LITERATURE REVIEW CONCERNING CELL AND SKIN SUBSTITUTE CULTURES OBTAINED BY MEANS OF TISSUE ENGINEERING USED IN THE TREATMENT OF BURNS*

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The observed progress in recent years concerning multidisciplinary research in the field of tissue engineering brought results including novel skin substitutes and methods of their use. Scientific progress in the field of biomaterials, isolation of stem cells and growth factors, cellular and biomimetic environment differentiation created the unique opportunity for the production of laboratory tissues. However, despite the enormous progress in the development of new skin substitutes, which took place in the past three decades, there are still fundamental problems that must be overcome in an effort to create an optimal full-thickness skin substitute (1, 2, 3).

The successful treatment of burn wounds by means of skin substitutes requires a low antigenicity, ability of rapid vascularization, and stable scaffolding (regeneration matrix). It can be said that dermal regenerative matrices represent the initial and most promising solution, considering tissue engineering used during tissue reconstruction (3-6).

Another breakthrough in the treatment of burns might be the use of stem cells. They have two unusual features: the ability of potentially unlimited, multiple differentiation, and pluripotent cell line differentiation (7, 8, 9).

A very important element of any postnatal healing, due to burn wounds, injury, surgical procedures, and the integration of skin substitutes prepared by means of tissue engineering are scars. The above-mentioned pose a significant problem in the treatment of wounds. Uncontrolled cicatrization might lead to a cosmetic defect and loss of limb function, due to uncontrolled tissue overgrowth and ensuing contractures. The prevention of scarring is yet another problem, which should be solved after wound closure.

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In comparison, prenatal healing does not leave scars, which to some extent is attributed to reduced inflammatory reactions, limited fibrin clot formation, and platelet degranulation. The above-mentioned lead to the development of therapeutic procedures, the so-called scar-free wound healing (1). Intensive research concerning TGF-β group growth factors and their influence on the wound healing process and scar formation are under way. It was established that during fetal wound healing TGF-β1 and β2 isoforms are scarce or absent, while the concentration of isoform – β3 is significantly increased. However, during the wound healing process in adults the presence of TGF-β3 is scarce, while TGF-β1 and β2 levels are elevated, due to platelet degranulation and monocytic cell line synthesis during the inflammatory stage of healing.

The burn patient represents a complex polymorphic clinical problem, based on the systemic inflammatory reaction, affecting the functioning of many organs, burdened with a high risk of mortality. Extensive burn wounds generate large areas of skin necrosis (10, 11). Patients with a TBSA (total burn surface area) of 50% have only 50% of intact skin, comprising a potential area of donor grafts. In such cases donor area grafts should be considered as damaged skin, and thus, the entire surface of the skin is damaged. Additionally, in case of patients with severe burn wounds, the damaged epidermal barrier associated with limited immunological immunity might lead to bacterial sepsis, as the major complication during the management of burns (12, 13, 14). Donor fields heal without the elimination of cicatrization processes and are additionally painful, hence, the need to use strong pain medication. One should also pay attention to the fact that depending on the thickness of the skin, sampling from the same site is only possible 3-4 times. Recurrent skin sampling is possible after re-epithelialization at the donor site. In case of significant skin defects the number of grafts is limited or they are absent (2, 15, 16).

An alternative approach in the treatment of significant burns is the culture of autologous epidermal cells-keratinocytes and/or skin substitutes developed by means of bioengineering methods (17). The priority considering therapy consists in the removal of necrotic tissue, in order to limit the inflammatory reaction.

Skin cell culture

The culture consists in maintaining separated cells, tissues, and even organs under in vitro conditions for a period of more than 24 hours. The above-mentioned methodology creates the opportunity to observe the behavior of living cells, devoid of controlled and regulatory influence of other tissues and the donor organism (18, 19, 20).

Considering the general culture one may distinguish the following:
- cell cultures (without previously mentioned tissue structures),
- tissue cultures,
- organotypic cultures (embryonal and adult donor organism samples).

Other important concepts considering cell cultures include:
- primary culture (cell or tissue collection directly from the donor organism),
- cellular line (transfer of part of the primary culture to a separate culture). If the material-keratinocytes or fibroblasts is possible to transfer into other cultures, one may think of an established cellular line;
- the cellular strain (derived from the primary culture or cellular line by isolating cells having defined characteristics continued during subsequent strain transfers). The strains’ characteristic might relate to the chromosomal and antigenic presence, possibility to produce specific chemical compounds, lack of a given enzyme or organelles, as well as insensitivity towards specific chemical substances.

Skin substitute cultures

Skin substitutes are divided into biological substitutes and those obtained by means of tissue engineering (6, 21, 22, 23). Biological substitutes include allografts and skin xenografts. Skin allografts are usually obtained from cadavers. The allogenic corporeal skin is usually used as a temporary biological dressing in case of full-thickness burns. A similar type of temporary dressing is the xenograft prepared from animal skin. Skin xenografts are usually collected from the domestic pig. The disadvantage of the above-mentioned method is the risk of unknown consequences in the body of the recipient through the possibility of
transmitting zoonotic pathogens, such as *Toxoplasma gondii*, *Rhabdoviridae*, or *Paramyxoviridae* (24).

Apart from a few exceptions, both allografts and xenografts are temporary dressings which do not guarantee a successful cure. There is often the need for surgical intervention, in order to remove the undesired scars and adhesions.

Skin substitutes obtained by means of tissue engineering are used alone or in cooperation with structures replacing the epidermis. Since artificial skin does not induce keratinocyte growth, it is necessary to use it in conjunction with non-full thickness skin (WPSPG) or other type of epidermal covering (25). Most components of the epidermis include epidermal cells-keratinocytes obtained from in vitro cultures. In order to obtain the adequate amount of culture keratinocytes, a minimum of 21 days is required. Keratinocytes cultured under standard conditions proliferate slowly, and the need to passage them to larger cultures results in their necrosis. Frequent passage results in their assimilation to fibroblasts (26, 27). The dermal component might be composed of a variety of substances, such as collagen, glycosaminoglycans, and polyglycol or hialuronic acid. The above-mentioned component forms a matrix, which may be covered by dermal cells-fibroblasts obtained by means of in vitro cultures (28).

The cultured patient cells (autologous culture epidermal cells)-keratinocytes are an alternative to skin substitutes. After culture they are placed in the wound (14, 16, 29). From the collected skin sample of a surface of several centimeters one may obtain a number of keratinocytes sufficient to cover the entire body of an adult patient. However, the above-mentioned method has major drawbacks. The first includes the relatively slow proliferation of keratinocytes under standard culture conditions. As a result of frequent passage, keratinocytes lose their characteristics conformed to fibroblasts (16, 29). The second disadvantage lies in the fact that keratinocytes applied to the wound in the form of a cellular suspension form foci and it is impossible to precisely control their position. Additionally, keratinocytes are only one type of cells constructing the external surface of the skin, thus, the restoration of the deeper layers requires more complex procedures. Based on literature data their existence indicates the possibility of restoring all skin layers together with their functional elements, such as defense cells, hair follicles, etc. (17).

Another very promising method of culturing is the in vitro epidermal culture in the form of a panel/sheet with its application on a properly prepared wound (30). In order to achieve this it is necessary to have a matrix, which will be biocompatible with keratinocytes. The challenge for current research is therefore, the development of skin substitutes consisting of cultured cells colonizing a suitable matrix, for example on a collagen-type biopolymer (31, 32).

Another method of improving the functionality of artificial skin substitutes is to add signal molecules, in order to regulate the cell-cell and cell-matrix interactions, as well as accelerate the process of biointegration (33, 34) or adjust the process in accordance with wound healing phases. Such biomimetic hybrid material are referred to in English as „smart“. Their role is to influence the process of a more natural regeneration of skin (35).

In many studies one may observe the urgent need for a new approach to skin cultures, considering the bridge between clinical examinations and basic experimental trials in the field of skin physiology. Research is being conducted concerning an effective model where one will be able to carry out experiments on ex vivo cultures. The first ex vivo chamber models have been elaborated ensuring the maintenance of physiological and histological explant properties during a period of 4 weeks. The above-mentioned model showed a physiological validation. Epidermolysis was not observed, and both the basic layer and blood vessels were observed in all tissue samples (36, 37, 38).

Chinese investigators (15) observed the influence of the supernatant of culture keratinocytes on the proliferation and apoptosis of fibroblasts. They reconstructed the burn injury (in vitro model) by treating the culture keratinocytes with high temperature. Simultaneously, a standard keratinocyte culture was conducted. In order to investigate the influence of the „burned“ keratinocytic supernatant on fibroblasts it was added to the culture of dermal cells and examined for their proliferation and apoptosis. For comparison, we evaluated the influence of „non-burned“ keratinocytes on fibroblast cultures. The obtained results
showed better fibroblast proliferation of "burned" keratinocytes, as compared to the control group and "non-burned" keratinocytes.

The biomedical application of polymers

Materials sensitive to environmental stimuli, that is those having the ability to reversibly change their properties in response to external stimuli, represent one of the most promising materials. One of the basic "smart" materials is based on polymers, as a material easily customizable to the specific needs, as compared to metal and ceramics. Polymer "smart material" might react to external stimuli, such as pH, temperature, ionic strength, magnetic and electric fields, light and/or chemical and biological stimuli, which in consequence enables a wide range of practical applications, including sensor technology, gene and drug transport, as well as tissue engineering (39).

Polymers sensitive to temperature have found application in drug and gene transport and tissue engineering.

Tissue engineering is an interdisciplinary field applying principles of engineering and biological sciences to develop biological substitutes repairing damaged tissues or improving their functioning (40). Its aim was to regenerate or replace the damaged or diseased biological tissue, or create substitute organs for many diseases, such as heart diseases, diabetes mellitus, tissue and bone necrosis.

The paradigm of tissue engineering considers the use of matrices within which it is possible to disseminate cells, and in effect obtain a mature tissue. This requires the use of material/biocompatible matrices, natural material, such as proteins or synthetic polymers with the required type D structure providing adequate mechanical support, and with the ability of both nutritional and growth factor transport to the encapsulated cells. The use of synthetic polymers as material capable of developing the matrix has focused the attention of many investigators, due to their numerous advantages. As compared to natural products it is easy to adjust them to specific needs, by modifying their mechanical and chemical properties (39). Synthetic polymers used in tissue engineering should be non-toxic, easily accessible, and relatively cheap.

Considering tissue engineering, thermo-controlled polymers are usually used in two cases: as substitutes enabling cellular growth and replication, as well as gel used for the injection of the scaffoldings (in situ).

In case of the first application the thermo-controlled polymer properties are used in the regulation of cellular adhesion (adhesion or detachment from the polymer layer) (41, 42). In case of some studies the surface of the polymer was repeatedly used for cellular cultures (86).

The latter use considered spatial cellular encapsulation (3D) in the body of the patient. The in situ cellular scaffold is in contrast to the cellular in vitro construction, enabling the delivery of encapsulated cells, nutritional contents, and growth factors to the damaged tissue using minimally invasive techniques. The basic in situ idea consists in the mixing of the thermo-controlled polymer and room temperature cells (23°C), followed by the injection of the above-mentioned mixture into the selected area of the patient’s body. After injection the mixing temperature increases to body temperature (approximately 37°C), above LCST (lower critical solution temperature) so that the polymer turns to gel. Cells become encapsulated within the three-dimensional structure of the gel (39).

Regardless of the nature of the surface of the polymer, the degree of preparation for cellular culture, the key issue considering the cellular response include the phenomena observed at the interface: cell-synthetic surface (43). The complexity of the mechanisms that must precede such processes, as adhesion and proliferation, makes the nature of the above-mentioned not fully understood (44). The knowledge of the structure and functioning of cells, as well as ability to apply diagnostic means (biochemical, immunochemical, and fluoro-immunochemical methods) enables to specify its functional condition, including the ability of adhesion (flattening) and proliferation, and thus, differentiation and migration of cells (30, 32, 40, 45). Analysis of the behavior of cells on the surface of synthetic material enables not only to formulate conclusions concerning the cellular response (in vivo conditions), but first of all enables to determine the composition and synthetic surface in such a direction as to predict or stimulate tissue behavior on their surface, and consequently receive biocompatible material for the recon-
Construction of pre-planned biological property skin (6, 10, 14).

Bioconstructions, biocompatible material available in the reconstruction of skin obtained by means of tissue engineering methods

Situations in which normal autografts cannot be used to replace damaged skin lead to increased risk of patient death, prolonged hospitalization, and represent higher costs of treatment for the health care system. There is a great demand for skin substitutes obtained by means of tissue engineering methods. Multicenter intensive studies are being conducted. In recent years one may observe substantial progress in the development and clinical implementation of different skin layer components elaborated by means of bioengineering methods. Constant access on the market of such constructions or the production of adequate amount of material for immediate use in the permanent closure of wounds, are often the only hope for the patient with significant skin defects (35, 36).

All skin substitutes, bioconstructions obtained by means of genetic engineering methods should meet three basic criteria: 1) be safe for the patient, 2) clinically effective, and 3) comfortable during transport and use. The properties of an „ideal” skin substitute to be used in vivo were described. MacNeil reviewed the above-mentioned (46).

Generally, products of such type should not exhibit toxic or immunogenic properties, or cause an acute inflammatory response. They should additionally lack or be characterized by a minimal level of disease transmission. Biomaterials for the reconstruction of skin should be biodegradable, repairable, and able to support the reconstruction of normal tissue, and possess similar physical and mechanical properties as skin. They should also relieve pain, protect the surface of the wound from loss of moisture and heat, as well as protect the wound from infection (46). The big advantage of such a substitute would be its low cost, wide availability, easy use, and long storage period. Currently, there are no products that meet all the above-mentioned criteria.

The available skin substitutes, including dermal and epidermal constructions, although imperfect, fill a specific niche, considering the complex approach to the treatment of severe burns, improving patient survival and their quality of life after the burn injury (46). These products are designed to meet only limited, specific tasks during the process of wound healing. First of all, they serve as a temporary, active, biological dressing, supplier of cytokines and structural molecules required for structural wound healing, while the patients’ own skin is regenerated for future autografts. Products based on autologous keratinocyte and fibroblast cultures possess better properties currently offered to supplement the skin substitutes. The obtained clinical study results are very encouraging (9, 10, 11, 14). However, all experts agree on the fact that these products are not fully able to replace the damaged skin (46).

Currently available skin substitutes might only partially play the role of a protective skin barrier. Other features, such as sensitivity to temperature, perspiration, UV radiation protection, thermoregulation, synthesis, not to mention esthetic properties are non-replaceable by the currently available skin substitutes (2, 5, 6).

The market offers the following skin substitutes requiring the use of in vitro cellular skin cultures:

- Apligraf™ – in vitro cultured allogenic fibroblasts and keratinocytes of neonatal origin. Type I animal matrix, collagen gel. All the above-mentioned form a confluent outer layer construction mimicking the normal structure of the human skin. Initially, an organo-type skin substitute it may be used as a temporary, bioactive dressing. It supplies ECM components (extracellular matrix) to the wound, as well as cytokines and growth factors, such as type α and β interferons, PDGF, interleukins 1, 6, 8. There exist literature data concerning its use in case of burns (47). Many Authors consider Apligraf as an alternative to traditional skin grafts in case of burn wounds. However, the product cannot be used to permanently occlude full-thickness skin wounds, due to the temporary character of allogenic cell grafts. Thus, the need to perform a simultaneous autogenous epithelial cell graft. Considering the high cost of the substitute (approx. $28/cm²), short storage time (<5 days), safety measures (risk of
disease transmission by means of allogenic elements), and temporary character of the dressing, it is unlikely that the substitute be widely used in the management of burn and chronic wounds.

A similar product was created on the basis of experimental bioengineering. It is based on sterilized human skin covered by autologous keratinocytes and fibroblasts. It can be a definite solution to the skin substitute, since autologous cells are not rejected by the host. The only limiting factor is the slow progression of cells.

- **OrCell ™** – in vitro cultured allogenic fibroblasts and keratinocytes of neonatal origin. Type I animal matrix, collagen sponge covered by fibroblasts and a smooth collagen-gel layer with an additional layer of keratinocytes, in order to create the confluent construction. Patented in 2001 for the purpose of treating wounds, and recessive, dystrophic pemphigus. This skin substitute shows a reduction in scarring and shortens the healing process. Allogenic cells constitute its composition. It is designed to be a temporary substitute, reabsorbable within 7-14 days (like Apligraf). No evidence of OrCell cellular DNA is observed in the wound, 14-21 days after its application.

- **PolyActive ™** – bi-laminar substitute based on *in vitro* cultured autologous fibroblasts and keratinocytes seeding the PolyActive matrix. The porous matrix contains a soft tereftalat polyethylene component preventing the shrinkage of the polymer. Synthetic scaffolding, PEO/PBT material. The product uses autologous cells and does not pose a potential danger typical of allogenic material (risk of cross infection or acute immunological response). PolyActive is applied as a temporary, biologically active dressing used in the management of non, full-thickness burn wounds. The cost of PolyActive is higher than that of OrCell or Apligraf. Additionally, due to the presence of autologous cells its storage time is limited (<4 days).

- **TissueTechAutograftSystem ™** (Laserskin & Hyalograft 3D) – a system consisting of two biomaterials applied consecutively to the wound: Hyalograft 3D dermal and Laserskin epidermal matrix. It is based on autologous keratinocytes and fibroblasts cultured on HAM. Based on available literature data the system enables to successfully manage diabetes foot and other ulcerations (70.4% wound closure index) where the wound exceeded 5 cm². The recurrence index did not exceed 8.2%. Although the system may be used for definitive wound closure it is not a two-layer substitute, where both the dermal and epidermal layers are present. The procedure in this case involves the implantation of two above-mentioned components, which is complicated under clinical conditions.

- **Epicel ™** – in vitro cultured autogenous keratinocytes (confluent cellular sheets). It is a permanent substitute.

- **EpiDex ™** – in vitro cultured autologous keratinocytes collected from hair bulbs (confluent cellular sheets). It is a permanent substitute.

- **Epibase ™** – in vitro cultured autologous keratinocytes (confluent cellular sheets). It is a permanent substitute.

- **MySkin ™** – in vitro cultured autologous keratinocytes (subconfluent cellular sheets). Synthetic silicone layer covered by a special formula. It is a permanent substitute.


- **Bioseed-S ™** – in vitro cultured autologous keratinocytes (subconfluent cellular suspension). Allogenic- fibrin glue matrix. It is a permanent substitute.

- **CellSpray ™** – non-cultured/cultured autologous keratinocytes (subconfluent cellular suspension). Permanent substitute.


The following in vitro cultured biomaterials are potential skin substitutes, being under investigation:
- **PermaDerm™** – dermo-epidermal construction. In vitro cultured autologous keratinocytes and fibroblasts. Allogenic matrix with veal collagen. It is a permanent substitute.


- **Allox™** – dermo-epidermal construction. Spray comprising a fibrin suspension of allogenic in vitro keratinocytes and fibroblasts. Temporary substitute.

- **Karocell™** – epidermal construction from in vitro autologous keratinocytes and fibroblasts. Permanent substitute.


- **Polycaprolactone Collagen Nanofibrous Membrane™** – dermal construction, in vitro allogenic dermal fibroblasts. Allogenic matrix with synthetic collagen combined with polycaprolactone nanofiber scaffolding. Temporary substitute.

- **Tegaderm™** – dermal construction with nanofiber for the in vitro culture of allogenic fibroblasts. Xenogenic-synthetic matrix with polycaprolactone collagen nanofiber. Temporary substitute.

Composite skin substitutes currently available on the market use only two types of cells: keratinocytes and fibroblasts, therefore, cannot fulfill all the functions of the skin, due to lack of innervation, sweat gland immune cells, and hair follicles. Multicenter investigations are under way considering the enrichment of the number of cells (endothelial Langerhans cells) incorporated into skin substitutes, in order to increase the functionality of their products.

Many investigators also acknowledged that any bioengineering products based on „natural” mechanisms of wound healing will give effect in the form of scarring with limited functionality, rather than full function skin regeneration (6, 12, 13, 44, 46).

**CONCLUSIONS**

Based on the presented literature review considering skin substitutes obtained by means of tissue engineering methods, one may come to the conclusion that there is no ideal composite product on the market used for permanent wound closure. All epidermal and dermal bioengineering products require multistage surgical procedures or autologous skin grafts for the definitive wound epithelialization.

Rapid progress in tissue engineering and the diversity of the biomaterials as skin substitutes, including stem cells, may give hope that in the near future, such a great product will be developed.

Current research concerning mature stem cells are still in preliminary stage, despite potential target for the research in the field of tissue engineering for the future treatment of severe burns and chronic wounds. Great public interest may result in the coming years in significant progress considering skin substitutes.

However, it should be noted that no matter how complicated the method we use to develop the skin substitute based on postnatal cell material, success is not assured. Such products apply reconstruction mechanisms, rather than tissue regeneration mechanisms. Proper wound healing requires the repair of damaged areas. The above-mentioned process evolved over thousands of years, together with the evolution of the human being, ensuring an effective procedure in the management of skin wounds, most of which were the result of a bite or mechanical injuries. These wounds were contaminated with saliva, blood, dirt, and microorganisms. There were no chemical antiseptic preparations, and therefore, natural, specific and unspecific immunological factors
evolved. Fibrin clots with a provisional fibrin matrix helped in stopping bleeding. Acute inflammatory reactions controlled bacteria and other biological factors contaminating the wound. Rapid tissue granulation progress enabled to close any loss of skin quite rapidly, thus ensuring the survival of a given individual and species.

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