different from each other. The capsule and inner regions, however, were consistently different from each other. This indicates that the middle regions should probably only be divided in half. This, however, may depend on the actual conditions of ingrowth which could vary with other implantation parameters.

The coefficients of variation for the directly measured implants ranged between 0.1 to 0.15. In order to get a C.V. of 0.05 approximately

4 times as many samples would need to be counted. In direct measurement, this could be done by increasing the number of sections per implant counted or increasing the implants counted by four. In this study 24 implants were measured and it would be easier to measure more sections than to do more implantations. In this study approximately a 17–25% change in the mean would be needed to show a significant difference.

From this study it is clear that direct measurement is the method of choice for several reasons. First, direct measurement gives you quantitative data which permits parametric statistical comparisons on vascular ingrowth through an implant. Second, it allows implants to be compared based on several factors (blood vessel size, blood vessel number, or blood vessel size distribution) unlike for casting. Therefore this type of data is quantitatively more powerful because it allows comparisons of both parameters. This data can be used to show how ingrowth proceeds into the porous implant. Although this makes direct measurement better for comparisons of vascular ingrowth between different types of implants, the labor intensive nature of the evaluation may make it impractical for large numbers of implants. Microcasting data was easier to obtain, but suffers from problems in obtaining good samples for evaluation. This leads to inaccuracies in data; and will make the data suspect; and a high data variation, which requires a large sample size to obtain a reasonable coefficient of variation.

CONCLUSIONS

Quantification of vascular ingrowth in soft tissue biomaterials, as shown in this study, should be done by direct measurement. The decision should be based on the number of animals and implants available for analysis. In addition, direct measurement of histological sections allows for other cell parameters to be measured.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation (ECS-8204597) and the Bioengineering Program at Texas A&M University.

REFERENCES

1. Feldman, D. S. and T. D. Estridge. 1984. "Factors Affecting Soft Tissue Ingrowth in Porous Implants," *Transactions of the Society for Biomaterials*, 20:37.

2. Wesolowski S. 1962. *Evaluation of Tissue Prosthetic Vascular Grafts.* Springfield, IL: Charles C. Thomas.
3. Davis, J. C. and T. K. Hunt. 1988. *Problem Wounds–The Role of Oxygen.* New York: Elsevier.
4. Murakami, T., O. Ohtani, A. Ohtsuka and A. Kikuta. 1990. "Injection Replication and Scanning Electron Microscopy of Blood Vessels," in *Biomedical Research Applications in Scanning Electron Microscopy.* London: Academic Press, Inc., pp. 1–30.
5. Weibel, E. 1979. *Practical Methods for Biological Morphometry, Vol. 1.* New York: Academic Press, Inc.
6. Dineen, P. 1991. *The Surgical Wound.* Philadelphia: Lea & Febiger.

ABOUT THE AUTHOR

Trudy D. Estridge

Trudy D. Estridge received a B.S. degree in 1983 and an M.S. degree in 1988 from Texas A&M University in Bioengineering. She received the Ph.D. degree in Biomedical Engineering from the University of Alabama at Birmingham, in 1991. Currently, she is a research scientist for Collagen Corporation, Palo Alto, California.