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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Dennis Lee Salzmann entitled Macrophage Response to Polymeric Vascular Grafts and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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ABSTRACT

The use of materials for replacement or repair of biological tissue and organs has been attempted for thousands of years. Regardless of material used or site of implantation all biomedical materials elicit a foreign body response by the host characterized by the presence of macrophages and foreign body giant cells with the polymer for the duration of the implant. This inflammatory response is believed to be responsible for the lack of biocompatibility of implanted materials. Furthermore, each type of biomedical device suffers from specific problems that may lead to the ultimate failure of the implant. Synthetic polymeric vascular grafts fail primarily due to the inherent thrombogenicity of the material and anastomotic neointimal thickening. In an attempt to create a non-thrombogenic lining on the blood contacting surface of vascular implants, the promotion of an endothelial lining on the luminal surface of vascular grafts has been investigated. This can be accomplished by both artificial and natural mechanisms. Regardless, it is believed that the inflammatory response elicited by the implant influences the angiogenic mechanisms and neointimal thickening associated with the implant. The relationship between inflammation and angiogenesis associated with biomedical implants remains to be delineated. Studies in this dissertation attempt to determine this relationship by examining the inflammatory response and inflammatory cytokines released by cells associated with polymeric implants and how these bioactive molecules influence the angiogenic response. Furthermore, an advancing technology in vascular repair, endovascular grafts, was tested in two vascular models to assess the general healing characteristics, inflammatory response and the formation of blood vessels associated
with the device. The results from these studies suggest that the inflammatory response plays a fundamental role in the formation of blood vessels around polymeric implants and neointimal thickening on the luminal surface of vascular implants. From these experiments a greater understanding of the healing response associated with vascular grafts has resulted.
1. INTRODUCTION

The use of biomedical devices dates back to B.C. where archeological records from both China and Egypt describe the use of stone and ivory for tooth replacements (Lemons, 1986). Since then, numerous materials have been introduced for use in a very diverse range of medical applications (Table 1.1). The limiting step for the introduction of biomedical polymers has been the technology used for their development. Regardless of material used or application, biocompatibility of these implants was based upon host tolerance to determine success or failure. Still today, success of biomedical implants is based upon and trial-and-error protocol. Even with the current advances in biotechnology, the engineering behind biomedical devices relies very little on biological data.

Worldwide, over 500,000,000 biomedical devices interact with biological tissues each year (Table 1.2) (Ratner, 1993), with the majority of these stimulating a foreign body reaction possibly leading the ultimate failure of the device. Yet very little time or energy has been spent on understanding the role the inflammatory reaction plays in the healing associated with biomedical implants. In order to engineer biomedical devices that stimulate a desired host response leading to a predicted outcome, the differences between normal wound healing and wound healing following biomedical device implantation must be delineated.

Normal Wound Healing

Wound healing is a continuous process with concurrent and overlapping events which can be divided into 4 general phases: inflammation, tissue repair, tissue remodeling and
resolution of inflammation (figure 1.1). The four phases of wound healing are not mutually exclusive and each of the processes can be occurring with no distinct borders separating the individual phases. Inflammation occurs following the initial trauma and, in the absence of infection, persists for up to 15 days (Clark, 1988; Clark, 1996). Following and concurrent with inflammation, the tissue repair process necessary for complete healing begins around day 3 and continues for approximately 30 days (Clark, 1988; Clark, 1996). The third phase of healing, tissue remodeling, can last for up to 2 years after the initial insult (Clark, 1988; Clark, 1996). Finally, resolution of inflammation begins with the removal of inflammatory stimuli, cessation of enhanced leukocyte emigration, restoration of the normal vasculature, and
Table 1.2. Number of biomedical devices implanted per year (U.S. only)

<table>
<thead>
<tr>
<th>Application</th>
<th>Numbers Used per Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraocular lenses</td>
<td>1,400,000</td>
</tr>
<tr>
<td>Retinal surgery implants</td>
<td>50,000</td>
</tr>
<tr>
<td>Prostheses after enucleation</td>
<td>5,000</td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>350,000</td>
</tr>
<tr>
<td>Arteriovenous shunts</td>
<td>150,000</td>
</tr>
<tr>
<td>Heart valves</td>
<td>75,000</td>
</tr>
<tr>
<td>Pacemakers</td>
<td>130,000</td>
</tr>
<tr>
<td>Blood bags</td>
<td>30,000,000</td>
</tr>
<tr>
<td>Breast prostheses</td>
<td>100,000</td>
</tr>
<tr>
<td>Nose, chin</td>
<td>10,000</td>
</tr>
<tr>
<td>Penile</td>
<td>40,000</td>
</tr>
<tr>
<td>Dental</td>
<td>20,000</td>
</tr>
<tr>
<td>Hips</td>
<td>90,000</td>
</tr>
<tr>
<td>Knees</td>
<td>65,000</td>
</tr>
<tr>
<td>Shoulders, finger joints</td>
<td>50,000</td>
</tr>
<tr>
<td>Ventricular shunts</td>
<td>21,500</td>
</tr>
<tr>
<td>Catheters</td>
<td>200,000,000</td>
</tr>
<tr>
<td>Oxygenators</td>
<td>500,000</td>
</tr>
<tr>
<td>Renal dialyzers</td>
<td>16,000,000</td>
</tr>
<tr>
<td>Wound drains</td>
<td>3,000,000</td>
</tr>
<tr>
<td>Sutures</td>
<td>20,000,000</td>
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</tbody>
</table>

removal of all other components not normally found within the tissue (Clark, 1988; Clark, 1996). Each of these four phases is discussed below.

Upon insult to tissue, damage to blood vessels results in the extravasation of blood components and initiation of the intrinsic and extrinsic pathways for blood coagulation. Blood platelets facilitate the formation of blood clots, release a plethora of growth factors and chemotactic molecules which attract inflammatory cells to the site of injury (Terkeltaub and Ginsberg, 1988). Other factors within the blood, for example complement and serum
FIGURE 1.1 Normal wound healing begins with an early, transient inflammatory response characterized by the infiltration of polymorphonuclear leukocytes (PMN's) followed the infiltration of macrophages. An transient increase in fibroblasts and vasculature leads to the formation of a scar. (Adapted from Clark, 1988).
PMN's  Macrophages  Fibroblasts

Relative Intensity

Time (days)
proteins, and cytokines released by various cells, can activate endothelial cells leading to increased expression of adhesion molecules enhancing the attraction of inflammatory cells to the wound (Bevilacqua et al., 1985a; Bevilacqua et al., 1985b; Magnuson et al., 1989). Activated leukocytes adhere and transmigrate through the endothelium increasing the accumulation of inflammatory cells within the wounded tissue (Issekutz et al., 1981; Tozeren and Ley, 1992). The blood clot serves as a provisional matrix allowing cell migration into the wound space (Yamada and Clark, 1996). Inflammatory cells and fibroblasts utilizing cell surface integrins recognize fibrin, fibronectin and vitronectin, the major proteins of the clot matrix, and infiltrate the wound promoting the healing process (Cox et al., 1994; Hynes, 1992). Taken together, the initial response to insult is blood coagulation, endothelial and leukocyte activation followed by enhanced attraction and migration of inflammatory cells into the wounded tissue.

Inflammation can be divided into early and late phase responses characterized by the inflammatory cell type dominant within the wounded tissue (figure 1.1). The early inflammatory response is characterized by the infiltration of neutrophils. While monocytes are also attracted to the wound immediately, neutrophil emigration is much greater during early inflammation (Hunt et al., 1984), probably due to their greater concentration within the circulation (Clark, 1996). Several factors have been identified as chemoattractants for neutrophils including kallikrein (Kaplan et al., 1972), fibrino-peptides (Stecher and Sorkin, 1972; Senior et al., 1986), C5a (Fernandez et al., 1978; Marder et al., 1985), leukotrienes (Ford-Hutchinson et al., 1980), IL-8 (Koch et al., 1992), and platelet derived molecules (Deuel et al., 1981; Deuel et al., 1982). These factors have also been shown to activate
neutrophils leading to an increased expression of integrins on their surface facilitating migration from the blood to the tissue (Smith et al., 1989). Neutrophils, once at the site of insult, phagocytose and destroy by oxygen radical mediated mechanisms any bacterial or foreign material introduced into the wound (Tonnesen et al., 1988). Enzymes and toxic oxygen products released by neutrophils remove unwanted debris but may also further damage the tissue. Neutrophil infiltration is transient and will cease around day 3, assuming no infection, at which time an increase in monocyte accumulation is observed (Clark, 1996). This marks the end of the early phase of inflammation and the beginning of the late phase of inflammation.

The late phase of inflammation is characterized by continued macrophage accumulation (Hunt et al., 1984), mainly through monocyte emigration and differentiation into macrophages along with resident tissue macrophage proliferation (figure 1.1) (van Furth et al., 1985). Monocytes transmigrate across the endothelium utilizing specific adhesion molecules, particularly α4β1 and β2 integrins (Meerschaert and Furie, 1995; Beekhuizen and van Furth, 1993; Takahashi et al., 1994; von Andrian et al., 1991), into the tissue where they differentiate into macrophages. The process of monocyte to macrophage differentiation is poorly understood, however, integrin binding, particularly β2 integrins, to extracellular matrix proteins and specific soluble molecules have been implicated in this process (Proveddini et al., 1986; Miller et al., 1987; Riches, 1996; Riches, 1988). Certain chemoattractants have been identified which may be responsible for the continued emigration of monocytes to regions of inflammation and these include fragmented collagen (Postlethwaite and Kang, 1976), elastin
(Senior et al., 1980; Senior et al., 1984), fibronectin (Norris et al., 1982), thrombin (Bar-Shavit et al., 1983), C5a des arg (Doherty et al., 1987; Marder et al., 1985), and TGF-β (Pierce et al., 1989; Wahl et al., 1987). Effete neutrophils, along with other foreign materials including bacterial and tissue debris, are phagocytosed by macrophages (Newman et al., 1982). Macrophages continuously secrete chemotactic and growth factors along with enzymes which facilitate tissue debridement and are required for the formation of granulation tissue (Koch et al., 1986b). Simpson and Ross (1972a) demonstrated that neutrophils are not required for normal wound healing to occur, and therefore, the macrophage appears to play the critical role in the sequential transformation from inflammation to tissue repair.

The second phase of wound healing, tissue repair, is characterized by the formation of granulation tissue which consists of macrophages, fibroblasts, loose connective tissue and new blood vessels (figure 1.1) (Clark, 1988; Clark, 1996). The formation of granulation tissue is a finely tuned sequence of events relying on the interdependence of cells within the wound and their synthesis of extracellular matrix proteins, enzymes and cytokines. Macrophages secrete cytokines which stimulate the proliferation and migration of fibroblasts into the wound (Clark, 1988; Clark, 1996; Riches, 1996). These fibroblasts produce matrix proteins which provide a substrate for macrophages and new blood vessels to adhere and migrate further into the wound (Clark, 1988; Clark, 1996). Neovascularization of the tissue provides the required nutrients for further tissue repair and sustained cell viability (Clark, 1988; Clark, 1996). All cells act coordinately to promote the formation of granulation tissue and further the healing process enhancing tissue repair.
The third phase of healing consists of tissue remodeling and cell maturation (figure 1.1). Tissue remodeling actually begins simultaneously with tissue repair and is characterized by the initial deposition of a fibronectin based matrix that is eventually replaced with collagen type III followed by collagen type I (Kurkinen et al., 1980; Holund et al., 1982). Fibronectin provides an excellent substrate for cell migration and ingrowth since it is easily penetrated by cells due to its hydrated state (Clark, 1988). As suggested by studies evaluating the leading edge of wounds, fibronectin may also serve as a template for collagen deposition (Kurkinen et al., 1980). Seven days following implantation of cellulose sponges, the leading edge of cellular infiltration consisted of fibroblasts and fibronectin. Located behind the leading edge of cells and fibronectin the extracellular matrix was primarily types I and III collagen. Replacement of fibronectin by collagen increases the wounds tensile strength and structural support. It has also been suggested that matrix deposition has a profound effect on cell phenotype (Form et al., 1986b). For example, endothelial cells cultured on laminin proliferate and migrate, however, when cultured on type IV collagen they appear more quiescent (Form et al., 1986a). This would suggest that the deposition of collagen into the wound may serve to suppress other cells and cellular functions including fibroblast migration and secretion of matrix proteins. During this time, collagen is remodeled and reformed into larger collagen bundles that increase the tensile strength of the wound tissue. In addition fibroblasts transform into myofibroblasts causing contraction of the wound (Clark, 1988). Enhanced collagen synthesis and remodeling continues for up to 12 months following the initial injury and will return to normal when scar formation is complete (Clark, 1988).
For healing to be complete, the inflammatory response must resolve returning the tissue to a normal cellular state. To accomplish this, mediators responsible for the inflammatory response must be removed or inactivated. Multiple mechanisms including, but not limited to, cell desensitization, inactivating enzymes or inhibition of protein synthesis may lead to resolution of the inflammatory response (Haslett and Henson, 1996). Furthermore, immigration of inflammatory cells must cease. The mechanisms for this are poorly understood; however, many hypothetical mechanisms have been proposed (Haslett and Henson, 1996). For example, removal of the chemotactic factors from the injured tissues by enzymes and inhibition of future synthesis of these factors decreases the immigration of inflammatory cells. Excess fluids are removed by lymphatic vessels while proteins and debris are broken down by inflammatory cells and phagocytosed (Haslett and Henson, 1996). The inflammatory cells must also be removed from the inflamed site in order to achieve a normal cellular state. Neutrophils are believed to age and undergo cell apoptosis (Squier et al., 1995), or necrosis (Squier et al., 1995), allowing them to be recognized by macrophages as senescent cells resulting in their phagocytosis (Newman et al., 1982). The process of neutrophil apoptosis is believed to play an important role in control of inflammation, while neutrophil necrosis is believed to stimulate continued inflammation (Haslett and Henson, 1996). The mechanisms leading to the clearance of macrophages from the site of inflammation remain entirely unknown. The complicated interactions of multiple cells and factors and many individual processes must cease for a normal non-inflammatory state to be achieved.
Wound Healing Associated with Biomaterial Implants

Biomaterial implants have been demonstrated to exhibit an altered healing response (Anderson, 1988). The interactions between host tissue and biomaterial implants determines the materials biocompatibility and success or failure. Most commonly associated with biomaterial implants is an increased and chronic inflammatory response characterized by macrophages and foreign body giant cells.

Following initial implantation of biomaterials, proteins adsorb to the surface as described by the Vroman effect which predicts that initial protein adsorption is dependent on the concentration of proteins in the blood, however, temporal protein adsorption is dependent on the proteins affinity for the polymer (Leonard and Vroman, 1991). The inflammatory response is exacerbated in both intensity and duration compared to the inflammatory response of normal wounds (figure 1.2) (Anderson, 1988). This may in part be due to fibrinogens affinity for polymeric surfaces. Tang and Eaton (1993) demonstrated that severely hypofibrinogenemic mice fail to mount an inflammatory response against implanted polyethylene teraphthalate unless the material is coated with fibrinogen or fibrinogen is injected into the bloodstream of the animals. Studies performed to quantitate the early phase of inflammation have revealed a transient infiltration of neutrophils into the perigraft region (Schreuders et al., 1988). These polymorphonuclear leukocytes (PMN) are activated and produce cytokines and other biological active substances (Eriksson and Thomsen, 1991; Lundberg et al., 1995) that in turn attract other inflammatory cells including monocytes and macrophages. Similar to normal wound healing, the early phase of inflammation is transient and slowly dissipates while the late phase of inflammation, characterized by the accumulation
Figure 1.2 Wound healing associated with biomedical implants is characterized by the chronic presence of macrophages and the formation of foreign body giant cells. (Adapted from Anderson, 1988).
of macrophages, is initiated (Kovacs and DiPietro, 1994; van Beusekom et al., 1993; Anderson, 1988). The most characteristic observation of biomaterial implants is the elevated and chronic response by macrophages (Jansen et al., 1994). Macrophages can be found associated with implants for years following implantation. After 1 month and 12 months of implantation, agarose beads (80-120 μm diameter) injected under the skin of rats had many macrophages but no PMN within the healing tissue (Eppley et al., 1994). Unlike normal wound healing, the macrophages associated with biomaterials continue to produce and release biologically active molecules for the life of the implant. Therefore, the late phase of inflammation associated with biomaterial implants never resolves and the normal mechanisms of wound healing are altered leading to altered healing patterns. This chronic inflammatory response to biomaterial implants requires in depth studies to fully appreciate the consequences it may have on wound healing.

The second stage of wound healing, tissue repair and the formation of granulation tissue, may be dramatically altered following implantation of biomaterials (figure 1.2). Fibroblasts exhibit an increased production and deposition of matrix proteins possibly due to the release of cytokines by macrophages (Miller et al., 1989) and other activated cells found within the wound. This may lead to the complete encapsulation polymeric implants by extracellular matrix proteins (Miller et al., 1989). Neovascularization of biomaterials and the surrounding tissues may be increased or decreased depending on the production of specific cytokines produced by macrophages and other cells within the wound tissue. The overall response is highly dependent on the tissue site of implantation and the type of material implanted and can have drastic effects on repair of the tissue (Spilezewski et al., 1988;
The third stage of healing, remodeling and resolution, may never occur (figure 1.2). Inflammatory cells, mainly macrophages (Jansen et al., 1994) and foreign body giant cells (Miller et al., 1989; Kao et al., 1995; Kao et al., 1994) continue to be associated with biomaterials for the life of the implant. These results suggest that macrophages play a fundamental role in the altered healing response observed with biomaterial implants. Furthermore, the chemical and physical properties of the biomaterial may play a role in the stimulation of the inflammatory response.

Collagen

Collagen is a natural occurring biodegradable biomaterial commonly used for sutures and skin substitutes. Anselme et al. (1990) studied the healing patterns of Heamostagen, a type I collagen sponge prepared from the skin of young calves, implanted subcutaneously in rats. Eight hours postimplantation the collagen sponges became infiltrated by an amorphous ground substance (predominantly fibronectin) and had PMNs at the periphery of the implant. After 48 hours, PMNs had migrated into the implant and the collagen sponge had been partially degraded. Mononuclear cells began to replace the PMNs within the implanted collagen sponge after 8 days, granulation tissue had begun to be formed around the implant and foreign body giant cells were present. Fifteen days postimplant the major inflammatory cell within the collagen sponge was the macrophage. Fibroblasts were abundant and granulation tissue was also found within the implant. The majority of the implanted collagen
sponge had been replaced by newly synthesized collagen and fibronectin. At 1 month, a small amount of the implanted collagen sponge still remained. The implant was entirely vascularized and contained densely packed collagen fibrils and foreign body giant cells. All of the implanted collagen sponge had disappeared following 3 months implantation and only a few inflammatory cells remained.

Polyurethanes and Derivatives

Polyurethanes have been used for many biological applications from catheters to artificial hearts. Marchant et al. (1986b) implanted Biomer, a polyetherurethaneurea, into subcutaneous tissue of the rat and evaluated the inflammatory response over a 21 day period. Their results demonstrated that immediately following implantation, PMNs are the major cell type associated with the implant. The number of PMNs decreased with time while the macrophages increased until 2 weeks when their numbers began to decline. The authors suggest that the Biomer caused an elevated acute inflammatory response (1 week) and a slightly elevated chronic inflammatory response (2 and 3 weeks).

Spilezewski et al. (1988) implanted polyurethane compounds used in the manufacture of catheters subcutaneously within rats and found similar results as above. Following 4 days of implantation, PMNs, macrophages and lymphocytes were all associated with the implant. However, there were significantly more macrophages associated with the implants than other leukocytes. All leukocytes decreased in numbers over time, and by 21 days no significant differences were noted between cell concentrations when compared to control tissues. Their results suggest that the major inflammatory cell associated with polyurethane implants is the
macrophage.

Dacron (PET)

PET has been used in many clinical applications, for example urethral prostheses, eye reimplantations, artificial heart linings, artificial skin, and vascular prostheses. Schreuders et al. (1988) implanted porous Dacron velour subcutaneously in rabbits. Examination of 10-day implants revealed collagen, foreign body giant cells, vascular profiles, fibroblasts, macrophages and other inflammatory cells within the implant and the presence of a collagen capsule surrounding the implant. Twenty-eight day implants exhibited matrix (collagen III) (von Recum et al., 1993) and cells within and around the implanted Dacron similar to that of 10-day implants. Sham surgeries were almost completely healed after 10 days with the only differences being in the maturity of collagen compared to control skin samples. Sham surgeries had completely healed after 28 days and were indistinguishable from normal connective tissue. Miller et al. (1989) observed similar results when examining Dacron implanted subcutaneously in the rat. These results suggest an altered healing response following Dacron implantation characterized by a chronic inflammation.

expanded Polytetrafluoroethylene (ePTFE)

ePTFE is most commonly used for vascular prosthesis, however, it has also been used for other surgical applications including wound patches and suture. Healing characteristics of ePTFE are highly variable depending on the tissue that surrounds the implant. When implanted subcutaneously, ePTFE becomes encapsulated by dense connective tissue,
predominantly type I collagen. However, when implanted within adipose tissue no encapsulation occurs (Williams et al., 1997). Tissue ingrowth of ePTFE has been shown to be both type I and III collagen (Hirabayashi et al., 1992; Kohler et al., 1992), and likely deposited by fibroblasts (Clowes et al., 1985). It has also been demonstrated that the surrounding tissue dictates the degree of neovascularization. For example, implants within adipose tissue generally vascularize to a greater degree than subcutaneous implants. ePTFE elicits an acute inflammatory response with enhanced PMN cell infiltration (Lundberg et al., 1995) and a chronic inflammatory response characterized by activated monocytes and macrophages and foreign body giant cells (Hirabayashi et al., 1992; Kohler et al., 1992; Williams et al., 1997).

Healing associated with various biomedical devices has many similarities. However, each application has many unique healing patterns characteristic of particular devices. The focus of this dissertation has been the evaluation of biomedical materials used in the manufacture of synthetic vascular prosthetics. These devices are chronically exposed to blood flow on the luminal surface while the abluminal surface is exposed to one or more of several soft tissue environments. Due to this unique position, an overview of the healing associated with synthetic vascular prosthetics is appropriate.

Healing Associated with Synthetic Vascular Prosthetics

An observation that a strand of silk suture transversing the right ventricle of a dog became coated with a thrombus-free film lead to the idea that arterial defects could be bridge using polymeric prostheses (Voorhees et al., 1952). The first documented case of using a
synthetic biomaterial in a vascular position was by Voorhees et al. (1952). This group used Vinyon “N” cloth in the abdominal aorta of dogs as an intrapositional graft. This led into an era of using polymeric materials for the repair and replacement of diseased or destroyed blood vessels.

The two most widely used polymers for vascular reconstruction are ePTFE, a fluorinated hydrocarbon, and PET. The majority of these grafts fail due to 1) acute thrombogenesis (0-6 months) and 2) anastomotic neointimal thickening (6-24 months). While both ePTFE and PET suffer equally from anastomotic neointimal thickening, PET is both more thrombogenic and inflammatory compared to ePTFE.

Following placement of a synthetic vascular conduit, the blood contacting surface (luminal side) adsorbs proteins as predicted by the Vroman effect. These proteins interact with platelets and initiate the blood coagulation cascade, covering the luminal surface with a psuedointima composed of fibrin and platelets (Rubin et al., 1993). Furthermore, thrombin and factor Xa are deposited on the luminal surface of prosthetic grafts stimulating blood clot formation (Toursarkissian et al., 1997). The adhering platelets degranulate releasing bioactive substances stored in dense bodies and alpha granules. This psuedointima serves as a provisional matrix for inflammatory cells, initially neutrophils, to adhere and migrate into the clot. The function of these neutrophils in the healing associated with vascular grafts remains unknown, however, the consequences of their arrival can be predicted. These neutrophils release proteases, oxygen radicals, and other products that initiate the wound healing response. However, neutrophils are not believed to have a significant impact on the healing associated with vascular grafts due to their transient nature. Macrophages begin to
accumulate within this provisional matrix within the first three days and are believed to play a significant role in the healing associated with synthetic vascular grafts (Urayama et al., 1996).

At the anastomosis, endothelial cells with underlying smooth muscles cells begin to migrate as a uniform monolayer from the native vessel onto the polymeric surface (Clowes et al., 1985; Clowes et al., 1986a; Guidoin et al., 1993). This is termed pannus ingrowth and only continues for approximately 2-4 mm from both the proximal and distal anastomosis. The underlying smooth muscle cells continue to proliferate producing a focal stenosis which introduces flow abnormalities resulting in endothelial cell injury (Clowes et al., 1985; Clowes et al., 1986a; Reidy et al., 1986). These endothelial cells also proliferate exacerbating the stenosis and possibly leading to the failure of the implant. Furthermore, smooth muscle cells produce extracellular matrix, predominantly collagen type 3, which further thickens the lesion (Urayama et al., 1996). The endothelium may also attract platelets that release granules of growth factors, predominantly platelet-derived growth factor (PDGF) and transforming growth factor beta-1 (TGF-β1), which further stimulate both smooth muscle cell and endothelial cell proliferation. It has also been suggested that the injured endothelium and underlying smooth muscle cells produce and release PDGF which act on neighboring cells (Clowes et al., 1985; Clowes et al., 1986a). Furthermore, these lesions become rich with macrophages that release cytokines inducing the overlying endothelium to express adhesion molecules promoting the adherence of PMNs that can release oxygen metabolism products injuring the endothelium leading to increased proliferation rates.
The abluminal surface of polymeric vascular grafts has received less attention until recently. The healing associated with the material's abluminal surface is dependent on the surrounding tissue environment (Sterpetti et al., 1992). Most often, vascular grafts placed in humans are tunneled from the proximal to the distal anastomosis through subcutaneous tissue composed predominantly of adipocytes. The initial inflammatory cells associated with the abluminal surface are neutrophils and this acute response is transient in nature. Macrophages accumulate at the tissue-material interface and release enzymes, oxygen radicals, cytokines and growth factors. Following several days, macrophages fuse to form foreign body giant cells (Murch et al., 1982) leading to the common foreign body reaction associated with polymeric implants. It has been suggested that the macrophages and foreign body giant cells associated with the abluminal surface influence the healing characteristics observed on the luminal surface (Zacharias et al., 1987). Fibroblasts are also associated with the abluminal surface and have been demonstrated to form a dense connective tissue matrix that surround the polymer.

One of two primary goals directing research on polymeric vascular prostheses is the elimination of intimal thickening at the proximal and distal anastomosis leading to increased patency rates for small caliber (< 6 mm) vascular grafts. Several approaches including polymer modification (Chakfe et al., 1996; Becquemin et al., 1997), tissue engineering (Greisler et al., 1996), and gene therapy (Stopec et al. 1997) have been used, however, to date none have been completely successful. The premise behind each of these techniques is that promotion of an endothelial lining on the luminal surface of synthetic vascular grafts would provide an anti-thrombogenic surface and control the extent of neointimal thickening.
at both anastomosis. Endothelial cells can become associated with the luminal surface by artificial means (cell seeding and cell sodding) or by natural mechanisms that include 1) pannus ingrowth (Clowes et al., 1985), 2) fallout endothelialization (Scott et al., 1994; Shi et al., 1994), and 3) transmural migration (Shi et al., 1997).

The transplantation of endothelial cells onto the luminal surface of polymeric grafts has been performed using either cell seeding or cell sodding. With cell seeding, endothelial cells are isolated from arterial or venous segments and mixed with whole blood (Herring et al., 1979). This mixture is then put onto the luminal surface of the polymeric graft. The endothelial cells within the blood clot proliferate and spread on the luminal surface to form an endothelial cell monolayer. Cell sodding uses endothelial cells isolated from liposuction derived fat. This relatively pure endothelial cell isolate is then placed onto the luminal surface at a density that provides theoretical confluence thereby immediately forming a completely endothelialized graft surface (Jarrell et al., 1986). Both methods have demonstrated success in human studies (Deutsch et al., 1997).

Several natural healing mechanisms have also been proposed as means for endothelialization of polymeric vascular grafts. As stated earlier, pannus ingrowth is limited to the anastomotic region with no evidence to suggest this as a possible mechanism for complete endothelialization of polymeric materials. Fallout endothelialization has been hypothesized to occur when circulating endothelial progenitor cells adhere to the polymeric surface, spread, and proliferate to form islands of endothelial cells eventually leading to complete endothelialization. Support for this method is minimal, however, reported cases of fallout endothelialization have appeared in the literature (Scott et al., 1994; Shi et al., 1994).
Transmural migration of endothelial cells occurs when blood vessels associated with the abluminal surface become angiogenic and grow through the interstices of the polymer (Clowes et al., 1986b; Golden et al., 1990; Kohler et al., 1992). These blood vessels open onto the luminal surface and spread to form a completely endothelial lined surface. Many reports in the literature have suggested that polymer porosity plays a role in the promotion of transmural migration (Golden et al., 1990; Hirabayashi et al., 1992; Kohler et al., 1992; Nagae et al., 1995; Brauker et al., 1995). Furthermore, it has been suggested by several investigators that the inflammatory response associated with the abluminal surface may play a fundamental role in the transmural migration of endothelial cells (Greisler et al., 1991; Zacharias et al., 1988), however, very little effort has been spent on abluminal healing characteristics and delineating the relationship between the macrophage’s influence on neovascularization via transmural migration of polymeric materials.

Methods to Improve Healing Associated with Biomedical Polymers

Due to the lack of biocompatibility of synthetic biomedical polymers the search for methods of improving the healing characteristics associated with polymeric implants continues. The most commonly used approaches to improve healing include surface modification, impregnation of polymers with bioactive components, tissue engineering and gene therapy.

One of the most commonly used surface modifications is the placement of the RGD amino acid sequence on the polymer (Glass et al., 1994; Lin et al., 1992). RGD has been identified as an amino acid sequence involved in active binding of cells to extracellular matrix
proteins (e.g. fibronectin and fibrinogen). The hypothesis is that cells will recognize this sequence, adhere and spread to incorporate the material in a more normal healing fashion. Other peptide sequences and proteins have also been utilized in an attempt to improve the healing associated with biomedical implants (Nicol et al., 1992).

Another commonly used method in an attempt to improve the healing associated with biomedical polymers is impregnation of the polymer with bioactive components (Langer and Moses, 1991; Bonzon et al., 1995; Greisler et al., 1987; Fournier and Doillon, 1996). For example, basic fibroblast growth factor (bFGF) has been impregnated into ePTFE grafts using collagen and an immobilization agent (Greisler et al., 1992). The data from these results demonstrate that using growth factors in conjunction with biomedical implants influences the healing response observed.

Tissue engineering encompasses the broadest spectrum of methods used to improve the biocompatibility of medical plastics. Tissue engineering involves the use of both cells and polymer to produce and superior device. For example, a polymeric vascular graft can be sodded with endothelial cells in an attempt to provide an anti-thrombogenic surface that inhibits blood coagulation (Jarrell et al., 1986). Other examples include scaffolds seeded with chondrocytes to grow cartilage (Puelacher et al., 1994; Grande et al., 1997) or neurocytes to grow nerves (Woerly et al., 1996).

Gene therapy is emerging as a new method for improving the biocompatibility of medical devices (Gilbert et al., 1993). This is a modification of tissue engineering in that the cells placed on the biomaterial are genetically modified, that is, have genes for certain bioactive molecules artificially incorporated into their nucleus. These genes allow the cell to
produce a specific product that will improve the healing response. For example, our laboratory has demonstrated that endothelial cells transfected with the gene that codes for interferon gamma (IFN-γ) produce and secrete the protein (Stopeck et al., 1997). When transfected endothelial cells are co-cultured with vascular smooth muscle cells a decrease in proliferation rates of the smooth muscle cells is observed (Stopeck et al., 1997). By sodding synthetic vascular grafts with transfected endothelial cells it is believed that the extent of neointimal thickening could be decreased by inhibiting cellular proliferation.

Regardless of the method selected in an attempt to improve the healing response an inflammatory reaction is still associated with the biomedical implant. Again the macrophage is believed to play the most significant role in the healing associated with biomedical implants and therefore discussion of this cells involvement in the healing process is pertinent.

Macrophage and Wound Healing

During inflammatory conditions, macrophages involved with wound healing are derived from circulating monocytes recruited into the tissue by chemoattractants (Leibovich and Ross, 1975b). Monocytes originate from monoblasts, a committed progenitor cell, further differentiate to become promonocytes, large cells (up to 18 μm in diameter) which divide twice during their development into monocytes (van Furth and Diesselhoff-den, 1970; Goud et al., 1975; Stein and Keshav, 1992). Mature monocytes have a half-life between 12 and 100 hours, comprise 3-7% of the leukocytes in blood and are the largest of the circulating leukocytes (13-18 μm) (van Furth and Diesselhoff-den, 1970; Goud et al., 1975; Stein and
Under steady-state conditions, monocytes continually migrate through the tissue for surveillance purposes (Riches, 1988; Daems, 1980). Inflammatory stimuli induce monocytes to differentiate into macrophages and promote wound healing. This was demonstrated by Leivovitch and Ross in 1975 by systemically administrating hydrocortisone, an inducer of monocytopenia, to guinea pigs and then inducing wounds (Leibovich and Ross, 1975b). This resulted in a 66% reduction in the number of macrophages within the wound. This suggests that the majority of macrophages associated with wounds are derived from circulating blood monocytes. Macrophages within the wound secrete enzymes and cytokines that play important roles in wound repair (Campbell et al., 1987). Many enzymes have been identified since the 1970s and include elastase, collagenase and plasminogen activator. Their role in extracellular matrix debridement and remodeling have been studied extensively. Also, cytokines released by macrophages play an important role in fibroplasia, matrix synthesis and angiogenesis (Montesano et al., 1984; Koch et al., 1986b).

**Macrophage-Biomaterial Interactions**

Many studies have been performed to determine which factors are released by macrophages in response to biomaterials. Most approaches have utilized in vitro techniques by culturing monocytes or macrophages with different biomaterials. Results from these experiments demonstrate that the factors released by macrophages in response to biomaterials differ depending on the type of material. Anderson et al. (1995) reported that supernatants obtained from macrophages cultured on polydimethylsiloxane (PDMS), polyethylene (PE) or polystyrene (PS) contained differing concentrations of interleukin 1 beta (IL-1β), IL-6 and
tumor necrosis factor alpha (TNF-α). Similarly, macrophages cultured on other biomaterials (e.g. PET and ePTFE) also exhibit differing concentrations of IL-1 and TNF-α in the supernatant (Miller and Anderson, 1988; Cardona et al., 1992; Bonfield and Anderson, 1993; Miller and Anderson, 1989; DeFife et al., 1995). Other factors which can alter the macrophage's release of cytokines are proteins adsorbed to the surface of the material (Bonfield et al., 1989; Bonfield et al., 1991; Bonfield et al., 1992a; Yun et al., 1995). Bonfield et al. (1992b) reported that when immunoglobulin G (IgG), fibrinogen or fibronectin were preadsorbed onto the biomaterials surface, monocyte culture supernatants contained differing concentrations of IL-1β, IL-6 and TNF-α.

Fewer studies have examined which cytokines are released by inflammatory cells in response to biomaterials in vivo. Eriksson and Thomsen (1991) implanted titanium and PTFE chambers in the abdominal wall of adult male rats and evaluated the number of leukocytes within the chambers from day 1 through 9 along with the concentrations of leukotriene B4 (LTB4) and IL-1 within the supernatant formed. Their results support the dogma that biomaterials stimulate a chronic inflammatory response characterized by the continual accumulation of leukocytes within the chambers. Their results also demonstrate that LTB4 and IL-1 secretion by inflammatory cells is altered by the type of material implanted. Lundberg et al. (1995) using a similar model, also demonstrated that LTB4 release by inflammatory cells can be modulated by the type of biomaterial implanted. These data, along with another study (Hunt et al., 1984) suggest a correlation between the healing response following biomaterial implantation and the types and concentrations of cytokines produced.
by inflammatory cells. Results from these experiments demonstrate that the type of material influences cytokine production by inflammatory cells that may then lead to an altered healing response. The role these cytokines play in modulating angiogenesis associated with synthetic vascular graft material remain unknown.

Angiogenesis

Angiogenesis is the formation of new vessels from existing blood vessels (figure 1.3). Microvessel endothelial cells are the major cell type involved with this process and exhibit different characteristics from large vessel endothelial cells (Madri et al., 1996). For example, large vessel endothelial cells respond to injury by sheet migration over regions of damaged vessel (Madri et al., 1996). In contrast, injury or stimuli to the microvasculature initiates an angiogenic response characterized by local disruption of the basement membrane, migration of endothelial cells, proliferation of endothelial cells, tube formation and stabilization which leads to the formation of a new blood vessel (Madri et al., 1996). These behaviors have lead to the hypothesis that endothelial cells can be phenotypically altered in response to injury.

The initial step in angiogenesis is activation of endothelial cells by enzymes, cytokines (Magnuson et al., 1989; Pober, 1988), and extracellular matrix molecules (Form et al., 1986a). Depending on several factors, for example, cell-cell interactions and local extracellular matrix composition, the response elicited by activation can vary. For example, (bFGF) added to endothelial cell cultures stimulates proteolysis resulting in the formation of a vasculature with patent lumina. However, bFGF and TGF-β1 when used together stimulate the formation of solid cords (Madri et al., 1996). Furthermore, activation of endothelial cells
FIGURE 1.3 Diagram illustrating angiogenesis depicting the four main cellular events required for new blood vessel growth. Stage I: The basement membrane is destroyed and endothelial cells form a neovessel sprout. Stage II: The sprout extends by migration and proliferation of the endothelial cells. Stage III: The new vessel matures and reforms the basement membrane. (Adapted from Hoying, 1994).
stage I

stage II

stage III
increases the number of adhesion receptors expressed on their luminal surface which enhances inflammatory transmigration (Beekhuizen and van Furth, 1993; Meerschaert and Furie, 1995; Takahashi et al., 1994). Activation must also lead to the release of endothelial cells from the basement membrane for migration to occur. This is accomplished through the production of proteases (e.g. collagenase, plasminogen activator and elastase) by endothelial cells which enzymatically break down the basement membrane (Gross et al., 1983; Bacharach et al., 1992; Pepper and Montesano, 1990; Kalebic et al., 1983). Once the endothelial cells have been released from the basement membrane, integrins on their surfaces are able to interact with extracellular matrix components facilitating endothelial cell migration (Brooks et al., 1994).

Migration of endothelial cells into the injured tissue is necessary for revascularization of the wound to occur. The endothelial cells at the leading tip of the angiogenic sprout migrate through the matrix in order to increase the vascularity of the tissue. Migration is modulated by cytokines (Groenewegen et al., 1985; Saksela et al., 1987), extracellular matrix proteins (Form et al., 1986a), proteases and protease inhibitors (Pepper et al., 1992; Pepper et al., 1993), and endothelial cell-endothelial cell interactions (Albelda et al., 1991; Takeichi, 1991). Cytokines have been described as being either stimulators or inhibitors of endothelial cell migration. For example, bFGF has been shown to stimulate migration of endothelial cells (Tsuboi et al., 1990; Abraham and Klagsbrun, 1996), whereas TGF-β1 can inhibit migration (Roberts and Sporn, 1996). However, recent experiments have demonstrated that the cytokines influence on migration can also be modulated by the type of angiogenic model. Even though a cytokine may inhibit migration when utilizing two-dimensional cell culture, the
same cytokine may stimulate migration when three-dimensional cell culture is used. Thus, the extracellular matrix must play a critical role in modulating migration of endothelial cells. It has been demonstrated that while fibronectin stimulates migration of endothelial cells, type IV collagen inhibits migration of endothelial cells (Madri et al., 1996). The endothelial cells interact with the extracellular matrix utilizing integrins which recognize specific amino acid regions of the proteins. The binding of integrins to matrix may serve to initiate intracellular mechanisms which regulate migratory functions of cells through both “outside-in” and “inside-out” signaling pathways (Shaw et al., 1993; Lub et al., 1995). Another important determinant in endothelial cell migration is the cell-cell interactions mediated by cadherins (Takeichi, 1991) and other transmembrane proteins. For example, platelet-endothelial cell adhesion molecule (PECAM-1), a membrane glycoprotein, is found between borders of endothelial cells (Albelda et al., 1991). Migrating endothelial cells lose their localization of PECAM-1 and therefore is thought to play an important role in regulating migration (Madri et al., 1996). Additionally, some inhibitors of endothelial cell migration stabilize the localization of PECAM-1 (Madri et al., 1996). Therefore, cytokines, extracellular matrix molecules and adhesion molecules all appear to play an important role in the migration of endothelial cells.

In order for new blood vessels to be formed, endothelial cells must proliferate to produce the extra cells needed to sustain the new vessels. Proliferation of endothelial cells is believed to occur only at the leading tips of angiogenic vessels just behind the migratory cells (Madri et al., 1996). Like migration, proliferation of endothelial cells is regulated by cytokines (Madri et al., 1996), extracellular matrix molecules (Form et al., 1986b), and cell-
cell interactions (Madri et al., 1996). However, the exact effects of each of these factors is controversial due to conflicting data within the literature. Again, cytokines may have multiple effects depending on the model system used. For example, proliferation of endothelial cells cultured in two-dimensional assays is inhibited by TGF-β1, while in three-dimensional cultures no effect on proliferation is observed, but TGF-β1 stimulates the formation of tube-like structures (Merwin et al., 1990). Extracellular matrix proteins serve as extracellular signals which modulate the endothelial cell's response. When endothelial cells are cultured (two-dimensional) on type IV collagen, a major basement membrane protein, proliferation rates are much less than cells cultured on fibronectin (unpublished results). However, few, if any, experiments have utilized three-dimensional culture models to determine the role of specific extracellular matrix proteins on endothelial cell proliferation. Therefore, little data have been obtained and further studies need to be performed to determine the role extracellular matrix molecules may play on the proliferation of endothelial cells.

The formation of tubes during angiogenesis is a complex process involving interactions with cytokines (Madri et al., 1988), cell-matrix interactions (Form et al., 1986b; Iruela-Arispe et al., 1991), proteolytic balance (Pepper and Montesano, 1990), and cell-cell interactions. In order for new capillaries to form, endothelial cells must recognize other endothelial cells and form stable junctions. These junctions are maintained by specific adhesion molecules, for example PECAM-1 and cadherin 5. The extracellular matrix also promotes the formation of tubes from the sprouting endothelial cells (Iruela-Arispe et al., 1991). Once the new vessel is formed, it must produce a basement membrane in order to
stabilize the vessel. The mechanisms by which this is brought about are not completely understood, however cytokines and the extracellular matrix have been suggested to play an important role.

Angiogenesis, which leads to revascularization of wounded tissue, has many influential regulators including cytokines. These cytokines affect the formation of new blood vessels by stimulating cells to produce extracellular matrix, enzymes and cytokines. Biomaterials induce an altered wound healing response characterized by the chronic association of inflammatory macrophages. Macrophages have been shown to produce a plethora of cytokines with the potential to dramatically effect the neovascularization of biomaterials by affecting angiogenic mechanisms. Therefore, the role macrophages play in the stimulation or inhibition of angiogenesis is critical when addressing neovascularization of biomaterial implants.

Inflammation and Angiogenesis

Inflammatory cells, particularly macrophages since depletion of PMNs has little effect on healing (Leibovich and Ross, 1975a; Simpson and Ross, 1972b; Stein and Levenson, 1966), appear to be an important component in angiogenesis during wound healing (Sunderkotter et al., 1994; Pulido et al., 1991; Hakkert et al., 1991). The macrophage's role in angiogenesis has been demonstrated by utilizing in vivo and in vitro techniques. Hunt et al. (1984) examined the role of wound macrophages on angiogenesis and collagen synthesis. Their results suggest that macrophages produce the cytokines which are the major signals for angiogenesis and collagen synthesis within the wound. They also suggest that the wound is not by itself the maximal stimulus for repair since macrophages can have an increased
stimulation when other factors (e.g. endotoxin, bacterial products, and other stimulants) are added to the wound. These results suggest that macrophages can be activated to differing levels by various synthetic polymers that in turn may lead to altered healing responses. Polverini et al. (1977) along with others utilizing similar techniques, also concluded that wound macrophages provide signals which can stimulate angiogenesis (Koch et al., 1986a; Greenburg and Hunt, 1978; Mostafa et al., 1980a; Mostafa et al., 1980b). For example, supernatant collected from cultured monocytes stimulate the formation of blood vessels within the cornea, normally an avascular tissue (Polverini et al., 1977). Cytokines have been implicated as the signals which stimulate endothelial cells to perform the functions (e.g. proliferation and migration) involved in angiogenesis.

Cytokines that stimulate angiogenesis can be categorized by their effect on migration, proliferation, and tube formation (Sunderkotter et al., 1994; Madri et al., 1988). Table 1.3 (compiled from Sunderkotter et al., 1994; Sunderkotter et al., 1991; Klagsbrun and D’Amore, 1991; Koch et al., 1992; Friesel et al., 1987) summarizes which cytokines released by macrophages have been associated with angiogenesis. Interestingly, some of these factors can either stimulate or inhibit angiogenesis depending on various conditions. Factors which exhibit this characteristic include TNF-α, IL-1, IL-6 and TGF-β (Sunderkotter et al., 1991). This evidence indicates the importance of cytokines released in response to differing wound conditions, for example biomaterial implantation, and how these factors affect healing.

Cytokines are biologically active polypeptides produced by a variety of cells that act on cells and regulate specific functions, such as cell proliferation and differentiation, protein synthesis and the production of other cell products. Cytokines have been suggested to play
Table 1.3. Angioactive factors released by macrophages.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Stimulates Migration</th>
<th>Inhibits Migration or Proliferation</th>
<th>Mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IL-8</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Angiotropin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PDGF</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IGF-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TGF-α</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Interferon-α</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TSP 1</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IL-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-6</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td>+</td>
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<tr>
<td>TGF-β</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>MECIF</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MD-ECI</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

An important role in wound healing, coordinating cellular functions and attracting increasing numbers of inflammatory cells (Movat et al., 1987). The major source of cytokines within "normal" wounded tissue appears to be the macrophage. Only a limited number of cytokines released by biomaterial activated macrophages have been identified: IL-1, IL-6 and TNF-α. These cytokines have been demonstrated to have profound effects on endothelial cell migration, proliferation and function (Stolpen et al., 1986).

IL-1 has been associated with both stimulation and inhibition of angiogenesis, probably due to differences in experimental design and concentration-dependent effects. Stimulation of angiogenesis has been demonstrated within the rabbit cornea and mammalian
brains (Giulian et al., 1988). However, inhibition of angiogenesis in vivo by IL-1 has also been demonstrated and is believed to be mediated through down regulation of FGF binding sites (Cozzoli et al., 1990; Cozzolino et al., 1990), and other growth factors (Norioka et al., 1987). These variable effects may be due to interactions with other angiogenic mediators and/or dose dependence and IL-1's importance in wound healing remains to be clarified.

IL-6 has been shown to both inhibit endothelial cell proliferation and stimulate migration. In vitro experiments have concluded that IL-6 inhibits endothelial cell growth (May et al., 1989) or has no effect (Podor et al., 1989). However, during embryonic development IL-6 appears to play a role in blood vessel formation (Motro et al., 1990). Due to conflicting results on inhibitory and stimulatory effects, the role of IL-6 in wound healing remains unanswered.

TNF-α has a wide range of biological activities and has been associated with several phases of angiogenesis. For example, TNF-α has been shown to up-regulate the expression of cellular adhesion molecules, particularly intercellular adhesion molecule 1 (ICAM-1), on endothelial cells (Le and Vilcek, 1987) and many contradictory results have been obtained on the effects TNF-α has on angiogenesis since experiments have shown both inhibitory and stimulatory effects. In vitro, TNF-α antagonizes the mitogenic effects of bFGF thereby inhibiting angiogenesis (Sunderkotter et al., 1994). TNF-α has also been shown to stimulate the migration of endothelial cells and the formation of capillary tube-like structures (Leibovich et al., 1987). TNF-α may stimulate angiogenesis by enhancing the production of proteases and/or cytokines by endothelial cells. Effects from TNF-α appear to be biphasic, with small
doses stimulating angiogenesis (Piguet et al., 1990; Leibovich et al., 1987) and large doses inhibiting angiogenesis (Sato et al., 1968). Conflicting results may be due to dose and/or time of exposure. For example, small, sustained doses of TNF-α have been shown to stimulate angiogenesis (Piguet et al., 1990) while single, high doses appear to be cytotoxic to endothelial cells by inducing apoptosis (Robaye et al., 1991; Stolpen et al., 1986; Piguet et al., 1990). Furthermore, in vitro and in vivo results differ. While TNF-α has been shown to inhibit endothelial cell proliferation in vitro, this cytokine stimulates angiogenesis in vivo (Frater-Schroder et al., 1987). The role of TNF-α in angiogenesis associated with biomaterials remains to be defined.

Significance

Biomaterial implantation leads to a foreign body response characterized by chronic inflammation and fibrous encapsulation. The major inflammatory cell associated with the chronic inflammatory response following biomaterial implantation is the macrophage. Macrophages can release a plethora of cytokines that have multiple effects on the wound healing process. These cytokines have also been demonstrated to have a profound effect on angiogenesis. Biomaterials activate macrophages and stimulate them to produce and secrete particular cytokines. The relationship between the cytokines released by macrophages in response to biomaterials and angiogenesis remains unknown. The goal of this dissertation was to delineate a relationship between macrophages and angiogenesis associated with biomedical implants. The overriding hypothesis for this dissertation was that macrophages
influence the angiogenic response associated with the abluminal surface of polymeric biomedical implants. The following two questions were the focus of the studies: Which soluble angiostatic factors are released from macrophages in response to biomedical polymers? How does modulation of these factors affect neovascularization of implanted materials? Four specific aims have been established to address this goal:

**Specific Aim #1.** Evaluate the healing characteristics of synthetic polymer implants as compared to control wound healing tissue. *Hypothesis 1: Synthetic polymers alter the normal healing response elicited by the host.* A subcutaneous tissue, adipose tissue and endovascular implant model was used to quantitate the differences between normal wound healing and wound healing following synthetic polymer implantation using histochemical and immunocytochemical techniques.

**Specific Aim #2.** Evaluate the differential activation of macrophages by various base polymers and determine the relationship between macrophages and neovascularization of these materials. *Hypothesis 2: Synthetic polymers elicit different macrophage responses depending on the chemical and physical structure of the polymer and these responses influence the neovascularization of the material.* A subcutaneous tissue, adipose tissue and endovascular implant model was used to quantitate the activated macrophage response and neovascularization of synthetic polymers using histochemical and immunocytochemical techniques.
Specific Aim #3. Identify specific angioactive cytokines expressed by activated macrophages in response to synthetic polymer implantation. **Hypothesis 3:** Angioactive cytokines are expressed by macrophages in response to synthetic polymers. Identification of cytokines expressed from synthetic polymer activated macrophages in vivo was performed utilizing RNase protection assays and in situ hybridization was used to localize and identify cells producing messenger RNA of inflammatory cytokines.

Specific Aim #4. Evaluate the effect(s) angioactive factors identified in specific aim #3 have on angiogenesis. **Hypothesis 4:** Modulation of angioactive factors will affect angiogenesis. Angiogenesis will be evaluated following addition of products released by macrophages in response to synthetic polymers in two-dimensional proliferation assays.
2. HEALING RESPONSE ASSOCIATED WITH POLYMERIC VASCULAR GRAFT MATERIAL

Introduction

Vascular complications requiring replacement of existing vessels has promoted the search for an acceptable biocompatible vascular prosthesis. Initially, autografts, most often the saphenous vein, were used, but often individuals requiring replacement vessels had profound cardiovascular disease and no acceptable vein could be located. Additionally, multiple surgical procedures on the same individual may lead to the depletion of all available autologous vessels. This has, in part, lead to the search for a biocompatible synthetic vascular graft that performs acceptably as a blood conduit. However, biocompatibility of synthetic materials remains to be defined. Initially, inertness of implants leading to no intolerable clinical responses was considered the ultimate in biocompatibility. More recently, however, biocompatibility has been defined as the ability of a material to stimulate a specific host response leading to desirable functions and playing an active role in the healing response (Didisheim, 1993).

The luminal and abluminal healing characteristics associated with synthetic vascular grafts differ. Luminal healing is characterized by the initial deposition of fibrin and platelets on the polymeric surface (Rubin et al., 1993). This pseudointima functions as a provisional matrix for inflammatory cells, primarily neutrophils, to adhere and infiltrate. At the anastomosis, endothelial cells and smooth muscles begin to migrate from the native vessel onto the polymeric surface (pannus ingrowth). Pannus ingrowth only occurs in the first 2-4
cm at both the proximal and distal anastomosis. Initially, the endothelium and underlying smooth muscle cells from the native vessel spread onto the polymeric surface (Clowes et al., 1985; Clowes et al., 1986a). These cells proliferate and produce extracellular matrix proteins that lead to the thickening observed at both the proximal and distal anastomosis of polymeric vascular implants.

Abluminal healing characteristics differ dramatically from luminal healing. Most noticeably, activated macrophages and foreign body giant cells are observed at the tissue-graft interface. These cells have the potential to release molecules that may influence the healing associated with the luminal and the abluminal surfaces, particularly transmural migration of endothelial cells onto the luminal surface leading to an endothelial cell monolayer. It is believed that the formation of an endothelial cell monolayer on the polymeric surface would inhibit the inherent thrombogenicity of the polymer by creating an anti-thrombogenic lining and furthermore inhibit the intimal thickening by regulating the proliferation and production of extracellular matrix proteins by the underlying cells.

Endothelialization of porous synthetic vascular grafts via transmural migration of blood vessels has been demonstrated in non-human animal models (Clowes et al., 1985; Clowes et al., 1986b). The process of transmural migration of endothelial cells is composed of several independent steps leading to the formation of an endothelial cell monolayer on the polymer’s luminal surface. The foreign body response associated with polymeric implants has been suggested as playing a fundamental role in the overall healing of biomedical implants, including the angiogenic mechanisms responsible for initiating transmural migration of endothelial cells (Clowes et al., 1986b; Lansdown et al., 1995; Schreuders et al., 1988;
Zacharias et al., 1987; Ziats et al., 1988). Delineating the interactions between the inflammatory response and angiogenesis may unlock the key to optimization of conditions resulting in spontaneous endothelialization of polymers.

The following studies were performed to evaluate the relationship between chemical composition, porosity, inflammation and neovascularization of polymers used in the manufacture of artificial blood vessels. The hypothesis was synthetic polymers alter the normal healing response by the host. Two corollary hypothesis were 1) synthetic polymers elicit different macrophage responses depending upon the chemical and physical characteristics of the polymer and 2) inflammation and neovascularization are inversely related.
Materials and Methods

Animal Selection and Welfare

All animal studies were performed following protocols approved by the University of Arizona Health Sciences Center animal care and use committee and according to the National Research Council “Guide for the Care and Use of Laboratory Animals” (1996). All surgeries were performed and animals housed in American Association for Accreditation of Laboratory Animal Care approved facilities.

Biomedical Polymers

Expanded polytetrafluoroethylene (ePTFE), 30 μm, 60 μm, and 100 μm internodal distance, from Impra, Inc. (Tempe, AZ), Gore-Tex ePTFE (20 μm internodal distance) from W.L. Gore & Associates (Flagstaff, AZ), Meadox weave nit and woven Dacron vascular graft material from Meadox Medicals Inc. (Oakland, NJ), Hemashield microvel and woven Dacron vascular grafts from Meadox Medicals Inc., and Golaski microknit Dacron vascular graft material from Golaski Labs, Inc. (Philadelphia, PA) were used in this study. Samples 6 mm in diameter were prepared using a circular punch. All polymers, except the Hemashield Dacron, were packaged and steam sterilized. Hemashield Dacron grafts were gas sterilized.

Implant Procedures

Fifteen male rats (260-286 g) were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. The abdominal and flank areas were prepared for surgery.
The abdomen was opened and the right and left epididymal fat pads located. A small hole was made in the serosal layer of the fat pads and the appropriate sample, determined randomly, was implanted by suturing fat around the graft. The linea alba was closed with an absorbable suture and the overlying skin was closed with staples. The rat was then placed on his abdomen and small incisions were made over the right and left haunch. The appropriate sample was placed abluminal side toward the skin, determined by the curvature of the sample and the incision closed with a staple. The rats were allowed to recover and returned to the animal facility.

Previous studies have established that the healing of biomedical implants is highly dependent on implant site (Williams et al., 1997; Sterpetti et al., 1992). Thus, the study of implant healing in models which mimic different implant sites is especially important for polymeric devices that may come into contact with multiple tissues. The majority of synthetic vascular grafts are tunneled subcutaneously between the proximal and distal anastomosis. Human subcutaneous tissue is composed predominantly of adipocytes. In order to best mimic this environment an implant site similar to human subcutaneous tissue was selected. Histologically, the rat epididymal fat pad is similar to human subcutaneous tissue and therefore was used as one of the implant sites. The other site chosen was rat subcutaneous tissue which has historically been used to assess the healing associated with biomedical polymers.
Explant Procedures

The grafts were removed five weeks after implantation. After the animals were anesthetized with sodium pentobarbital injected IP, the abdomen was opened and the right and left fat pads located. The implants were isolated and removed with the surrounding fat. Each implant was placed in a glass vial containing Histochoice™ (AMRESCO, Solon, OH). The rat was then placed on his abdomen and the subcutaneous implants were removed with surrounding tissue and similarly fixed. All grafts were processed through graded alcohols, followed by xylene, and finally paraffin embedded. Samples were sectioned (6-8 µm thick), and dried onto poly-L-lysine coated slides.

Histology

Sections were deparaffinized and stained with hematoxylin and eosin to examine gross morphology. Samples were examined on a Nikon Optiphot microscope with a 40x water-immersion objective lens and 10x eyepiece lens.

Immunohistochemistry

Sections were deparaffinized and reacted with Griffonia simplicifolia (GS1) lectin (Harlan Bioproducts for Science, Inc., Indianapolis, IN) which shows specificity for endothelial cells, monocytes, and macrophages (Hansen-Smith et al., 1992; Hansen-Smith et al., 1995), or primary antibody ED1 (Harlan Bioproducts for Science, Inc., Indianapolis, IN) which shows specificity for activated monocytes and macrophages (Dijkstra et al., 1985) and visualized using peroxidase-conjugated secondary antibody. Nuclei were lightly stained using
methyl green. Sections were then evaluated using a Nikon Optiphot microscope with a 40x water immersion lens.

Quantification

Sections were analyzed with a 40x water immersion objective lens and a 10x eyepiece containing a 6x6 grid. Each area within the grid corresponded to a 27 μm x 27 μm area (729 μm²). The tissue located next to the polymer interface was examined for inflammation and neovascularization. For both GS1 and ED1 forty grid areas were analyzed. If within the 27 μm x 27 μm area there occurred one or more positively stained elements, the area was designated "1". If no positively stained elements occurred then the area was designated "0". For each cytchemical stain the total number of positive areas was divided by the total number of areas analyzed. This calculation determined the index and was derived from the following equation:

\[
\text{index} = \frac{\text{number of areas positively stained}}{\text{total number of areas counted}}
\]

After the GS1 index (derived from the analysis of GS1 stained sections) and the ED1 index (derived from the analysis of ED1 stained sections) were calculated, a neovascularization index was determined using the following equation:

\[
\text{neovascularization index} = \frac{(\text{GS1 positive areas}) - (\text{ED1 positive areas})}{\text{total number of areas counted}}
\]
To determine the inflammation index, sections reacted with ED1 antibody were examined on a Nikon Optiphot microscope using a 40x water-immersion objective lens. Inflammation index was determined by capturing images using a CCD camera mounted on the microscope and connected to a Gateway computer containing Metamorph™ Image Analysis software. Five regions at the tissue-graft interface, 27 μm x 27 μm wide, were captured. For each image an inflammation index was calculated by inverting the captured image (allowing positively stained cells to be thresholded), thresholding the image and measuring the percentage of thresholded area. This value was then expressed as a fraction and is referred to as the inflammation index.

Scanning Electron Microscopy

Polymer samples were mounted on aluminum stubs using silver glue-stick tape, sputter coated using a gold target and examined with a JEOL 820 scanning electron microscope (25 kV accelerating voltage). Both sides of all materials were examined under scanning electron microscopy.

Statistical analysis

ANOVA values were calculated using SPSS for Windows (SPSS Inc.) and were used to compare different treatment groups implanted within the same tissue. Values with $p \leq 0.05$ were considered significantly different.
Results

Scanning Electron Microscopy

Scanning electron micrographs of each material are shown in figure 2.1. The Impra ePTFE polymers having differing porosities were examined to determine significant differences in internodal distance, interfiber distance, and fiber width (Table 2.1). Figure 2.2 defines the morphological determinants of internodal distance, interfiber distance, and fiber width. Interestingly, the interfiber distance was the greatest for 60 μm ePTFE, followed by 30 μm and finally 100 μm ePTFE. The fiber width for 30 μm ePTFE was significantly less than the other porosity ePTFE. Furthermore, the morphological appearance of the fibers spanning the internodal distance differed between the three materials (figure 2.3).

Table 2.1. Structural characteristics of 30 μm, 60 μm and 100 μm ePTFE.

<table>
<thead>
<tr>
<th>Manufacturer's Stated Internodal Distance (μm)</th>
<th>Polymer Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Internodal Distance 1</td>
</tr>
<tr>
<td></td>
<td>(μm)</td>
</tr>
<tr>
<td>30</td>
<td>26.5 ± 5.2 b^2</td>
</tr>
<tr>
<td>60</td>
<td>48.1 ± 10.1 b</td>
</tr>
<tr>
<td>100</td>
<td>67.5 ± 18.2</td>
</tr>
</tbody>
</table>

^1 defined by line a, figure 2.2.
^2 defined by line b, figure 2.2.
^3 defined by line c, figure 2.2.

^ significantly different compared to 60 μm ePTFE (p<0.05).
^‡ significantly different compared to 100 μm ePTFE (p<0.05).
FIGURE 2.1 Scanning electron micrographs of a) Impra 30 µm ePTFE (luminal and abluminal side identical), b) 60 µm ePTFE (luminal and abluminal side identical) and c) 100 µm ePTFE (luminal and abluminal side identical), d) Gore-Tex ePTFE luminal side, e) Gore-Tex ePTFE abluminal side, f) Hemashield microvel Dacron luminal side, g) Hemashield microvel Dacron abluminal side, h) Hemashield woven Dacron (luminal and abluminal side identical), i) Meadox weavenit Dacron (luminal and abluminal side identical), j) Meadox woven Dacron (luminal and abluminal side identical), and k) Golaski microknit Dacron (luminal and abluminal side identical). Bar = 10 µm.
FIGURE 2.2 Scanning electron photomicrographs of 60 µm ePTFE illustrating measurement parameters of a) internodal distance, b) interfiber distance and c) fiber width (insert). Bar = 14 µm for a,b; Bar = 0.6 µm for c.
FIGURE 2.3  Scanning electron photomicrographs of a) 30 μm ePTFE internodal fibers, b) 60 μm ePTFE internodal fibers and c) 100 μm ePTFE internodal fibers. Bar = 0.6 μm.
Histology

Subcutaneously implanted Impra 30 \( \mu m \) and Gore-Tex ePTFE samples exhibited the formation of a predominantly acellular fibrous capsule (figure 2.4), or a hypercellular capsule (figure 2.4). Beyond the fibrous capsule, microvessel profiles within a loose connective tissue were observed. Few cells had migrated and little connective tissue was observed within the interstices of the graft material. Impra 30 \( \mu m \) ePTFE (figure 2.5) and Gore-Tex ePTFE (figure 2.5) implanted within adipose tissue had numerous cells located at the tissue-graft interface. While no fibrous capsule was observed associated with Impra ePTFE, an acellular fibrous capsule was observed associated with Gore-Tex ePTFE. Microvessel profiles were occasionally observed at the tissue-graft interface of implants not encapsulated, however, no microvessel profiles were observed within the material.

Changing the porosity of ePTFE had an additional effect on the healing associated with the polymer. Samples from subcutaneous tissue were surrounded by a dense fibrous capsule surrounding the ePTFE implants. The 30 \( \mu m \) ePTFE implants had the thickest dense fibrous capsule, while both the 60 \( \mu m \) and 100 \( \mu m \) ePTFE had much thinner, less dense fibrous capsules. Few cells had migrated into the graft material of 30 \( \mu m \) or 60 \( \mu m \) ePTFE while 100 \( \mu m \) ePTFE contained numerous cells within the interstices. Profiles of capillaries were rarely observed in the fibrous capsule or within the graft interstices. Samples from epididymal fat pads were surrounded by a thin fibrous capsule. Similar to the subcutaneous implants, few cells had migrated within the graft interstices of 30 \( \mu m \) or 60 \( \mu m \) ePTFE while
FIGURE 2.4  Light micrograph of hematoxylin and eosin-stained sections of a) Hemashield microvel Dacron, b) Hemashield woven Dacron, c) Meadox weavenit Dacron, d) Meadox woven Dacron, and e) Golaski microknit Dacron, f) Gore-Tex ePTFE abluminal side, g) Impra 30 μm ePTFE, h) 60 μm ePTFE, i) 100 μm ePTFE discs following 5 weeks implantation in subcutaneous tissue. P = polymer, F = fibrous capsule, MV = microvessel. Bar = 23 μm.
FIGURE 2.5  Light micrograph of hematoxylin and eosin-stained sections of a) Hemashield microvel Dacron, b) Hemashield woven Dacron, c) Meadox weavenit Dacron, d) Meadox woven Dacron, and e) Golaski microknit Dacron, f) Gore-Tex ePTFE abluminal side, g) Impra 30 μm ePTFE, h) 60 μm ePTFE, i) 100 μm ePTFE discs following 5 weeks implantation in adipose tissue. P = polymer, F = fibrous capsule, MV = microvessel. Bar = 23 μm.
100 μm ePTFE contained numerous cells within the graft interstices. Capillary profiles were observed within close approximation to the implant and within the perigraft tissue (figure 2.6).

Subcutaneously implanted Meadox weavenit (figure 2.4) and woven (figure 2.4) Dacron, and Hemashield microvel (figure 2.4) and woven (figure 2.4) Dacron were all surrounded by a hypercellular capsule or limited fibrous encapsulation of varying thicknesses. Many multinucleated giant cells were observed at the tissue-graft interface and many cells were found within the graft material of all implants. Microvessel profiles were observed mainly outside of the hypercellular capsule, but infrequently could be found within the hypercellular capsule. The Golaski microknit Dacron implanted within subcutaneous tissue (figure 2.4) healed much differently compared to the other Dacron materials. First, fewer inflammatory cells surrounded the implant (figure 2.7) and second, microvessel profiles were observed in close association with the material (figure 2.7). All samples of the Dacron materials implanted within adipose tissue (figure 2.5) healed in a similar manner when compared to each other. A hypercellular capsule surrounded the implant and some evidence of fibrous encapsulation, even though more cellular in nature, existed. Many multinucleated giant cells were observed at the tissue-graft interface and many cells were found within the graft material. Again, microvessel profiles were most frequently observed outside the hypercellular capsule, however few were observed within this capsule and none were observed within the graft material.
FIGURE 2.6 Light micrographs of *Griffonia simplicifolia* (GS1) lectin stained sections of 30 μm ePTFE discs following 5 weeks implantation in adipose tissue. a) Capillary profiles in the ePTFE interstices and b) in the perigraft tissue can be seen (arrows). Bar = 23 μm.
FIGURE 2.7  Light micrographs of sections reacted with either ED1 antibody (a,b,e,f) or GS1 lectin (c,d,g,h) illustrating inflammation and neovascularization, respectively.  a) Meadox weavenit Dacron, largest inflammatory index, b) Golaski microknit Dacron, smallest inflammatory index, c) Golaski microknit Dacron, largest neovascularization index, and d) Meadox weavenit Dacron, smallest neovascularization index for 5 week subcutaneous tissue implants.  e) Meadox weavenit Dacron, largest inflammatory index, f) Golaski microknit Dacron, smallest inflammatory index, g) Golaski microknit Dacron, largest neovascularization index, and h) Meadox weavenit Dacron, smallest neovascularization index for 5 week adipose tissue implants.  Bar = 23 µm.
Inflammation and Neovascularization

To determine inflammation differences between material types, an inflammation index was calculated as described in the methods. Results from these calculations are reported in figure 2.8, 2.9, 2.10 and 2.11. Generally, Dacron material implants were more inflammatory than all other implants. One exception was the Golaski microkint Dacron which was more similar to the ePTFE implants when implanted within subcutaneous tissues. GS1 lectin was utilized to detect the presence of endothelial cells in the form of longitudinal and cross-sectional microvessel profiles as well as single endothelial cells. Samples implanted within adipose tissue exhibited many more microvessel profiles when compared to samples implanted within subcutaneous tissue. GS1 lectin reacts with both endothelial cells, activated monocytes, activated macrophages and foreign body giant cells. For this reason, ED1 antibody which reacts specifically with activated monocytes, activated macrophages and foreign body giant cells was used thereby allowing a neovascularization index (figure 2.8, figure 2.9) to be calculated for each sample as described in the methods. These data suggest an inverse relationship between the inflammatory and extent of neovascularization since only those polymers having a low inflammation intensity exhibited a significant neovascularization index.
FIGURE 2.8 (●) Inflammation and (○) neovascularization index for polymeric implants following 5 weeks of implantation within subcutaneous tissue. * depicts significant differences, p<0.05.
Internodal Distance

Index

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

30 µm 60 µm 100 µm

*
FIGURE 2.9  (●) Inflammation and (○) neovascularization index for polymeric implants following 5 weeks of implantation within subcutaneous tissue. * depicts significantly different from Impra, p<0.05.
FIGURE 2.10 (●) Inflammation and (○) neovascularization index for polymeric implants following 5 weeks of implantation within adipose tissue. * depicts significant differences, p<0.05.
FIGURE 2.11 (●) Inflammation and (○) neovascularization index for polymeric implants following 5 weeks of implantation within adipose tissue. * depicts significant differences from Impra, p<0.05.
Discussion

The biocompatibility of synthetic prostheses remains the single most important factor in determining the long-term function of polymeric vascular grafts. The development of a truly biocompatible vascular graft requires the identification of materials which exhibit an appropriate host response resulting in long term implant function. The most common host responses observed following vascular graft implantation are fibrotic encapsulation (Miller et al., 1989), chronic inflammation characterized by the presence of activated macrophages and foreign body giant cells (Jansen et al., 1994), and neointimal thickening (Rekhter et al., 1993; Clowes et al., 1985). These responses may lead to the complete failure of the biomedical device rendering the implant useless. A complete understanding of the healing responses following vascular graft implantation is therefore necessary in order to improve the development of artificial blood conduits. In this study, the relationship between inflammation and neovascularization associated with clinically used vascular polymeric materials and two experimental polymers (60 μm and 100 μm ePTFE) was explored.

The most common implant site used for polymer biocompatibility evaluation has been subcutaneous tissue in animal models. The choice of this location is due in part to the easy access of subcutaneous tissue and the ease of surgically placing the implant. However, with respect to vascular grafts, it remains unclear whether polymer healing in subcutaneous tissue of rats accurately predicts the healing response predicted when these polymers are implanted in humans. Accordingly, both subcutaneous and adipose tissue implant sites were evaluated. The choice of implanting in adipose tissue was driven by the fact that many vascular grafts in humans are implanted in subcutaneous adipose tissue using tunneling devices. By contrast,
previous studies have determined that the subcutaneous tissue of animals is essentially devoid of adipose tissue and is more represented by loose connective tissue. This loose connective tissue contains numerous fibroblasts and elastin fibers along with other extracellular matrix proteins. Conversely, human subcutaneous tissue contains predominantly adipocytes and endothelial cells in the form of microvessels. Rat adipose tissue is similar to human adipose tissue in that it is predominantly composed of adipocytes and microvessel endothelial cells. Therefore, when evaluating the biocompatibility of materials used in vascular grafts, implantation within rat epididymal fat pads may better mimic the healing response observed in subcutaneous vascular grafts in humans.

In previous studies, our laboratory examined the role anatomic site of implantation plays in the healing response observed following polymer implantation (Williams et al., 1997). These results clearly demonstrated that ePTFE placed within subcutaneous tissue of rats healed dramatically different when compared to ePTFE implanted within adipose tissue of rats. Specifically, subcutaneous implants were encapsulated and had few if any microvascular structures within the interstices of the graft while implants within adipose tissue were not encapsulated and microvascular profiles were occasionally observed within the graft material. In the current experiments, gross morphological examination of explanted materials demonstrated different healing responses when the same polymer was placed within different tissue sites. Site of implantation appeared to be most important in determining the extent of fibrous encapsulation. While the subcutaneously implanted polymers had a thicker and less cellular capsule, the adipose tissue implants had thinner and more cellular capsules. Fibrous encapsulation of vascular grafts may not lead to failure of the implant, however, this may
decrease the extent of neovascularization thereby inhibiting the formation of an endothelial cell lining on the vascular grafts luminal surface. These findings suggest the healing of biomedical materials must be examined within a tissue that structurally parallels the cellular environment where the device will reside.

For this study, an inflammation index was calculated to quantify differences observed between polymeric material types. Typically, the ePTFE materials were less inflammatory than the Dacron materials, with the exception of Golaski microknit Dacron which was more similar to ePTFE implants. The inflammatory responses for Dacron materials were similar to each other at both tissue sites, except for the Golaski microknit Dacron. Subcutaneously implanted Golaski microknit Dacron was less inflammatory when compared to all subcutaneous implants and adipose tissue implants. Possible explanations for the differences in inflammation observed between the various polymeric implants include, but are not limited to: the Vroman effect (Leonard and Vroman, 1991), surface structure of the material (Hunt et al., 1996; Kohler et al., 1992), and material impurities (Andrade, 1973). The Vroman effect states that proteins adsorb onto the surface of polymers depending on the proteins relative concentration in the medium. However, with time, proteins with the greatest affinity for the polymer of interest will replace other proteins found on the surface of the polymer. The inflammatory response may be dependent on which proteins are associated with the implant by influencing the extent and intensity of the inflammatory response (Bonfield et al., 1992a). Furthermore, proteins adsorbed to the surface of polymeric materials may have an altered tertiary configuration (possibly denatured) leading to an altered healing response. Surface structure may also play an important role in modulating the inflammatory response
to material implants, possibly through altering the Vroman effect or by directly affecting inflammatory cells via surface receptor-polymer interactions. Furthermore, impurities introduced during the manufacturing process may be influencing the inflammatory response (Andrade, 1973). For example, Hemashield Dacron™ grafts are impregnated with a bovine type I collagen crosslinked using fixatives. Both the bovine collagen and the fixatives are released by enzymatic actions following implantation which may lead to differential healing responses when compared to other non-modified polymeric materials.

The importance of the inflammatory response following placement of vascular grafts has yet to be determined. Even though a certain level of inflammation may protect the implant from bacterial infection (Strachan, 1995), intense inflammatory reactions may have adverse effects on vascular grafts. For example, activated macrophages are known to produce and release growth factors which can stimulate cellular proliferation and matrix deposition (Miller et al., 1989; Zenni et al., 1993). Cellular proliferation and matrix deposition on the luminal surface of vascular grafts may promote neointimal thickening leading to device failure (Clowes et al., 1985).

From the calculated neovascularization indexes, these data suggest that not only does the tissue surrounding the implant and base material influence neovascularization, but the inflammatory response has a direct effect on neovascularization. Kobayashi et al. (1994) and Polverini et al. (1977) observed that monocytes and macrophages affect endothelial cell migration. A correlation between the number of activated monocytes and macrophages associated with implants and neovascularization was observed. Those polymers exhibiting a high inflammatory response had a corresponding low neovascularization index. These data
suggest that monocytes and/or macrophages or a secretion product of monocytes and/or macrophages may inhibit transinterstitial migration of endothelial cells. In this experiment, only those materials having a low inflammation index neovascularized to any significant extent. This suggests that inflammatory cells may be producing angioactive factors which determine the extent of neovascularization. These angioactive molecules may be acting on many various aspects of the angiogenic response as well as other cells or molecules which influence angiogenesis (Sunderkotter et al., 1994). Vascular grafts may benefit by the promotion of angiogenesis around the implant. If new blood vessels could be stimulated to grow through the grafts interstices, the formation of an endothelial cell monolayer on the vascular grafts luminal surface may follow, leading to a more biocompatible implant (Clowes et al., 1986b; Kohler et al., 1992; Clowes et al., 1987).

Another factor which may be important in the host response is the physical characteristics of the material and how this affects healing. When studying ePTFE, the major characteristic which can be altered is the porosity of the material. Porosity of polymers can be defined by indirect and direct measures which include: 1) water flow through the material, 2) internodal distance, and 3) fiber density between the nodes. In previous work, ePTFE porosity has been defined as the internodal distance and expected cellular responses are correlated with this porosity measurement. Other investigators have postulated that the more porous graft, defined by internodal distance, will support greater ingrowth of blood vessels through the polymer. Often not considered are that differences in the internodal distance may alter the inflammatory response resulting in differential neovascularization. The data demonstrate that 60 μm ePTFE implanted within both adipose and subcutaneous tissue
exhibited a greater number of blood vessels associated with the polymer than 30 μm and 100 μm ePTFE. Moreover, 60 μm and 100 μm ePTFE exhibited comparatively thinner fibrous capsules. Intuitively, one would assume that the more porous the material would permit more tissue and vascular ingrowth. However, that is not the case with ePTFE. Previously, Golden et al. (1990) observed that 60 μm ePTFE intrapositional vascular grafts in baboons healed with a complete endothelial lining while both 30 μm and 90 μm ePTFE implants failed to completely endothelialize. Upon SEM examination of all three ePTFE porosity polymers a differences between the apparent densities of fibers spanning the internodal space along with differences in fiber structure was noticed. These data suggest that one limiting factor of cell migration into the polymer may be the density of fibers spanning the internodal space. The microporosity of the fibers may also be influencing the migration of cells into the polymer and therefore should be examined in more detail.

The relationship between inflammatory response and neovascularization of biomedical materials is beginning to be delineated. The data presented in this chapter support the initial hypotheses. These results indicate that synthetic polymers alter the normal healing response and intense inflammatory responses may lead to decreased neovascularization of vascular grafts. Therefore, designing biomedical polymers or modifying existing polymers through matrix protein or growth factor impregnation to inhibit the inflammatory response or stimulate desired healing characteristics may lead to a more biocompatible material. Furthermore, the design of biomedical devices which stimulate an inflammatory response that enhances implant healing properties will vastly improve the biocompatibility of polymeric
materials.

Results from these experiments raise questions concerning the means by which cells associated with biomedical polymers may be influencing angiogenesis around implants. To address this question, tissues associated with biomedical polymers were examined for expression of common inflammatory cytokines and how these factors may be influencing the angiogenic process.
3. INFLAMMATORY CYTOKINES EXPRESSED BY CELLS ASSOCIATED WITH BIOMEDICAL IMPLANTS. INVOLVEMENT OF MACРОPHAGES AND IMPLICATIONS ON ANGIOGENESIS

Introduction

The orchestration of cellular events during wound healing involves various interactions that remain unknown. However, many of the molecular components utilized during wound healing have been delineated. With advances in molecular biology, understanding how polymers influence the healing response and modulate wound repair is beginning to be understood. Determination of which cells are responsible for directing the healing associated with polymeric implants will serve to direct new approaches to the treatment of failing artificial devices implanted within biological tissues.

Several investigators have examined which bioactive substances are released by cells associated with polymeric implants. Marchant et al. (1983) developed the cage implant model for the analysis of exudate formed by cells associated with polymeric materials. Some of the bioactive compounds include, but are not limited to, IL-1β, IL-6, and TNF-α. This model has been employed by many researchers and has served as a valuable tool for determination of bioactive components produced by cells associated with biomedical polymers (Hunt et al., 1996; Goodman et al., 1996; Marchant et al., 1986a; Marchant et al., 1984; Freyria et al., 1991; Lundberg et al., 1995; Eriksson and Thomsen, 1991; Miller et al., 1989). Many in vitro studies have demonstrated which proteins can be produced by macrophages or macrophage-like cells in response to biomedical polymers (DeFife et al., 1995; Anderson et al., 1995;
Cardona et al., 1992; Zenni et al., 1993; Bonfield and Anderson, 1993; Bonfield et al., 1992a; Bonfield et al., 1992b; Miller and Anderson, 1989; Bonfield et al., 1991; Miller et al., 1989; Miller and Anderson, 1988; Bonfield et al., 1989). However, very little information is known on which cells in vivo are directly responsible for the production of these bioactive compounds. Hunt et al. (1996) demonstrated that around intramuscular poly(ether)urethane implants macrophages express TNF-α message and Goodman et al. (1996) demonstrated the expression of various cytokines by cells associated with hip prosthetics. However, to date, very little research has been directed toward determining cellular expression of inflammatory cytokines associated with polymeric vascular grafts. Griesler, along with others, has suggested that the macrophage is the primary producer of cytokines and enzymes found around vascular polymeric implants and possibly responsible for the failure of many these prostheses.

Understanding the macrophages role in the healing associated with synthetic vascular grafts requires delineating which molecules are expressed by macrophages and then determining the effects these molecules may have on the wound healing process. The hypotheses to be tested were 1) cytokine message is expressed by macrophages in response to synthetic polymers and 2) factors released by cells associated with polymeric implants influence angiogenesis. The following studies use a rat subcutaneous implant model to isolate RNA for determination of cytokine mRNA expression, identification of cells expressing specific cytokine mRNA, and collection of wound fluid associated with biomedical polymers.
Materials and Methods

Animal selection and welfare

All animal studies were performed following protocols approved by the University of Arizona Health Sciences Center Animal Care and Use Committee and according to the National Research Council "Guide for the Care and Use of Laboratory Animals" (1996). All surgeries were performed and animals housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care.

Polymer Preparation

Impra ePTFE (4 mm i.d., 30 μm internodal distance), Gore-Tex ePTFE (wrapped 4 mm i.d., 20 μm internodal distance), Golaski microknit Dacron (4 mm i.d.), and Hemashield microvel Dacron (4 mm i.d.) were purchased from Impra Inc. (Tempe, AZ), W.L. Gore & Associates (Flagstaff, AZ), Golaski Labs, Inc. (Philadelphia, PA), and Meadox Medical, Inc., (Oakland, NJ), respectively. Each material was cut into 2 cm tubes in length. One end of the tube was heat-sealed using a hot hemostat (252°C). The polymeric tube was then filled with sterile saline and the other end heat-sealed. All polymeric tubes were then placed into sterile saline and steam sterilized.

Implant Procedure

Nineteen Sprague Dawely rats (250 g) were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. The flank areas were prepared for surgery. The
rat was placed on his abdomen and a small incision, just off midline, was made. Each rat received one sample and the incision closed with a staple. The rats were allowed to recover and returned to the animal facility.

Explant Procedures

The grafts were removed five weeks after implantation. After the animals were anesthetized with sodium pentobarbital injected IP, the skin was dissected away from the polymeric samples. A needle (25 G) and syringe (1 cc) were used to remove any wound fluid that had collected within the polymeric tube. This wound fluid was immediately placed in a microfuge tube and frozen in liquid nitrogen. Each polymer sample was then removed and cut into 3-4 equal size pieces. One piece was placed into a glass vial containing Histochoice™ (AMRESCO, Solon, OH) while the others were immediately placed into cryotubes and frozen in liquid nitrogen. All grafts fixed in Histochoice™ were processed through graded alcohols, followed by xylene, and finally paraffin embedded. Samples were sectioned (6-8 μm thick), and dried onto poly-L-lysine coated slides.

Histology

Sections were stained with hematoxylin and eosin to examine the healing characteristics associated with the polymeric implants.
RNase Protection Assay

To determine which mRNA species were present in the tissues associated with the implants, an RNase Protection Assay (rCK-1 RPA kit, Pharmingen, San Diego, CA) was performed on each of the samples. This kit allows for the quantification of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, TNF-β, IFN-γ, GAPDH, and L32 mRNA species.

Total RNA was isolated from tissues surrounding the polymeric implants using Trizol reagent (Life Technologies, Grand Island, NY). Tissue was homogenized using a pestle in a cryotube in 1 ml Trizol per 100 mg tissue. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform per 1 ml Trizol reagent was added and the tubes vigorously shaken by hand for 15 seconds. Samples were then incubated at room temperature for 3 minutes. Samples were then centrifuged at 6,000 x g for 40 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains in the upper aqueous phase. The aqueous phase was transferred to a fresh tube and RNA precipitated by mixing with 0.5 ml isopropl alcohol per 1 ml Trizol reagent used in the initial homogenization. Samples were then incubated at room temperature for 10 minutes and then centrifuged at 6,000 x g for 40 minutes at 2 to 8°C. The supernatant was then removed and the RNA pellet washed once with 1 ml 75% ethanol per 1 ml Trizol reagent used in the initial homogenization. The sample was vortexed and centrifuged at 6,000 x g for 5 minutes at 2 to 8°C. The supernatant was removed and the RNA pellet air dried. The RNA pellet was
then dissolved in RNase-free water and stored at -70°C.

**Probe synthesis:** 10 μl [α-32P]UTP, 1 μl GACU nucleotide pool, 2 μl DTT, 4 μl 5X transcription buffer, and 1 μl RPA template set (rCK-1) at room temperature and 1 μl RNasin, and 1 μl T7 polymerase were mixed in order in a 1.5 ml Eppendorf tube and incubated at 37°C for 1 hour. The reaction was terminated by adding 2 μl DNase, mixing and incubating at 37°C for 30 minutes. Then 26 μl 20 mM EDTA, 25 μl Tris-saturated phenol, 25 μl chloroform:isoamyl alcohol (50:1), and 2 μl yeast tRNA were added in order to the tube. This mixture was vortexed into an emulsion and spun in a microfuge (12,000 x g) for 5 minutes at room temperature. The upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube and 50 μl chloroform:isoamyl alcohol (50:1) added. This mixture was then vortexed and spun in a microfuge (12,000 x g) for 2 minutes at room temperature. The upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube and 50 μl 4 M ammonium acetate and 250 μl ice cold 100% ethanol added. The samples were then inverted to mix and incubated at -70°C for 30 minutes. Samples were then spun in a microfuge (12,000 x g) for 5 minutes at 4°C. The supernatant was carefully removed, the pellet washed with 100 μl 90% ethanol, and the samples spun in a microfuge (12,000 x g) for 5 minutes at 4°C. The supernatant was removed and the pellet air dried for 5 minutes. The pellet was dissolved in 50 μl of hybridization buffer. Duplicated 1 μl samples were quantitated in a scintillation counter with an acceptable minimum value of approximately 3 x 10^3 Cherenkov counts/μl.

**RNA Preparation and Hybridization:** 10-20 μg of sample RNA was added to each 1.5 ml Eppendorf tube and frozen for 15 minutes at -70°C. The samples were then dried
completely using a vacuum evaporator centrifuge (no heat). 8 μl of hybridization buffer was added to each sample and the pellet solubilized by gently vortexing for 3-4 minutes. The probe was diluted to the appropriate concentration (4.0 x 10^3 cpm/μl) in hybridization buffer. 2 μl of diluted probe was added to each RNA sample and mixed by pipetting. A drop of mineral oil was placed in each tube, followed by a quick spin, and then the samples were placed in a heat block pre-warmed to 90°C. The temperature was immediately turned to 56°C and the samples incubated for 12-14 hours. The heat block was turned to 37°C 15 minutes prior to RNase treatment. 100 μl of an RNase cocktail (2.5 ml RNase buffer, 6 μl RNase A + T1 mix) was added to each sample, the samples microcentrifuged for 10 seconds, and incubated for 45 minutes at 30°C. The samples were transferred to a new Eppendorf tube containing 18 μl of a Proteinase K cocktail (390 μl Proteinase K buffer, 30 μl Proteinase K, 30 μl yeast tRNA), vortexed, spun on a microcentrifuge, and incubated for 15 minutes at 37°C. 65 μl Tris-saturated phenol and 65 μl chloroform:isoamyl alcohol (50:1) were then added to each sample, vortexed into an emulsion and spun in a microcentrifuge for 5 minutes at room temperature. The upper aqueous phase was transferred to new tubes and 120 μl 4 M ammonium acetate and 650 μl ice cold 100% ethanol added. This mixture was incubated for 30 minutes at -70°C, and then spun in the microfuge for 15 minutes at 4°C. The pellet was then washed with 90% ethanol and spun on a microfuge for 5 minutes at 4°C. The pellet was air dried and 5 μl 1X loading buffer added. The mixture was vortexed for 2-3 minutes, heated for 3 minutes at 90°C, and immediately placed in an ice bath. The samples were loaded in the wells of a polyacrylamide/bis (19:1) gel using 0.5X TBE as the running buffer.
Samples were run at 50 watts constant power until the leading edge of the Bromphenol Blue reached 30 cm. The gel was then dried onto gel blot paper and the dried gel exposed to film followed by phosphorimaging. Quantification of bands visualized using the phosphorimager was performed on a computer containing Imagequant Software. Bands were normalized by dividing the volume of a particular band of interest by the average volume of the two house keeping genes, GAPDH and L32. Each band volume was multiplied by the longest band length divided by its own length to offset differences due to quantity of labeling.

In Situ Hybridization

Tissue samples were fixed in Histochoice™ fixative, dehydrated through graded alcohols to xylene and embedded in paraffin. Sections were cut approximately 6-8 µm, dried onto Fisherbrand Superfrost glass slides, and placed in an oven at 60°C for 2 hours. The sections were then deparaffinized through xylene and rehydrated through graded alcohols and DEPC-water into APK Buffer (Ventana Medical Systems, Inc., Tucson, AZ). The slides were then loaded onto the Ventana automated in situ hybridization instrument and preprogrammed protocols performed (appendix A) for the in situ process. Briefly, samples were immersed in protease 3 (Ventana Medical Systems, Inc., Tucson, AZ) for 4 minutes then rinsed with APK buffer. Then, hybridization buffer, poly dT probe (1:200, Sigma), randomer probe (1:200, Keystone Laboratories, Camarillo, CA), or IL-1β probe (1:200, R&D Systems, Minneapolis, MN) were manually placed on individual samples for 1 hour at 65°C. All probes were labeled with digoxigenin. Samples included subcutaneously implanted Hemashield
Dacron, Golaski microknit Dacron, Impra ePTFE, Gore-Tex ePTFE and rabbit thoracic aorta (negative control). To be certain probes were binding to RNA, some samples were also treated with RNase prior to probe application. Next, samples were rinsed with APK buffer and a series of reagents added for detection (1:200 biotin labeled anti-digoxigenin, 1:200 anti-biotin antibody, 1:250 avidin labeled alkaline phosphatase). Stringency washes were carried out at 50°C using 0.1 x SSC buffer. Probe was then visualized using a mixture of 4-nitro blue tetrazolium chloride (Boehringer Mannheim), 5-bromo-4-chloro-3-indoly1-phosphate (Boehringer Mannheim) and Genius buffer III (Ventana Medical Systems, Inc, Tucson, AZ) was then placed on each slide for 1 hour. Finally, slides were removed from the Ventana instrument, washed in Dawn water to remove oil-based coverslip, placed in APK buffer, and reacted with NBT for 2 hours for further detection. After detection products were visualized, slides were dehydrated through acetone to xylene and coverslipped.

**Proliferation Assays**

All procedures were performed using sterile technique under a laminar flow hood. Male Sprague-Dawely rats (250 g) were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. The abdomen was shaved and cleaned with 70% ethanol. The abdomen was opened and the epididymal fat pads located. The distal two-thirds of the fat pad were removed by cutting away with a scissors. The fat pads were minced in a small beaker. In an Erlenmeyer flask, 15 ml of minced fat and 15 ml of collagenase [2.5 mg collagenase and 2.5 mg bovine serum albumin (BSA) per 1 ml Dulbecco’s cation-free phosphate buffered saline(DCF-PBS)] was mixed by shaking (64 RPM) for 30 minutes in a 37°C water bath.
Following the digestion, the mixture was put into sterile 15 ml centrifuge tubes and centrifuged at 700 x g for 5 minutes. The supernatant was poured off and the pellets resuspended in 20 ml of DCF-PBS containing 0.1% BSA and transferred into two clean tubes. The suspension was centrifuged at 700 x g for 4 minutes and the supernatant aspirated. The pellets were resuspended in 20 ml of sterile DCF-PBS containing 0.1% BSA. The cells can then be plated at the desired density.

After the fourth passage, MVEC were used to seed gelatinized 96 well plates at a density of $1 \times 10^4$ cells/cm$^2$ and allowed to adhere for 1 hour. Next, the medium was removed and cells were assigned one of the following treatment groups: rat complete medium, rat complete medium + wound fluid (9:1), Dulbecco’s modified eagle medium (DMEM) + 0.1% BSA, or DMEM + 0.1% BSA + wound fluid (9:1). After four days half of the wells were used to count cells using an electron cell counter and determine cell density. The medium from the other wells was removed and identical medium replaced into the wells. Following seven days the remaining wells were used to determine cell density.
Results

Histology

The Hemashield Dacron, Golaski Dacron, Gore-Tex ePTFE, and Impra ePTFE tube implants were surrounded by an inflammatory response characterized by macrophages, foreign body giant cells and fibrous encapsulation (figure 3.1). Hemashield and Golaski Dacron had both inflammatory cells and extracellular matrix within the center of the polymeric tubes while only the Golaski Dacron implants had vascular profiles within the centers of the tubes. The Impra and Gore-Tex ePTFE had evidence of a fibrin clot within the centers of the tubes.

RNase Protection Assay

To examine levels of cytokine mRNA expression a RNase protection assay was employed. All samples contained similar expression of RNA species protected from enzymatic digestion and visualized using radioactivity. All treatment groups expressed mRNA for IL-1β in the greatest quantity when compared to all other mRNA species probed (figure 3.2). Other cytokine mRNA expressed by cells associated with polymeric implants and listed in order from greatest to least highly expressed were TNF-α, IL-10, and IL-6. One RNase protection assay performed using Impra ePTFE and Golaski microknit Dacron also had bands corresponding to IFN-γ, IL-5, IL-4 and IL-1α (figure 3.3). No significant differences were identified when comparing the same cytokine between different polymeric implants.
FIGURE 3.1  Light micrograph of hematoxylin and eosin-stained sections of a) Impra 30 µm ePTFE, b) Gore-Tex ePTFE, c) Hemashield microvel Dacron, and d) Golaski microknit Dacron tubes following 5 weeks implantation within subcutaneous tissue. Bar = 10 µm.
FIGURE 3.2 Illustration of relative expression of IL-1β mRNA by cells associated with subcutaneously implanted polymeric tubes. Expression was calculated by the following equation: 

\[
\frac{\text{IL-1β band volume}}{(\text{GAPDH band volume}) + (\text{L32 band volume})}/2.
\]
FIGURE 3.3  Representative RNase protection assay gel developed on x-ray film. Bands are visible at lengths specific for IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IFN-γ and TNF-α. Each lane represents one Impra explant from different rats.
In Situ Hybridization

Sections hybridized with randomer probe or treated with RNase prior to hybridization with the IL-1β probe demonstrated no positively staining cells and little background. Furthermore, sections of normal rabbit aorta hybridized with IL-1β probe demonstrated no positively staining cells. Cells reacting positive for the IL-1β message probe were localized to regions associated with polymeric material while regions distant from the implant (<100 μm) had fewer cells expressing IL-1β message (figure 3.4). Cells observed as positive for IL-1β message expression included macrophages, foreign body giant cells, vascular endothelial cells of arterioles, and fibroblast-like cells. Interestingly, not all cells of the same type reacted with the IL-1β message probe suggesting subtypes of these cells associated with polymeric implants (figure 3.4). For example, some foreign body giant cells associated with Dacron fibers expressed IL-1β message while others did not. The Gore-Tex ePTFE was surrounded by foreign body giant cells, nearly all expressing IL-1β message. Some cells within the lumen of the Dacron implants also expressed IL-1β message, particularly the vascular channels. Interestingly, the arterioles appeared to express more IL-1β message than venules (figure 3.4). Within the lumen of some blood vessels, IL-1β message positive cells were observed, presumably monocytes (figure 3.4).

Proliferation Assay

Following addition of wound fluid collected from ePTFE polymeric tubes implanted within the subcutaneous tissue of rats for 5 weeks to rat complete medium, rat microvessel
FIGURE 3.4  Light micrographs of IL-1β probed sections of Hemashield microvel Dacron implanted within subcutaneous tissue for 5 weeks.  a) only those cells located within approximately 100 μm of polymer expressed IL-1β mRNA.  Bar = 40 μm.  b) however, not all cells associated with the polymer expressed IL-1β mRNA (arrow, foreign body giant cell).  Bar = 10 μm.  c) arterioles appeared to express more IL-1β mRNA compared to veinules (arrow, arterioles; arrowhead, veinules).  Bar = 10 μm.  d) mononuclear cells expressing IL-1β mRNA were observed within the lumen of blood vessels.  Bar = 10 μm.
endothelial cells had a significant increase in proliferation rates. After 4 days and 7 days the cellular density of RCM + wound fluid wells was approximately double the cellular density of all other groups (figure 3.5). All other treatment groups did not demonstrate any significant changes in proliferation rates when compared to the respective controls. Interestingly, at 4 days, all wells appeared nearly confluent, therefore, the bioactive molecules within the wound fluid may release the cells from contact inhibition thereby allowing increased densities of cells per well.
FIGURE 3.5  Proliferation assay on endothelial cells treated with (▼) rat complete medium (RCM), (●) DMEM + FBS (depleted medium), (♦) RCM + wound fluid (SN), (▲) RCM + DMEM, or (■) depleted medium + SN. After 4 and 7 days, endothelial cell density was significantly greater in the RCM + wound fluid group compared to all other groups (* depicts significantly different from RCM group, p≤0.05).
Discussion

The literature examining the inflammatory response associated with biomedical polymers has begun to determine the importance cytokines play in the healing response. Anderson's group at Case Western Reserve University has demonstrated the production of cytokines by monocytes cultured in vitro with polymers and the production of cytokines by cells associated with polymers in vivo. However, the cells responsible for the production of specific cytokines associated with polymers in vivo is not know. In this chapter, methods for the identification of cytokine message produced by cells associated with polymers in vivo and identification of cells responsible for the production of IL-1β message are presented. Furthermore, how these cytokines and other factors released by cells in response to biomaterial implantation effect the proliferation of endothelial cells is demonstrated.

The cage implant model was introduced by Merchant et al. (1983) to evaluate the healing associated with biomedical polymers. This model has been useful in identifying specific cytokines produced by cells in response to polymeric implants since wound fluid produced by cells in response to the implant can be collected and analyzed. However, the cage implant model utilizes a titanium chamber to hold the polymeric material while implanted within biological tissue. This titanium cage may in fact be stimulating an inflammatory response, skewing the results obtained using this model (Rosengren et al., 1996). The Vroman effect predicts that proteins will adsorb to the surface of not only polymers, but any foreign substance placed within biological systems. Therefore, even the titanium chamber will adsorb proteins which may have an influence on the healing associated with the implant. To address this problem, a model for the analysis of wound fluid produced by cells associated
with biomedical polymers was developed. Polymeric tubes are heat-welded shut at the ends and then implanted within biological tissues. Wound fluid collects within the lumen of the tube and can be evaluated for a number of criteria. Furthermore, the polymeric tubes can then be analyzed using histochemical and molecular techniques. Theoretically, the inflammatory response observed associated with these polymeric tube implants are in response to only the polymer of interest.

Following histological analysis of the tubular implants, the importance of porosity becomes clear. The less porous polymers (Gore-Tex ePTFE, and Impra ePTFE) had very few cells (mainly red blood cells) within the lumen of the tube and little if any matrix deposition. In contrast, inflammatory cells and extracellular matrix were observed within the lumens of Hemashield and Golaski Dacron implants while only the Golaski implants had vascular profiles in the centers of the tubes. Research on synthetic vascular conduits has focused on the promotion of spontaneous endothelialization via transmural migration. The data presented here would suggest that only the highly porous polymers would permit the ingrowth of cells leading to the formation of an endothelial cell lining on the luminal surface.

To determine which cytokines may be playing a role in the healing associated with these tubular implants RNase protection assays were performed on the tissues surrounding the implants. The results from these experiments demonstrate that the message for several cytokines were expressed by cells associated with the polymeric implants. These cytokines may each be playing separate roles in the healing response. IL-1β and TNF-α, the two most highly expressed message using this assay, have been implicated in stimulating endothelial cell expression of adhesion molecules, tissue factor, and shifting endothelial cells from an anti-
thrombogenic to a thrombogenic nature (Dinarello, 1994; Tracey, 1994). TNF-α has also
been demonstrated to induce IL-1β expression in endothelial cells. Furthermore, IL-1β is
released into the circulatory system and may account for the presence of mononuclear cells
expressing IL-1β message within the lumen of vessels (Dinarello, 1994). This systemic
elevation may also activate both PMNs and monocytes stimulating the expression of surface
adhesion molecules leading to the recruitment of inflammatory cells to the implant. Therefore, IL-1β may be responsible for recruitment of other inflammatory cells and the
increase in thrombogenicity characteristic of anastomotic endothelial cells. IL-6 is elevated
in patients following organ transplantation and is an important cytokine in B- and T-cell
differentiation (Hirano, 1994). The significance of this is unknown, however, the presence
of IL-6 and IFN-γ could possible suggest the involvement of an immune response associated
with these biomedical polymers (Hirano, 1994; De Maeyer and De Maeyer-Guignard, 1994).
IL-4 has been demonstrated to play a fundamental role in the formation of foreign body giant
cells (Kao et al., 1995), endothelial cell proliferation (Toi et al., 1992), recruitment of T cells
via VCAM 1 upregulation on endothelial cells (Schleimer et al., 1992; Thornhill et al., 1991;
Masinovsky et al., 1990), and inhibition of ICAM-1 expression by endothelial cells
(PielaSmith et al., 1992). IL-10 is thought to play an important role in the resolution of
inflammation, possibly by inhibiting the secretion of several cytokines by macrophages and
suppression of cell-mediated-immunity (de Waal Malefyt et al., 1991; Fiorentino et al., 1991;
Mosmann, 1994). The role these molecules play in the healing response requires further
analysis. The most surprising finding was that there were no significant differences in the
expression of cytokine mRNA between the different types of implanted polymeric materials.

Identification of cells responsible for the production of specific cytokines may lead to improved therapies for wound healing. In an effort to identify the cells expressing IL-1β mRNA, in situ hybridization was utilized. IL-1β was selected for several reasons. First, IL-1β has been demonstrated to either stimulate or inhibit angiogenesis depending on concentration and assay system. Second, IL-1β has been demonstrated to stimulate the upregulation of tissue factor and adhesion molecules on endothelial cells, stimulate the proliferation of smooth muscle cells and fibroblasts and stimulate the production of extracellular matrix proteins by cells. All of these functions of IL-1β may play a significant role in the healing associated with both the luminal (neointimal thickening) and the abluminal (neovascularization and recruitment of inflammatory cells) surfaces of vascular implants. To date, one other report in the literature concerning biomedical devices has used this method for the identification of cytokine message expressed by cells associated with bone cement used in hip prostheses (Goodman et al., 1996). However, the healing associated with hip prosthetics and vascular prosthetics differs and therefore warrants evaluation. The in situ results demonstrate that various cell types express IL-1β mRNA and these include endothelial cells, macrophages, foreign body giant cells and fibroblast-like cells. These results suggest that cell subtypes exist in association with vascular polymers. This may prove useful in treatments of problems associated with polymeric vascular implants. For example, many polymeric arteriovenous access grafts placed in diabetics become encapsulated with a dense fibrous capsule rendering the implant useless. By inhibiting this capsule formation, possibly
by turning off IL-1β expression in fibroblasts, these implants may remain accessible for longer periods of time.

The products produced by cells associated with polymeric implants direct the healing and determine the success or failure of polymeric implants. The promotion of an endothelial cell lining via transmural migration of endothelial cells is one of the processes most likely influenced by these products (Lepidi et al., 1996). The examine the role these products may play in angiogenesis, wound fluid that formed within polymeric tubes implanted subcutaneously in rats was collected. The results from these experiments demonstrate that the wound fluid increased proliferation rates of rat microvessel endothelial cells in two-dimensional culture. This goes against the hypothesis that the inflammatory cells associated with vascular grafts inhibit neovascularization (chapter 2). However, it is reasonable to suggest that the wound fluid is not representative of the environment in vivo cells see. The products secreted by cells in this experiment diffused across an approximate 700 μm thick wall of polymeric material. Not only may the molecules possibly degrade in this time, it is likely that the products are diluted. The wound fluid collected in this experiment may be more representative of what anastomotic cells see. In this case, an increase in proliferation associated with the wound fluid may be representative of the increased proliferation rates observed at anastomotic neointimal thickening of polymeric vascular grafts.

The data presented in this chapter support the hypotheses tested. Macrophages, along with other cells, associated with polymeric implants express message for inflammatory cytokines. Furthermore, products released by these cells increase proliferation of microvessel cells.
endothelial cells in vitro. Unexpectedly the cells expressing IL-1β message appear to be separated into subclasses since not all cells of each type expressed message for IL-1β. Further evaluation of the roles each cytokine play in the healing process is required.

Results presented in chapter 2 and 3 suggest that several factors determine the healing associated with biomedical polymers. Of particular interest concerning biomedical devices is that the materials structural properties, for example porosity, has a dramatic effect on the healing response even when the base polymer is held constant. This was demonstrated best by the differences in healing observed with 30 μm, 60 μm and 100 μm ePTFE. This suggests that alterations of polymeric implants due to placement of the device may lead to an altered healing response. Therefore, the following studies were performed to assess structural changes of ePTFE due to the deployment process of an emerging technology, endovascular grafts, and how these alterations may influence the healing response.
4. EFFECTS OF BALLOON DILATATION ON STRUCTURAL CHARACTERISTICS AND HEALING ASSOCIATED WITH ePTFE

Introduction

The introduction of medical devices fabricated from clinically available prosthetics requires evaluation of the new device to demonstrate acceptable performance and biocompatibility with biological tissues. Endovascular grafts, one such hybrid, consist of rigid, often metallic, stents surrounded by polymeric vascular graft material, often ePTFE or PET (Palmaz et al., 1995; Marin et al., 1996; Chalmers et al., 1994a). Endovascular grafts have been proposed as a method to treat aneurysms, occlusive disorders, and other diseases within the vascular system. The deployment procedure expands the stent, which anchors the device within the vessel, and stretches the ePTFE beyond its original dimensions. Palmaz et al. (1995) demonstrated that balloon dilatation of endovascular grafts utilizing ePTFE did not significantly alter the internodal distance of the polymer. However, as demonstrated in chapter 2 of this dissertation, internodal distance is only one of several physical parameters of ePTFE. Other parameters include wall thickness, nodal width and fiber width which may be altered following balloon dilatation and therefore require evaluation. As demonstrated in chapter 2, fiber width significantly differed between ePTFE having different internodal distances, and the healing may have been altered by these differences. Therefore, it is unknown if balloon dilatation of ePTFE will have any effect on the wound healing response following implantation.
In 1986 McClurken et al. (1986) developed standards for evaluating static and dynamic physical properties of synthetic vascular grafts. Static physical properties include inside diameter, wall thickness and internodal distance. In the present study these static physical properties are addressed along with the addition of nodal width, interfiber distance and fiber width. Physical properties may influence the healing associated with ePTFE as demonstrated by studies examining the impact porosity had on healing (Nagae et al., 1995; Hirabayashi et al., 1992; Kohler et al., 1992; Clowes et al., 1986b; Golden et al., 1990). Furthermore, Holubed et al. (1992) demonstrated that deformation of ePTFE induced by surgical instruments alters the healing response associated with the polymer. Therefore, changes induced by dilating ePTFE mandate a reassessment of the healing characteristics associated with this prosthetic when used in an application that differs from previously documented experiences (Marin et al., 1995). The hypotheses to be tested were 1) the physical properties of ePTFE are altered following balloon dilatation and 2) these changes influence the healing associated with implants.
Materials and Methods

Polymer

Samples of 30 μm internodal distance ePTFE of both 3 mm i.d. thin wall and 4 mm i.d. standard wall were purchased from Impra, Inc. (Tempe, AZ). The vascular grafts were cut into samples measuring approximately 50 mm, 70 mm or 82 mm in length in order to completely cover the various angioplasty balloons used during dilatation. All experiments were performed at room temperature and were video recorded on VHS using a CCD camera mounted on a copy stand. The camera/VCR system was attached to a Gateway computer containing Metamorph™ Image Analysis software (Universal Imaging, West Chester, PA). Metamorph™ Image Analysis was used to capture an image of the pre-ballooned ePTFE (figure 4.1). The angioplasty balloons (Meditech Corp., Watertown, MA) were made of noncompliant polyethylene teraphthalate, and therefore consistency in inflated balloon diameter between experiments was maintained. For each angioplasty balloon (2 cm length by 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, 9 mm or 10 mm diameter), the following protocol was followed. The balloon was inserted into the luminal space of the graft, centered from the ends, fully inflated and held at 12 atm for 30 seconds. This insured complete dilatation of the ePTFE around the balloon. Metamorph™ Image Analysis was used to capture an image of the inflated graft (figure 4.1). After 30 seconds, the balloon was deflated and moved toward one end. The balloon was inflated again (12 atm) in order to dilate half of the ePTFE previously expanded along with some non-dilated ePTFE. Metamorph™ Image Analysis captured a
FIGURE 4.1 Photograph illustrating protocol for dilatation of ePTFE will angioplasty balloons. A) initial outer diameter is measured and balloon catheter is inserted into the lumen of the graft. B) balloon is inflated to 12 p.s.i. and the resulting outer diameter measured. Following 30 seconds, the balloon is deflated. C) then the balloon is moved toward one end of the graft and again inflated to 12 p.s.i. with the outer diameter of the previously dilated material measured (recoiled outer diameter). Following 30 seconds, the balloon is deflated and removed.
third image (figure 4.1), and after 30 seconds the balloon was deflated and removed from the lumen of the ePTFE. Measurements were made from the captured images of the initial outer diameter, the outer diameter with inflated balloon in place, and the outer diameter following removal of the balloon. All samples were then cut in half longitudinally to expose the luminal surface or circumferentially to expose the wall surface. Samples were mounted on aluminum stubs using silver glue-stick tape, sputter coated with a gold target and examined using a JEOL 820 scanning electron microscope at 25 kV accelerating voltage.

Additionally, the effect of multiple balloon dilatations on ePTFE diameter was examined. The balloon (2 cm length by 8 mm diameter) was inserted within the lumen of 4 mm i.d. standard wall ePTFE as outlined above and repeatedly balloon dilated (8 times) using Metamorph Image Analysis to capture images during each dilatation as outlined previously. The outer diameter (balloon inflated) and outer diameter (balloon removed) was measured to evaluate the materials ability to recoil.

Physical properties measured using scanning electron microscopy were wall thickness, internodal distance, nodal width, interfiber distance and fiber width. Except for wall thickness, all measurements were made on the luminal surface of the ePTFE. Average values for internodal distance, nodal width, interfiber distance and fiber width were calculated from 20 randomly chosen areas and are defined in figure 4.2. Average wall thickness (figure 4.2) was calculated from 4 random points, approximately 90 degrees apart.
FIGURE 4.2 Scanning electron photomicrograph defining A) a) internodal distance, b) nodal width, c) interfiber distance, and d) fiber width and B) a) wall thickness.

Bar = 5 μm for a,b,c; Bar = 0.33 μm for d; Bar = 40 μm for e.
Animal selection and welfare

All animal studies were performed following protocols approved by the University of Arizona Health Sciences Center Animal Care and Use Committee and according to the National Research Council "Guide for the Care and Use of Laboratory Animals" (1996). All surgeries were performed and animals housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care.

Biomedical polymers

ePTFE (4 mm i.d., 30 μm internodal distance) was purchased from Impra, Inc. (Tempe, AZ). The ePTFE was placed over angioplasty balloons (Meditech Corp., Watertown, MA) made of noncompliant polyethylene teraphthalate and having an inflated diameter of 6, 8, 10, 12, or 18 mm. The angioplasty balloons were fully inflated (12 atm) within the luminal space of the ePTFE samples. After 30 seconds, the balloons were deflated and removed providing samples of ePTFE balloon dilated to various diameters. The tubular grafts were then cut in half longitudinally and samples 6 mm in diameter were prepared using a circular punch. All ePTFE was packaged and steam sterilized. Steam sterilization of the polymers had no noticeable effect on structure based upon scanning electron microscopy analysis.

Scanning Electron Microscopy

Samples of control and balloon dilated ePTFE were mounted on aluminum stubs using
silver glue-stick tape, sputter coated with a gold target and examined using a JEOL 820 scanning electron microscope at 25 kV accelerating voltage.

Implant procedures

Four male rats (260-290 g) were anesthetized with an intraperitoneal (IP) injection of 50 mg/kg sodium pentobarbital. The abdominal and haunch areas were prepared for surgery by clipping away fur and cleansed with a sterile spray. The abdomen was opened and the right and left epididymal fat pads were located. A small hole was made in the serosal layer of the fat pads, and the appropriate ePTFE sample, determined randomly, was implanted by suturing the fat around the ePTFE. The linea alba was closed with an absorbable suture and the overlying skin was closed with staples. The rat then was placed on his abdomen and small incisions made over the right and left haunch. The appropriate ePTFE sample was placed abuminal side toward the skin, determined by the curvature of the sample, and the incision closed with a staple. The rats were allowed to recover and were returned to the animal facility.

Explant procedures

The ePTFE samples were removed five weeks after implantation. The rats were anesthetized with sodium pentobarbital injected IP, the abdomen was opened and the right and left fat pads located. The implants were isolated and removed with surrounding adipose tissue. The subcutaneous implants also were removed with surrounding tissue. All implants were placed within separate glass vials containing Histochrome™ fixative (AMRESCO, Solon,
All samples were processed through graded alcohols followed by xylene, and then they were paraffin embedded. Samples were sectioned (6-8 µm thick) and dried onto Fisherbrand® Superfrost®/Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Histology

Sections were deparaffinized and stained with hematoxylin and eosin to examine cellular histology and fibrous capsule formation. Samples were examined on a Nikon Optiphot microscope with a 40x water-immersion lens. Fibrous capsule thickness was determined by capturing images using a CCD camera mounted on the microscope and connected to a computer containing Metamorph™ Image Analysis software (Universal Imaging, West Chester, PA) and measuring the thickness of the capsule at four random points around the implant.

Immunochemistry

Sections were deparaffinized and reacted with horse radish peroxidase-conjugated Griffonia Simplicifolia (GS1) lectin (Harlan Bioproducts for Science, Inc., Indianapolis, IN) which show’s specificity for endothelial cells, activated monocytes, activated macrophages and foreign body giant cells or with primary antibody ED1 (Harlan Bioproducts for Science, Inc., Indianapolis, IN), which shows specificity for activated monocytes and activated macrophages. The ED1 was visualized using a peroxidase-conjugated secondary antibody. Nuclei were lightly counter stained using methyl green. Sections then were evaluated using a Nikon Optiphot microscope with a 40x water-immersion lens.
Inflammation and Neovascularization Quantification

Sections were examined using a 40x wet objective lens and an 10x eyepiece containing a 6 x 6 grid with each grid area corresponding to a 27 \( \mu \text{m} \times 27 \mu \text{m} \) area. For both GS1 and ED1, fifty-nine grid areas for each sample were examined at the tissue-graft interface. If a grid area contained a positively stained element, the area was designated “1.” If no positively stained elements occurred, the area was designated “0.” For each cytochemical stain an index was determined from the following equation: \( \text{index} = \frac{\text{number of areas positively stained}}{\text{total number of areas counted}} \). A measure of neovascularization can be determined by calculating a neovascularization index from the following equation: \( \text{neovascularization index} = \frac{\text{GS1 positive areas}}{\text{total number of areas counted}} - \frac{\text{ED1 positive areas}}{\text{total number of areas counted}} \).

Statistical analysis

Measurements are reported as mean ± standard deviation (SD) for wall thickness, nodal width, interfiber distance and fiber width and statistical analysis were performed using a one-way ANOVA (SPSS for Windows, SPSS, Inc.). To assessed differences between inflammation and neovascularization a one-way ANOVA (SPSS for Windows, SPSS, Inc.) was used. Values with a \( p \leq 0.05 \) were considered significantly different.
Results

Graft Diameter

The external diameter of the ePTFE was quantified by image analysis at two times, first, when grafts were dilated by the inflated balloon, and second, immediately following deflation (refer to figure 4.1). As demonstrated in figure 4.3, the changes observed for both 3 mm and 4 mm i.d. grafts followed similar patterns of recoil. In these figures, when the balloons were inflated, the external diameters were a measurement of the balloons diameter plus the ePTFE’s wall thickness. Therefore, the external diameter of a 3 mm i.d. ePTFE graft dilated with a 4 mm angioplasty balloon would measure approximately 5 mm. As demonstrated by figure 4.3, regardless of inflated balloon diameter, the material recoiled approximately 20% after the balloon was deflated.

To evaluate whether repeated balloon dilatation results in loss of the material’s ability to recoil, 4 mm i.d. ePTFE was balloon dilated to 2x it’s initial diameter 8 times and the resulting diameter measured. Figure 4.4 illustrates the ability of 4 mm i.d. ePTFE to recoil, however, the amount of recoil was decreased after repeated balloon dilatations. Even after 8 dilatations, the ePTFE graft material still recoiled approximately 10%.

Scanning Electron Microscopic Evaluation of ePTFE

Structural differences between control and maximally dilated ePTFE can be observed in figure 4.5. Differences in nodal thickness are apparent. Furthermore, nodes located within
FIGURE 4.3 Scatter-plot illustrating recoil of A) 3 mm i.d. ePTFE and B) 4 mm i.d. ePTFE following balloon dilatation. Each point represents a different piece of graft material. (●) represents outer diameter when balloon is fully inflated within the lumen of the graft, (■) represents recoil outer diameter after removal of the balloon from the lumen of the material.
Inflated Balloon Diameter (mm)

A

B

Inflated Balloon Diameter (mm)
FIGURE 4.4 Scatter-plot illustrating recoil of ePTFE following repeated balloon dilatation to 2x the material's initial inside diameter. (●) represents outer diameter when balloon is fully inflated within the lumen of the graft, (■) represents recoil outer diameter after removal of the balloon from the lumen of the material.
FIGURE 4.5  Scanning electron micrographs of A) control 3 mm i.d. ePTFE, B) 3 mm i.d. ePTFE dilated using a 10 mm diameter balloon, C) control 4 mm i.d. ePTFE, and D) 4 mm i.d. ePTFE dilated using a 10 mm diameter balloon. Bar = 5 μm.
the wall of the graft appear beneath the internodal fibers following balloon dilatation of the ePTFE. This is believed to occur from the dilatation process which results in a decreased wall thickness, forcing nodes within the material’s walls to move closer together.

Wall Thickness

The wall thickness of 3 mm i.d. and 4 mm i.d. ePTFE was significantly decreased when balloon dilated with either an 8 mm and 10 mm angioplasty balloons (figure 4.6). These figures demonstrate an almost linear relationship between wall thickness and extent of dilatation. Also noted during the analysis of wall thickness was the decrease in the radial internodal distance of the inner 1/3 of the vascular graft (figure 4.7). The decrease in wall thickness from balloon dilatation forces the nodes closest to the luminal surface to move together until side-by-side. Dilatation appears to have little effect, if any, on the radial internodal distance of the outer 2/3’s of the vascular graft (figure 4.7).

Internodal Distance

In these experiments, ePTFE graft material was radially dilated and no change in graft length was observed. Therefore, due to the circumferential orientation of the nodes we do not believe balloon dilatation of the material had any dramatic effect on the internodal distance of the luminal surface. However, when inflated balloon diameters (i.e. 9 mm and 10 mm) were much greater than the material’s initial internal diameter, slight increases in
FIGURE 4.6 Scatter-plot illustrating wall thickness of a) 3 mm i.d. ePTFE and b) 4 mm i.d. ePTFE following balloon dilatation. Each point represents a different piece of graft material. * significantly different from control (p≤0.05).
FIGURE 4.7 Scanning electron photomicrograph of A) control 4 mm i.d. ePTFE (cross section) and B) 4 mm i.d. ePTFE dilated using a 10 mm diameter balloon (cross section). Note the decrease in distance between radially orientated nodes. Bar = 7 μm. C) 4 mm i.d. ePTFE dilated using a 10 mm diameter balloon (cross section). Arrow demarcates wall-luminal surface junction, arrow-head demarcates wall-abluminal surface junction. Note that the radial internodal distance closest to the luminal surface is most affected by balloon dilatation. The radial internodal distance furthest from the luminal surface appears unaffected. Bar = 30 μm.
internodal distance occurred as demonstrated in Tables 4.1 and 4.2. Furthermore, as stated above, the radial internodal distance appears to be dramatically affected following balloon dilatation.

Table 4.1. Summary of Physical Properties of Control and Balloon Dilatated 3 mm i.d. ePTFE

<table>
<thead>
<tr>
<th>Inflated Balloon Diameter (mm)</th>
<th>Internodal Distance (µm)</th>
<th>Nodal Width (µm)</th>
<th>Interfiber Distance (µm)</th>
<th>Fiber Distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>23.8 ± 3.8</td>
<td>10.8 ± 3.4</td>
<td>3.1 ± 1.6</td>
<td>0.53 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>30.5 ± 5.2</td>
<td>9.6 ± 2.2</td>
<td>2.9 ± 1.3</td>
<td>0.40 ± 0.12*</td>
</tr>
<tr>
<td>5</td>
<td>25.0 ± 3.9</td>
<td>5.9 ± 1.9*</td>
<td>1.8 ± 0.7*</td>
<td>0.32 ± 0.14*</td>
</tr>
<tr>
<td>6</td>
<td>29.3 ± 3.0</td>
<td>7.8 ± 1.7*</td>
<td>1.6 ± 0.6*</td>
<td>0.31 ± 0.10*</td>
</tr>
<tr>
<td>7</td>
<td>25.1 ± 3.7</td>
<td>7.8 ± 2.0*</td>
<td>2.9 ± 1.5</td>
<td>0.42 ± 0.19</td>
</tr>
<tr>
<td>8</td>
<td>29.1 ± 4.5</td>
<td>7.5 ± 2.8*</td>
<td>1.5 ± 0.6*</td>
<td>0.33 ± 0.08*</td>
</tr>
<tr>
<td>9</td>
<td>26.8 ± 3.5</td>
<td>5.9 ± 2.3*</td>
<td>1.4 ± 0.8*</td>
<td>0.29 ± 0.12*</td>
</tr>
<tr>
<td>10</td>
<td>28.0 ± 4.1</td>
<td>4.8 ± 1.0*</td>
<td>1.7 ± 0.5*</td>
<td>0.29 ± 0.18*</td>
</tr>
</tbody>
</table>

* significantly different from control (p<0.05)

Nodal Width

Measurements of nodal width revealed similar results for both 3 mm and 4 mm i.d. ePTFE (Tables 4.1 and 4.2, respectively). The nodal width of 3 mm i.d. ePTFE was significantly decreased after balloon dilatation with a 5 mm or greater diameter balloon. Nodal widths of 4 mm i.d. ePTFE were significantly decreased after balloon dilatation with 9 mm and 10 mm diameter balloons.
Table 4.2. Summary of Physical Properties of Control and Balloon Dilated 4 mm i.d. ePTFE

<table>
<thead>
<tr>
<th>Inflated Balloon Diameter (mm)</th>
<th>Internodal Distance (µm)</th>
<th>Nodal Width (µm)</th>
<th>Interfiber Distance (µm)</th>
<th>Fiber Distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>27.7 ± 4.4</td>
<td>7.6 ± 1.2</td>
<td>2.1 ± 0.9</td>
<td>0.32 ± 0.12</td>
</tr>
<tr>
<td>5</td>
<td>32.3 ± 3.2</td>
<td>7.0 ± 2.4</td>
<td>1.3 ± 0.7*</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>6</td>
<td>25.8 ± 4.3</td>
<td>10.1 ± 2.4*</td>
<td>1.9 ± 0.6</td>
<td>0.33 ± 0.13</td>
</tr>
<tr>
<td>7</td>
<td>30.1 ± 5.3</td>
<td>6.3 ± 2.5</td>
<td>1.9 ± 1.0</td>
<td>0.45 ± 0.25</td>
</tr>
<tr>
<td>8</td>
<td>27.5 ± 4.2</td>
<td>7.2 ± 1.8</td>
<td>2.1 ± 0.7</td>
<td>0.31 ± 0.11</td>
</tr>
<tr>
<td>9</td>
<td>26.1 ± 5.3</td>
<td>5.2 ± 1.7*</td>
<td>1.3 ± 0.5*</td>
<td>0.27 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>25.4 ± 3.6</td>
<td>4.8 ± 1.5*</td>
<td>1.8 ± 0.6*</td>
<td>0.31 ± 0.18</td>
</tr>
</tbody>
</table>

* significantly different from control (p≤0.05)

Interfiber Distance

Interfiber distances for 3 mm i.d. ePTFE significantly decreased when a 5 mm, 6 mm, 8 mm, 9 mm or 10 mm diameter balloons were used (Table 4.1). Significant decreases in the 4 mm i.d. ePTFE interfiber distance were limited to balloon diameters of 5 mm, 9 mm or 10 mm (Table 4.2). Examination of scanning electron micrographs suggests that decreases in the interfiber distance was caused by the splitting of fibers during the dilatation process, not only to stretching of the fibers. Fibers of control ePTFE (figure 4.8) appear as if they are composed of 3-5 individual fibers that have not fully separated during the manufacturing process. Upon dilatation, these fibers split apart from each other (figure 4.8) and form individual fibers thereby decreasing the interfiber distance.
FIGURE 4.8  Scanning electron photomicrograph of fibers spanning the internodal space of
A) control 3 mm i.d. ePTFE, B) 3 mm i.d. ePTFE dilated using a 10 mm
diameter balloon, C) control 4 mm i.d. ePTFE, and D) 4 mm i.d. ePTFE
dilated using a 10 mm diameter balloon. Bar = 0.33 μm.
Fiber Width

Differences in fiber width of the 3 mm i.d. ePTFE showed a similar pattern of changes as seen for interfiber distances (Table 4.1). Fiber widths for 3 mm i.d. ePTFE were significantly decreased after dilatation with all balloons except the 7 mm balloon. Fiber widths for 4 mm i.d. ePTFE were not significantly altered when balloon dilated (Table 4.2). Differences between 3 mm i.d. and 4 mm i.d. data most likely reflect differences in the extent of radial dilatation. The 3 mm i.d. ePTFE experienced much greater radial strain then the 4 mm i.d. ePTFE when angioplasty balloons of equal diameter were employed for dilatation of the material. Therefore, fibers from a 3 mm i.d. ePTFE vascular graft would split apart to a greater extent than those of a 4 mm i.d. ePTFE vascular graft when the same diameter balloons are utilized for dilatation. Therefore, due to reasons stated above (interfiber distance section), the fiber width decreased for the 3 mm i.d. ePTFE but not for the 4 mm i.d. ePTFE.

Internodal Fibers

Fibers spanning the internodal space appeared ridged and had holes penetrating fully through the fiber. As the ePTFE was balloon expanded it appeared as if the fibers split longitudinally as well as broke apart (figure 4.8). Furthermore, the larger diameter the balloon used for dilatation the greater number of fibers that appear split and broken.

Fore-shortening

A phenomena observed when dilating ePTFE vascular grafts with large diameter balloons (9 mm and 10 mm in this study) is known as fore-shortening (figure 4.9). At the
FIGURE 4.9 Scanning electron photomicrograph of 4 mm i.d. ePTFE which had been dilated using a 10 mm diameter balloon. "Fore-shortening" occurs at the ends of the graft during inflation of the balloon. Internodal fibers can be seen transversing nodes which have been forced toward the luminal surface during the ballooning process. Bar = 5 μm.
macroscopic level, this was observed as a region graft which resisted dilatation and did not dilate to the same extent as other regions of the material. Graft regions which macroscopically appeared fore-shortened consisted of node-node interactions that decreased the effective internodal distance. The internodal distance within these regions was approximately 10 μm and the nodal width was approximately equal to that of control material.

Histology

Control ePTFE implanted within subcutaneous tissue was consistently surrounded by an acellular dense extracellular matrix fibrous capsule, while balloon dilated ePTFE was surrounded by a cellular loose connective tissue (figure 4.10). None of the ePTFE implants within adipose tissue were encapsulated (figure 4.11). The density of cells around and within the implants appeared related to the extent of balloon dilatation with control material having extremely few cells associated with the implant and ePTFE ballooned to 10, 12, and 18 mm having greater numbers of cells. Furthermore, both control and balloon dilated ePTFE contained regions of high cell density within the graft interstices (figure 4.11).
FIGURE 4.10 Light micrographs of hematoxylin and eosin stained sections from a) control ePTFE, and ePTFE balloon dilated to b) 6 mm i.d., c) 8 mm i.d., d) 10 mm i.d., e) 12 mm i.d., and f) 18 mm i.d. implanted within subcutaneous tissue. Bar = 25 μm.
FIGURE 4.11 Light micrographs of hematoxylin and eosin stained sections from a) control ePTFE, and ePTFE balloon dilated to b) 6 mm i.d., c) 8 mm i.d., d) 10 mm i.d., e) 12 mm i.d., and f) 18 mm i.d. implanted within adipose tissue. Bar = 25 μm.
The inflammatory response dramatically differed depending on tissue environment surrounding the implant. Control ePTFE implanted within subcutaneous tissue was more inflammatory than control ePTFE implanted within adipose tissue (figure 4.12), in contrast, balloon dilated ePTFE implanted within adipose tissue was more inflammatory than balloon dilated ePTFE implanted within subcutaneous tissue (figure 4.13). Furthermore, subcutaneously implanted ePTFE balloon dilated to greater than 2.5 times its original internal diameter had a significantly decreased inflammation index. For adipose tissue implants, balloon dilatation of the ePTFE resulted in a significantly greater inflammation index compared to control ePTFE. Unexpectedly, ePTFE balloon dilated to 18 mm had a significantly smaller inflammatory index when compared to material dilated to 12 mm. Significant neovascularization was only observed with ePTFE balloon dilated to 6 mm and implanted within adipose tissue.
FIGURE 4.12 (●) Inflammation and (○) neovascularization index for subcutaneous implanted control and balloon dilated ePTFE. * denotes significantly different compared to control implants (p ≤ 0.05).
FIGURE 4.13 (●) Inflammation and (○) neovascularization index for control and balloon
dilated ePTFE implanted within adipose tissue. * denotes significantly
different compared to control implants (p ≤ 0.05).
Discussion

The two most commonly used biomaterials for vascular repair are Dacron™ and ePTFE. However, traditional vascular reconstruction utilizing these materials involves trauma to obtain exposure and repair of the diseased vessels. In an attempt to improve current methods of treatment for arteriosclerotic occlusive diseases, aneurysms and other vascular disorders, the endovascular graft, a hybrid consisting of a metallic or alloy stent surrounded by vascular graft material, has been developed. With the introduction of the endovascular graft, several aspects of current treatment methods are expected to be improved upon. First, vascular grafts can be placed either percutaneously or by limited cut down incisions remote from the site of disease, thereby avoiding extensive open surgical incision. Furthermore, the stent acts as a fixation device at the anastomosis, eliminating the need for securing with suture. Moreover, by combining these two materials it is possible that the healing response following treatment can be positively affected compared to that seen with either material alone. For example, intimal thickening occurs at the graft-vessel anastomosis with standard implants and leads to failure of the device. Endovascular grafts, on the other hand, have been shown to have decreased intimal thickening at the graft-vessel anastomosis (Chalmers et al., 1994b; Chalmers et al., 1994a). However, endovascular grafts, when deployed, are dilated and therefore the material surrounding the stent also becomes dilated. The possibility exists that balloon dilatation and it's resultant effect on the physical properties of ePTFE will markedly affect the healing properties. In this study structural alterations occurring after balloon dilatation of 30 µm intermodal distance ePTFE (3 mm i.d. thin wall and 4 mm i.d. standard wall) and the effects these alterations may play in the healing response were
Altering the physical properties of polymeric materials is known to influence the healing response associated with biomedical implants. To determine if balloon dilatation of ePTFE altered the physical properties of the material, several parameters were examined. After balloon dilatation, the ePTFE recoiled approximately 20% regardless of polymer i.d. or balloon inflated diameter. This recoil could be decreased by approximately half when the material was dilated multiple times. In an in vivo application using this process to deploy an endovascular graft composed of ePTFE, the inherent recoil of the material will decrease the luminal diameter and subsequently induce blood flow characteristics possibly leading to adverse healing conditions.

Dilating a tubular structure intuitively results in a decreased wall thickness. Palmaz et al. (1995) compared wall thickness of ballooned dilated ePTFE stent-grafts implanted for 3, 6, and 12 months and concluded that wall thickness does not significantly change following implantation. However, no comparison to control ePTFE was performed leaving questions to whether the wall thickness of balloon dilated ePTFE is changed. The results indicate that wall thickness is significantly decreased when ePTFE is balloon dilated. The consequences of this decrease in wall thickness are apparent when examining the space between the luminal and abluminal surfaces. The radially orientated nodes are forced together following balloon dilatation resulting in the loss of cellular ingrowth and possible changes in the healing characteristics associated with the material.

The internodal distance has historically been the measure of porosity for ePTFE and therefore the most commonly examined physical property. The results demonstrate that
balloon dilatation of ePTFE appears to have no significant effect on internodal distance when this is calculated by measuring the mid-node to mid-node distance. The differences observed in internodal distance were most likely due to variations within the graft material. However, the nodal widths for both 3 mm i.d. and 4 mm i.d. ePTFE significantly decreased with increasing balloon diameter. The significance in this finding is important when considering neovascularization of the polymer. As the nodes become thinner, the effective region cells can migrate through increases and therefore it can be predicted that dilated ePTFE will enhance cellular invasion into the material. However, balloon dilatation also decreases wall thickness resulting in a decreased space between radial nodes. Therefore, cellular invasion of the polymer may increase when smaller balloons are used, but as larger diameter balloons are utilized, cellular invasion would decrease due to the loss of the space within the walls of the graft. Another parameter that may have an influence on migration of cells into the polymer is interfiber distance. As larger diameter balloons were used to dilate the ePTFE, the interfiber distance decreased significantly, especially with the 3 mm i.d. thin wall ePTFE. The decrease in interfiber distance appears to be due to the longitudinal splitting of the fibers. When the interfiber distance decreases, the region through which cells can migrate is reduced. Examination of the fiber width revealed that only the 3 mm i.d. thin wall ePTFE fiber width was significantly decreased. Furthermore, as the ePTFE was dilated with larger balloons more fibers appeared to be broken and split apart. This loss in integrity may result in a decreased tensile strength and the ultimate failure of the endovascular graft.

Another important finding in this study was the fore-shortening of the ePTFE when the balloon extends past the end of the material. This fore-shortening can be characterized
by the close association of nodes that effectively decreased the internodal distance to 10 \( \mu \text{m} \). This will likely have a significant effect on the healing within this region of the graft, most likely with the loss of cellular transmigration at regions of fore-shortening.

The healing characteristics of ePTFE have been directly associated with the physical properties of the polymer, most often the internodal distance. Porosity of ePTFE, as defined by internodal distance, has been shown to be an important determinant in the healing response observed of biological models. Many studies have suggested that an internodal distance of 60 \( \mu \text{m} \) offers optimal healing as characterized by neovascularization, neointimal formation and matrix formation (Golden et al., 1990). However, at this time, only ePTFE having an internodal distance on the luminal surface of less than 30 \( \mu \text{m} \) has been approved for clinical use and therefore current manufacture of devices utilizing ePTFE must be designed using this porosity. Following deployment of endovascular grafts, the structural changes of the ePTFE due to balloon dilatation may lead to an altered healing response when compared to a non-dilated implant. The data suggest balloon dilatation of ePTFE alters the healing characteristics associated with this polymer and that tissue site of implantation is critical in determining the body's reaction to biomedical devices.

Control ePTFE implanted within subcutaneous tissue became encapsulated by an acellular, dense extracellular matrix which resulted in little tissue ingrowth into the polymer's interstices. Balloon dilatation of ePTFE resulted in the formation of loose connective tissue capsules surrounding the implants and an increased ingrowth of cells within the polymer interstices. Surprisingly, the cells found around and within dilated ePTFE were not activated
macrophages, resulting in a significantly decreased inflammatory response following balloon dilatation to at least 2.5 times the material's original diameter. Implantation of balloon dilated ePTFE into adipose tissue resulted in an increased number of cells around and within the interstices of the polymer and an inflammatory response increasing with increasing balloon diameter, except at 18 mm, which was still significantly greater than control, but less inflammatory than 8, 10, and 12 mm. These results suggest that the healing response following deployment of endovascular grafts within the vasculature will be dependent on the difference between the original and final diameter of the graft material. This is significant when considering the design of endovascular grafts consisting of ePTFE. The material surrounding the stents must be engineered so that following deployment within a vascular environment, the healing response initiated maintains appropriate implant function providing the patient with the best possible chance of a successful vessel reconstruction.

One possible explanation for the differences observed in the healing response include changes in surface properties following balloon dilatation. For example, the polymers inherent hydrophobicity may be altered from manipulation and exposure of new material following the dilatation process. These variations in the surface hydrophobicity may result in an altered healing response following dilatation of ePTFE (Woerly et al., 1993; Hunt et al., 1997). Another possibility includes differential affinities for proteins following balloon dilatation (Leonard and Vroman, 1991). Again, as new material is exposed, different proteins may have a greater affinity for the surface of the polymer leading to an altered healing response as observed with these results. Furthermore, balloon dilatation may cause the release of impurities embedded within the polymer which influence the healing response
Further studies must be performed analyzing the surface microstructure and chemical characteristics to better understand the alterations of ePTFE following balloon dilatation.

The differences observed between tissue site of implantation and the healing response associated with polymeric materials have been demonstrated in previous studies (Williams et al., 1997). Results presented in this chapter suggest that the healing response associated with polymeric implants is dependent on both the physical characteristics of the material and the cellular environment surrounding the implant. Specifically, ePTFE implanted within subcutaneous tissue becomes less inflammatory following balloon dilatation while just the opposite occurred for implants placed within adipose tissue. These data suggest that biomedical devices must be evaluated within a system which mimics the healing response of the tissue the implant will reside in humans.

The hypotheses tested in this chapter are supported by the results. Following balloon dilatation, many of the physical properties of ePTFE are altered. Not only may these alterations affect the integrity of the material, but the data suggest they influence the healing response. Therefore, design of polymeric tubes for use in endovascular technology must consider the deployment process a probable cause of detrimental healing and design materials to counteract these effects.

The ultimate testing of a biomedical device must be performed in a tissue site similar to the intended site in the host. Endovascular grafts are deployed within the vascular system using catheters introduced at a distance from the lesion. The proceeding studies were performed to assess the healing associated with endovascular grafts in two vascular sites,
aortic and peripheral.
5. RABBIT MODEL FOR THE ANALYSIS OF THE HEALING ASSOCIATED WITH ENDOVASCULAR GRAFTS

Introduction

Treatment of medical complications with the least invasive protocol benefits the patient by expediting recovery and also decreasing the risk of complications. Furthermore, high-risk patients unable to undergo general anesthesia may benefit from minimally invasive surgical interventions requiring only local anesthesia. In this study, the potential of using stent, polymeric graft material, and cell transplantation technology as a new method for the treatment of vascular occlusive diseases was examined. The hypothesis to be tested is that endothelial cell transplantation can be used with endovascular grafts in an attempt to improve the patency rates. A corollary hypothesis to be tested was that endovascular grafts stimulate a foreign body reaction typical of biomedical implants.

New model development for biomedical device analysis requires optimization of surgical procedures to ensure reproducible experiments. In order to analyze healing associated with endovascular grafts for use in the treatment of occlusive disorders, the New Zealand white rabbit offers many advantages. First, the rabbit has been used extensively to model human atherosclerosis (Kritchevsky, 1974; Vesselinovitch, 1979; Jokinen et al., 1985) a major contributor to occlusive disorders. Second, the size of the rabbit’s abdominal aorta (4-6 mm) allows deployment of endovascular grafts utilizing interventional equipment used in human surgeries. Finally, the low cost of purchasing and maintenance of New Zealand white rabbits is more cost effective than large animal models (e.g. dog or pig) for studies
The use of stents for treating occlusive disorders is well documented in the literature. One common problem with the stenting of blood vessels remains restenosis following deployment. This may lead to failure of the device requiring a second procedure. Covering stents with polymeric materials may lead to improved long-term patency and improved healing characteristics by inhibiting the neointimal thickening commonly observed with metallic stent placement. Furthermore, certain surgical procedures may be vastly improved with the use of endovascular grafts. For example, cerebral occlusive disease (COD) requires major surgery and long recovery. Treatment of COD by carotid endarterectomy leads to the formation of a thrombogenic surface suggested to contribute to restenosis (Gagne et al., 1993; Stoney and String, 1976) and treatment by stent deployment leads to thromboembolic complications and late restenosis due to intimal hyperplasia (Becker, 1994). The use of polymeric grafts has not been considered in part due to the thrombogenic surface of the material. Our laboratory has hypothesized that the establishment of an endothelial cell lining on the luminal surface of polymeric grafts will be associated with a reduced thrombogenicity and produce a lining similar to that of a native artery (Williams et al., 1989). Previously, our laboratory has established methods for the transplantation of endothelial cells onto polymeric surfaces and this has been shown to create an antithrombogenic endothelial cell lining on the graft which is characteristically similar to the native lining of vascular vessels (Herring et al., 1978; Graham et al., 1979; Park et al., 1990; Sterpetti et al., 1988; Hunter et al., 1983; Allen et al., 1984). Endovascular grafts, which can be positioned and deployed via the femoral artery using only a local anesthetic, would not only decrease the risk to the patient, but would
also decrease the recovery time required by the patient.
Materials and Methods

Animal Selection and Welfare

All animal studies were performed following protocols approved by the University of Arizona Health Sciences Center animal care and use committee and according to the National Research Council “Guide for the Care and Use of Laboratory Animals” (1996). All surgeries were performed and animals housed in American Association for Accreditation of Laboratory Animal Care approved facilities.

Endovascular Grafts

Expanded polytetrafluoroethylene (ePTFE) was generously provided by Impra, Inc., Tempe, AZ and Palmaz balloon expandable stents (P188) were generously provided by Johnson & Johnson Corp. (Warren, NJ). Endovascular grafts were made by placing one suture (6-0 suture) tie through the thin wall ePTFE (3 mm i.d.) and around one of the Palmaz stents tines. The ePTFE was approximately 4 mm longer than the stent on the proximal end and 6 mm longer than the stent of the distal end.

Sodding of Endovascular Grafts

New Zealand white rabbits (10-12 lbs.) were pre-anesthetized with an intramuscular injection of xylazine/acepromazine/ketamine. Rabbits were then intubated and maintained on inhalation anesthesia using halothane. In order to harvest endothelial cells, a laparotomy was performed and approximately 20 g of uterine horn fat was removed and minced. To the minced fat, an equal volume of collagenase (4 mg/ml) was added and the mixture placed in
a flask in a 37°C shaking water bath for 30 minutes. The slurry was then placed in a centrifuge tube and centrifuged at 700 x g for 4 minutes. A resulting endothelial cell pellet formed in the bottom of the tube. This pellet was resuspended in sodding media [DMEM, 0.1% FBS (fetal bovine serum)] and again centrifuged at 700 x g for 4 minutes. The resulting pellet was resuspended in a volume of sodding media equal to the volume of the endovascular graft. The suspension was pipetted into the endovascular graft and four times the graft volume forced through the interstices of the graft. This forces the endothelial cells into the interstices of the ePTFE. The cells were permitted to interact with the ePTFE for 30 minutes before the endovascular graft was placed on the angioplasty balloon.

In Vitro Cell Viability Test

To determine if balloon dilatation of sodded ePTFE affects the viability of cells, 4 mm ePTFE was sodded, as described above, with microvessel endothelial cells derived from epididymal fat pads of male Sprague Dawely rats. Sodded ePTFE was balloon dilated using a 10 mm x 20 mm angioplasty balloon catheter (Meadox Medical, Oakland, NJ). The ePTFE was then cut into rings approximately 5 mm wide and placed into organculture medium [DMEM, 15% FBS, endothelial cell growth factor (ECGF), heparin, L-glutamine]. Following 0 hours, 1, 3, 5, and 7 days the samples were removed from culture and a Live/Dead Cytotoxicity Kit (Molecular Probes) was used to determine cell viability. The rings were cut on one side and placed flat between two glass microscope slides in order to expose the luminal surface. Using fluorescence microscopy, the number of live cells per unit area was determined using Metamorph Image Analysis software (Universal Imaging, West Chester,
PA) and a statistical comparisons made using a student t test (p ≤ 0.01).

Animal Surgery

New Zealand white rabbits were prepared for surgery as described above. Either the left carotid artery (n=2) or abdominal aorta (n=6) was exposed for introduction of the endovascular graft.

Left Carotid Artery: The neck was prepared for surgery by shaving and cleansing. The carotid artery was exposed and vessel loops placed around the artery to maintain control. Five minutes prior to arteriotomy, heparin (100 U/kg) was administered intravenously. An arteriotomy was preformed and an 11F introducer sheath placed within the vessel. The introducer was removed and the angioplasty balloon catheter with endovascular graft introduced into the vessel. The endovascular graft was to be placed, monitored by fluoroscopy, in the thoracic aorta just proximal to the diaphragm. However, due to the anatomy of the rabbits vasculature, this was never accomplished using the carotid artery as the entry site. The sheath was removed and the carotid artery ligated and the animals euthanized.

Abdominal Aorta: The abdomen was prepared for surgery by shaving and applying a cleanser. The abdominal aorta, just proximal to the iliac bifurcation, was exposed and vessel loops placed around the artery to maintain control. Five minutes prior to aortotomy, heparin (100 U/kg) was administered in a bolus intravenously and prostacycllin (85 ng/kg/min) administered intravenously through the marginal ear vein via an infusion pump for the duration of the surgery to prevent hind limb paralysis due to spinal cord ischemia. An
arteriotomy, perpendicular to blood flow, was performed and an 11F introducer sheath placed within the vessel. The introducer was removed and the angioplasty balloon catheter with endovascular graft introduced into the vessel. The endovascular graft was placed, monitored by fluoroscopy, in the thoracic aorta just proximal to the diaphragm. The angioplasty balloon was inflated (12 atm) until the stent was fully deployed. The angioplasty balloon was then deflated and removed. Following removal of the angioplasty balloon catheter, the sheath was removed and the aorta closed using 6-0 suture and blood flow established. The abdomen was closed in two layers using 2-0 suture. The rabbits were allowed to recover and returned to the animal facility.

**Explant of Endovascular Graft**

Endovascular grafts were removed following 24 hours (n=3) and 48 hours (n=2), or 5 weeks (n=1, sodded graft). Rabbits were pre-anesthetized with an intramuscular injection of xylazine/acepromazine/ketamine. Rabbits were then intubated and maintained on inhalation anesthesia using halothane. The rabbits abdomen and neck area were prepared for surgery by shaving and cleansing with aseptic spray. The left carotid artery was exposed and cannulated with an 18 gauge catheter. Contrast medium was injected, and using fluoroscopy, aortic vessel patency determined at both the endovascular graft placement site and site of introduction (abdominal aorta). Next, the abdomen and chest were opened, endovascular graft isolated, removed, cut in half longitudinally, and half placed into Histochoice™ fixative (AMRESCO, Solon, OH) while the other half was placed into 3% buffered glutaraldehyde. The rabbit was euthanized by an intracardiac injection of euthasol.
Histological Analysis

All grafts were processed through graded alcohols, followed by xylene, and finally paraffin embedded. Samples were sectioned (6-8 µm thick), and dried onto poly-L-lysine coated slides. Sections were deparaffinized, rehydrated and stained with hematoxylin and eosin or immunocytochemical markers.

Immunocytochemistry

Sections were reacted with RAM11 primary antibody, specific for rabbit macrophages, and visualized using peroxidase-conjugated secondary antibody. Nuclei were lightly stained using methyl green. Sections were evaluated using a Nikon Optiphot microscope with a 40x water immersion lens.

Scanning Electron Microscopy

Samples placed into 3% buffered glutaraldehyde were processed through graded acetone, critical point dried, and sputter coated using a gold target. The samples were evaluated, and photomicrographs obtained using a JOEL 820 scanning electron microscope.
Results

In Vitro Cell Viability

After transplanting endothelial cells onto the luminal surface of ePTFE vascular grafts, the grafts were balloon dilated and the number of live cells remaining on the surface quantified over a 7 day period. As demonstrated in figure 5.1, there were no significant decreases at any time-point in cell viability of the sodded balloon dilated graft when compared to sodded control graft. Both control and balloon dilated grafts lost a large proportion of cells on day 0, most likely due to poor attachment of the cells to the polymers surface. However, over the next 7 days, the remaining cells on the grafts surface proliferated to near a confluent density.

Access via Left Carotid Artery

Upon access of the left carotid artery, an 11F introducing sheath was placed within the lumen of the vessel. The use of a guide-wire was required to position the endovascular graft within the thoracic aorta. After the guide-wire was successfully placed within the aorta, the endovascular graft was introduced through the introducing sheath into the left carotid artery. When the polymeric material left the sheath and was within the lumen of the vessel, forward progress was impossible due to the size difference between the ePTFE and the vessel wall. Furthermore, upon reaching the carotid artery-aortic arch junction with the balloon catheter, the angle between the two vessels proved too great for placement of the
FIGURE 5.1 Scatter-plot of cell density on the luminal surface of ePTFE sodded with microvessel endothelial cells and then cultured in vitro for up to 7 days. (●) control ePTFE and (▼) balloon dilated ePTFE. No significant difference was observed in cell density following balloon dilatation (p≤0.05).
endovascular graft within the thoracic aorta. Due to the size and inflexibility of the stent, placement of the endovascular graft within the thoracic aorta via the left carotid artery was not possible.

Access via Abdominal Aorta

An 11F introducing sheath was easily placed within the abdominal aorta (figure 5.2). Care was taken to not damage the arteries that feed the spinal cord and branch off the aorta at the introduction site. The endovascular graft could be easily placed within the thoracic aorta just above the diaphragm, deployed, and the angioplasty balloon catheter and introducing sheath removed within 2 minutes without the use of a guidewire (figure 5.2). The aorta was reapproximated using 6-0 suture by first securing both lateral sides of the vessel with a tie. Then each consecutive suture was placed to decrease the opening in the aorta. When the final suture was placed, blood flow was reestablished. This method of closure provided the quickest closure, most optimal reapproximation of the aorta, and greatest lumen diameter after reestablishment of blood flow. Following the implantation period, the endovascular graft was located within the thoracic aorta and removed.

Histology and Immunocytochemistry

At 24 hours following deployment, the luminal surface of the ePTFE was covered with a fibrin clot approximately 150 μm thick. Within this fibrin matrix, many PMNs were observed (figure 5.3). No cells or matrix were observed within the interstices of the ePTFE. Furthermore, no RAM11 positive cells (macrophages) were associated with the implants.
FIGURE 5.2  Photographs showing procedure for the placement of endovascular grafts into the thoracic aorta via the abdominal aorta.  a) an 11 french introducing sheath (arrow) was placed within the lumen of the abdominal aorta and the angioplasty balloon catheter was positioned in the aorta via the introducing sheath and the endovascular graft deployed in the thoracic aorta.  Bar = 3 mm.  
b) fluoroscopic image showing endovascular graft (arrow) following deployment in the thoracic aorta.  Bar = 8 mm.
FIGURE 5.3  Light micrographs of hematoxylin and eosin (a,b,c) and RAM11 (d) stained sections of endovascular graft implanted for a) 24 hours, b) 48 hours and c,d) 5 weeks.  a,b,c: P = polymer, arrows = PMN, F = fibrous clot, M = tunica media, N = neointima.  d: P = polymer, arrows = macrophages, N = neointima.  Bar = 20 μm for a, 10 μm for b, and 40 μm for c,d.
The abluminal side of the ePTFE was pressed against the native blood vessel wall. PMNs were observed at the native vessel tunica media and adventitia.

Forty-eight hours post deployment, the fibrin clot on the luminal surface had partially resolved. Between the stent tines, the clot was approximately 40 μm thick, while around the tines the clot was 150 μm thick. PMNs were observed within the fibrin clot, but at a lower density than observed at 24 hours (figure 5.3). Within the ePTFE interstices, PMNs and red blood cells were observed. On the abluminal side of the ePTFE, PMNs and inflammatory cells were observed separating the polymer from the native vessel wall and at the tunica media-adventitia interface.

Five weeks after deployment of the sodied endovascular graft, dramatic neointimal thickening, approximately 270 μm thick, had occurred on the luminal side of the implant (figure 5.3). The neointimal tissue closest to the ePTFE was composed of many nucleated cells, of these approximately 10% were RAM11 positive (macrophages). The neointimal tissue closest to the blood flow surface was composed of extracellular matrix with nucleated cells. Within these areas were regions which demonstrated either a hyperplastic response or a hypertrophic response. The abluminal side of the implant had many cells at the material-tissue interface. Approximately 20% of these cells were RAM11 positive (macrophages) (figure 5.3). Characteristic of the chronic inflammation associated with polymeric implants, foreign body giant cells were observed associated with the abluminal surface. The native vessel tunica media was disrupted and was infiltrated with many inflammatory cells.
Scanning Electron Microscopy

Following 24 hours, the stent surface was entirely covered with platelets while the ePTFE surface was sparsely covered with platelets, fibrin clot, and red blood cells (figure 5.4). After 48 hours, the stents and ePTFE are completely covered with fibrin clot. The five week sodded implant appeared to have an endothelial cell surface partially covered with fibrin clot (figure 5.4). Furthermore, it appeared that the thickened region had areas of dissection between the neointima and ePTFE allowing blood flow around the neointima (figure 5.4).
FIGURE 5.4 Scanning electron micrograph of the luminal surface of endovascular grafts implanted within the rabbit aorta. While the luminal surface of the a) 24 and 48 hour implants are covered by primarily red blood cells, platelets and fibrin, the b) 5 week implant was completely covered with what appeared to be a confluent endothelial cell layer (arrow, ePTFE). Bar = 2 μm for a and 200 μm for b.
Discussion

Endovascular grafts have been suggested for the treatment of aneurysms, occlusive diseases, and other vascular complications. Depending on the application, an appropriate model must be delineated to best approximate the healing associated with these devices. The New Zealand white rabbit has been used extensively as a model of human cardiovascular disease (Kritchevsky, 1974; Vesselinovitch, 1979; Jokinen et al., 1985). Cerebrovascular occlusive disease (COD), one such disorder due to luminal narrowing of the carotid arteries, can be treated by the introduction of an endovascular graft to reestablish adequate flow and thereby prevent a stroke. Therefore, we chose the New Zealand white rabbit to model the healing associated with endovascular grafts.

The current accepted treatment for cerebrovascular occlusive disease (COD) is carotid endarterectomy. Complications related with this procedure include perioperative technical errors that may lead to stroke, and following recovery, late recurrent stenosis. It has been suggested that late recurrent stenosis is due to damage inflicted to the intima during carotid endarterectomy. Other alternative therapies have been suggested, for example, balloon peripheral transcutaneous angioplasty (PTA) and intravascular stenting. However, neither offer added benefits since both have associated complications: balloon PTA-fear of embolic complications, and intravascular stenting-thrombosis, thromboembolic complications, and late restenosis due to hyperplasia. Our success with cell transplantation onto polymeric surfaces has lead us to utilize this technology for the treatment of COD and also reduce the occurrence of late recurrent stenosis. Introduction of an endothelial cell monolayer onto the endovascular grafts surface eliminates the inherent thrombogenicity of the polymer and may
prevent failure of the device due to later recurrent stenosis.

Initially, it had to be determined if balloon dilatation of cell transplanted ePTFE resulted in the loss of the cellular lining. Cells may be damaged or killed during inflation of the balloon and scrapped from the surface with the balloons removal. At all time points, no significant difference in the number of viable cells found on the luminal surface of sodded grafts was found between control and balloon dilated groups. These data demonstrate that balloon dilatation does not result in the loss of a significant number of cells due to the ballooning procedure. Additionally, these data support the hypothesis that ePTFE can be sodded with endothelial cells and these cells will proliferate to form a confluent monolayer of cells on the luminal surface.

The placement of endovascular grafts within the vascular requires access via a blood vessel that will accommodate the introducing apparatus. An 11F introducing sheath was required for this procedure, therefore the carotid arteries and the abdominal aorta were chosen and possible access sites. The left carotid artery, although large enough to accommodate the introducing sheath, would not permit easy movement without massive damage to the vessel of the endovascular graft through the vessel. Furthermore, the stent could not be directed beyond the aortic arch-carotid artery junction. The abdominal aorta proved much more successful. This vessels large diameter accommodated the introducing sheath and offered no resistance to placement of the endovascular graft within the thoracic aorta. One critical aspect of using the abdominal aorta distal to the renal arteries was the level of vessel occlusion during the procedure. The rabbit has been used as a model of spinal cord ischemia since clamping the aorta distal to the renal arteries prevents blood flow to the
arteries feeding the lower spinal cord. When placing vessel loops around the aorta, caution had to be taken to be distal to these arteries feeding the spinal cord. For further protection, prostacyclin has been demonstrated to protect against spinal cord ischemia and therefore this prostaglandin was administered.

The healing associated with the endovascular grafts followed the current dogma of polymer healing characterized by acute thrombogenicity and chronic inflammation. In the experiments, after 24 hours, the luminal surfaces were covered with fibrin clots infiltrated by PMNs. After 48 hours, these fibrin clots had partially resolved and PMNs were still present. PMNs were also observed within the interstices of the polymer and associated with the abluminal surface of the implant. Long-term implantation of the endovascular graft resulted in neointimal thickening and chronic inflammatory cells, macrophages and foreign body giant cells, associated with the polymer. This neointimal thickening may be due to secretion of cytokines from macrophages associated with both the luminal and abluminal surface of ePTFE.

The results demonstrated that the rabbit model can be used as a model for endovascular graft healing. Deployment via the abdominal aorta can be achieved with acceptable recovery of the animal. The hypotheses tested were supported by the data. The healing associated with these implants follows the current dogma of polymeric healing in a vascular site. Finally, the data demonstrated the efficacy of sodding endovascular grafts with endothelial cells to possibly improve the biocompatibility of polymeric implants.
6. EVALUATION OF ENDOVASCULAR GRAFTS AND STENTS IN THE ILIAC ARTERY OF PIGS. RELATIONSHIP BETWEEN INFLAMMATORY RESPONSE AND NEOINTIMAL THICKENING

Introduction

In 1969, Dotter introduced endovascular therapy for occlusive vascular disease (Dotter, 1969) and since then, various designs have been developed for use in endovascular treatment. Currently, balloon angioplasty is the most commonly used endovascular therapy. Modifications of angioplasty include deployment of metallic stents during the procedure to mechanically open occluded blood vessels. Neointimal thickening has been associated with metallic stent deployment leading to failure of a majority of implants. Surrounding the stent with polymeric material has been suggested as a means to help prevent the growth of cells around and over stents thereby preventing neointimal thickening. However, vigorous testing of these devices using this design has not demonstrated any significant reduction in neointimal thickening. The hypothesis was that macrophages and foreign body giant cells associated with polymers used in the design of endovascular grafts are partly responsible for the neointimal thickening observed on the luminal surface. The objectives of this study were to evaluate and characterize the healing response associated with endovascular grafts and endovascular stents.

Very little data have been reported on the inflammatory response associated with endovascular grafts. Most studies have focused on the luminal healing characteristics and the efficacy of using these devices for the treatment of aneurysmal disorders and vascular disease.
Marin et al. (1995) is one of the only publications to address the abluminal healing characteristics associated with transluminally placed endovascular grafts in humans. All of the grafts analyzed were reported to having an inflammatory response associated with the abluminal surface of the graft material. While the authors suggest that the inflammatory response may in part control the anastomotic healing, no suggestion to an involvement in neointimal thickening was made. The following study was performed to compare the healing associated with endovascular grafts to endovascular stents. The hypothesis tested was that the inflammatory response promotes neointimal thickening commonly observed with vascular prosthetics.
Materials and Methods

Animal Selection and Welfare

All animal studies were performed following protocols approved by the University of Arizona Health Sciences Center animal care and use committee and according to the National Research Council "Guide for the Care and Use of Laboratory Animals" (1996). All surgeries were performed and animals housed in American Association for Accreditation of Laboratory Animal Care approved facilities.

Endovascular Grafts and Stents

Expanded polytetrafluoroethylene (ePTFE) grafts were generously provided by Impra, Inc. (Tempe, AZ) and Palmaz balloon expandable stents (P204 and P188) were generously provided by Johnson & Johnson (Warren, NJ). Endovascular grafts were constructed by suturing (7-0 suture) thin wall ePTFE (3 mm i.d.) to Palmaz stents supporting each end of the ePTFE (6 cm in length). The proximal stent protruded approximately 3-4 mm from the lumen of the material while the ends of the distal stent matched the end of the graft. Approximately 2.5 cm of the ePTFE was not supported by the stents. Endovascular stents used for this study were Palmaz P308 or P294 generously provided by Johnson & Johnson. These stents when deployed to 8 mm measure approximately 2.5 - 3 cm in length.

Medication

Ten domestic swine (75-85 kg) were used in this study. One day prior to surgery, each swine was started on aspirin (325 mg) and maintained on this regimen for the duration
of the study.

Endovascular Graft and Stent Deployment

Each swine was anesthetized using ketamine/xylezine/acepromazine and maintained using isoflurane. The left common carotid was isolated for introduction of the endovascular graft and stent. Each swine received heparin (5000 U) 5 minutes prior to cannulation of the artery. The left common carotid was cannulated and a 0.035 inch guidewire introduced. Over the guidewire, an 11F introducing sheath was placed into the lumen of the carotid. The endovascular grafts or stents were mounted on an 8 mm x 40 mm angioplasty balloon catheters (Olbert, Medi-Tech, Boston, MA) and introduced into the carotid through the introducing sheath. Arteriograms were performed at the aortic bifurcation using a c-arm fluoroscope to position the endovascular graft and stent in opposite iliac arteries. After positioning of the endovascular graft or stent, the device was deployed within the iliac artery. Following deployment of both devices, a second arteriogram was performed to verify patency and success of the deployment process. The angioplasty catheter, introducing sheath, and guidewire were then removed and the left common carotid ligated. The neck was closed in two layers and the animals allowed to recover.

Explant

Endovascular grafts and stents were retrieved following 1, 5, and 12 weeks. Swine were anesthetized and maintained on isoflurane for the entire explant procedure. A midline laparotomy was used to gain access to the endovascular devices. Prior to removal of the
implants, the abdominal aorta was cannulated and an arteriogram performed to assess patency. Heparin (5000 U) was then administered and the endovascular graft removed with the attached aorta and iliac vessel. The explant was gently irrigated with DCF-PBS containing 0.1% BSA. The samples were then divided longitudinally with one half placed in Histochoice™ fixative and the other half placed in 3.0% buffered glutaraldehyde. Gross morphological assessment was performed at this time.

Scanning Electron Microscopy

Samples fixed in glutaraldehyde were prepared for SEM by dehydrating through a graded series of acetone, critical point drying, and sputter coated using a gold target. Samples were mounted on aluminum stubs and examined on a JEOL-820 scanning electron microscope.

Histology

The stents were dissected away from the samples fixed in Histochoice™ fixative and then dehydrated through a graded series of alcohol to xylene and paraffin embedded. Samples were sectioned (6-8 μm) and dried onto glass slides. Sections were then stained with hematoxylin and eosin and Masson’s tri-chrome or reacted with von Willibrand factor (vWF) antibody or smooth muscle cell (smc) alpha actin antibody. vWF and smc alpha actin were visualized using a horseradish peroxidase-conjugated secondary antibodies and nuclei lightly stained using methyl green.
Results

All endovascular devices were successfully deployed. One pig died shortly after surgery, presumably due to a reaction to the anesthesia.

Patency and Gross Examination: Endovascular Grafts

After one week, only 1 of 3 endovascular grafts was patent (Table 6.1). One of the occluded grafts was retrieved from a pig suffering from severe pneumonia. The other occluded graft was from a pig that had acutely thrombosed the graft prior to deployment. The graft was made patent by maceration of the clot using the guide wire, however, suboptimal flow was established following this procedure. Upon gross examination, red thrombus was observed on the surface of the patent endovascular graft.

After 5 weeks, 2 of 3 endovascular grafts were patent (Table 6.1). One of the patent grafts had mid-graft luminal narrowing due to recoil of the ePTFE following the deployment process. A translucent white layer covered the stents of the patent endovascular grafts, presumably an endothelial layer.

After 12 weeks, 3 of 3 endovascular grafts were patent (Table 6.1). Again, narrowing at the mid-graft region was observed, probably due to recoil following the deployment procedure. The entire surface of the endovascular grafts were covered by a white layer, again presumably endothelial cells.

Patency and Gross Examination: Endovascular Stents

Two of three stents were patent following one week. The occluded stent was
Table 6.1. Summary of patency and complications associated with endovascular grafts and stents.

<table>
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<th>Time</th>
<th>Implant</th>
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<th>Percentage Patent</th>
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<td>2</td>
<td>33</td>
<td>Pneumonia, acute thrombosis</td>
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<td>stents</td>
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explanted from the pig suffering from severe pneumonia. Whether this had any effect on patency is unknown. Red thrombus was observed on the metallic tines of the patent stents.

At 5 weeks and 12 weeks following implantation all of the stents placed within the iliac arteries were patent. A glistening white layer almost completely covered the tines of the 5 week explants, while the 12 week explants exhibited a similar layer covering the stents completely.

Scanning Electron Microscopy

Pannus ingrowth of endothelial cells was observed on the one week endovascular graft explants (figure 6.1). The rest of the surface was covered by platelets on fibrin. Stents implanted for 1 week demonstrated fibrin and platelet deposition between the tines (figure 6.1). The entire surface of 5 week endovascular graft and stent and 12 week endovascular
FIGURE 6.1  Scanning electron micrograph of luminal surface of endovascular grafts implanted for a) 1 week, b) 5 weeks and c) 12 weeks. Only the 5 and 12 week implants demonstrated the formation of a complete endothelial cell layer. Platelets and fibrin were observed on the surface of 5 week implants suggesting activation of the endothelial cells. Bar = 10 μm.
stent implants was covered by endothelial cells (figure 6.1). Associated with areas of the monolayer on endovascular grafts were regions of white blood cells, platelets and fibrin adhering to the surface (figure 6.1). Twelve week endovascular graft explants were completely endothelialized and no white blood cells, platelets, or fibrin was observed adhering to the monolayer (figure 6.1).

Histology

Following one week, the luminal surface of endovascular grafts was covered predominantly with fibrin into which PMNs had migrated (figure 6.2). The tunica media of the native vessel was associated with the abluminal surface of the ePTFE. Where the material had buckled, an inflammatory response was observed between the abluminal surface of the ePTFE and the internal elastic membrane (figure 6.2). The tunica adventitia appeared disrupted and inflamed. Following 1 week of implantation, a neointima had formed (figure 6.2). This neointima was approximately 100 μm thick around the stents and 50 μm thick between the tines. There were mononucleated cells, presumably macrophages associated with the metallic tines.

Following 5 weeks, neointimal thickening (100 μm thick) was observed on the luminal surface of endovascular grafts and stents (figure 6.3). Where the graft had buckled, this neointima was approximately 450 μm thick. The neointima was composed of smooth muscle cells covered by an endothelial monolayer (figure 6.3). Associated with endovascular graft polymer on the luminal side were inflammatory cells and foreign body giant cells (figure 6.3).
FIGURE 6.2 Light micrograph of hematoxylin and eosin stained sections of endovascular grafts (a,b) and endovascular stents (c) implanted for 1 week. a) the luminal surface was covered by fibrin incorporated with PMN leukocytes (arrows). Bar = 20 μm. b) an inflammatory response was observed between the abluminal surface of the ePTFE and the internal elastic membrane (arrowhead) of the iliac artery. Bar = 10 μm. c) the neointima of endovascular stents was approximately 50 - 100 μm thick. Mononuclear cells were associated with the metallic tines (arrows). Bar = 40 μm. P = polymer, M = tunica media, N = neointima.
FIGURE 6.3  Light micrograph of hematoxylin and eosin stained sections of endovascular grafts (a, b) and endovascular stents (c) implanted for 5 weeks.  a) the luminal surface was covered by a neointima approximately 100 - 450 μm thick.  Bar = 10 μm.  b) an inflammatory response separated the abluminal side of the polymer from the tunica media.  Bar = 10 μm.  c) the neointima of stents was approximately 100 - 300 μm thick.  A mild inflammatory response was associated with the metallic tines (arrows).  Bar = 20 μm.  P = polymer, N = neointima, M = tunica media, S = stent space.
On the abluminal side, inflammatory tissue separated the ePTFE from the tunica media (figure 6.3). This inflammatory tissue was composed of smc alpha actin positive cells, macrophages, and vWF positive cells. The inflammatory response within the tunica adventitia had resolved. Stents explanted after 5 weeks exhibited a neointima approximately 300 μm thick around the tines and 100 μm thick between the tines (figure 6.3). A mild inflammatory response was associated with the metallic tines.

Following 12 weeks, the neointimal thickening associated with the endovascular grafts was approximately 1000 μm thick where the graft had buckled and 300 μm thick elsewhere (figure 6.4). The neointima was composed of smc alpha actin positive cells covered by vWF positive cells. No inflammatory response was observed associated with the luminal side of endovascular grafts. However, the abluminal surface of the endovascular graft had either 1) an intense inflammatory response consisting of foreign body giant cells and macrophages (figure 6.4), or 2) a mild inflammatory response and large neovessels between the tunica media of the native vessel and the ePTFE (figure 6.4). The neointima associated with the mild inflammatory response was only 300 μm where the graft buckled and 50 μm thick elsewhere. The neointimal thickening associated with endovascular stents was more uniform in thickness (120 μm) throughout the length of the stent (figure 6.4). No significant inflammatory response was observed with the endovascular stents.
FIGURE 6.4 Light micrograph of hematoxylin and eosin stained sections of endovascular grafts (a,b) and endovascular stents (c) implanted for 12 weeks. The neointima of endovascular grafts was 50 - 1000 μm thick depending on the intensity of the inflammatory response and buckling of the graft. a) the neointima of highly inflammatory endovascular grafts was 300 - 1000 μm thick. Bar = 40 μm. b) the neointima of the mildly inflamed endovascular graft was only 50 - 300 μm thick. Bar = 40 μm. c) the neointima of endovascular stents was approximately 120 μm thick. Bar = 40 μm. P = polymer, M = tunica media, N = neointima, S = stent space.
Discussion

Wound healing involves several temporally coordinate cellular responses which include acute and chronic inflammation, granulation tissue formation, and extracellular matrix deposition leading to scar formation (Clark, 1996). The coordination of these responses, both temporally and with respect to intensity, lead to the development of new tissues similar in structure and function to the original tissues. Upon material implantation within a biological tissue a primary inflammatory response, similar to normal wound healing, is observed (Anderson, 1988; Schreuders et al., 1988). However, polymeric materials have been shown to exacerbate the inflammatory response at the implant site leading to a foreign body reaction and the formation of foreign body giant cells. Placement of synthetic materials, specifically vascular grafts, results in an altered healing response which differs in intensity as well as duration (Anderson, 1988). Artificial blood vessels fail predominantly due to thrombogenicity of the polymer and anastomotic neointimal thickening. In this study, endovascular grafts and stents were evaluated in a temporal fashion to evaluate the luminal and abluminal healing characteristics.

Endovascular stenting has been one of the most important advances in interventional therapy since balloon angioplasty. When placed properly stenting resists elastic recoil of the vessel wall and reestablished blood flow to ischemic tissue. However, late restenosis caused by intimal thickening occurs in 50% of femoral and coronary placed stents. The restenosis may be due to both response to injury and inflammatory cells associated with the stents. Bai et al. (1994) observed macrophages associated with stents placed in rabbit aortas for two months. However, they suggest that the macrophages were not playing a significant role in
neointimal thickening since proliferating smooth muscle cells were not associated with the macrophages. They later suggest that the neointima formed contains significant amounts of extracellular matrix secreted by both smooth muscle cells and endothelial cells. The authors do not suggest that the macrophages may be responsible for stimulating these cell types to produce matrix. The hypothesis was that macrophages associated with biomedical implants have the potential to stimulate extracellular matrix production by cells through cytokine induced pathways. In the third chapter of this dissertation, it was demonstrated that macrophages associated with polymeric materials express various cytokine message, many of these having been demonstrated to stimulate matrix production by cells.

In this study, endovascular stents removed after 1 week had neointimal thickening (100 μm) around the stents while between the tines thickening was only 50 μm. Furthermore, the inflammatory response was limited to the stent region suggesting a correlation to inflammation and neointimal thickening. A similar pattern was observed with the stents removed after 5 weeks. Around the stents the neointimal thickening was approximately 300 μm while between the stents the thickening was only 100 μm. Again, the inflammatory response was limited to the stent region. At 12 weeks implantation, the neointimal thickening was more uniform in thickness (120 μm) and no significant inflammatory response was observed.

Endovascular grafts healed in a somewhat different manner. Endovascular grafts removed after 1 week had red thrombus (100 μm thick) with infiltrated PMNs on the luminal surface of the material. An inflammatory response was observed on the abluminal surface
only where the graft material had buckled and in the tunica adventitia. After 5 weeks, the intimal thickening was approximately 450 \( \mu \text{m} \) thick in regions where the graft had buckled and 100 \( \mu \text{m} \) thick elsewhere. An intense inflammatory response was associated with the luminal and abluminal surfaces. Following 12 weeks implantation, the neointima was over 1000 \( \mu \text{m} \) thick where the graft had buckled and 300 \( \mu \text{m} \) thick elsewhere. An intense foreign body reaction characterized by macrophages and foreign body giant cells was observed associated with the abluminal surface. However, one of the 12 week implants did not have an inflammatory response associated with the abluminal surface. Instead, neovessels and loose connective tissue was observed between the ePTFE and the tunica media of the native vessel. Interestingly, the neointima was only 300 \( \mu \text{m} \) thick where the graft had buckled and 50 \( \mu \text{m} \) elsewhere. These data support the hypothesis tested and suggest a correlation between the inflammatory response and neointimal thickening.

In this study, the endovascular stents healed in a more favorable fashion, however, endovascular grafts may have more potential for treatment using tissue engineering. The placement of endothelial cells on the luminal surface of polymeric vessels has demonstrated limited success. The endovascular graft may benefit from tissue engineering. The major drawback to the covering of stents with polymeric materials may be the increased inflammatory response due to the polymer. Decreasing the inflammatory response through tissue engineering and/or polymer modification may lead to more appropriate healing responses following endovascular graft placement.
Four overriding hypotheses were tested to determine the influence macrophages play in the healing associated with synthetic vascular prostheses. The first hypothesis stated that synthetic polymers alter the normal healing response elicited by the host. Evidence presented in chapters 2, 5, and 6 support this hypothesis. Eight different synthetic biomedical polymers used in the manufacture of vascular prostheses were implanted in a rat wound healing model to evaluate the healing characteristics associated with biomedical polymers. Furthermore, endovascular grafts were evaluated to determine healing characteristics in a vascular position. Each of the polymers in every position stimulated a chronic inflammatory response characterized by activated macrophages and foreign body giant cells. Normal wounds were not distinguishable from normal tissue by the 5 week time point. During these studies a relationship between inflammation and neovascularization was determined. From these results, a second hypothesis was developed and tested. Synthetic polymers elicit different macrophage responses depending on the chemical and physical structure of the polymer and these responses influence the neovascularization of the material. Support for this hypothesis was presented in chapters 2 and 4. Those polymers which elicited a highly intense inflammatory response had very few blood vessels associated with the polymeric interface. Furthermore, changing the structural characteristics of the polymer (e.g. internodal distance or nodal width) affected the inflammatory response associated with the implants. To determine which factors may be playing a role in the healing associated with polymers, RNase protection assays and in situ hybridization techniques were utilized in testing the third
hypothesis: angioactive cytokines are released by macrophages in response to synthetic polymers. Results presented in chapter 3 support this hypothesis. Every polymer evaluated using the RNase protection assay stimulated cells to express the message for IL-1β, TNF-α, IL-10, and IL-6 cytokines. Other cytokine message expressed include IL-1α, IL-4, and IFN-γ. Many of these cytokines have been demonstrated to influence angiogenesis and stimulate the formation of foreign body giant cells characteristically associated with biomedical implants. To determine a possible role of the products produced by cells surrounding implants and angiogenesis, a fourth hypothesis was tested: products released by cells associated with biomedical implants influence angiogenesis. Evidence for this hypothesis is reported in chapter 3. Wound fluid collected within the lumen of tubular implants increased the proliferation rates of endothelial cells in in vitro two-dimensional culture. These results suggest the inflammatory response may stimulate angiogenesis, however, very few microvessels were ever observed within the interstices of the polymers. Therefore, the polymers structure may be a limiting factor in transmural migration of endothelial cells. The observations presented in this dissertation allow insight into the healing characteristics associated with synthetic polymeric materials.

Currently, there is a general lack in information pertaining to the mechanisms responsible for the healing associated with the abluminal surface of synthetic vascular grafts. Most studies concerning vascular grafts only rate the inflammatory response associated with the abluminal surface using an arbitrary scale (Lee et al., 1997; Marin et al., 1995; Mary et al., 1997; Hirabayashi et al., 1992; Kadoba et al., 1992). This dissertation, in an attempt to
delineate some of these mechanisms, presents evidence supporting the involvement of inflammatory cytokines in mediating both angiogenic and anastomotic neointimal thickening responses. Previous studies concerning synthetic vascular grafts have been directed toward enhancing spontaneous endothelialization and eliminating neointimal thickening (Nagae et al., 1995; Kohler et al., 1992; Kraiss et al., 1991; Kohler et al., 1991; Golden et al., 1990; Kadoba et al., 1992; Hirabayashi et al., 1992; Clowes et al., 1986b). The majority of these studies have used trial-and-error strategies in an attempt to obtain successful outcomes. The studies presented in this dissertation may be the first to examine the involvement of abluminal healing characteristics and the role inflammatory cytokines play in the angiogenic and anastomotic healing response associated with synthetic vascular grafts.

The initial experiments examined the tissue response to synthetic vascular graft materials, both clinically available and experimental. The most critical healing characteristics evaluated were fibrous encapsulation, inflammation, and neovascularization in two tissue sites, subcutaneous and adipose. Historically, rat subcutaneous implants have been the standard for characterization of the healing response associated with biomedical polymers. This site may be adequate for biomedical devices implanted within tissues that histologically resemble subcutaneous tissue of the rat. However, the majority of polymeric vascular conduits used in human cases are tunneled through subcutaneous tissue between the anastomosis. Human subcutaneous tissue is composed predominantly of adipocytes and does not resemble rat subcutaneous tissue (Williams et al., 1997). In an effort to maximize histological similarities, the epididymal fat pads of the rat were chosen. Histologically, this site resemble human subcutaneous tissue and is therefore a better model for healing associated
with polymeric vascular grafts (Williams et al., 1997). When comparing the healing characteristics of the polymeric material between the two sites many differences were observed. Mainly, subcutaneous implants were more prone to become surrounded by a dense fibrous capsule, less inflammatory, and had fewer blood vessels associated with the polymer when compared to implants within adipose tissue. However, regardless of site of implantation, all polymers stimulated a foreign body reaction characterized by the association of giant cells with the implants.

Not only was site of implantation an important factor in predicting the healing response, but the chemical and physical characteristics of the polymer played a fundamental role. Most notably were the differences observed in the inflammatory response between types of polymers. The Dacron™ implants generally had more activated macrophages and foreign body giant cells associated with the fibers when compared to ePTFE materials. One exception to this was Golaski microknit Dacron™ which was significantly less inflammatory when compared to the other Dacron™ materials. The manufacturing process of this material remains proprietary. To examine physical properties, samples of ePTFE having three different internodal distances (30 μm, 60 μm, 100 μm) were evaluated. Intuitively, the most porous graft (greatest internodal distance) would have the greatest number of blood vessels associated with the implant. However this was not the result. The 60 μm ePTFE had the greatest numbers of vascular elements and the least number of inflammatory cells associated with the polymer regardless of tissue site of implantation. Unexpectedly, neither the 60 μm or 100 μm ePTFE when implanted within subcutaneous tissue became encapsulated by a
dense connective tissue matrix. The mechanisms responsible for these differences remain unknown. Regardless of the material type or structure, one general trend was observed for all polymeric implants in both tissue sites. Those which stimulated an intense inflammatory response had few blood vessels associated with the polymer-tissue interface. This lead to the hypothesis that inflammatory cells, predominantly activated macrophages and foreign body giant cells, secreted factors in close approximation to the polymers that were angioinhibitory. It was noted, however, that anywhere from 50-100 μm from the inflammatory capsule that blood vessels were observed in great abundance. This lead to the hypothesis that the products released by inflammatory cells associated with polymeric implants were inhibitory to the growth of blood vessels near the implants. However, after these products had diffused away from the polymer, the concentration of the products became such that angiogenesis was the net effect. This lead to the formation of blood vessels at this distance from the polymer. Regardless the products released from these cells remain unknown.

Other studies have evaluated the products produced by monocytes cultured with biomedical polymers in vitro (Cardona et al., 1992; Bonfield and Anderson, 1993; Bonfield et al., 1992a; Bonfield et al., 1992b; Miller and Anderson, 1989; Bonfield et al., 1991). These experiments have demonstrated the capability of the monocyte to modulate the concentrations of cytokines in response to polymeric implants. Fewer studies have examined which products can be released (Eriksson and Thomsen, 1991; Lundberg et al., 1995) or expressed (Goodman et al., 1996; Hunt et al., 1996) by inflammatory cells associated with biomedical polymers in vivo. However, as pointed out in chapter 3, the titanium cage implant model may add a variable to the experiment (Rosengren et al., 1996). Regardless, these studies
demonstrated that cells associated with biomedical polymers in vivo secrete similar products. In an attempt to determine a greater number of inflammatory cytokines and compare expression levels RNase protections assays were performed. This assay allows for the evaluation of 11 different cytokine expression levels in one experiment. This tool is important when considering the importance of both synergistic and opposing effects of products released at a wound site. The results from experiments in this dissertation demonstrate that several inflammatory cytokines are expressed in tissues surrounding polymeric implants that include IL-1β, TNF-α, IL-10, IL-6, IL-4, IL-1α, and IFN-γ. Interestingly, regardless of polymer type, similar expression and levels were observed for these messages. Still, the importance these cytokines play in the healing response must not be underplayed. IL-1β has been demonstrated to stimulate both smooth muscle and endothelial cell proliferation, increase extracellular matrix deposition by cells, upregulate the expression of adhesion molecules and tissue factor on endothelial cells (Dinarello, 1994) all important factors involved in the healing associated with polymeric implants. Possible roles for the other cytokines are: TNF-α, similar to IL-1β (Tracey, 1994); IL-10, immunity and resolution of inflammation (Mosmann, 1994); IL-6, rejection of implants and immunity (Hirano, 1994); IL-4, formation of foreign body giant cells (Kao et al., 1995), adhesion molecule expression by endothelial cells and endothelial cell proliferation (Banchereau and Rybak, 1994); IFN-γ, immunity (De Maeyer and De Maeyer-Guignard, 1994). Additional experiments demonstrated that IL-1β was expressed in various cells types that included macrophages, foreign body giant cells, endothelial cells, and fibroblasts. However, not all cells of a particular class expressed IL-1β
message suggesting subclasses of cells associated with the polymeric implants. The significance of this may be most important when seeking alternative means of treatment of patients with failing vascular grafts. Possible control of specific cell types within the wound may lead to treatments to both save and keep synthetic vascular grafts from failing.

The importance of the factors released by cells associated with polymeric implants was demonstrated by results presented in chapter 3. It has been suggested that cells associated with polymeric implants release mitogenic factors which act on both endothelial and smooth muscle cells leading to neointimal thickening (Lepidi et al., 1996; Zacharias et al., 1988). For this dissertation, wound fluid was collected from the lumen of close-ended tubes implanted within subcutaneous tissue of the rat. This wound fluid was then mixed with rat complete medium, used in the culture of rat microvessel endothelial cells, to determine the effects it may have in cellular proliferation. The results from these studies suggest that the factors in this wound fluid stimulate endothelial cell proliferation. For this dissertation, this is important in both the luminal and abluminal healing characteristics. The wound fluid collected from the lumen of the polymeric tubes would be essentially what cells on the luminal surface of polymeric vascular grafts would “see”. It is then reasonable to suggest that the diffusion of products from cells associated with the abluminal surface through the polymer to the luminal surface would stimulate cellular proliferation leading to neointimal thickening, one of the main modes of failure of synthetic vascular grafts. Furthermore, if IL-1β is one of the products within this fluid, it is likely that endothelial cells on the luminal surface would express tissue factor, increasing the potential for blood clots. The abluminal healing would be similarly affected. As the fluid diffused away from the implant, the concentration of factors in the fluid
would stimulate angiogenesis. Interestingly, a distance from the polymer surface, an overabundance of vascular profiles is observed. However, again if IL-1β is one of the factors within this fluid, then these blood vessels would express adhesion molecules for inflammatory cells promoting the recruitment of more macrophages to the polymeric implant site. This would then lead to the association of macrophages with the polymer for the life of the implant.

Another important aspect of this dissertation was the evaluation of a cardiovascular device, the endovascular graft, in vivo. These devices are a hybrid of stent and polymeric graft technology. In the configuration used for these studies, the polymeric material had to be balloon dilated for proper deployment within the vascular tree. Other studies have demonstrated that polymeric structure plays an important role in the healing response associated with implants (Brauker et al., 1995; Hirabayashi et al., 1992; Golden et al., 1990; Lepidi et al., 1996; Kohler et al., 1992; Nagae et al., 1995). Prior to the in vivo analysis, both the alterations of the material following balloon dilatation and effects these alterations may have on general healing were assessed. Significant changes were observed in nodal width, wall thickness, interfiber distance, and fiber width of the ePTFE following the ballooning process. Data presented concerning changes in porosity and how these changes may influence the healing process lead to the hypothesis that balloon dilatation of ePTFE would alter the healing response associated with the material. Indeed this was the case, however, the results obtained were not expected. In this study, tissue site of implantation played a significant role in the healing response. Balloon dilated material implanted within subcutaneous tissue had a significantly decreased inflammatory response compared to control. Furthermore, balloon
dilated material did not become surrounded by a dense fibrous capsule like the control implants. In contrast, balloon dilated material implanted within adipose tissue had a significantly increased inflammatory response when compared to control implants. The mechanisms responsible for these findings remain unknown, however possibilities include alterations in the Vroman effect (Leonard and Vroman, 1991), leaching of impurities (Andrade, 1973), and fragments of polymer being dislodged following the ballooning process. Regardless the significance of these findings support the need to test materials in tissue environments similar to the intended applications.

The penultimate testing of a device requires in vivo analysis. In this dissertation, the endovascular grafts were evaluated in two models, the thoracic aorta of the rabbit and the iliac artery of the pig. Results were similar between the two models. Initially (24-48 hr), a red thrombus infiltrated with PMNs formed on the luminal surface of the implant. On the abluminal side, the tunica adventitia appeared disrupted and inflamed. After 1 week, the luminal surface was still covered with red thrombus and the beginning of an inflammatory response was observed between the abluminal surface of the ePTFE and the tunica intima. At five weeks, the luminal surface was covered by a neointima and both the luminal and abluminal surfaces had macrophages and foreign body giant cells associated with the polymers. These inflammatory cells have the potential to release factors that can stimulate cellular proliferation and extracellular matrix deposition leading to increased neointimal thickening. Following 12 weeks, the inflammatory cells associated with the luminal surface had disappeared while those associated with the abluminal surface were still present. When evaluating metallic stents alone, the inflammatory response was only associated with the
sts at all time points. Furthermore, the neointimal thickening was greatest associated with inflammatory cells. These data suggest that the inflammatory response may in part be responsible for the process of neointimal thickening. In support of this, one of the endovascular grafts evaluated at 12 weeks had a minimal inflammatory response associated with the polymer. The neointimal thickening associated with this implant was significantly thinner when compared to the other endovascular grafts. Further experiments will be required to determine the importance of the inflammatory response in the process of neointimal thickening.

All of the data presented in this dissertation leads to a model for healing associated with polymeric vascular grafts. Initially following implantation, inflammatory cells are recruited to the site of injury due to trauma and due to activation of the leukocytes by the polymer or proteins associated with the polymer (Swartbol et al., 1996). The PMNs remove any infection however do not play a significant role in the foreign body response commonly observed with polymeric implants. The macrophages are recruited by products released by PMNs and debris for the process of tissue repair and remodeling. However, due to the presence of the polymer the macrophages can not successfully repair the tissue. Therefore, the macrophages release IL-1β, TNF-α, IL-6, and IL-4 (along with other molecules not addressed in this dissertation) possibly leading to the recruitment of more inflammatory cells and the formation of foreign body giant cells. As a consequence of these inflammatory cells secreting bioactive molecules, the cells on the luminal surface proliferate and secrete extracellular matrix proteins leading to neointimal thickening. The healing on the abluminal surface is directed by macrophages that secrete bioactive molecules. As these factors diffuse
away from the tissue-polymer interface, their concentration becomes such that angiogenesis is promoted leading to the increased vasculature 50-100 μm from the abluminal surface. With the continuous secretion of factors from the macrophages and foreign body giant cells the vasculature becomes activated and expresses adhesion molecules on their surface enhancing the recruitment of inflammatory cells indefinitely. These processes continue indefinitely due to the presence of the polymer and lead to the failure of the vascular graft.

In summary, the data presented in this dissertation support the four overriding hypotheses: 1) polymeric materials alter the normal wound healing response, 2) polymeric materials elicit different macrophage responses depending on the chemical and physical structure of the polymer and these responses influence neovascularization of the material, 3) macrophages associated with polymeric implants express angioactive cytokines, along with many others, and 4) the bioactive products released by cells associated with polymeric implants stimulate microvessel endothelial cell proliferation. Further studies to examine specific roles products released by both macrophages and other inflammatory cells play in the healing associated with biomedical implants must be performed. Furthermore, molecules responsible for the foreign body reaction characteristic of biomedical implants must be identified.
APPENDIX A

Procedure = USER++

Start Untimed Steps
Warmup rinse to 42 C
Select APK wash solution
Dual rinse
Apply low tem cover slip
Warmup slides to 40 C
Hold at temp for 4 min.
Start timed steps
Dual rinse
Apply low temp cover slip
Apply 1 drop digestion enzyme
  Protease 3, 4 min.
Dual rinse
Apply low temp cover slip
Start timed steps
Select SSC wash solution
Dual rinse
Slide wipe - inner only
Apply high temp cover slip
Skip past reagent dispense station
Man app : MAN APPLY PROBE
Disable vortex mixers
Start unttimed steps
Enable vortex mixers
Hold at temp for 4 min.
Start timed steps
Warmup slides to 65 C
Hold at temp for 4 min.
Warmup slides to 42 C
Apply short low temp cover slip
Incubate - hybridization
  60 min.
Dual rinse
Apply low temp cover slip
Start timed steps
Dual rinses
Slide wipe - inner only
Rinse with 0.1 x SSC
Apply high temp cover slip
Warmup slides to 50 C
Skip past reagent dispense station
Dual rinse
Slide wipe - inner only
Rinse with 0.1 x SSC
Apply high temp cover slip
Warmup slides to 50 C
Skip past reagent dispense station
Dual rinse
Slide wipe - inner only
Rinse with 0.1 x SSC
Apply high temp cover slip
Warmup slides to 50 C
Skip past reagent dispense station
Dual rinse
Apply low temp cover slip
Start timed steps
Warmup slides to 40 C
Select APK wash solution
Dual rinse
Apply low temp cover slip
Apply 1 drop of det. step 1
Reagent 7, 32 min.
Dual rinse
Apply low temp cover slip
Apply 1 drop of det. step 2
Reagent 1, 32 min.
Dual rinse
Apply low temp cover slip
Apply 1 drop of det. step 3
Reagent 2, 32 min.
Dual rinse
Apply low temp cover slip
Apply 1 drop of det. step 4
Reagent 8, 32 min.
Dual rinse
Apply low temp cover slip
Apply 1 drop of user substrate
substrate 2, 32 min.
Hold at temp for 28 min.
Dual rinse
Apply low temp cover slip
Start timed steps
Start untimed steps
Dual rinse
Dual rinse
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