Tissue Engineering for Wound and Organ Repair: Angiogenesis as a Mechanism of Action

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ABSTRACT

Over the last two decades, skin substitutes have been developed which have found application in the treatment of acute and chronic wounds. Laboratory investigation of the mechanism of action of these agents has revealed that they depend for their action on the production of growth factors, on the provision of a substrate on with keratinocyte migration can take place and in the modification of the inflammatory response. The angiogenic activity of tissue engineered products, such as Dermagraft®, which is a three-dimensional, scaffold-based fibroblast culture system, has lead to their application to the important problem of reperfusion of the heart made ischemic by coronary arterial occlusion. Recent studies in experimental animals have demonstrated that Dermagraft® application to a heart in which the coronary circulation has been occluded, causes the generation of new blood vessels, both arterioles, venules and capillaries, and partially restores the heart function. In the future, optimization of such a system, in terms of cell type, scaffold architecture and delivery systems holds promise for a new type of therapeutic angiogenic device.

1. INTRODUCTION

Over the last decade, tissue-engineered skin substitutes have been developed to treat chronic wounds. Initially, these products were considered artificial skin grafts, with the expectation of closing the wound much as autologous split skin. It soon became evident, however, that the mechanism of action of these devices was rather more complex and involved angiogenesis as well as re-epithelialization, cell proliferation and changes in inflammatory processes. In the case of Dermagraft®, early studies demonstrated effective angiogenic activity, both in terms of synthesis of mRNA for angiogenic factors, secretion of the factors themselves, angiogenic activity in *in vitro* assays, activity in the chick chorioallantoic membrane (CAM) assay *in vivo* and in a clinical setting. As a result of these studies, other applications were investigated for such an angiogenic device, among them restoration of blood flow to the ischemic heart and in the peripheral circulation.

Such a device has to meet certain criteria in order to be used as a therapeutic agent. First, it should be angiogenic in itself, and with minimal inflammation. Certain inflammatory agents, such as IL1, IL-6 have been demonstrated to be angiogenic [1] and inflammatory cells, such as macrophages are certainly able to generate angiogenic factors. However, it is not satisfactory to induce inflammation solely for the purpose of attendant angiogenesis. The device should, therefore, generate angiogenesis directly. Secondly, the device should be able to control angiogenesis and switch it off when adequate. Continued angiogenesis may lead to deleterious consequences [2]. Thirdly, the device should be able to survive and continue to produce angiogenic factors until it can provide itself with adequate nutrition. Fourthly, it should produce a vascular plexus. Angiogenesis is a complex process, taking place in a context of cytokines, and involving the development of capillary, arteriolar and venular systems. An angiogenic device useful outside the simplest applications should be able to perform all of these.

2. SKIN

The tissue engineering of skin substitutes has a long history. The earliest attempts to grow keratinocytes were performed by Karasek [3] but the method was developed into a routine procedure by Green's laboratory [4]. This procedure was applied to the production of autologous epidermal sheets for the treatment of severe epidermal loss as in major burns or blistering diseases [5] and developed into a service by Genzyme Tissue Repair.

The first composite artificial skin, including both dermal and epidermal components was developed by Bell [6], based on fibroblasts grown in a collagen gel, overlain by cultured keratinocytes. Since those early studies, tissue engineering of skin has advanced considerably, until, currently, it has reached a mature stage from which it can branch out into other areas [7]. At present, several tissue-engineered therapeutic products have achieved regulatory approval in the United State, including TransCyte® for burn wounds, Apligraf® for venous and diabetic ulcers, Orcel for epidermolysis bullosa and Dermagraft® for diabetic foot ulcers. The range of each of these products is being extended, Transcyte® into pressure ulcer, Dermagraft® into venous ulcers and all of the products into a wide range of less common chronic wounds.

Raised to the air-liquid interface [8], composite cultures will form structures anatomically very similar to skin, although differentiation is incomplete, and have been developed into a product used for the treatment of ulcers and other skin conditions by Organogenesis Inc., as Apligraf®. A similar product, based on an artificial dermis grown in a collagen sponge, has been developed by Ortec.

A related approach has developed epidermis grown on non-living de-epidermized dermis [8, 9]. The idea is that this material constitutes a closer preparation than bovine collagen gels to the dermal extracellular matrix, without problems associated with allogeneic cells. It also possesses basement membrane components, which aid in keratinocyte attachment and migration. This product is available from Lifecell Corp. In another application of extracellular matrix, the submucosa of the small intestine has been found to act as a scaffold which can be remodeled in a wide variety of ways [10]. This material has been used by Cook Biotech for the treatment of wounds.

Early experiments indicated that keratinocytes are capable of secreting a wide range of growth factors [11, 12] and were thought to play the dominant role in the control of wound healing. However, it has become evident fibroblasts also play a major role [13] [14] and, recently, a product, Dermagraft®, has been approved for the treatment of diabetic foot ulcers based on fibroblasts alone. This material is based on the growth of fibroblasts on a three-dimensional scaffold, when the cells lay down quantities of extracellular matrix, which forms a dermal structure resembling wound healing or fetal tissue. The reason for the extensive deposition of this material is not clear. The concentration of the mRNA for collagen type 1 is not greatly different from that present in monolayer culture, which does not show collagen deposition to the same degree. The difference does not appear to be in deposition as only a small amount of soluble collagen is found in the supernatant of monolayer cultures. It appears that culture in three dimensions, or,

possibly, a foreign body response alters to a large extent the amount of collagen synthesized and secreted from a rather similar amount of collagen mRNA. In addition, the fibroblasts in three dimensional culture deposit many other extracellular matrix proteins, including fibronectin (the splice variant found in wound healing), tenascin, collagen III, collagen VI, a little collagen IV and VII and decorin [15]. Dermagraft® has been found to improve the healing of diabetic foot ulcers [16] through several routes [15]. A major feature is that fibroblasts in three-dimensional culture, like keratinocytes, secrete several angiogenic factors, including the diffusible form (121 amino acids) of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and angiogenin. It will cause the formation of new blood vessels, both in experimental systems, such as the CAM assay [17] and aortic ring assay [18] and in a clinical setting [19]. Since a major complication of these ulcers is infection by anaerobic organisms, stimulation of blood supply may be expected to inhibit such bacteria as well as improving wound healing. In addition, Dermagraft® provides a good substrate for keratinocyte adhesion and migration. Krejci-Papa has shown in a murine excisional wound system that keratinocyte migration on Dermagraft® is superior to that on the wound bed [20]. Chronic wounds possess and abundance of activated keratinocytes at the wound margin [21] which seem unable to migrate onto the wound bed. Dermagraft®, thus, supplies a route for them to use. Dermagraft® is supplied in frozen form at –70ºC to provide time for its manufacture and distribution. The cryopreservation process constitutes a cell stress, both osmotic stress during exposure to cryopreservative, and freezing. In investigation gene expression during this process, we have found that the cells respond both with the induction and the repression of genes [22]. Among a well defined group of induced immediately after thawing is VEGF and HGF which presumably activate angiogenesis quickly. Dermagraft®, Apligraf® and Orcel® all use allogeneic cells and much discussion has been directed at the possibility of immunological response. The clinical experience has been that no acute rejection has been observed in several thousand patients treated with these materials and, for these tissue-engineered cell types, no immunological response has been reported. This is in sharp contrast to allogeneic skin grafts that provoke acute rejection within about 2 weeks. An acute immunological response of this type is caused by recognition of antigens, mainly of the HLA class II (*e.g.* HLADR) in humans, together with the co-stimulatory molecules CD80 and CD86 on the donor cells by CD28 on recipient lymphocytes. In the absence of □-interferon, fibroblasts and keratinocytes do not express HLA class II antigens or the co-stimulatory molecules. The expression of CD80 and CD86 is stimulated in many cells by engagement of CD40 on the target cell with CD154 on the lymphocyte. The expression of CD40 on fibroblasts and keratinocytes is limited. However, expression of HLA class II and CD40 is stimulated by □-interferon, which is likely to be present in the wound environment. However, while monolayer fibroblasts or fibroblasts in collagen gel culture show this induction, a majority of the fibroblasts in three-dimensional scaffold-based culture, as in Dermagraft, fail to respond [23]. It has also been shown that lymphocytes will not respond to allogeneic fibroblasts without engagement of CD28 by an activating antibody, while with allogeneic keratinocytes, engagement of both CD28 and CD154 is required [24]. This supports

findings that fibroblasts in their native dermal matrix do not activate lymphocytes [25]. The reason for the lack of rejection, is thought to be related to the lack of antigen presenting ability by fibroblasts and keratinocytes. Rejection is provoked by cells one of whose major physiological functions is the presentation of antigen, such as dendritic cells (dermal dendritic cells and Langerhans cells in the epidermis) and endothelial cells. These cells either constitutively express HLADR or induce it very easily, but are absent in the cultured products of tissue engineering. It thus appears that tissues composed solely of non-antigen-presenting cells may be resistant to acute rejection. The extent to which this phenomenon applies is not yet defined, but it appears that cells such as chondrocytes and smooth muscle cells may well show similar characteristics. If this is true, acute rejection may become a much less important question for tissue engineered products than has hitherto been thought. It must, however, be emphasized that this conclusion is restricted to acute rejection. Other types of immunological response, such as chronic rejection, are not addressed, although slow replacement of allogeneic cells by autologous ones may be without clinical consequences.

The same type of three-dimensional fibroblast culture has also been applied to the treatment of acute wounds, particularly burns. The original concept was to develop a replacement for cadaveric skin, which is used to cover severe burns after debridement of the eschar. Allogeneic skin is only useful for about 2 weeks, when it is rejected. TransCyte® comprises the extracellular matrix secreted by fibroblasts in three-dimensional culture, without living cells. It has been applied to a debrided burn, in a similar manner to cadaveric skin, for up to 120 days without rejection. However, it has been found that, beyond its use as a temporary covering for severe burns, it also enhances the healing of partial thickness burns [26], both in rate of healing and cosmetic result.

In the treatment of severe burns, it has long been thought that replacement of the dermis, either by means of a scaffold alone [27] or by a scaffold preseeded with keratinocytes, with our without fibroblasts [28, 29] [30]. Both of these systems have been developed into highly successful treatments for severe burns [31].

Beyond the therapeutic applications of tissue-engineered skin substitutes, test systems have been manufactured, including keratinocytes alone, fibroblasts alone, composite tissues containing both dermal and epidermal elements and systems containing other cells types such as melanocytes [32], a vascular system [33] and Langerhans cells.

In its application to angiogenic problems elsewhere in the body, Dermagraft® has some very attractive qualities. First, it is undoubtedly angiogenic, and angiogenic through its secretion of known, direct angiogenic factors rather than through indirect affects. Secondly, it retains its complete complement of cellular control systems, so it is able to respond to its environment and modulate its secretion of active agents according to the circumstances. In principle, this enables to avoid the potential problems associated with excessive angiogenesis. Thirdly, the cells are comparatively hardy and well able to survive adverse conditions. In terms of oxygen supply, they tend to grow best under mild hypoxia [34], and are capable of surviving considerable periods of low oxygen tension. Fourthly, as discussed below, they are

capable of inducing several types of blood vessels. Thus, Dermagraft® shows considerable promise as an angiogenic device for application in such circumstances as peripheral vascular occlusion or cardiac ischemia.

3. TISSUE ENGINEERING FOR THE HEART

Normal systolic and diastolic function of the heart is an elegant process that relies upon a variety of physiologic processes. One of these processes being adequate blood perfusion into the myocardium, helps to support the metabolic demands of the heart as an organ. The heart is an organ that continues to need a blood supply for the lifetime of the host. However, when perfusion of the myocardium is compromised, numerous pathologic conditions are initiated. Therefore, in the area of "heart repair" many investigators have focused upon re-establishing the vital blood perfusion into myocardium of the heart. These therapeutic efforts have utilized a wide range of treatment modalities that recently have begun to utilize tissue-engineering approaches including angiogenesis as a mechanism of action.

3.1. Heart Disease

Heart disease continues to represent a leading killer among the world's population. Therefore, maintenance of the heart as a functional organ continues to be a major focus of clinical and research efforts. Specifically within heart disease, arteriosclerosis represents a disease state that affects the normal function of the vasculature, specifically small caliber arteries such as coronary arteries. Progression of this disease can result in narrowing or occlusion of the coronary vasculature[35]. This can ultimately lead to reduced blood flow, induction of areas of cardiac ischemia, and an increased risk of myocardial infarction, where normal function of the myocardium is compromised. The loss of myocytes due to poor perfusion of the myocardium is an important mechanism in the development of cardiac failure[36]. Mechanisms of myocyte death and secondarily, fibrosis of the ventricular wall, have been attributed to both necrosis of the myocardium and/or apoptosis signals [36]. Necrosis of myoctes is characterized by the depletion of ATP, damage to intracellular organelles, cell swelling, and rupture of cell membranes[37],[38]. Apoptosis, on the other hand, is an energy-requiring process that involves active intracellular signaling pathways. It involves loss of cell surface contact, cell shrinkage, and the condensation of chromatin at the nuclear periphery[37]. However, both the mechanisms of necrosis or apoptosis lead to myocyte death, scar formation, and myocardial remodeling. These conditions are ultimately triggered by limitations in coronary perfusion[39], [40] and heightened mechanical stress [41].

Some of the early efforts to repair damaged myocardium in the 1930's began with the Beck procedure [42, 43]. This procedure was used to stimulate the formation of a new collateral network within damaged myocardium. The Beck procedure involved initiating an inflammatory induced angiogenic response on the heart's surface by rubbing the epicardium with sandpaper or an emery board [43]. By inducing an angiogenic response, it was thought that a new vascular supply to the damaged myocardium would help to increase local

perfusion and ultimately lead to improved cardiac function. Later in the 1940's and 1950's, Arthur Vineberg used the internal mammary artery (IMA) to re-direct arterial blood flow into the left ventricular myocardium [44],[45]. The IMA was transected and detached from its chest wall bed and then placed within a tunnel in the ventricular myocardium. An anastomosis was observed to develop between the implanted IMA and the left coronary circulation. These observations were made from injection studies, radiographs, plastic casts, and serial sections [45]. In both of these early surgical studies, efforts were made to use a means of angiogenesis as an agent of therapy.

3.2. Current Treatments

Current treatments of coronary heart disease focus on re-establishing coronary perfusion to reduce angina (chest pain) and to prevent ischemic regions from becoming infarcted tissue or to prevent expansion of the existing infarct area. Clinical cases where coronary vessels are narrowed and regions of myocardial perfusion are diminished focus on preventing a loss in myocardial perfusion [46], [47], [48] [49]. Conditions where infarcted tissue is already present in a diseased heart may benefit by increasing myocardial perfusion into this damaged myocardium. This may help to prevent continued progression of the infarct condition by re-vascularizing ischemic (reversible or hibernating) myocardium that borders the infarct area [50], [51]. This ischemic or hibernating myocardium represents an underperfussed tissue that can be revascularized to achieve functional recovery [52]. Hibernating myocardium may be present in up to 50% of patients with significantly impaired left ventricular function and evidence of heart failure [53]. Additionally, recent data indicate that hibernating myocardium is present in about 78% of patients after acute myocardial infarction [54]. Importantly, these conditions of reduced cardiac function can be prevented or reversed by lessening the ischemic burden through re-establishing myocardial perfusion into the hibernating region[53].

Widely utilized treatment modalities for re-establishing myocardial perfusion include the coronary artery by-pass graft (CABG) procedure and percutaneous transluminal coronary angioplasty (PTCA). The CABG procedure involves the use of native vessel, donor vessel, or synthetic conduit [55] to by-pass the occluded or narrowed coronary vessel. In contrast PTCA uses an intravascular approach to "balloon-open" the narrowed coronary vessel. Both of these procedures allow for distal perfusion of the myocardium to resume and have demonstrated their effectiveness in the treatment of coronary heart disease patients [56], [57]. Recent studies have demonstrated the ability of both CABG and PTCA to rescue hibernating myocardium thereby increasing left ventricular function [58], [51].

3.3. New Treatment Modalities

The high incidence and risk of cardiovascular disease has motivated the development of new therapeutic angiogenesis strategies to help treat the associated pathologies. Specifically, single growth factor therapies such as VEGF [59], [49] or bFGF [60], [48] have been injected into the coronary vasculature to stimulate myocardial collateral flow. In these studies, angiogenic therapies have been successful at

simulating new microvessel growth or an increase in myocardial collateral blood flow. Other studies have demonstrated improved cardiac function following angiogenic growth factor therapy, as measured by echocardiography (specifically ejection fraction and ventricular wall motion) [47] [61]. Additionally, transmyocardial laser revascularization (TMLR) studies have demonstrated increased microvessel density values within infarcted myocardium and an increase in regional myocardial perfusion [62], [63]. However, no significant global ventricular function improvements have been reported in these studies where cardiac function was evaluated using echocardiography techniques [62] or stroke work index calculations from starling relationships [63]. However in limited examples TMLR techniques have been reported to significantly improve regional myocardial function as measured by percent segmental shortening [62] with no significant difference in global ventricular function.

Recent studies using myocyte transplantation into infarcted myocardium have demonstrated more encouraging results with improvements in cardiac function. In rat studies, fetal ventricular cardiomyoctyes have been transplanted into infarcted myocardium to stimulate greater systolic pressure in the transplant group in comparison to controls [64]. In other rat studies, autologous bone marrow cells have been used as cardiomyocyte precursors in ventricular scar tissue. These transplant studies have also demonstrated improved systolic pressure over control animals [65]. Additionally, recent rabbit studies have used autologous skeletal myoblast transplantation into infarcted myocardium [66]. These data demonstrate improved ventricular function in animals that received myoblast transplantation. Ventricular function parameters were reported as pre-load recruitable stroke work (PRSW) values (the relationship between regional stroke work and end-diastolic segment length) [66].

Collectively, current and newly developed treatments for coronary heart disease and myocardial infarction have demonstrated that re-establishing myocardial perfusion following episodes of ischemia or conditions of infarction can function to restore regional or global ventricular function. Recent work aimed at replacing lost cardiomyocytes in infarcted myocardium have demonstrated a new treatment modality that may be combined with re-vascularization therapies to allow for the replacement and maintenance of functional myocardium. While the therapeutic goal of cell transplantation therapies was not originally to stimulate new blood vessel formation, these studies were the first to use viable cells in the treatment of heart damage. However, recently a revascularization therapy has demonstrated the use of a tissue-engineered patch (consisting of viable cells and a co-polymer) to be effective in stimulating angiogenesis in damaged cardiac tissues.

3.4. Tissue-Engineered Cardiac Patch, Dermagraft®

As described previously, a critical feature in the mechanisms that Dermagraft® uses to induce wound repair is angiogenesis. The viable cells synthesize a number of angiogenic growth factors (including VEGF, bFGF, and hepatocyte growth factor [HGF]) and has been shown to stimulate angiogenic activity [67]. While Dermagraft® has been used in the treatment of chronic leg wounds, more recently Dermagraft® has been shown to stimulate an angiogenic response in other

wound sites such as ischemic or infarcted cardiac tissue [68]. In these studies, a coronary occlusion of a branch of the left anterior descending coronary artery was performed in severe combined immunodeficient mice. Dermagraft® with or without viable cells were sized to the damaged area, implanted in replicate mice onto the epicardium at the site of tissue injury, and compared with animals that received infarct surgery but no implant. Fourteen and 30 days after surgery, the damaged myocardium receiving viable Dermagraft® exhibited a significantly greater angiogenic response (including new arterioles, venule, and capillaries) than nonviable and untreated control groups [68]. In this animal model, viable Dermagraft® stimulated angiogenesis within a region of cardiac infarction and augmented the repair response in damaged tissue. Therefore, a potential use for Dermagraft® is the repair of myocardial tissue damaged by infarction.

3.5. Summary

The adult human heart is a muscular organ that experiences approximately 100,000 heart cycles each day, pumping approximately 8,000 liters of blood. When blood perfusion to the heart organ is compromised, the ability of the heart to maintain its life-sustaining role is severely compromised. Therefore within the field of "heart repair" a major focus area remains-- to restore or rescue myocardial perfusion. These therapeutic efforts have utilized a wide range of treatment modalities that recently have begun to utilize angiogenesis as a mechanism of action. Specifically, single or dual growth factor therapies have demonstrated promise as angiogenic treatments. However, recent tissue-engineering approaches have demonstrated the success of Dermagraft® to stimulate mature microvascular formation within an area of damaged cardiac tissue. The advantages that tissue-engineered cardiac patches offer over single or dual growth factor therapies include the continued or sustained release of an angiogenic milieu. Additionally, the viable cells release an angiogenic environment that includes growth factors, cytokines, and extracellular matrix components that are essential for the initiation, maturation, and maintenance of the new microvasculature. These recent tissue-engineering approaches in which viable cells and surrounding tissue are developed into a cardiac patch may prove to be a new emerging field that combines heart repair and tissue engineering.

4. Future Directions

Application of the angiogenic concept exemplified by the use of Dermagraft® to improve perfusion of the ischemic heart has many applications. For instance, peripheral vascular occlusion, caused by thrombosis or atherosclerosis is a major problem that might be alleviated by the use of an angiogenic device to induce development of collateral blood vessels. While this occurs to some extent without external stimulation, the process is generally inadequate in humans, and might be greatly improved.

The possibility of the use of Dermagraft® as a cardiac revascularization device was not considered initially and represents development of the product in a direction for which it was not initially designed. With the possibility of its use in this way established, a purpose-designed device can be considered. From a tissue-engineering point

of view, an ischemic reperfusion device can be optimized in many ways.

Dermagraft® is based on dermal fibroblasts. While angiogenesis is important to these cells, other cells might have better angiogenic potential, for instance smooth muscle cells or skeletal muscle cells. The polymer scaffolds, on which the cells are grown are another area I which considerable optimization might be attempted. The Vicryl™ (Ethicon, USA) scaffold, on which Dermagraft® is grown is a knitted lactate glycolate copolymer fabric, which was selected because it was known to allow the proliferation of cells, was commercially available and had regulatory approval. It might not be optimal for a reperfusion device and other scaffold structures need to be investigated.

Delivery of an angiogenic device to the heart is clearly a challenge and sheets of Dermagraft®, placed on the organ during thoracic surgery, are not an ideal solution. Ideally, an optimized ischemic reperfusion device should be delivered in a minimally invasive manner, not requiring major surgery. Thus the design of a suitable delivery device will be a major advance.

At this stage of the development of reperfusion devices, we have relied on the inherent cellular angiogenic activity to provide the therapeutic molecules. It may, in the future, prove beneficial to enhance this artificially by the use of genetically-modified cells. This might be performed either by increasing the production of an angiogenic factor, using a plasmid, or the use of cells generating a factor which is itself enhanced, by slower degradation, increased receptor affinity matrix binding and so forth. Measures such as these must be undertaken great caution. Just as one needs adequate angiogenesis, too much can be equally deleterious. It is essential that such enhanced agents be carefully controlled. It is one advantage of a tissue-engineered product, such as Dermagraft®, that the cells have intact control systems and switch themselves off at the appropriate time. Further, angiogenesis is a complex process, requiring the appropriate expression of different factors in appropriate amounts at appropriate times. We do not yet understand the process sufficiently to be able to achieve this, although the cells themselves presumably can. We might well upset the process, generate too much of one component, or, while getting the attention of the cells, leave them unable to respond, in the manner of a stun grenade. Thus, while genetic modification of tissue-engineered devices for angiogenesis is likely to be a future development, it should not be undertaken without careful consideration and understanding of the process being modified. The potential to use the angiogenesis properties of Dermagraft® for multiple different applications is large. This may include skin, oral cavity, cardiovascular, vascular, optic, nerve, and musculoskeletal opportunities. It remains to be determined if these opportunities can be converted into clinical treatments.

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