

The In Vitro Growth of a Three-Dimensional Human Dermal Replacement Using a Single-Pass Perfusion System

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Received June 25, 1993/Accepted November 1, 1993

A human dermal replacement has been developed by seeding human neonatal dermal fibroblasts onto a biosorbable polyglactin (polyglycolide/polyacid) mesh and culturing in a bioreactor. The mesh provides the proper environment for the cells to attach, grow in a three-dimensional array, and establish a tissue matrix over a 2- to 3-week culture period. The dermal replacement has been characterized and found to contain a variety of naturally occurring dermal matrix proteins, including fibronectin, glycosaminoglycans, and collagen types I and III. To efficiently and reproducibly produce this dermal tissue equivalent, a closed, single-pass perfusion system was developed and compared with a static process. In the single-pass perfusion system, growth medium (containing ascorbic acid) was perfused around the 4×6 in. pieces of mesh at specific flow rates, determined by nutrient consumption and waste production rates. The flow rates used for this system indicate that a diffusion-limited regime exists with a mean residence time greater than 1 h for essential nutrients and factors. By controlling glucose concentrations in the system to a delta of 0.70 g/L from the inlet to the outlet of the bioreactor, it took 6 fewer days to grow a tissue similar to that produced by the static system. © 1994 John Wiley & Sons, Inc.

Key words: human dermal replacement • neonatal dermal fibroblasts • biosorbable polyglactin mesh • matrix proteins • bioreactor design • tissue engineering

INTRODUCTION

The science of growing human tissues and organs in vitro ("tissue engineering") has emerged over the past several years in part because of our increased understanding of mammalian cell culture.^{8,29,30} Many advances have taken place in the culture of cells in vitro, including the development of appropriate medium formulations (serum and serum-free)^{1,2,10,12,16} and design of reactor systems for the long-term growth of cells.^{4,5,9,22,23} However, in the past, the major emphasis has been on growing these cells for the production of proteins and not for the formation of tissue. The past decade has shown great advances in the area of growing tissues and organs in vitro.¹⁵ To take advantage of these various new disciplines for the growth of a tissue, Naughton et al.¹⁸ developed a culture system

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Biotechnology and Bioengineering, Vol. 43, Pp. 740-746 (1994)
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have developed a novel single-pass perfusion system that For the growth of this human dermal equivalent, we autograft skin and cultured epithelial sheets.³

mini-pigs and athymic mice as a support for both meshed dermal replacement has been successfully implanted in both environment for the development of the dermal tissue. This cells on a three-dimensional scaffold provides a natural commonly used in implantable sutures. The growth of the polyglycolic acid (PGA)/polylactic acid (PLA) copolymer and cultured onto a biodegradable mesh consisting of a our laboratory.^{3,11} Cells are isolated from human foreskins tissue, we have developed a human dermal replacement in To provide a uniform, reproducible, readily available collagen and other extracellular matrix proteins.^{7,26}

basement membrane, and the synthesis and degradation of regulate the growth of keratinocytes, the production of their in the dermis normally secrete critical growth factors that skin grafting is the availability of a dermal layer. Fibroblasts or could benefit from a skin graft. A critical component for decubitus (pressure) ulcers and either require skin grafts year in the United States suffer from venous, diabetic, and skin grafts. In addition, two to three million patients per 100,000 people suffer burns, 25% to 50% of whom require Every year in the United States, between 70,000 and nology has important potential therapeutic application.^{3,11}

The ability to grow human skin products using this tech- and skin.^{14,21,28}

however, it has been applied also to the growth of liver²⁰ originally designed for the growth of bone marrow.^{18,19} for the full differentiation of the tissue. This system was The resulting system provides an "in vivo" environment is ready for the seeding of the parenchymal cells of interest. grown and has developed into a three-dimensional tissue, it and removal of waste products. When the stromal tissue has adequate diffusional environment for delivery of nutrients cell-cell and cell-matrix communication as well as an culture milieu provides the necessary microenvironment for development of a three-dimensional tissue. This static cell matrix and deposit growth factors that contribute to the grow in this environment, they produce an extracellular of interest onto a porous substrate. As the stromal cells that involved seeding the stromal cells from the tissue

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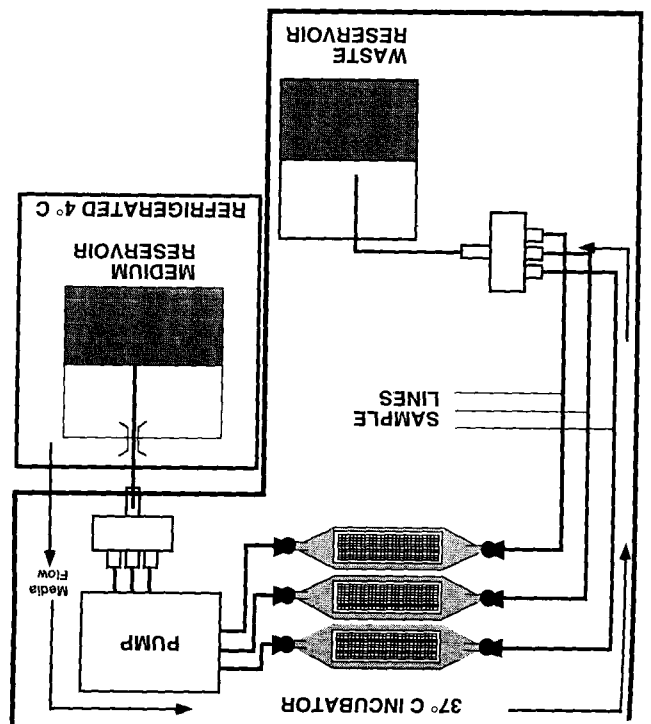
and implementation of a scale-up process for manufacture of our human dermal skin replacement.

Packaging and Freezing

The cryopreservative used was a solution of Dulbecco's phosphate-buffered saline (PBS; Gibco) containing 10% DMSO (v/v) (Cryoserv Research Industries, Salt Lake City).

The perfusion system consisted of attaching two media reservoir bags (Gibco) filled with a total of 40 L of ascorbate growth medium (75 μ g/mL ascorbate). The 16 bags were connected to silicone tubing via polypropylene connectors (Value Plastics, Fort Collins, CO). The 16 inlet and outlet lines were hooked up to two manifolds: the inlet manifold had one inlet and 16 outlets; the outlet manifold had one outlet and 16 inlets. The single port of the manifold was connected with silicone tubing to the medium bag, which was stored at 4°C to increase the half-lives of ascorbate and L-glutamine. The 16 inlet ports of the manifold were connected to sets of Bioprene pump tubing (Bacon Technical Industries, Marblehead, MA), which were placed into a 16-channel peristaltic pump (Watson Marlow, Falmouth Cornwall, England). The outlet was then connected using silicone tubing to a 100-L waste bag (Gibco). The reactor system was placed into an incubator, and the pump was turned on at a flow rate of 5.0 mL/h per bag (the volume inside each bag was 120 mL). To facilitate the increase in nutrient consumption rates, the flow rate per bag was increased over time from 5.0 to 8.5 mL/h (see Results). On day 16, the system was disassembled.

Figure 2. Closed single-pass perfusion system. In the actual system, 16 bags are attached in parallel. Growth medium refrigerated at 4°C is pumped through multiple bioreactors which have been seeded with fibroblasts and are incubated at 37°C. The effluent medium is collected in a closed reservoir (see Materials and Methods).



Assays

The determination of fibroblast mitochondrial activity by the MTT assay has been described previously and applies to three-dimensional tissue cultures.³¹ The assay involves the conversion of MTT (a tetrazolium salt) (Sigma, St. Louis, MO) to an insoluble formazan by intramitochondrial dehydrogenase enzymes. The formazan is solubilized and extracted using isopropanol. Optical density (540 nm) of the extracted dye is measured using a 96-well microplate reader (Molecular Devices, Menlo Park, CA) and is considered to be an indication of fibroblast cell number.

Process variables	Static	Single-pass perfusion
Process Time	22 days	16 days
Method	Static	Constant perfusion
Total media	1 L/mesh (fed 6 times)	1.7 to 2.7 L/mesh
Ascorbate	50 μ g/mL (added 6 times)	25 μ g/mL constant
PBS interval	4 times	None

Table 1. A comparison of the methods of growth between the static and perfusion processes.

Histology

A YSI glucose/lactate analyzer (Model 2700, YSI, Yellow Springs, OH).

Glucose and lactate concentrations were measured using the measurement of collagen content.

was measured using a microplate reader and was related to used to extract the dye, and the optical density (595 nm) fixed in formalin and stained with aniline blue. Ethanol was and is used to stain mature collagen. Tissue samples were a component of the Masson's Trichrome stain kit (Sigma) performed using the aniline blue assay.²⁷ Aniline blue is An in situ measurement of total collagen matrix was considered to be an indication of fibroblast cell number.

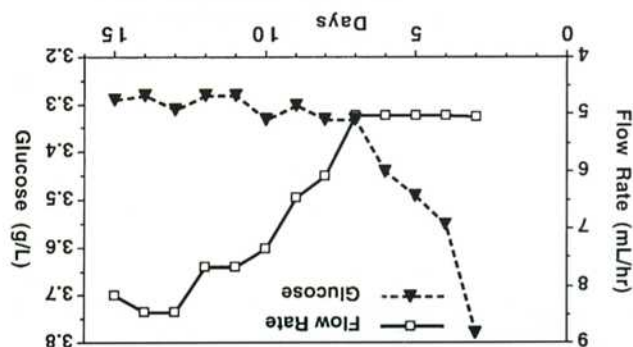
reader (Molecular Devices, Menlo Park, CA) and is considered to be an indication of fibroblast cell number. the extracted dye is measured using a 96-well microplate extracted using isopropanol. Optical density (540 nm) of dehydrogenase enzymes. The formazan is solubilized and extracted using isopropanol. Optical density (540 nm) of the extracted dye is measured using a 96-well microplate reader (Molecular Devices, Menlo Park, CA) and is considered to be an indication of fibroblast cell number.

RESULTS

Growth of Cells on Mesh

To grow a three-dimensional dermal tissue, human foreskin fibroblasts are seeded onto PGA/PLA mesh. These cells

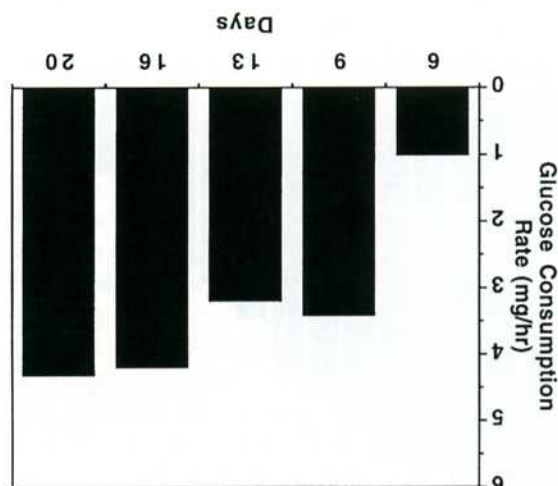
Figure 5. Flow rate (mL/h) and outlet glucose concentration (g/L) versus time (days) for the single-pass perfusion system. Flow rate and glucose data were pooled from seven perfusion runs.



We next wanted to measure the change of cell growth during the growth of the dermal tissue using the perfusion system. Figure 7a is a graph of MTT vs. days of growth of

through 21 days (data not shown).
shown that this glucose consumption rate remains constant flow rate in the system of 8.5 mL/h; other experiments have the glucose consumption rate leveled off near 5.5 mg/h at a to 5.5 mg/h at day 11 (Fig. 6). After day 11 in this study, glucose consumption rate increased from 1.1 mg/h at day 3 inlet glucose concentration was constant at 4 g/L. The outlet glucose concentration was constant at 4 g/L. (The glucose across the mesh from day 7 through day 15. The was increased to control the differential concentration of outlet glucose concentration, shows how the perfusion rate pH 6.8 at the outlet). Figure 5, a graph of flow rate vs. outside of our acceptable range (pH 7.4 at the inlet to 3.3 g/L at the outlet of the bag) created pH gradients Previous experiments have indicated that a delta greater than 0.7 g/L (4.0 g/L glucose concentration at the inlet and lactate concentrations by adjusting the perfusion rate. perfusion culture system was employed to control glucose might be useful to facilitate waste removal, the single-pass shown). Therefore, because more frequent media changes

Figure 4. Glucose consumption rates (mg/h) versus time (days) for the static process. Glucose data were pooled from eight separate 12-bag lots.



matly 2.0 g/L at the end of the culture period (data not such as lactate, which increased from 0 g/L to approxi-

The depletion in glucose concentrations in the static process also correlated with an increase in waste products

increased from 1 mg/h at day 6 to 4.3 mg/h at day 20. glucose consumption rate of a static grown tissue gradually and for glucose consumption rates. Figure 4 shows that the

ized the static process for changes in glucose concentration

is more efficient than the static process, we first character-

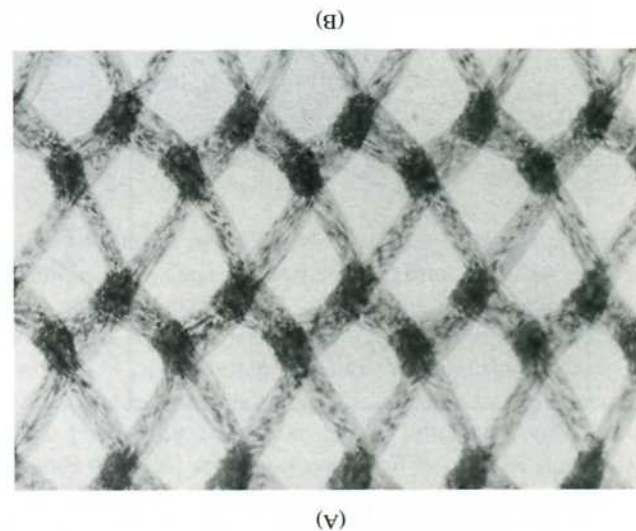
To develop a process for culturing human dermal tissue that

Development of a Perfusion System

a three-dimensional dermal tissue.
point, the culture is 8 to 12 layers thick and begins to form confluent within 4 to 5 days after seeding (Fig. 3a) and become stretch across the mesh interstices (Fig. 3a) and become deposition to occur in a three-dimensional array. The cells environment for cell growth and for extracellular matrix contact inhibition limits cell growth, the mesh provides an

pletely in less than 24 h. Unlike monolayer cultures, where adhere rapidly to the mesh scaffolding and attach com-

Figure 3. (A) Photomicrograph (40 \times) of fibroblasts stretching across the mesh substrate 1 to 2 days postseeding. (B) Photomicrograph (40 \times) of confluent cells on the mesh substrate 4 to 5 days postseeding.



A new perfusion system was used to develop a functional, three-dimensional dermal tissue. The successful development of a human dermal skin equivalent to be utilized in wound healing requires an understanding of the role

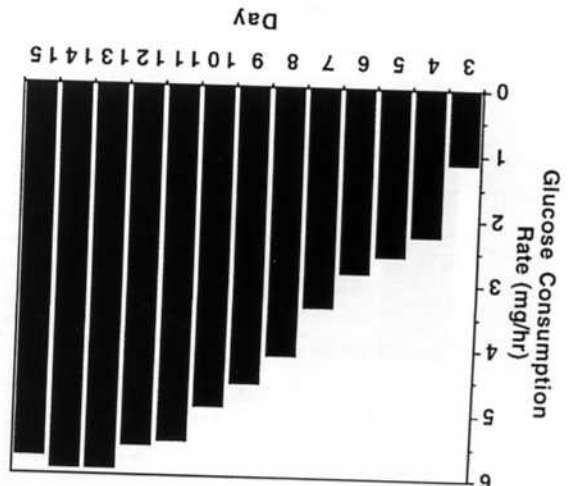
DISCUSSION

Histologic comparisons between products from perfusion and static systems. comparable with that obtained after 22 days growth in a static system. that in 16 days, the perfusion process can grow a tissue on both fresh and cryopreserved mesh. These data indicate are derived from MTT and aniline blue assays performed for growing a dermal replacement. The data for this table the final products from the perfusion and the static systems Table II summarizes and compares the characteristics of

Comparison of the Perfusion and Static Processes

toward the end of the culture. transitioning to an emphasis placed on matrix deposition an emphasis on cell proliferation at the start of the culture indicate a transition in metabolic activity over time with sampled product. The MTT and aniline blue data may the assays were performed on noncryopreserved, freshly from triplicate samples pooled from two separate lots, and throughout the process (Fig. 7b). These data were obtained sured by the aniline blue assay, increased fairly constantly after day 11. In contrast, collagen production, as measured by the aniline blue assay, increased fairly constantly throughout the process (Fig. 7b). These data were obtained from triplicate samples pooled from two separate lots, and the assays were performed on noncryopreserved, freshly sampled product. The MTT and aniline blue data may indicate a transition in metabolic activity over time with an emphasis on cell proliferation at the start of the culture transitioning to an emphasis placed on matrix deposition toward the end of the culture.

Figure 6. Glucose consumption rate (mg/h) versus time (days) for the single-pass perfusion process. Glucose data were pooled from seven separate perfusion runs.



Assay	Static	Single-pass perfusion
MTT-fresh	1.04 (n = 6)	0.92 (n = 13)
MTT-frozen (-70°C)	0.60 (n = 24)	0.63 (n = 4)
AB	0.39 (n = 24)	0.45 (n = 13)

Table II. Comparison of dermal tissues from the 22-day static and 16-day perfusion processes. Values shown are optical density units for MTT and aniline blue (AB) assays (see Materials and Methods). *n* is equal to the number of dermal replacements used per data point. All standard deviations were within 10% of the mean.

Figure 7. (A) Optical density (540 nm) versus time (days) from MTT assays of dermal tissues grown in perfusion system. The MTT assay standard deviations of triplicate samples from two lots. Error bars represent deviations of triplicate samples from two lots. Aniline blue stains collagen and correlates to relative amounts of extracellular matrix (see Materials and Methods).

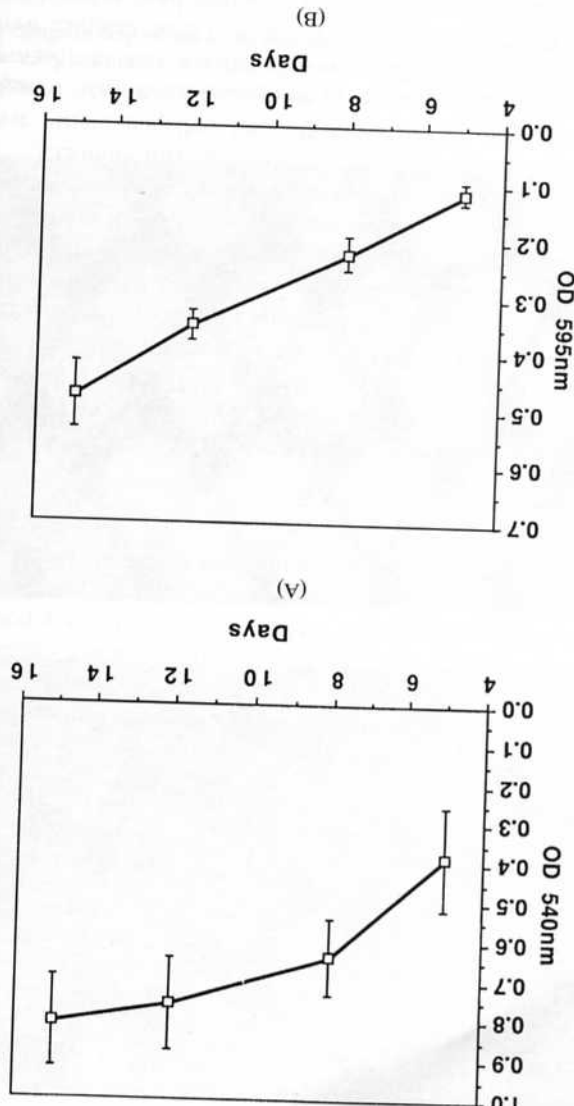
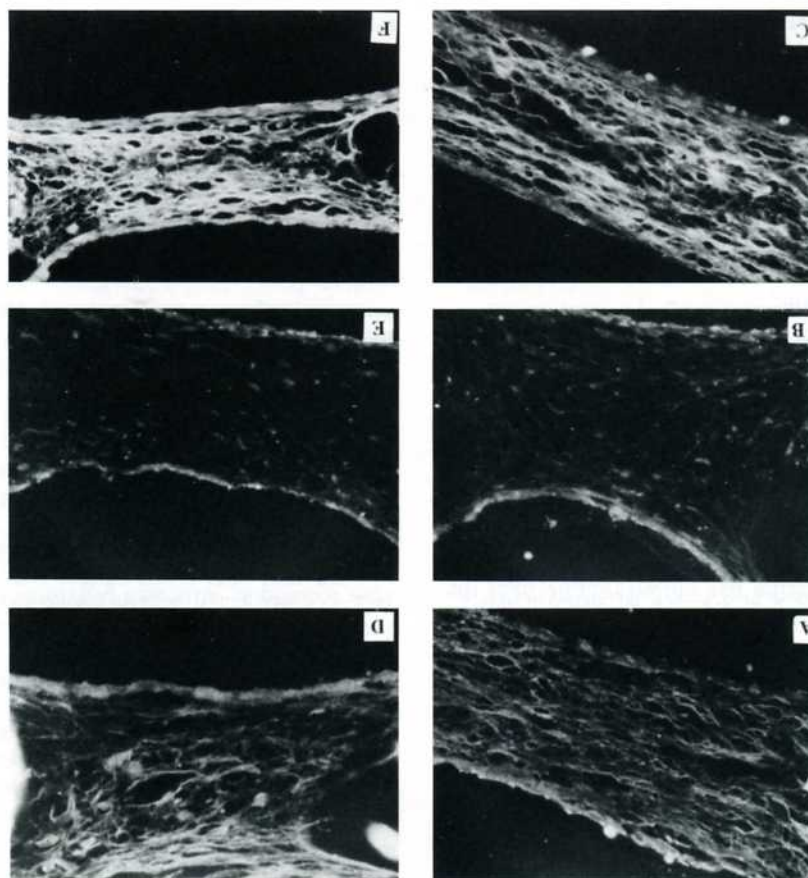


Figure 8. Photomicrographs (400 \times) of human dermal replacements grown under static (A, B, and C) and single-pass perfusion (D, E, and F) processes examining the production of collagen type I (A and D), fibronectin (B and E), and decorin (C and F) using immunofluorescence for detection (see Materials and Methods).



of the dermis in vivo. The dermis is an organized, three-dimensional hydrogel of fibroblast cells in an extracellular matrix consisting primarily of proteins (collagen, fibronectin, elastin); glycoproteins; and glycosaminoglycans (chondroitin sulfates and hyaluronic acid).³² The matrix molecules bind to each other²⁴ and have varying degrees of affinity for resident growth factors. During wound healing the dermal fibroblasts and many other cell types are coordinately regulated in a cascade of cellular responses. Therefore, the production of a living dermal replacement is challenging on many levels, because the dermis produced must be not only structurally similar but also functionally equivalent to native dermis.

We have established a method to grow cells on a three-dimensional scaffold which enables them to grow in an *in vivo*-like configuration. Using glucose and lactate concentrations to regulate perfusion flow rates has been shown to be a good indicator of the overall environmental conditions of the system.^{4,6,13,17} Therefore, we employed this analysis to maintain a constant delta of 0.7 g/L from the inlet to the outlet of each bag, thereby providing an environment for the growth of the cells without creating deleterious nutrient gradients. Unlike conventional bioreactor systems, which are designed to ensure uniform distribution of nutrients to the cells, the perfusion system described here creates long residence times not only for the nutrients but also for autocrine and paracrine factors.

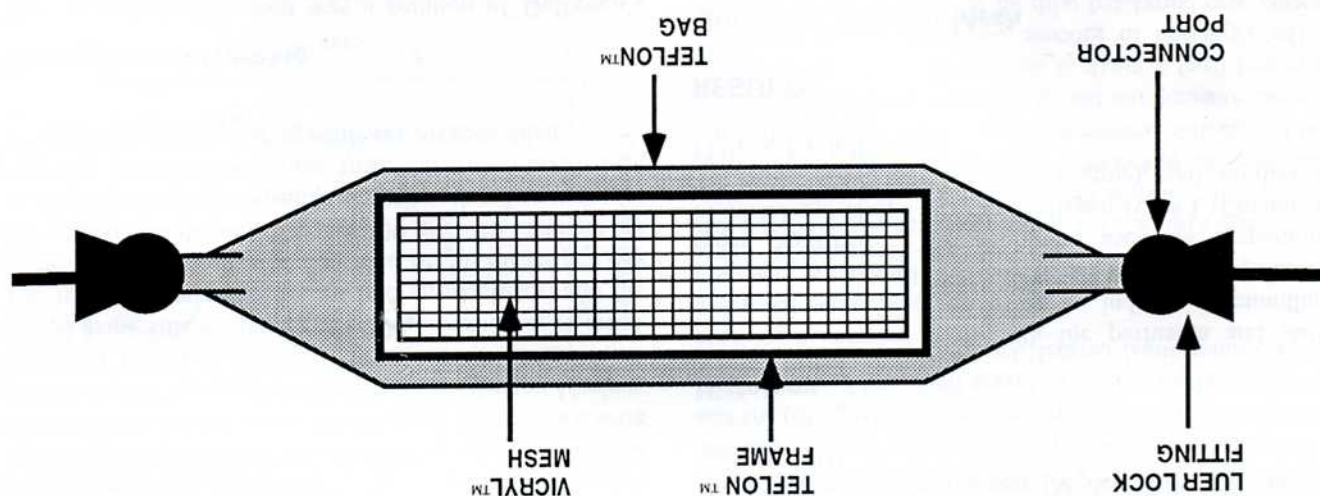
These longer residence times provide a microenvironment necessary for tissue development. As a result, the deposition of extracellular matrix proteins by these cells in a three-dimensional structure provides opportunities for the cells to organize the matrix and for the cells to interact in a manner which more closely represents that which occurs *in vivo*. Both the static and perfusion systems promoted the deposition of fibronectin, decorin, and collagen types I, which are all found in normal human skin. However, as shown in Table II, the perfusion system produced the tissue, as measured by cell viability and matrix, in 6 fewer days than the static system.

A reproducible, easy-to-operate system that controls the microenvironment is necessary for the successful manufacture of three-dimensional, living human dermal replacement. We have found that a single-pass perfusion system can produce this tissue more efficiently and effectively than a static system. To further enhance the development of this tissue, we are developing on-line monitoring and control of both the macro- and microenvironments. Because of this efficiency, the perfusion system makes feasible the design

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Figure 1. Teflon bag bioreactor housing the polyglactin mesh. The 4×6 in. mesh is attached to a Teflon frame with Vicryl sutures and the mesh/frame assembly is free-floating in the bioreactor (see Materials and Methods).



After the mesh had been seeded, lots of 12 to 36 bags were grown for 21 days under static conditions using ascorbate growth medium (DMEM, 10% [v/v] iron-supplemented calf serum [Hyclone], L-glutamine, NEAA, and 50 $\mu\text{g}/\text{mL}$ ascorbate).¹⁴ For the operation of the single-pass perfusion system, 16 bags were seeded and then attached in parallel (Fig. 2). Table I lists the methods of growth for these two processes.

Growing the Dermal Tissue

Fibroblasts were harvested from the roller bottles using trypsin and were counted using a hemocytometer. They were then injected into 16 bags at a concentration of 6.0×10^5 cells/mL (3.0×10^7 cells per mesh) in FBS growth medium. The bags were transferred to a roller bottle apparatus at $37^\circ\text{C}/5\% \text{CO}_2$, where they were rotated at 0.25 rpm for 24 h to facilitate attachment of the cells to the mesh. After the bags were rotated, they were placed flat on an incubator shelf where they remained for an additional 24 h.

Seeding of Biodegradable Mesh in Teflon Bags

Biodegradable mesh composed of PGA/PLA copolymer (Vicryl; Ethicon Inc., Somerville, NJ) was used for the three-dimensional growth of the fibroblasts. This mesh has $>95\%$ porosity (1.0×0.5 mm, diamond-shaped pores) and 0.19-mm thickness. The mesh was cut into 4×6 in. pieces and was subsequently placed between two sheets of Dupont Teflon FEP (fluorinated ethylene propylene copolymer; American Fluoroseal, Silver Spring, MD). The sheets were laser-welded to form a bag with openings at each end where tubing ports were placed (Fig. 1). The Teflon bag was then sterilized with ethylene oxide (EtO) (Dravon, Clackamas, OR). Removal of EtO (until levels were less than 25 ppm) was achieved by vacuum outgassing.

Preparation of the Mesh and Culture Bag

Human dermal fibroblasts were aseptically isolated from a circumcised neonatal foreskin after obtaining informed consent of the mother. The epidermis and dermis were separated by incubation in 0.25% trypsin/0.2% EDTA (Gibco, Grand Island, NY) for 1 to 2 h at $37^\circ\text{C}/5\% \text{CO}_2$. After the epidermis was mechanically removed, the remaining dermis was minced and digested with 0.35% collagenase B (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 to 2 h at $37^\circ\text{C}/5\% \text{CO}_2$. The resulting cell suspension was washed, and the cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT; MEM nonessential amino acids (NEAA; Gibco); L-glutamine (Gibco); gentamicin (Gibco); and amphotericin B (Squibb, Princeton, NJ). The cells were tested at passage one for viral pathogens, mycoplasmas, sterility, and karyotype analysis. If all tests were negative or normal, the cell strains were accepted for use in the final product. Fibroblast cultures were expanded into roller bottles using DMEM with 10% FBS (v/v), NEAA, and L-glutamine (FBS growth medium) at $37^\circ\text{C}/5\% \text{CO}_2$.

Cell Strain

MATERIAL AND METHODS

enhances delivery of nutrients and removal of waste products through continuous movement of fluid. The perfusion system consists of 16 self-contained bioreactors, which contain 4×6 in.-fibroblast-seeded mesh connected in parallel to a media-feed stream and a waste-removal line. Upon completion of tissue growth, the tissue substitute can then be aseptically cryopreserved, stored for a period of time, and then shipped to a hospital or clinic for transplantation. This report shows that the single-pass perfusion system can grow tissue that is equivalent to a static-grown product in 6 fewer days than the static system.