2 - Experimental model of cultured keratinocytes[1]

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ABSTRACT - The bioengineering research is essential in the development of ideal combination of biomaterials and cultured cells to produce the permanent wound coverage. The experimental model of cultured keratinocytes presents all steps of the culture, since the isolation of the keratinocytes, preparation of the human acellular dermis, preparation of the composite skin graft and their elevation to the air-liquid interface. The research in cultured keratinocytes model advances in two main ways: 1. optimization of the methods in vitro to the skin cells culture and proliferation and 2. developing biomaterials that present similar skin properties.

KEY WORDS: Keratinocyte. Human acellular dermis. Skin transplantation. Fibroblasts. Cultured cell.

Introduction

Extensive lack of skin, as degloving injuries, aplasia cutis congenital, epidermolysis bullosa, giant congenital nevi, pyoderma gangrenosum, have a precise indication to the utilization of the cultured keratinocytes to permanent wound coverage when limited autograft availability exists. Although, theses pathologies normally do not determine total body surface suffering as often occurs in burns.[1]

The proliferative keratinocytes are small cells that are on the top of the basal membrane and normally present a low division rate. The new cells serve to substitute the ones that suffer terminal differentiation and desquamation. It is necessary 3 to 4 weeks to the new cell goes from basal stratum to the corneum stratum.[2]

The knowledge of this differentiation process is important, because when the cells are separeted from others by enzymes to form the cell suspension to the culture, many cells are incapable of colony formation because they started the terminal differentiation. Within isolated epidermal cells to the culture only 3 to 4% proliferate and form colonies in optimal culture conditions. The cells suffer the normal terminal differentiation process in the central area of the colony, and with the colony confluence, this occurs in the entire surface of it.[3] The dermis shows the elasticity and resistance, essentials characteristics that determine the functional and aesthetic aspects of the skin. Several late alterations, especially related to the skin function and aesthetic become determinants when the dermis is destroyed and not reconstituted properly. In this way, the dermis structure is extremely important in the production of any material to wound coverage.[4]

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The area of skin that are necessary to the keratinocytes culture is small. A 1 cm biopsy can be expanded more than 5000 in 3 to 4 weeks, generating sufficient epithelium to the adult body surface coverage.[2]

The utilization of a cultured keratinocyte sheets in the wound showed the disadvantage of low rate of graft integration and the consequent prolonged period of immobilization to the wound healing. And, after this process has finished, there still have the possibility of occurrence of new wound by the coverage fragility.[5]

In spite of this, should remark the importance of the use of the cultured keratinocytes sheets that detemined the possibility of patient survival with burns, that in the past decades they evolved to death and they continue evolving in that way in places which there have not this kind of technology.

This kind of fragile wound healing is consequent by the direct contact between the cultured keratinocyte sheet and the receptor area determining more easily of the graft nutrition, but more difficult adherence and anchorage to the profound structures by the lack of the basal membrane.[6]

The reconstruction of the basal membrane occurs rapidly when there is any dermal structure in the wound coverage, as in the partial or total skin auto-grafting. In the cultivated epithelium is necessary wait many months or years until the reconstruction of the basal membrane.

So, the researchers have interest in the study of the association of cultured keratinocytes to one dermal analogue, as a composite skin graft, by the dermis architectural similar to the normal skin, and it is not necessary so many time to the reconstruction of the basal membrane.

The bioengineering research is essential in the development of ideal combination of biomaterials and cultured cells to produce the permanent wound coverage. With the improvement of this materials and the clinical use, the scar aspect has great importance.

The wound coverage is permanent when it is utilized the human acellular dermis with autologous cultured human keratinocytes.

The utilization of the human acellular dermis a dermal analogue shows as favorable factor the low cost when compared to the production of synthetic materials compound by collagen and glicosaminoglycan that depend on high technology, and they do not have got a basal membrane.

The fundamental advantage of these skin substitutes, in the near future, is the possibility of the utilization of cultured cells genetically modified and a dermal analogue to the permanent coverage and the consequent modulation of the wound healing by the production of growth factor that interfere in the normal or altered wound healing, skin congenital diseases and systemic diseases.

In this way, many researchers are interested at the present time in the development and production of the composite graft, utilizing many dermal analogues, a human acellular dermis, synthetic dermis or others in research phase.[7,8,9,10,11]

Proposition

The experimental model of cultured keratinocytes presents all steps of the culture, since the isolation of the keratinocytes, preparation of the human acellular dermis, preparation of the composite skin graft and their elevation to the air-liquid interface.

Method description

Culture of human cells

Normal human keratinocytes derived from neonatal foreskins are isolated and cultured following described methods by Green *et al* (1979), revised in 1985, and this is the protocol presented here. The procedures with the cells and tissues from human are realized in laminar flood.

To culture keratinocytes are necessary co-cultivated them with 3T3-J2 mouse fibroblasts, as feeding layer (originally provided by H.Green, Harvard Medical School, Boston, MA).

The developing and maintenance of the feeding layer is initiated by one cryo preserved vial of 3T3-J2

cells to generate fresh stock. The preparation of the 3T3-J2 to the keratinocyte culture is initiated with proliferation of this cells in 175 cm^2 flasks with $5x10^5$ cells per flask with 40 cc of 3T3-J2 culture medium.

The 3T3-J2 culture medium is set up by Dulbecco s'Modified Eagle s'medium (DMEM) (high glucose [4,5 g/L], L-glutamin [584 mg/L], sodium piruvate [110 mg/L]), with bovine calf serum (BCS) 10% (iron supplemented), and Penicillin/Streptomicin (100 UI/ml - 100µg/ml) and buffered with sodium bicarbonate (1N), determining a red medium, by the presence of red phenol, showing a pH of 7,2. This permits the gross evaluation of the medium pH by the color change and proved by the pH meter.

After cells put in the flask, the gaseous phase of the culture is prepared with a mixture of 10% CO_2 and air for 10 seconds and the flask cap is firmly closed and the flask is put inside the humidified incubator with 5% CO_2 at 37°C for 2 h. After this, the flask cap is opened permitting the exit of the gas excess produced and returns to incubator with the cap partially closed to maintain the flask pH in balance with the incubator pH, maintained in 7,2.

Swiss mouse 3T3-J2 cells were routinely passaged in DMEM (high glucose) supplemented with 10% bovine calf serum (Hyclone, Logan, UT) and penicillin-streptomycin (100 IU/ml-100 µg/ml) incubated in a humidified 10% CO_2 atmosphere at 37° C.

The culture medium change is realized each 2 or 3 days. The cell proliferation maintain its lineage and the presence of this cells to the moment of the keratinocytes isolation (Figure 1).

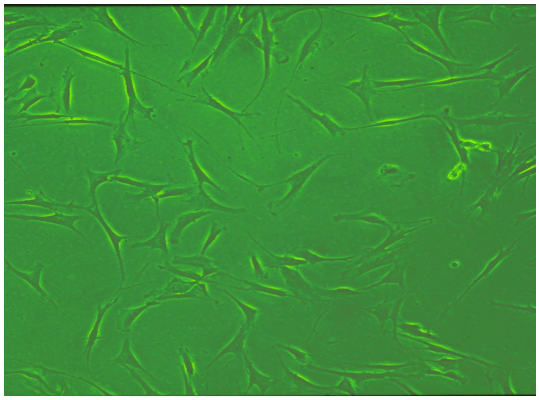

FIGURE 1 Feed layer of 3T3-J2 fibroblasts.

At the day before of the keratinocytes isolation, approximately 18 hours before the isolation beginning, the 3T3-J2 cells are passed to 75 cm^2 flask with $2x10^6$ cells per flask with 15 cc of the same culture medium and put in the humidified incubator with 5% CO_2 at 37°C, with flask cap partially closed. Each flask is prepared to each skin sample to the isolation.

At morning of the day of the isolation, the 3T3-J2 cells present 50% of confluence in the flask. So, the cells are prepared with 15 µg/ml mitomycin C (Boehringer Mannheim Co., Indianapolis, IN), taking off the normal culture medium and putting in the flask the culture medium supplemented by mitomycin C. This substance limits the 3T3-J2 proliferation and produces a viable feed layer, determining sufficient space in the flask to the aderence of the keratinocytes.

This mitomycin C treatment takes exactly 2 hours and it is removed and the 3T3-J2 are washed 3 times with DMEM. After this, the keratinocyte culture medium is put in the flask without epidermal growth factor (EGF), at minimum 2 hours before the keratinocytes isolation.

Keratinocyte culture medium (KCM) is a 3:1 mixture of Dulbecco s'Modified Eagle s'medium (DMEM) (high glucose) and Ham s'F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 24 mg of adenin (6-aminopurina hidrocloreto) prepared fresh diluting in 20 cc of the mixture DMEM:F12 of HAM with final concentration of $1.8x10^{-4}$ M; 1 cc of cholera toxin (Vibrio Cholerae, Type Inaba 569 B) with final concentration of 10^{-10} M; 2 cc of penicilin/streptomicin 100 UI/ml-100µg/ml; 2 cc of

hydrocortisone with final concentration of 0,4 µg/ml; 1 cc of transferrin/triiodo-L-tironin (T/T3) (human transferrin, partially iron satured) with final concentration of 5 µg/ml, (3,3 ,5 triiodo-L-tironin, sodium salt) with final concentration of $2x10^{-9}$ M; 1,3 cc of insulin (100 U/ml or 3,8 mg/ml) with final concentration of 5 µg/ml; the pH is adjusted approximately in 7,2; sterilized with 0,45 µm filter, and put in refrigerator from 2 to 4°C, showing the red color.

At the morning of the keratinocyte isolation is necessary to produce the fresh trypsin constituted of D-dextrose 0,1%, Trypsin (1-300) 0,1 %, in saline solution phosphate buffered (PBS) with pH of 7,5 and utilized 0,45 µm filter to sterelization.

Versene or EDTA solution 5mM in PBS is other component produced. These two solutions are put at 4°C, and half hour before the beginning of the experiment they are put in 37°C.

In the morning of the isolation day, at operation room, the foreskins are obtained. The children are submitted to circumcision, removing approximately 1 cm^2 of foreskin. The fragment is sent to the laboratory in 50 cc conic tube with 30 cc of keratinocyte culture medium without epidermal growth factor in ice. The fragments are processed until 6 or 8 hours after the circumcision.

The fragment is put in 100 mm culture dish with 1 cc of PBS and is removed the subcutaneous tissue and part of the dermis, to the separation process of epidermal cells easy obtained. After this, the fragment is put in 50 cc conic tube with 35 to 40 cc of PBS and the tube is vigorously agitated by 45 seconds, and this procedure is repeated 8 times in 8 different tubes with PBS, to remove contaminants.

In a 60 mm culture dish are put 3 cc of trypsin and the fragment, starting the sectioning in many little pieces with approximately 0,5 mm^3, procedure that demands around 15 minutes (Figure 2).

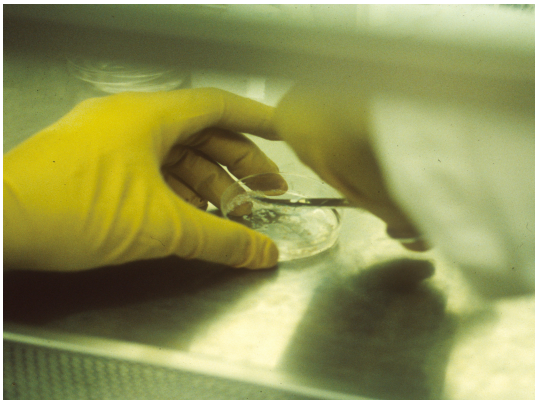

FIGURE 2 Fragment sectioning in trypsin.

The fragments are put in an agitator flask that should be pre heated (37°C) and in its lateral arm is pre humidified with 1 cc of Versene to not allow the fragments stick in the glass (Figure 3).

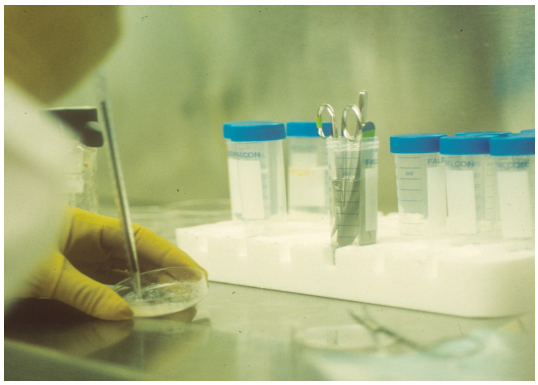

FIGURE 3 Fragments and trypsin aspiration.

Five cc of Versene are added to the fragments in the dish and this volume and the pieces are transferred to the agitator flask. More three cc of trypsin are added to the 60 mm dish to remove the reminiscent pieces. This mixture is added to the agitator flask resulting in a 12 cc total volume (Figure 4).

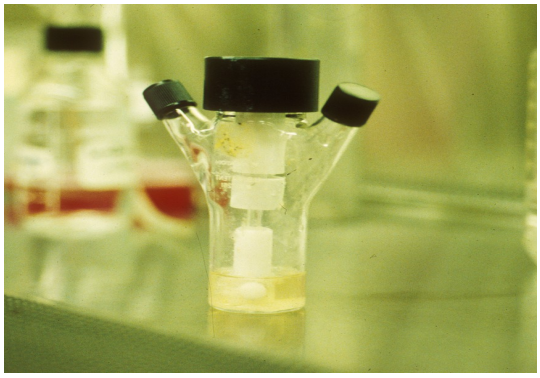

FIGURE 4 Agitator Flask with fragments, trypsin and EDTA.

The agitator flask presents one central vertical bar stick to the cap and it exists in its inferior part another horizontal magnetic bar that when it is placed in a platform heat/agitator make the bar rotate. This procedure is realized in slow rotation (120 rotations/minute) in 37°C, in association with trypsin and Versene action determines the cell separation presents in the fragments.

After 30 minutes of this procedure, it is removed the supernatant of the agitator flask solution, wherein the cells are suspended (Figure 5).

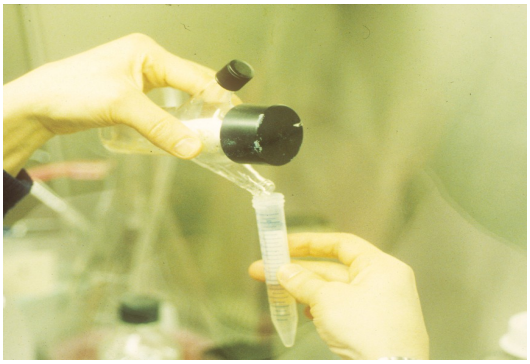

FIGURE 5 Supernatant decantation.

It is centrifuged (Figure 6) and the cells resuspended in tree cc of keratinocyte culture medium removed from the 3T3-J2 flask previously prepared to the isolation (Figure 7).

FIGURE 6 – Centrifuged cells in the tube bottom.

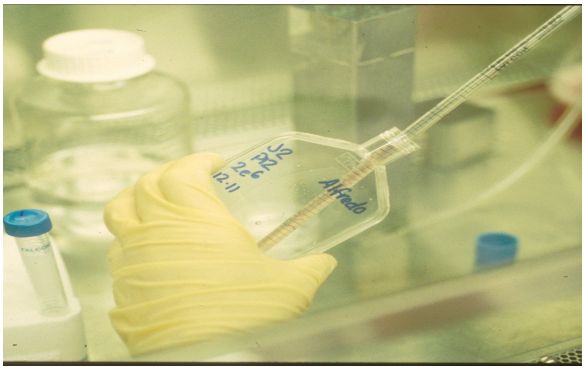

FIGURE 7 – Feed layer flask receiving keratinocytes isolated from skin fragment.

Cell count is realized with phase hemocitometer in a inverted microscopic with phase objectives, and the flask is put in humidified incubator with 5% CO_2 and 37°C.

To the agitator flask with the skin pieces not yet separated is added 6 cc of trypsin and 6 cc of versene and a new 30 minutes session is realized to complete the keratinocytes isolation. After this second time, the flask is maintained in humidified incubator with 5% CO_2 and 37°C, and evaluated daily regard the contamination and it is realized culture medium change when it is indicated. The flask is evaluated also regard the colony presence after 5 days of the isolation (Figure 8 and 9).

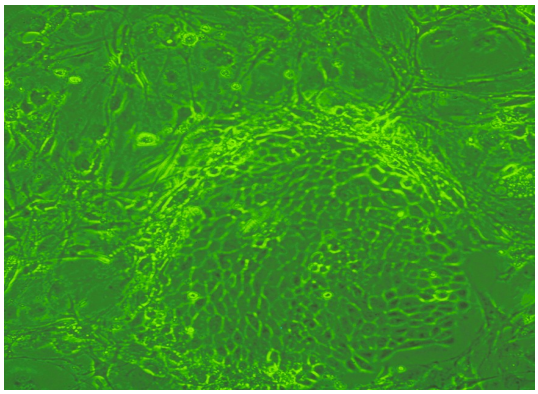

FIGURE 8 New keratinocyte colony after 5 days of isolation.

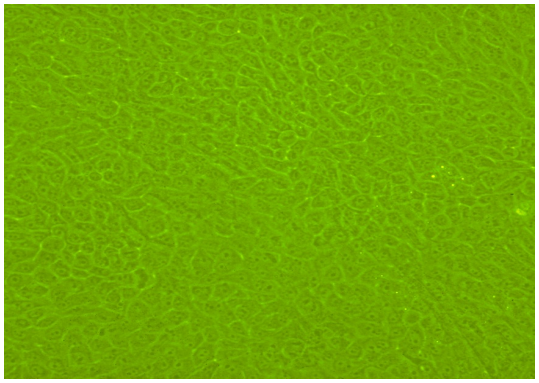

FIGURE 9 Confluence of keratinocyte colonies.

The culture medium is changed each 3 days, added of the epidermal growth factor, with 0,1 cc of the factor to 100 cc of the keratinocyte culture medium. This supplementation is realized on the first culture medium change, with final concentration of 10 ng/cc.

In the keratinocyte culture, the melanocyte survive as passenger cells with no need of special procedures to maintain them.

Preparation of the Acellular Dermis

Cadaver skin with an average thickness of 0,03 cm is obtained from skin bank. It had been cryopreserved according to the bank s'protocol. To separate the epidermis from the dermis and render the dermis acellular, the skin is subjected to three rapid freeze-thaw cycles in liquid nitrogen to devitalize the cells, washed three times in sterile phosphate buffered saline (PBS), and incubated at 37^{o} C for 1 week in sterile PBS with antibiotics (gentamycin at 100 µg/ml, ciprofloxacin at 10 µg/ml, amphotericin B at 2,5 µg/ml, and penicillin-streptomycin at 100 IU/ml-100 µg/ml).

After this, the epidermis could be gently stripped from the dermis with forceps. The dermis is maintained 4 weeks in antibiotic solution at 4^{o} C to remove any remaining cells. The dermis becoming complete acellular after this procedure, but retain the architecture with elastic and colagen fibers and membrane basal proteins.

Preparation of the Composite Skin Grafts

Prior to use, the acellular dermis is washed three times with DMEM to remove residual antibiotics. The acellular dermis is cut into 1,25-1,5 cm^{2} pieces, and each piece is placed into a 35 mm tissue culture dish, with the papillary side up. Second passage cultured keratinocytes in keratinocyte culture medium are seeded onto

the surface of each piece of dermis (2,5 x 10^5 cells/cm^2), and the composite grafts are maintained in culture for 10 days prior to grafting (Figure 10).

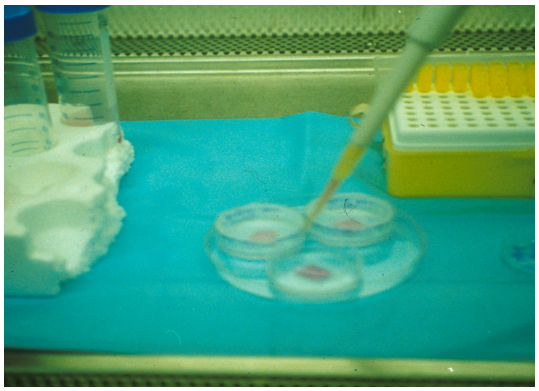

FIGURE 10 Keratinocytes put in human acellular dermis.

Elevation to the air-liquid interface

Keratinocytes are cultivated in human acellular dermis in air-liquid interface (A/L) following the protocol related by Ponec (1997). Three culture medium formulations are used : 1. medium used in the keratinocyte seeding over the acellular dermis for 24 hours, 2. intermediate medium used for 48 hours, between seeding and the elevation to air-liquid interface and 3. air-liquid medium used after the elevation of the graft and it should be changed each two days.

At first day of preparation of the composite graft, the dermis is placed in a 35 mm dish culture, where the internal surface of the dish is scraped to improve the adherence of the dermis to the plastic.

The dermis adhered to the dish is placed in the incubator for one hour and after this is seeded 250.000 keratinocytes resuspended in 100 µl of the seeding medium, put over surface dermis without leakage to the dish and maintained for 2 hours in the incubator to permit the cells adherence to the dermis. After 2 hours, are added more 3 cc of the seeding medium with care to not take off the cells above the dermis. In the next day, the medium is changed to the intermediate medium that remained 48 hours.

On the third day, the composite graft is elevated to the interface air-liquid by the remove of the culture medium and with the use of the 15 scalpel is performed the release of the margins of the graft (Figure 11).

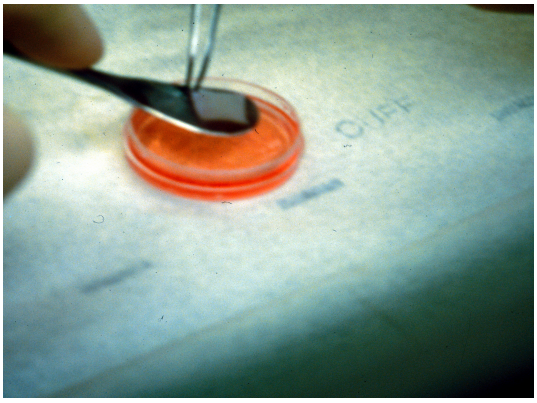

FIGURE 11- Release of the composite graft from the dish to the elevation to the air-liquid interface.

After the graft elevation with a spatula, it is placed a steel meshed screen with a media width of 2 cm^2 into dish culture and above this screen it is placed the graft. The air-liquid medium fulfill the dish space until the inferior limit of the graft, and the dish is maintained in CO2 humidified incubator (Figure 12).

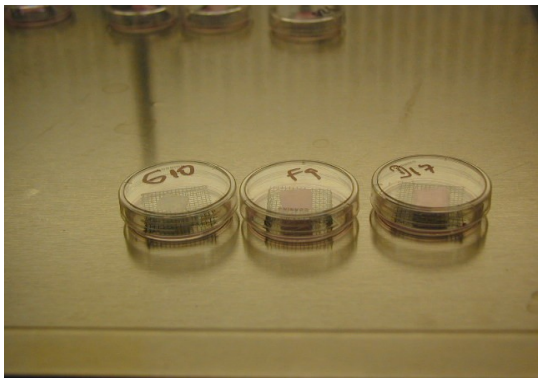

FIGURE 12 Composite graft over steel mesh screen.

The seeding medium is composed by a mixture 3:1 DMEM and HAM F12 medium with 1% FBS, cholera toxin 10^{-10} M, hydrocortisone, 200 ng/cc, insulin, 5 µg/cc, ascorbic acid (Vitamin C), 50 µg/cc, e penicillin / streptomycin, 100 UI/cc-100 µg/ml.

The intermediate medium is similar to the seeding one, with addition of albumin of bovine serum (BSA) (24µM), a cocktail of oleic acid (25 µM), linoleic acid (15 µM), aracdonic acid (7 µM) and palmitic acid (25 µM); and addition of L-carnitina (10 µM) and L-serina (1,0 mM).

Finally, the air-liquid medium presents the same composition of the intermediate one, with addition of the EGF, 1,0 ng/ml, and removed the FBS. The change of this medium is realized each 48 hours.

Perspectives

The research in cultured keratinocytes model advances in two main ways: 1. optimization of the methods in vitro to the skin cells culture and proliferation and 2. developing biomaterials that present similar skin properties.

In association to the continuous efforts to obtain an ideal combination of the cultured epithelial cells and the expansion of biomaterials possibilities, the nowadays research explore the knowledge of the genetic therapy technique. The cell genetic modification used in substitutes of the skin determines a new dimension and promises in the future be useful in the skin congenital diseases treatment, with production of growth factors to modulate the local process of wound healing and the systemic release of proteins.

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RESUMO - A pesquisa em bioengenharia é primordial no desenvolvimento da combinação ideal de biomateriais e células cultivadas para produzir a cobertura definitiva das lesões. O modelo experimental da cultura de queratinócitos apresenta toda as etapas do cultivo, desde o isolamento dos queratinócitos, preparação da derme acelular humana, do enxerto composto e da sua elevação à interface ar-líquido. A pesquisa em modelo de cultura de queratinócitos desenvolve-se em duas vias principais: 1. otimização dos métodos in vitro para cultivo e proliferação de células da pele e 2. desenvolvimento de biomateriais que mimetizem as propriedades da pele.

DESCRITORES -Queratinócitos. Derme acelular humana. Transplante de pele. Fibroblastos. Cultura de células.

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