Tissue-Engineered Skin Constructs and Application of Stem Cells for Creation of Skin Equivalents (Review)

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Development and introduction of new biotechnological analogues (equivalents) of tissues and organs into clinical practice, such as human skin equivalents (SE), designed for temporal or permanent replacement of damaged or destroyed tissue, remains an urgent problem of regenerative medicine. Currently, full-thickness SE as well as separate skin layers, which include living cells of different types, are being created and investigated. Since the ideal skin substitutes have not been created, the efforts of researchers in many countries are aimed at solving this problem.

In our review, we present a comparative analysis of existing SE, both commercial and those being at the stage of preclinical study, analyze their structure and feasibility of application for solving experimental and clinical tasks. Characteristics of the three main variants of SE have also been considered. Examples of stem cell application for creation of skin equivalents have been given. The main advantages of using stem cells as a cell component of skin equivalents have been described.

Key words: bioengineered skin substitutes; skin equivalents; biomaterials; tissue engineering; wound healing; stem cells.

Skin is the largest organ of mammals serving as a barrier at the interface between the human body and environment. Due to its boundary location, the skin is constantly affected by potentially detrimental microbiological, thermal, mechanical and chemical factors [1]. When skin is damaged, restoration of the barrier properties becomes the main task of the organism, which is primarily associated with the partial or full regeneration of the skin structure, as the structure and function of this organ are closely interconnected [2].

Impairment of the normal biological reaction to the skin injury due to a disease, trauma or operation inevitably leads to significant complications. Wound healing is an extremely complex process and, in case of chronic wounds, is often multifactorial [3]. A regenerative capacity in humans is greatly limited: in contrast to animals, the integument cannot recover by primary intention, and marginal epithelization is difficult. Currently, incomplete understanding of molecular, cellular, and physiological mechanisms regulating wound healing is often the cause of disappointing results of treatment.

The most important and quickly developing direction of the present regenerative medicine is application of cellular technologies in the treatment of acute and chronic wounds, diabetic ulcers, burns. The task of the cellular technologies, in this case, is not only to transplant living cells in the defect area but fully restore the structure and function of the skin, stimulate regenerative processes and create micro-environment to realize the potential

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of patient's own tissues and cells. Methods of tissue engineering are used for solving these tasks.

The main trends in tissue engineering are isolation and growth of cell and tissue cultures *in vitro*, investigation of the stem cell properties, the role of defect microenvironment, as well as feasibility of using biologically compatible synthetic materials. The works in this direction resulted in creation of histotypical or functional analogues (equivalents) of tissue and organs, such as, for example, human skin equivalents (SE). Such equivalents are already being employed for studying and modeling the biological processes. Besides, they are also being applied in clinical practice to speed up the healing of acute and chronic wounds, and in pharmaceutical investigations as test-systems.

The structure of skin equivalents

Human skin equivalents are bioengineered constructs, skin substitutes, consisting of a cellular component, i.e. cultured human skin cells, and a substrate (matrix, scaffold), being an analogue of the extracellular matrix [4].

The majority of tissue-engineered substitutes of a living skin are created by culturing the skin cells in the laboratory and combining them with a scaffold. SE are used to restore the structure and, consequently, the barrier function of the skin (the main goal of burn patient treatment), and for the initiation of wound healing (in chronic non-healing wounds). Employment of SE hastens wound healing, reduces a pain syndrome, inflammation, and prevent formation of scars, contracture or pigment defects [4].

The main requirements to SE are their biological and toxicological safety, efficacy, and absence of immunogenicity. It is important to take into consideration that any cellular material bears a risk of viral, bacterial or other infection, and scaffolds may be individually intolerant for patients or cause a strong inflammatory reaction.

Besides, it is desirable for SE to be biodegradable and to promote regeneration of the normal tissue with similar physical and mechanical properties inherent to the replaced human skin. Of great importance is also economic efficiency of biomaterial production, its availability and a long storage life [2, 5].

A cellular component

Fibroblasts and keratinocytes are the dominant types of cells in the mammalian skin. Accordingly, in the vast majority of investigations on wound healing one or both types of these cells is used as a cellular SE component.

Epidermal keratinocytes represent the main part of the skin cells. During keratinization, synthesis of special proteins takes place in them: acidic and alkaline types of keratins, filaggrin, involucrin, keratolinin and others, resistant to mechanical and chemical actions [6, 7]. Keratinocytes of the basal layer are capable of active division, and as differentiation proceeds, the cells move from the basal layer to the superficial skin layers. So, every 3–4 weeks, the epidermis is renewed (physiologically regenerated) [8, 9].

The first keratinocyte culture was obtained in 1975 by Reinwatd and Green [10]. Initially, the keratinocytes were cultured using feeder cells — murine fibroblasts of 3T3 Swiss line. Further on, feeder cells were replaced by adding growth factors and extracellular matrix proteins to the culture medium. Some of them turned out to facilitate keratinocyte growth, and could be used as substrates for culturing [11–13].

Dermal fibroblasts represent heterogeneous cell population of mesenchymal type, and play a key role in regulation of cell interactions and maintenance of skin homeostasis. Dermal fibroblasts produce all main components of intercellular matrix: collagen, glycosaminoglycans, proteoglycans, and are also responsible for the continuous process of matrix remodeling [14]. Owing to these properties fibroblasts are widely used for SE creation.

Melanocytes are one more numerous type of human skin cells, an average number of which in relation to keratinocytes in the epidermis amounts to 1:36. In a human, epidermal melanocytes form a structural and functional epidermal unit in the complex with the cells of the basal and spinous layers of epidermis, keratinocytes. By way of phagocytosis, these keratinocytes capture melanosomes, synthesized by melanocytes, and regulate thereby the speed of melanin synthesis, the main pigment of the human skin, which provides photoprotection of the skin from harmful sun radiation [15]. Because of the uniqueness of these cells, melanocytes are used for creation of pigmented SE, which are the model for investigating the regulation of the skin pigment system work and mechanisms of photoprotection from detrimental ultraviolet radiation [16]. This type of cells may be also used to impart pigmentation to SE. However, a high liability of the melanoblasts to tumor formation should be taken into account (melanomas).

Investigations on creation of prevascularized SE via introduction of ***endothelial cells*** within its structure can be found in the literature. In 2014 there was published a work, in which a population of endothelial cells isolated from a stromal vascular fraction of the adipose tissue and cultured in the gel consisting of the collagen and fibrin, was used for creation of the model of a full-layer SE [17]. At the same time, a group of scientists under the guidance of Marino [18] obtained a full-layer SE from the cultures of dermal microvascular endothelial cells of the human foreskin immersed in the three-dimensional gel. Such cultures were composed of the mixture of the lymphatic and endothelial cells of blood vessels. Owing to this *in vitro* formation of functional blood and lymphatic capillaries in the hydrogel were observed. Further on, SE-containing keratinocytes, fibroblasts and rudimental vascular network in the gel were transplanted

on the artificially simulated wound of immunodeficient rats. As a result, both teams of authors report positive effects observed after transplantations of these SEs: in the *in vivo* equivalent such layers as stratified epidermis and vascularized derma are formed, formation of anastomoses with the recipient vessels during four days is observed. Equivalent contraction was absent owing, most likely, to effective blood supply and substantially rapid establishment of epidermal homeostasis.

One more concurrently evolving direction of developing tissue-engineered constructions is application of ***stem cells*** (SCs). These cells are characterized by the capacity of a long-term self-renewing, and differentiation into tissue-specific cells by asymmetric division [19, 20]. It is this ability of differentiation into the cells of various tissue types that can help solving the problem with creation of such skin components, which are absent in the traditional equivalents and, thereby, improve their efficacy [21].

Immunocompetent cells are known to penetrate via blood in the wound area during the inflammatory phase of wound healing. There also exist data that bone marrow SCs are present in the wound bed either [22, 23]. Besides, heavy trauma was shown to result in the increase of circulating SCs [24]. This phenomenon was also confirmed in the experiments with simulated injuries of various tissues. For example, Badiavas et al. [25] used a model of the skin wound in mice, which were transplanted a bone marrow labeled with a green fluorescent protein (GFP). They found GFP-labeled cells, which were differentiating into tissue-specific skin cells, in the wound area. In the similar experiments of Fathke et al. [26], bone marrow SCs are reported to promote restoration of dermal fibroblast population in skin damage. Such data testify the importance of SCs migration in the process of wound healing, which is not yet fully studied and demands further exploration.

Development of methods of trauma treatment and wound healing using SCs is mainly associated with SCs of adults, in particular, with multipotent stromal cells or, as they are also called, *mesenchymal stem cells* (MSCs). MSCs are of great importance for regenerative medicine not only because of their multipotency, trophic and immunomodulating properties but owing to their stability during cultivation as well [27].

In *in vitro* studies, MSCs have demonstrated a number of properties, which can facilitate tissue regeneration and even accelerate this process, including synthesis of some growth factors, cytokines, collagen and matrix metalloproteinases, angiogenic factors [26, 28, 29]. Besides, they are capable of providing migration of other skin cells such as keratinocytes [30].

Preclinical and clinical studies of MSCs effect on tissue regeneration and wound healing showed a good result. Thus, in one investigation the authors studied the impact of bone marrow, placed on the collagen matrix, on the wound healing in mice. As a result of their research work, a substantial increase of angiogenesis has been found [31]. In the work of Falanga et al. [32] a variant of this approach was applied consisting in the use of polymer fibrin spray with autologous MSCs, obtained from the bone marrow aspirate, to speed up healing of acute and non-healing skin wounds in mice, and thereafter in humans. Such approach represents one more optional version of introducing cells into the wound area. Badiavas and Falanga [33] published the clinical results with application of autologous bone marrow cells used for healing chronic skin wounds in three patients resistant to standard therapy for more than a year. Several days after cell introduction, improvement of wound condition was observed in all patients, showing a stable reduction of the wound size, increase of the dermal layer thickness and wound vascularization. Another investigation of diabetic foot chronic ulcers [34] included 29-day treatment using a transplant composed of autologous dermal fibroblasts in combination with autologous MSCs inoculated on biodegradable collagen substrates, which were applied directly on the wound, injections of cell suspension to the wound margins were also made on days 1, 7 and 17. As a result, diminishing of the wound size, and increase of derma thickness and vascularization were observed. Yoshikawa et al. [35] employed autologous bone marrow MSCs to treat 20 patients with diverse non-healing wounds (burns, lower limb ulcers, and pressure sores) with or without autologous skin transplantation. In 18 of patients wounds healed completely, and histological examination showed that addition of MSCs contributed to more rapid regeneration of the native tissue.

The results of MSCs application are fascinating, but this approach to therapy requires, as a rule, a large quantity of cellular material. It is not a problem if small wounds are to be treated but it may become a challenge in case of vast injury treatment.

Another type of cells can also be considered as one of the SC components, i.e. *dermal papilla* (DP) *cells,* which are a part of the skin hair follicles. These cells are known to be one of the main cellular population of the hair follicle determining its viability and functionality. For the previous 20 years it has been found that the main property and chief biological role of DP cells are their ability to induce and regulate morphogenesis of a hair follicle [36]. However, findings of the investigation [37] demonstrated that DP are capable of differentiating in osteogenic and adipogenic direction, therefore, they may be referred to the classical mesenchymal fibroblast-like cells with special functions. It is the reference of DP cells to MSCs gives grounds to suggest that a skin equivalent containing DP cells will speed up neoangiogenesis, remodeling of extracellular matrix, formation of granulation tissue and skin regeneration. This is proved by the results presented in the work of Leirós et al. [38]. The researchers created SE, in which they used only SCs of hair follicles or SCs hair follicle together with human DC cells as a cellular component. After transplantation of SE to immunocompromised mice,

they assessed the structure of the tissue-engineered skin, SCs survival, hair follicle regeneration and graft take. Presence of DP cells was shown to facilitate formation of more structured, multilayer, stratified epidermis with basal p63-positive cells and crests more exactly mimicking the normal skin architecture. Besides, presence of DP cells in SE contributes not only to a good graft take and its remodeling after transplantation but also to formation of new vessels and maturation of the nonvascular network, which leads to the reduction of the inflammatory process, effective healing with less scarring and wound contraction. Interestingly, that only DP-containing constructs showed rudiments of hairs, precursors of epithelial cells, and expression of the hair differentiation marker. Thus, these results have assessed the important role of DP cells in the correct skin repair.

Works are actively being carried out to study the application of *induced pluripotent stem cells* (iPSCs) as an alternative cell component.

Investigations of recent years have shown that usage of iPSCs makes it possible to obtain effective tissue-engineered products for regenerative medicine including treatment skin wounds [39]. A good illustration is the work of Itoch et al. [40] in which they elaborated a protocol of IPSCs differentiation into keratinocytes and dermal fibroblasts to be used in the treatment of recessive dystrophic epidermolysis bullosa. Besides, they created 3D equivalents of human skin consisting of keratinocytes and fibroblasts obtained from iPSCs only. Gledhill et al. [41] reported in their work the creation of functioning pigmented 3D skin equivalent composed of differentiated derivatives of iPSCs (keratinocytes, fibroblasts, melanocytes) and containing functional epidermal melanin units. For the first time, the process of melanin synthesis by IPSCs derivatives was described, as well as the process of melanin transferring between melanocytes and keratinocytes. This study is an important direction in the creation of more complicated patient-specific skin models, which, among other things, may become a useful tool for searching novel pharmaceutical preparations in the epoch of personalized medicine.

Biopolymer matrices

Biomaterials, acellular natural or synthetic substances, are used as carriers for SE during creation of tissue-engineered constructs. They imitate extracellular matrix.

Examples of natural substances are polypeptides, hydroxyapatites, hialuronas, glycosaminoglycans, fibronectin, collagen, chitosan and alginates.

Synthetic fully degradable substances are polyglycolides, polylactides, poly(lactide-co-glycolide), polytetrafluoroethylenes, polycaprolactones, polyethylene terephthalates, and the most common nondegradable matrix is polyurethane [42]. Current technologies of 3D printing and electrospinning make it possible to produce biomaterials with exactly specified form, a definite pore size and other necessary parameters. The main drawback of synthetic materials is absence on them the recognition signals for the cells. One way to overcome this disadvantage is inclusion adhesion peptides in the matrix using, for example, RGD-sequences. The so-called friendly matrices consist of the materials capable of controlling cellular metabolism and differentiation, essentially accelerating regeneration [43]. For example, hydrogels of polyethylene glycol, which acts as an inert framework, are included in the substrate, since glycogen hydrophilic is inert in regard to protein absorption. The gel can be modified by attaching to it adhesion RGD-sequences or functional domains. Besides, the degree of biodegradability can be regulated including protease-sensitive oligopeptides [44–46].

Not only the composition but also the shape of the graft used influences material biocompatibility. Matrices for the cells may be in the form of gels, micro- and nanospheres, fibers, films, various 3D constructs [47, 48].

Matrices of the natural origin. Decellularized derma or other stromal structures may be considered to be similar to the native tissue to the most extent. However, they have some limitations: availability of the material, difficulty of standardization and manipulation in the course of culturing (infeasibility of microscopy), risk of infecting patients. The most familiar examples of natural matrices, which are not only included in the SE composition but produced as separate products, are acellular lyophilized matrices of the human skin (AlloDerm) and porcine skin (Permacol). These materials are obtained by removing epidermis and intradermal cellular elements preserving, at the same time, the structure of the native derma. The advantage of these materials is in their natural dermal porosity necessary for rapid regeneration and vascularization of the graft. Investigations *in vitro* showed that matrices from decellularized derma promote adhesion, growth and functioning of several cell types [49, 50]. Additionally, during creation of these matrices the basal membrane is partially preserved, which may help in epidermal cell attachment [51]. Nevertheless, these products are known to be expensive and to have the risk for transmitting viral and other infectious agents [52].

Artificial collagen matrices. Collagen is the main protein of the extracellular matrix of the dermal skin layer. Medico-biological properties of the collagen, i.e. the capacity to accelerate wound healing, enhance thrombocyte adhesion and induce homeostasis, to be a natural substrate for patient skin cell migration without antigenicity, determined its wide application in the reconstructive surgery [53].

There exist three forms of collagen used during creation of SE: hydrogel, sponge, and mesh.

Collagen gel was used during creation of dermal equivalent to study the effect of cells on collagen contraction, which was later connected with wound contraction [54]. However, researches showed the

necessity of using collagen with higher viscosity and strength in the form of meshes and sponges. A collagen mesh, made of tightly twisted collagen fibers, represents a natural supporting bionetwork, which is formed by restructuring of collagen by fibroblasts. It was employed for imitation of a dermal skin layer *in vitro*. But after transplantation, a low degree of contraction of the collagen mesh increased the time of wound closure. Besides, the reduction of collagen synthesis due to cell disunity was found [55, 56]. Collagen sponges, as a rule, are obtained by lyophilization of collagen solution or collagen gel [57]. Their important properties are a specified mechanical strength, which provides the possibility to use them as a base for tissue-engineered constructs, and ability to biodegradation in the body. Such equivalents on the sponge matrix are effectively employed for skin regeneration in the experiments on animals and in treating skin wounds in patients [58–60].

Various modifications of collagen substrates using combinations of collagen with other natural or synthetic polymers such as glycosaminoglycans, chitosan, polycaprolactone and copolymer of the lactic and glycolic acids are used to improve mechanical properties, biostability, to prevent collagen matrix contraction in the course of wound healing, and to prolong clinical life-time of the graft [61, 62].

Chitosan matrices. Chitosan is one more natural polymer most widely used alongside with collagen for wound healing. It possesses numerous advantages including biocompatibility with biological tissues, biodegradation, hemostatic activity and antibacterial properties [63–65]. Chitosan can stimulate collagen synthesis and bind to fibroblast growth factor, that may increase the rate of wound healing [66, 67]. Materials from chitosan with diverse structure are easy to produce. However, chitosan is easily degraded in the human tissues, especially in the acid medium, which is often formed in wound healing [68]. Different chitosan modifications are used to enhance structural stability, including a combination with other polymers, e.g. with collagen, gelatin, and glycosaminoglycans [69]. These modifications can increase biostability and improve mechanical properties of the matrix. But such disadvantages of chitosan as deformation and weak biostability in the human tissues prevent its application in the field of tissue engineering.

Other natural polymers, fibrin and gelatin, also possess a high biocompatibility but are effectively used only as an additional component with other polymers for mutual supplementation or alteration of general biological or mechanical properties of a biomaterial [70–73].

Synthetic polymer matrices. As a rule, synthetic polymers such as polyurethane and aliphatic polyethers on the basis of lactide, glycolide, □-caprolacton, have less expensive and more reliable sources of primary products. Using various methods of production, they may be imparted different physical properties [4].

Researches with application of synthetic polymers for SE creation were directed to the possibility of combining them with natural polymers. An example of such combined matrix is Integra preparation, which consists of bovine collagen and chondroitin-6-sulfate with a thin silicon substrate serving as a temporary replacement of epidermis. The preparation is reported to give good esthetic and functional results in treating burns [74]. Nevertheless, infection still remains the most common complication after using Integra [75–77]. A thorough preparation of the wound bed prior to the usage of this model (or a similar type of artificial biological materials) and absence of infection after application of the equivalent is of critical importance for a successful healing. Nowadays, owning to new dressing means such as Acticoat, which is applied over Integra as an additional bandage [78], and other antiseptic technologies [79–81] the risk of infection diminishes.

One more combined preparation, which has currently been spreading widely, is MatriDerm. It consists of bovine collagen and elastin hydrolysate. In contrast to Integra, which has antigenic properties due to the presence of chonroitin-6-sulfate, the combination of collagen and elastin in MatriDerm is supposed to spur vascularization facilitating cell ingrowth and vessel generation, improving stability and elasticity of regenerating tissue [78]. Besides, MatriDerm degrades more rapidly than Integra [82].

Types of skin equivalents

Skin equivalent may represent a monoculture and contain only a layer of epidermis, or only a layer of derma, or have a full-layer structure depending on the designation [2]. So, the existing types of SE can be divided into three main groups: epidermal, dermal, and full-layer.

An epidermal type of the equivalent

Keratinocytes are used to create this type of SE. Depending on the origin of the obtained cells, these equivalents may be autologous (the origin is patient's own skin) or allogeneic (cells are obtained from donor's skin). To isolate keratinocytes, it is enough to have a skin flap 1–2 cm^2 in size. With the help of enzymes and mechanical actions, epidermis is separated from derma, and thereafter by means of additional enzymatic treatment, suspension of separate keratinocytes is obtained. Primary keratinocytes are cultured for several weeks in the laboratory, and finally keratinocyte sheets, the area of which is several times greater the size of the donor skin flap, are obtained [1, 83]. Green et al. were the first to use the sheets of cultivated epithelium, created from autologous keratinocytes, for transplantation to two patients with extensive burns [84, 85]. Epidermal autografts (EA) were later used for constant coverage of extensive burns of two other

patients [85]. Transplantation of the grown epidermal strata was successfully performed in Russia as well to treat burn patients [86].

One of the chief drawbacks of EA is a weak graft take, mainly, on the wounds lacking dermal elements, even in case of correct keratinocyte culturing [87–89]. As early as in the middle of 1980s, Cuono et al. [90, 91] demonstrated the importance of dermal component availability, they reported a good take for EA placed on a healthy vascularized allogenic derma. But the method suggested by them has its own disadvantages. Firstly, in some countries, where transplantation of organs and tissues is not practiced yet, skin allografts may not be available [92, 93]. Secondly, flaps of allogenic (cadaveric) derma bear the risks of infection or rejection. Thirdly, it is difficult to coordinate two successive stages of transplantation: first placement of derma allografts on the wound and thereafter placement of EA. It was noted that in case of allogenic derma rejection before employment of cultured EA, this method of treatment becomes impossible [94]. And finally, a high cost of EA production is often indicated in many reviews as one of the main impediments for a wide use [95–97].

A dermal type of the equivalent

As a rule, it represents the cells of the connective tissue — fibroblasts together with collagen matrix (scaffold). The cells can occupy the surface and/or the entire substrate volume. Dermal equivalent can be produced on the basis of other cells of the connective tissue, MSCs, and practically any of the currently existing 3D substrates can be used as an extracellular matrix. According to the literature data, there are a lot of commercially available dermal equivalents and many of such products have been well analyzed and tested at the level of preclinical and clinical trials [78, 98–101]. The majority of current biocompatible dermal grafts are capable, to some extent, of simulating the main properties of human skin connective tissue, providing structural integrity, elasticity and presence of the bloodstream. Fibroblasts are easy to isolate and technologically culture, and at the same time, they are an active cellular component, able to structure derma collagen, stimulate wound granulation and secrete a number of growth factors accelerating skin regeneration. No wonder that dermal equivalents with fibroblasts are so widely used over the world.

Nevertheless, in case of dermal equivalent application a problem of epithelization of the large skin injuries remains unsolved, and in the majority of cases usage of such products is combined with application of skin autografts for constant covering [102].

Development of dermal equivalents using synthetic materials started in 1990s, but presently they are not so commonly used. TransCyte can be referred to these products. It is composed of allogenic fibroblasts, derived from human neonatal foreskin, bound to a silicon membrane and cultured on the porcine collagen covering a nylon mesh. Dermagraft, another equivalent, consists of human cryopreserved allogenic fibroblasts obtained from a foreskin of newborns, grown on a biodegradable mesh from polyglactin (vicryl) [103]. At present, these products are not presented in the market, but these technologies have been licensed by Advanced BioHealing for further improvement [78]. Unlike TransCyte, Biobrane has been in use until now as a synthetic skin equivalent for healing second-degree burns in many centers [104–106]. It is similar in structure to TransCyte but contains a less number of human neonatal fibroblasts. It is also used as a dressing material together with autografts in complex wound topology, as well as for keratinocyte culturing [105, 107]. Biobrane is popular owing to its multi-purpose application and low cost in comparison with TransCyte, being at the same time highly effective in treating second-degree burns [108].

Original dermal equivalent has been developed in Russia at the Koltzov Institute of Developmental Biology, Russian Academy of Sciences. Fibroblasts, previously grown on gelatin or collagen microcarriers, were incorporated into 3D collagen gel. Microcarriers are a specific three-dimension matrix in the form of tiny (50–70 μm) smooth or porous spheres with a longer period of biodegradation relative to gel. Such equivalent fills better full-layer deep defects of the connective tissue, for example, fistulas [109].

A full-layer type of the equivalent

A full-layer SE type, called also a living skin equivalent, consists of the epidermal and dermal layers.

A perspective autologous skin epidermal equivalent is a composite cultured substitute developed in Cincinnati (USA). This SE is composed of collagen-glucosamine glycan substrate, which contains autologous fibroblasts and keratinocytes. This product, known at present as PermaDerm [2], can be produced within 30 days and is able to provide constant replacement of dermal and epidermal skin layers. It is indicated for a large skin defect treatment, but has not been approved by FDA (United States Food and Drug Administration) for clinical applications [94, 110–114].

In 2009 a group of German scientists reported the development of a composite autograft using MatriDerm as a matrix for growing autologous fibroblasts and keratinocytes [115]. This full-layer skin equivalent was claimed to be homologous to a healthy human skin. The grounds for such declaration were the characteristics of the epidermal layer, comparison of differentiation and proliferation markers, availability of the functional basal membrane. Transplantation of this equivalent showed a good result with a full closure of relatively small (about 9$_{\times}$6 cm) wounds [116, 117].

The best-known of full-layer SE is Apligraf. This is a skin equivalent comprising a dermal component, matrix

Table 1

Commercial products of skin equivalents applied in clinical practice as transplants (according to: Vyas and Vasconez, 2014 [127] with amendments)

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
Epicel® Genzyme Tissue Repair Corporation, Cambridge, MA, USA (2007) Permanent skin substitute Living cell therapy Cultured epithelial autograft (CEA)	Autologous keratinocytes with murine fibroblasts are cultured to form epidermal autografts which are then processed into sheets and placed onto petroleum gauze It is used as an adjuvant to split-thickness skin grafts or alone if split-thickness skin grafts are not available due to the extent or severity of the burns	Recommended for treatment of deep dermal or full thickness burns (greater than or equal to 30% total body surface area); grafting after congenital nevus removal For treatment of diabetic and venous ulcers	*Burns:* No randomized control trials (RCT) have been conducted to evaluate the effectiveness of this product in improving health outcomes for deep dermal/full thickness burns In a large, single-center trial, Epicel CEA was applied to 30 burn patients with a mean total body surface area (37±17%). Epicel achieved permanent coverage of a mean of 26% total body surface area compared to conventional autografts (mean 25%). Final CEA take was a mean 69±23%; 90% of these severely burned patients survived [128]	*Advantages:* Use of autologous cells obviates rejection Permanent large area wound coverage, especially in extensive burns *Disadvantages:* Long culture time (3 weeks) Variable take rate Poor long-term results 1-day shelf life Expensive Risk of blistering, contractures, and infection
Laserskin® Fidia Advanced Biopolymers, Abano Terme, Italy Permanent skin substitute	Autologous keratinocytes and fibroblasts derived from a skin biopsy cultured on a laser-microperforated biodegradable matrix of benzyl esterified hyaluronic acid. Cells proliferate and migrate through the matrix Microperforations allow for drainage of wound exudate	For treatment of diabetic foot ulcers and venous leg ulcers, partial thickness burns, vitiligo	*Diabetic foot ulcers:* A multicenter RCT on patients with unhealed diabetic foot received treatment (Hyalograft-3D autograft and then Laserskin autograft after two weeks) and comparison with control (paraffin gauze) showed significant efficacy of treatment [129] *Wounds:* In a retrospective observational study in 30 patients with chronic wounds not responding to conventional therapy, keratinocytes on Laserskin to treat superficial wounds or fibroblasts on Hyalograft-3D to treat deep leg ulcers were applied; the wounds were then dressed with nanocrystalline silver dressing. A reduction in wound dimension and exudates and an increase in wound bed score was observed. The group treated with keratinocytes had a significantly greater degree of healing compared to those treated with allogenic fibroblasts [130] Collagen matrices such as Integra have been poor recipients of cultured keratinocytes, although some studies report successes in the use of Laserskin on the neodermis of Integra® after the silicone membrane is removed 14–21 days post-grafting [131, 132]	*Advantages:* Use of autologous cells obviates rejection Can be produced in shorter period of time than confluent epidermal sheets Does not require the use of the enzyme dispase to remove the sheets from culture flasks, in contrast to CEA Good graft take Low rate of infection Ease of handling during application Transparency allows wound to be visualized during dressing changes *Disadvantages:* Only available in Europe 2-day shelf life Expensive

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
TransCyte® Shire Regenerative Medicine, Inc., San Diego, CA, USA; Smith & Nephew, Inc., Largo, FL, USA (1997) Temporary skin substitute Composite matrix	Human allogeneic fibroblasts from neonatal foreskin seeded onto silicone covered bioabsorbable nylon mesh sponge and cultured *ex vivo* for 4–6 weeks, secreting components of the extracellular matrix and growth factors	Temporary covering of deep partial thickness and full thickness burn wounds For chronic leg ulcers diabetic foot ulcers lasting more than 6 weeks; venous and pressure ulcers	*Burns*: 33 children with partial-thickness burn wounds were randomized to receive TransCyte, Biobrane, or Silvazine cream. Mean time to re-epithelization was 7.5, 9.5 and 11.2 days, respectively. Wounds requiring autografting were 5, 17 and 24%, respectively. TransCyte promoted faster re-epithelization, required fewer dressings, and required less autograft compared to those treated with Biobrane or Silvazine [133] In a randomized prospective study of 21 adults with partial-thickness burn wounds to the face, patients treated with TransCyte had significantly decreased daily wound care time (0.35±0.10 vs. 1.9±0.5 h), re-epithelialization time (7±2 vs. 13±4 days), and pain (2±1 vs. 4±2) compared to patients treated with topical bacitracin [134] 20 pediatric patients with burns over 7% of the body were treated with TransCyte and compared to previous patients those who received standard therapy of antimicrobial ointments. Only one child required autografting in the TransCyte group, compared to 7 children in the standard treatment group. In addition, children treated with TransCyte had a significantly decreased length of stay (5.9 vs. 13.8 days) [135] 110 patients with deep partial-thickness burns were treated with dermabrasion and TransCyte and compared with data from the American Burn Association Patient Registry. Patients with 0–19.9% total body surface area burn treated with dermabrasion and TransCyte had a significantly shorter length of stay of 6.1 vs. 9.0 days [136] *Wounds:* A randomized prospective comparison study of TransCyte and silver sulfadiazine on 11 patients with paired wound sites was performed. Wounds treated with TransCyte had significantly quicker healing times to re-epithelialization (mean 11.14 *vs.* 18.14 days). Wound evaluations at 3, 6, and 12 months revealed that wounds treated with TransCyte healed with significantly less hypertrophic scarring than those treated with silver sulfadiazine [137]	*Advantages*: Easy to remove compared to allograft Widely used for partial-thickness burns Improved healing rate 1.5 year shelf life *Disadvantages*: Expensive

Continued Table 1

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
Dermagraft® Shire Regenerative Medicine, Inc., San Diego, CA, USA (2001) Permanent or temporary skin substitute Living cell therapy Allogenic matrix derived from human neonatal fibroblast	Cryopreserved allogenic neonatal fibroblasts derived from neonatal foreskin and cultured on bioabsorbable collagen on polyglactin (Dexon) or polyglactin-910 (Vicryl) mesh for several weeks The biodegradable mesh disappears after 3–4 weeks	For full-thickness diabetic lower extremity ulcers present for longer than 6 weeks extending through the dermis but not to the tendon, muscle, or bone [92] Chronic wounds, and noninfected wounds. It can be used as a temporary or permanent covering to support take of meshed split-thickness skin grafts on excised burn wounds	*Diabetic foot ulcers*: A multicenter RCT with 314 patients with chronic diabetic ulcers to Dermagraft or conventional therapy was performed. At 12 weeks, 30.0% of the Dermagraft patients had complete wound closure compared to 18.3% of control patients. Although the incidence of adverse events was similar for both groups, the Dermagraft group (19.0%) experienced significantly fewer ulcer-related adverse events (infection, osteomyelitis, cellulitis) compared to the control group (32.5%) [138] A prospective, multicenter RCT in 28 patients with chronic diabetic foot ulcers (more than 6 weeks duration) comparing intervention (Dermagraft + saline gauze) to control (saline gauze) was performed. By week 12, 71.4% of ulcers healed in the experimental group compared to 14.3% in the control. Wounds closed significantly faster in patients treated with Dermagraft [139] A randomized, single-blind DOLCE trial compared the differences among acellular matrices (Oasis), cellular matrices (Dermagraf), and standard of care in the treatment of diabetic foot ulcers using the primary outcome of complete wound closure by 12 weeks [140] A multicenter clinical trial of Dermagraft in the treatment of diabetic foot ulcers in 62 patients after sharp debridement was performed. Patients received dressing changes with saline gauze or polyurethane foam dressings weekly. By week 12, 44% patients had complete wound closure, and 52% healed by week 20. Median time to healing was 13 weeks. Dermagraft was safe and effective in the treatment of non-healing ulcers [141] A prospective multicenter randomized single-blinded study to evaluate wound healing in 50 patients with diabetic foot ulcers was performed. Patients were randomized into one of four groups (three separate dosages of Dermagraft and one control group). A dose response curve was observed and ulcers treated with the highest dosage of Dermagraft healed significantly more than those treated with conventional wound closure methods.	*Advantages:* Semitransparency allows continuous observation of underlying wound surface Cell bank fibroblasts have been tested for safety Easier to remove and higher patient satisfaction compared to allograft Equivalent or better than allograft for graft take, wound healing time, wound exudate and infection No adverse reactions, such as evidence of rejection *Disadvantages:* Used for temporary coverage 6-month shelf life *Contraindications:* Clinically infected ulcers Ulcers with sinus tracts Hypersensitivity to bovine products

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
			50% of the Dermagraft and 8% of the control ulcers healed completely [142] *Venous leg ulcers:* A prospective multicenter RCT to evaluate Dermagraft + compressive therapy vs. compressive therapy alone in the treatment of venous leg ulcers was conducted. For ulcers less than 12 months duration, 52% of patients in the Dermagraft group vs. 37% patients in the control group healed at 12 weeks. For ulcers less than 10 cm^2, complete healing at 12 weeks was observed in 47% patients in the Dermagraft group compared with 39% patients in the control group [143] A prospective RCT in 18 patients with venous leg ulcers treated with Dermagraft + compression therapy or compression therapy alone was performed. Healing was assessed through ulcer tracing and planimetry. The rate of healing was significantly improved in patients treated with Dermagraft [144]	
Apligraf®/Graftskin® Organogenesis, Canton, MA, USA (1998, 2001) Permanent skin substitute Living Cell Therapy Composite matrix	Epidermal allogeneic keratinocytes derived from neonatal foreskin cultured on a type I bovine collagen gel seeded with living neonatal allogeneic human fibroblasts in dermal matrix	For chronic partial and full thickness venous stasis ulcers and full thickness diabetic foot ulcers For epidermolysis bullosa, recurrent hernia repair, pressure sores, burn reconstruction	*Venous leg ulcers:* A Cochrane review concluded that a bilayer artificial skin used in conjunction with compression bandaging increases venous ulcer healing compared with a simple dressing plus compression [145] In a prospective multicenter RCT of 240 patients with hard-to-heal chronic wounds (more than 1 year) receiving either intervention with Graftskin plus compression or compression alone, treatment with Graftskin with compression was significantly more effective in achieving complete wound closure at 8 weeks (32 vs. 10%) and significantly more effective at 24 weeks (47 vs. 19%) [146]. A previously conducted prospective RCT by the same group revealed similar results [147] *Burns:* In a multicenter RCT of 38 patients with acute split-thickness skin grafts wounds, Apligraf flap was placed over meshed autograft while control sites were treated with meshed autograft only. There was no difference in the percent take of meshed split-thickness autograft with or without Apligraf. The Apligraf group demonstrated	*Advantages:* Small wounds require one application Improved cosmetic (scar tissue, pigmentation, texture) and functional outcomes in chronic wounds Primary role in treating chronic ulcers *Disadvantages:* Large wounds may require multiple applications 5-day shelf life Potential for viral transmission; mothers blood and donor's cells screened; cell banks screened for product safety Consider ethics with use of biological material: bovine collagen (Hindus, Buddhists; vegetarians); derived from foreskin (Quakers) *Contraindications:* Infected wounds Allergy to bovine collagen

Continued Table 1

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
			significantly improved vascularity, pigmentation, wound healing and Vancouver burn scar scores. A cosmetic and functional advantage of Apligraf compared to controls was demonstrated [118] *Donor site healing:* A RCT of 60 skin donor sites treated with meshed autograft, meshed Apligraf, or polyurethane film dressing was conducted. The healing time with Apligraf (7.6 days) was significantly shorter than with polyurethane film dressing In a multicenter RCT of 10 patients treated with Apligraf, Apligraf with dermis-only, and polyurethane film for acute split-thickness skin grafts donor sites, there were no differences among the treatment modalities including establishing basement membrane at 4 weeks [148] *Diabetic foot ulcers*: In a multicenter RCT of 72 patients comparing Apligraf and standard therapy vs. standard therapy alone in the treatment of ulcers, there was a significantly shorter time to complete wound closure in the Apligraf group (51.5%) compared to with standard treatment (26.3%) at 12 weeks [149] In a prospective multicenter RCT of 208 patients randomly assigned to ulcer treatment with Graftskin or saline-moistened gauze (control), (56%) of Graftskin patients achieved complete wound healing compared to 38% in the control at 12 weeks. Kaplan–Meier curve to complete closure was also significantly lower for Graftskin (65 days) compared to control (90 days). Osteomyelitis and lower-limb amputations were less frequent in the Graftskin group [150] Treatment with Apligraft plus good wound care for diabetic foot ulcers results in 12% reduction in costs during the first year of treatment compared to good wound care alone [151] *Wounds:* In a prospective RCT of 31 patients requiring full-thickness surgical excision for non-melanoma skin cancer	

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
			Apligraf reduced post-operative pain, but it was not determined whether it could decrease healing time or result in better aesthetic outcomes [152] In a prospective controlled clinical trial, 48 deep dermal wounds were created and Apligraf, split-thickness skin grafts, or dressing was applied. Apligraf demonstrated more cellular infiltrate but less vascularization compared to controls. Apligraf also demonstrated survival of allogeneic cells in acute wounds for up to six weeks and was recommended for the management of acute surgical wounds [153]	
OrCel® Forticell Bioscience, New York City, NY, USA (1998) Living Cell Therapy Composite matrix	Neonatal foreskin-derived keratinocytes and dermal fibroblasts cultured in separate layers into a type I bovine collagen porous sponge During healing, autologous skin cells replace the cells in the product	Approved in 2001 for use in patients with dystrophic epidermolysis bullosa undergoing hand reconstruction surgery to close and heal wounds created by surgery, including donor sites Approval for autograft donor sites in burn patients (overlay on split thickness skin grafts to improve cosmesis and function) For chronic diabetic and venous wounds	A randomized matched pairs study comparing treatment of split-thickness donor site wounds with OrCel or Biobrane-L revealed that scarring and healing times for sites treated with OrCel were significantly shorter than for sites treated with Biobrane-L [58]	*Advantages:* 9-month shelf life *Disadvantages:* Cryopreserved Cannot be used in infected wounds, in patients who are allergic to any animal products, or in patients allergic to penicillin, gentamycin, streptomycin, or amphotericin B
GraftJacket® Wright Medical Technology, Inc., Arlington, TX, USA, licensed by KCI USA, Inc., San Antonio, TX, USA Permanent skin substitute Human skin allograft derived from donated human cadaver	Micronized acellular human dermis with a dermal matrix and intact basement membrane to facilitate ingrowth of blood vessels	A pharmaceutical mean only for using as a constant skin replacement (deep and superficial wounds, sinus tract wounds, tendon repair, such as rotator cuff repair) Not subject to FDA pre-notification approval as it is a human cell or tissue-based product	*Diabetic foot ulcers*: Multicenter, retrospective study in the treatment of 100 chronic, full thickness wounds of the lower extremity in 75 diabetic patients revealed a 91% healing rate and suggested its use in the treatment of complex lower extremity wounds. No significant differences were observed for matrix incorporation or complete healing. Mean time to complete healing was 13.8 weeks [154] In a prospective multicenter RCT comparing GraftJacket with standard of care therapies for the treatment of ulcers in 86 patients for 12 weeks, the proportion of completely healed ulcers between the groups was statistically significant. The odds of healing in the study group were 2.7 times higher than in the control group [155]	*Advantages:* 2-year shelf life Pre-meshed for clinical application Single application Utilized in both deep and superficial wound healing *Disadvantages:* Cryopreserved

End of Table 1

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
			A prospective randomized study evaluating diabetic patients with lower extremity wounds demonstrated that patients treated with GraftJacket healed significantly faster than those with conventional treatment at 1 month In a prospective, randomized single-blind pilot study of 40 patients with debrided diabetic lower extremity wounds, GraftJacket was compared to the hydrogel wound dressing Curasol. At 4 weeks, there was a significant reduction in the ulcer size in the GraftJacket group compared to the controls. At 12 weeks, 85% of the patients with GraftJacket healed compared to 5% of controls [156] A prospective single center RCT comparing intervention (sharp debridement + GraftJacket + mineral oil-soaked compression dressing) to control (wound gel with gauze dressing) for the treatment of full-thickness chronic non-healing lower extremity wounds in 28 diabetic patients revealed that at 16 weeks in 12 of 14 patients treated with GraftJacket had complete wound closure compared to 4 of 14 patients in the control group. Significant differences were observed for wound depth, volume, and area [157] A retrospective multicenter series in 12 patients with diabetic foot ulcers and complex, deep, irregularly-shaped, tunneling sinus tracts treated with GraftJacket Xpress Scaffold (a micronized, decellularized flowable soft tissue scaffold that can be delivered through a syringe into the wound cavity) demonstrated complete healing in 10 of 12 patients at 12 weeks [158] In a prospective case series of 17 patients with debrided, non-infected, non-ischemic, neuropathic diabetic foot ulcers treated with a single application of GraftJacket with weekly silicone dressing changes, 82.5% of wounds had complete re-epithelialization in 20 weeks, with a mean time to healing of 8.9±2.7 weeks [159]	
PermaDerm® Regenicin, Inc., Little Falls, NJ, USA Permanent skin substitute	Autologous keratinocytes and fibroblasts cultured on bovine collagen scaffold	Pharmaceutical means to be used only as a permanent skin substitute in burns	No clinical trials available	*Advantages:* No risk of rejection Permanent substitute for massive burn injury

Skin substitutes	Product description	FDA and other indications	Clinical trials	Advantages/Disadvantages
				Disadvantages: No clinical trials or long-term studies available
StrataGraft® The Luminis Group, Ltd. for Stratatech Corp.	Immortalized patented NIKS® line keratinocytes and dermal fibroblasts on collagen scaffold In the process of healing native skin cells replace the product cells	A medicinal agent used as a constant skin substitute in burns and trophic ulcers	StrataGraft is in development phase III for the management of heavy full-thickness and partial burns. FDA approval is expected by 2020	No data on clinical or long-term trials are available

Table 2

Commercial and some laboratory (noncommercial products) of skin equivalents used for permeability study and toxicological analysis (no FDA approval)

Skin substitutes	Company	Product description
Episkin®	SkinEthic (France)	Comprised of human keratinocytes which are cultured on a collagen scaffold, and able to differentiate with formation of functional horny epidermis layer
EpiDerm®	MatTek (USA)	Neonatal epidermal keratinocytes capable of forming highly differential multilayer model of human epidermis are utilized
EpiDermFT®	MatTek (USA)	Co-culturing of neonatal dermal fibroblasts with neonatal epidermal keratinocytes is used to form highly differential multilayer model of human epidermis and derma
StrataTest®	Strata-Tech (USA)	NIKS cell line of human keratinocytes is used to create this full-layer human skin model
Epidermal Skin Test 1000® (EST1000)	CellSystems Biotechnologie, GmbH (Germany)	Reconstructed epidermis model, created from human keratinocytes, is presented by fully differentiated epidermis with the layers of viable and keratinized cells
Advanced Skin Test 2000® (AST2000)	CellSystems Biotechnologie, GmbH (Germany)	It is a full-layer human skin model consisting of fibroblasts as a basal skin layer and keratinocytes as the epidermis
Leiden epidermal skin model for the assessment of skin condition after its exposure to aggressive chemical agents	Thakoersing, Bouwstra et al. [161, 162], and El Ghalbzouri [163] (Netherlands)	It is a full-layer human skin model consisting of fibroblasts in 3D collagen matrix as a basal skin layer and keratinocytes as the epidermis
The model for investigation of a long-term skin regeneration and epidermal function	Boehnke et al. (Germany) [164]	It is a full-layer human skin model consisting of fibroblasts as a basal skin layer and keratinocytes as the epidermis

from bovine collagen, type 1, populated by human neonatal fibroblasts, and epidermal layer generated from cultured keratinocytes on the equivalent surface. Several multi-center randomized clinical investigations showed the efficiency of using Apligraf for treating slowly healing wounds, venous and diabetic trophic ulcers [118, 119], and this preparation became one of the first tissue-engineered equivalents approved by FDA for skin wound treatment.

In Russia, investigations in the development and application of living SE started from the end of the 90 s of the last century [120]. The works were mainly carried out in two directions: living SE with patient's autologous cells, which were incorporated into the damaged tissues, and living SE with allogenic cells engrafted at the site of transplantation for a short time sufficient for the normal course of reparative process and stimulation of the recipient tissue regeneration [121–123]. Such cellular constructs are used for restoration of various epithelial-mesenchymal defects. And their application is not limited by skin wounds only [124].

Engraftment of the described full-layer SE may be restricted by the absence of blood and lymphatic vessels, as well as skin appendages. It is because of these shortages that work at the level of preclinical trials are actively being carried out to develop the equivalents with the structure and properties similar to the normal human skin [8, 125].

In spite of the fact, that the application of already existing commercial SE has led to a significant progress in the field of regenerative medicine, their use has not yet become routine due to a high cost, limited efficiency, and inability to generate skin appendages [126].

The list of the most common commercial SE products having passed, fully or partially, clinical trials as transplants is presented in Table 1.

Tissue equivalents for skin modeling *in vitro*

Another sphere of SE application is connected with transdermal permeability study of the preparations and toxicological analysis of substances. At present, trials of cosmetic preparations on animals are forbidden in the European Union, even if there are no alternatives (Guidance Document for the Conduct of Skin Absorption Studies). Traditionally, flaps of human cadaveric skin or animal skin are used as models for evaluation of substance permeability through the skin [160]. A disadvantage of the former is difficulty in obtaining the material and great variability of sample-to-sample results. Animal skin is an easily-available material, but it is morphologically different from the human skin. This situation determines commercial demand in SE, which would serve as a suitable test-system for toxicological investigations. In Table 2, the basic commercially available equivalents being already used for testing pharmaceutical and cosmetological products are presented.

Conclusion

At present, as the presented data show, there is no ideal commercially available skin equivalent for wound healing. All epidermal and dermal products of bioengineering require either multi-step application procedure or autografts for complete wound epithelialization.

However, rapid advances in tissue engineering and the development of various approaches to creation of human skin substitutes, including application of the stem cells, give hope that such product will be constructed in the near future.

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