ENDOTHELIAL CELL BASED ENGINEERING OF CAPILLARIES-LIKE NETWORK IN A TISSUE-ENGINEERED SKIN SUBSTITUTE

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Abstract
Treatment of patients with burns, surgeries and accidents involving extensive skin replacement has long been a challenge for reconstructive surgery. Classical approaches like cadaver skin or xenografts have failed to provide a permanent solution to this ever increasing problem. Different tissue engineering techniques have been tried in the past but, clinically none has lived up to the early promise that in vitro studies showed mostly, due to limited vascular supply leading to cell death when grafts comprising of epidermis and dermis were used in deep wounds. More recently scientists have developed a new approach using endothelial cells for construction of pre vascularised tissue engineered skin grafts constituting epidermis and dermis. In this mini review we look at a few experiments and animal studies which have been conducted so far and have yielded promising results, both in vitro and in vivo. This new approach involving endothelial cells for engineering of capillary like network in tissue engineered skin substitute is the way forward and can potentially provide a solution to this problem which scientists and surgeons have been trying to address for the last half century.

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**Introduction**

Treatment of burn victims or patients with surgeries involving extensive skin removal is a complex procedure. The classic therapeutic options which have been tried like cadaver skin or xenografts have proved to be temporary solution\[1, 2\] \[3\]. In recent times tissue engineered skin has offered a therapeutic alternative \[4-6\]. Although human epidermis has already been developed in large quantities from skin biopsy by using techniques developed in tissue engineering \[7, 8\] \[9\] but if dermis is destroyed due to trauma, replacement by epidermis alone does not lead to optimal healing \[3, 8\]. In such instances, a bilayered tissue engineered substitute is sought \[10\].

Drawbacks for replacement of skin wounds with epidermal substitutes alone are many and include high sensitivity of mechanical damage and variable percentage of graft take which may be attributed to absence of underlying dermis. With engineered constructs consisting of both epidermis and dermis, many of these drawbacks will be potentially overcome \[3, 11\].

Researchers have tried to transplant skin substitutes with increasing thickness to accommodate for increased tissue loss in different clinical scenarios. Although there have not been many such transplantation experiments but, there have been studies which, show unfavourable results which may be due to insufficient vascularisation in dermis which in turn leads to deleterious effects on epidermis survival as this layer is dependent on diffusion of nutrients from dermis\[4, 12, 13\].

In most of the cases the graft failure is due to inadequacy of vascularisation which leads to hypoperfusion and ischemic injury \[4, 9, 14-16\]. The graft cells are dependent on diffusion of nutrients and oxygen from underlying wound which is generally insufficient for sustained survival of graft \[9\]. Cell located within 100-200 micro meter of a vessel can derive nutrients via diffusion \[17-21\]. As the distance increases, diffusion alone cannot sustain cells and so cell death increases.

Inosculation has been explained as the anastomoses or connection of capillaries present in graft with vessels in the host’s wounds \[4, 22, 23\]. In some studies it was demonstrated when human skin was grafted onto mouse, initial vascularisation was due to anastomoses between severed ends of human capillaries with mouse micro vasculature\[16, 19\]. Later mice endothelial cells replaced the human endothelial cells. This demonstrated that grafts with intrinsic blood supply may have a better long term prognosis and survival. Hence it was proposed that methods should be sought to replace skin with grafts containing intrinsic microvasculature \[13\]. This observation found the basis of idea to develop a tissue engineered skin substitute with intrinsic vascular component \[4\].

Many approaches are currently being investigated to increase vascularisation in tissue engineered skin substitutes to promote inosculation and hence survival of the transplanted engineered skin substitute \[19\]. One approach is to incorporate growth factors into scaffold biomaterial \[24\]. A different approach is to pre-encapsulate the growth factors in microspheres which are then transferred to the scaffold leading to steady and sustained released of growth factor \[25\]. This has been proven by studies like \[26\] who demonstrated that various biodegradable polymers can be developed and used for transplantation of isolated or encapsulated cells which would allow to form an engineered tissue with intrinsic vasculature \[26\]. Many studies have been carried out to promote vascularisation in engineered tissues through use of growth factors including Vascular Endothelial Growth Factor \[5, 6, 27-30\], Fibroblast Growth Factor \[29\] and Transforming Growth Factors \[31\]. But it has been shown in previous studies that systemic delivery can cause unwanted tissue growth and subcutaneous injections yield a very
limited promotion in neovascularisation [26]. Another approach is utilization of stem cells for creation of tissue engineered vascular grafts [24, 32, 33]. Studies involving genetic modification of cells have also been carried out. For instance study by [34]. In this study it was demonstrated that over expression of platelet derived growth factor A in tissue engineered skin substitutes by genetically modified keratinocytes and fibroblasts was not statistically significant compared to unmodified keratinocytes and fibroblast. It was also observed that Tissue Engineered Skin substitute was devoid of intrinsic microvasculature and this limitation can hypothetically be overcome by over expression of certain cytokines [35]. Lastly a different approach is to engineer micro vessels in vitro in scaffold using endothelial cells. After transplantation, the endothelial cells form blood vessels which develop interconnections with host blood vessels leading to adequate perfusion of the graft. However one issue needs to be addressed which is homogenous distribution of endothelial cells and resulting blood vessels [24]. This issue has been addressed in a few studies like [26]. Their paper hypothesizes that endothelial cells if seeded in tissue engineered scaffold would form capillary like tubular structures which can form the basis of intrinsic graft microvasculature which would increase the long term survival of graft by anastomoses between graft and host microvasculature [26]. Human endothelial cells have also been shown in previous studies to spontaneously sprout capillary like structures and form microvasculature when co cultured with fibroblasts and seeded on tissue engineered skin substitutes in vitro. For instance in a study by [36] conditions for HUVEC (Human Umbilical Vein Endothelial Cells) to form capillary like tubular structures with in a 3D scaffold were demonstrated which would allow HUVEC to form in vitro capillary like tubular structures within tissue engineered scaffold which can then be transplanted onto graft site on host.

In this mini review, we look at different studies which used endothelial cells in different models to increase the development of microvasculature in the engineered skin substitutes so as to promote inosculation and hence survival of the tissue engineered skin substitute.

In Vitro Experiment, First of its Kind
In a study by [4], the objective was to develop Endothelialized Skin Equivalent (ESE) in which capillary like structures could be reproduced in vitro. For this purpose Human Keratinocytes and Dermal Fibroblasts were isolated. As it has been shown by [37] that human dermal fibroblasts and keratinocytes produce a more well organised tissue engineered construct in comparison to bovine cells which results in the production of auto or self produced tissue engineered skin construct which would increase the tendency for ‘take’ of the graft. Human Umbilical Vein Endothelial Cells (HUVECs) were taken as endothelial cells. Three dermal equivalents were produced with seeding fibroblasts on top of biopolymer in one; HEVEC on biopolymer in second and endothelial-fibroblast dermal equivalent was prepared seeding both fibroblasts and HUVEC on biopolymer in third. Keratinocytes were seeded in all three dermal equivalents [4]. It was observed that HUVEC when cultured alone although expressed on the biopolymer scaffold but mostly showed features attributed to dying cells and produced very limited extracellular matrix. On the other hand fibroblast cultured alone attached to the biopolymer scaffold, proliferated and laid down extracellular matrix in appreciable amount. In equivalents with both cells, proliferation of fibroblasts with deposition of extracellular matrix was observed in which HUVEC migrated and formed new capillary like tubular structures. The capillary like tubular structures observed in
Endothelialized Dermis Equivalent (EDE) and Endothelialized Skin Equivalent (ESE) was not observed in case of absence of HUVEC.

**Taking in vitro results to in vivo experiments**

In another study by Tremblay et al. HUVEC seeded in dermis were used to develop microvasculature with engineered skin substitute and transplanted onto mice to take the in vitro technique developed by [4] to in vivo stage. This was compared with dermis without HUVEC.

Two different tissues were prepared. One the standard reconstructed dermis without endothelial cells and second reconstructed dermis with human umbilical vein endothelial cells (HUVEC). Adult male athymic mice were selected for graft transplantation. Excisions were made and deep wounds were created in three groups of mice. In one group human skin was transplanted, in other reconstructed skin without endothelial cells and in third endothelialized reconstructed skin was transplanted. Four mice in all three groups were sacrificed at day 4, 7 and 14 post-grafting for analysis. [16]

Capillary like Structures (CLS) were observed under epidermis in ERS before placement of graft while no such structures was observed in RS. Also red blood cells were detected in capillaries on fourth day after placement of skin graft. These red cells came from mice as no red cells were observed in capillaries of human skin before transplantation.

In ERS, after four days, blood containing vessels were seen underneath the epidermis. These vessels were identified via double staining which showed that vessels beneath epidermis contained human Endothelial Cells (ECs). It was also shown that mouse EC were only present in the lower compartment of graft and not in epidermis as seen with RS.

At 14 days after graft placement, a co-localization between human and mouse endothelial cells was seen in the graft through use of confocal microscopy. Presence of red blood cells in their lumen was a clear evidence of inosculation between graft capillary like structures and mouse capillaries.

Lastly total number of capillaries human and mouse were evaluated per tissue section for each type of grafts i.e. Human Skin, Reconstructed Skin without endothelial cells and Endothelialized Reconstructed Skin. There was a twofold decrease in number of human capillaries in Human Skin graft. This could be attributed to degeneration of supernumerary vessels that did not anastomose with the mouse capillaries. This type of decrease was not observed in Endothelialized Reconstructed Skin, in fact a statistically significant increase in number of mouse capillaries was observed in ERS [16].

In another study by Laschke et al., microvascular network was formed in the PLGA scaffold by placing the scaffold onto flank of donor mice. After 20 days when blood vessels had grown into the scaffold, forming a network of micro vasculature within the scaffold, the engineered scaffold was excised and transplanted onto the recipient mice. The results showed that preformed microvessels within tissue engineered skin substitute developed interconnections with host micro vasculature which resulted in accelerated vascularisation of cells in the graft compared to avascular grafts [25]

In 2010, a study was carried out by Gibot et al in which two substitutes were prepared. Endothelialized Reconstructed Skin (ERS) and RS (Reconstructed Skin) serving as control. The dermal layer in these models comprised of three layers of fibroblasts with inferior two plated with Human Umbilical Vein Endothelial Cells (HUVECs) in ERS and without HUVEC in RS. Graft site were prepared on athymic mice by removal of back skin and then tissue engineered skin was grafted onto the mice. [19]

After 30 days of in vitro culture, the Endothelialized Reconstucted Skin showed well developed and fully differentiated human
epidermis. In this model, human endothelial cells formed Capillary like Structures which has also been shown in previous studies. The lumens of micro vessels were well defined. Reconstructed skin endothelialized with either Human Umbilical Vein Endothelial Cells (HUVEC) demonstrated an organised network of Capillary like Structures (CLS) with a branching morphology.

The Endothelialized Reconstructed Skin developed its own intrinsic microvasculature where as Reconstructed Skin which was considered a negative control did not show development of such microvasculature. All the grafts showed a complete take at day. Both grafts, RS and ERS adhered completely with underlying hosts tissues at day but more vascularisation was observed in ERS compared to RS. It was also revealed that the human endothelial lined vessels transported mice red blood cells which demonstrated that functional anastomoses was established between graft microvasculature and hosts capillaries at. The human vessels formed were homogenously distributed in superficial and deeper layers ensuring complete perfusion of the entire graft [19].

**Discussion**

One of the main obstacles in wide spread use of Reconstructed Skin for wound coverage is delayed vascularisation due to thickness of dermis. But this thick dermis is important because it protects the reconstructed epidermis against mechanical, chemical and bacterial accumulations and also results in better healing. This however delays the complete vascularisation of the graft which ultimately culminates in necrosis of epidermis [16]

It took years to fully understand and appreciate the phenomenon of neovascularisation which is a key step in increasing take of graft and keeping it viable. It has been shown in previous studies that split thickness skin graft survives by diffusion, followed by inosculation and finally by neovascularisation. Composite skin substitutes without intrinsic blood supply cannot vascularise as the natural split thickness graft. Diffusion is responsible for supply of nutrients to the epidermis which is not sufficient to ensure the permanent implantation of grafted skin substitute until process of neovascularisation takes over. In study carried out by [4]which was the first study of its kind the aim was to develop capillary like network within the skin substitute in vitro, which may prove to be a cornerstone in tissue engineered skin substitutes [4]

The aim of the Black ET AL study was to develop in vitro endothelialized tissue engineered skin substitute which would demonstrate vascular like tube formation developed from human cells without the use of specific growth factors or carcinogenic agent like PMA. Their results were very promising and formed the basis of further trial of endothelial cells for formation of blood vessels within the Tissue Engineered Skin Substitute which could be tried in vivo.

The results of [4] study strongly suggest that HUVEC can organise and give rise to network of capillary-like structures when cocultured with dermal fibroblasts and keratinocytes. On the other hand these structures do not develop when each cell type is cultured independently. The results showed strong evidence that fibroblasts when present in conjunction with endothelial cells in a 3D culture medium lay down the extracellular matrix in which capillary like structures formed [4]

The study Black et al formed the basis of taking in vitro results to in vivo experiments carried out by Tremblay et al in which it was hypothesized that a reconstructed skin containing capillary like structures would promote faster vascularisation by anastomoses between graft vessels and hosts capillaries as had been shown by
transplantation of full thickness human skin on wounds in previous studies. In this study it was demonstrated that Human Umbilical Vein Endothelial Cells when added to the Reconstructed Skin accelerated the process of vascularisation as compared to conventional Reconstructed Skin.

Human capillaries filled with red blood cells were observed near the epidermis. These findings were in coherence with the previous work done by other groups of researchers. Same results were observed with ERS. Human capillaries containing mouse red blood cells were observed beneath epidermis after 4 days of graft placement and no mouse blood vessel was observed in the area in that time.

The filling of human capillary with mouse blood pointed to the fact that there were anastomoses or inosculations between the human and mouse vasculature. It was also observed that blood circulation establishment beneath epidermis was faster in ERS compared to RS and was as fast as seen in Human Skin.

The results showed that initial vascularisation observed at 4 days was due to inosculation of human capillary like structures with mouse vessels. Mouse capillaries extended by process of neovascularisation were not detected beneath epidermis of ERS until day 14. According to authors of study this was the first study which demonstrated inosculation process between human capillaries reconstructed in vitro through tissue engineering with wound bed vasculature [16].

The latest study in this regard using Endothelial Cells was carried out Gibot et al which was again based on the initial work done by Black et al. In this study, the aim was to develop an easy to handle ERS model, which could demonstrate in vitro formation of capillary like network by co culture of fibroblasts, endothelial cells (HUVEC) without use of external growth factors. This model could also be completely autologuos for future clinical applications. In this model, cell secreted extra cellular matrix and there was also cell-cell and cell-extracellular matrix interaction which created a physiological micro environment allowing endothelial cells to express capillary like structures. Also the thickness of model could be compared with human skin slit thickness graft generally used in clinical arena which is categorized as thin (130-300), intermediate (300-460) or thick (460-760) micro meter. The human microvasculature created in vitro anastomosed with host capillaries and became functional in less than 96 hours. Mouse red blood cells were found in lumen of human endothelial capillaries which demonstrated efficacious blood circulation. These anastomoses could only be established if hosts micro vessels grow in the graft and then establish inosculation with graft vessels.

Complete replacement of graft vasculature endothelial cells by host cells is key for skin graft revascularization and long term survival and acceptance of graft. In this study the graft was remodelled completely with host's endothelial cells. [19]

**Conclusion**

From use of Split thickness skin grafts, to grafts from cadavers and xenografts, the science of development of perfect Tissue Engineered Skin Substitute has come a long way. With in vitro development of microvasculature in Tissue Engineered Skin, the first steps towards perfect skin substitutes were taken.

“Although many studies have been conducted but it is safe to say that Tissue Engineering of Skin and its application in clinical practise is the beginning of a long journey. Now with improvement in cell culture techniques and advancement in knowledge, epidermis and dermis are being prepared separately from small biopsies taken from the recipient, in vitro. This is just the beginning, with final
aim of generating a full transplantable replica of skin with adnexa and vasculature” [38].

References


