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Evaluation of a fibrin-based skin substitute prepared in a defined keratinocyte medium

Metka Krasna[a,*,1], Franc Planinsek[b], Miomir Knezevic[a], Zoran M. Arnez[b], Matjaz Jeras[a,1]

[a] *Tissue Typing Center, Blood Transfusion Centre of Slovenia, Slajmerjeva 6, SI-1000 Ljubljana, Slovenia*
[b] *Department of Plastic Surgery and Burns, University Medical Centre, Ljubljana, Zaloska cesta 2, SI-1000, Slovenia*

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Abstract

The purpose of this study was to evaluate the influence of fibrin glue and aprotinin on the growth of adult human skin keratinocytes in defined serum-free conditions. The keratinocytes were cultured on cell culture plastics and on a fibrin matrix prepared from fibrin glue. The cell growth was measured by MTT assay, while the growth of clonogenic keratinocytes was evaluated by colony assay and expressed as colony-forming efficiency (CFE). The clonogenic potential of keratinocytes released from subconfluent and confluent cultures grown on fibrin glue was also studied by the colony assay. In comparison to a plastic culture surface the fibrin glue had significantly ($P < 0.05$) increased the clonogenic potential of keratinocytes, as well as enhanced their growth. Keratinocytes released from subconfluent cultures grown on fibrin glue attained a significantly ($P < 0.05$) higher percentage of clonogenic cells than their confluent parallels. At 75, 150, 300 and 450 KIU/ml aprotinin did not influence the growth of keratinocytes ($P > 0.2$). A fibrin-based skin substitute produced in the defined keratinocyte medium could be safely used to treat a number of skin defects.

Keywords: Human skin keratinocytes; Fibrin glue; Aprotinin; Colony-forming efficiency; MTT assay; Defined keratinocyte medium

1. Introduction

Various skin substitutes have been developed in order to treat acute and chronic skin defects (burns, scars, cutaneous ulcers and congenital cutaneous anomalies). They are based on different biocompatible and biodegradable matrices that support the growth of skin cells (Boyce, 2001). Fibrin-based cultured skin

* Corresponding author. Tel.: +386 1 5438 220; fax: +386 1 4312 304.
E-mail addresses: metka.krasna1@guest.arnes.si (M. Krasna), matjaz.jeras@ztm.si (M. Jeras).
[1] Both authors equally contributed to this work.

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substitutes use fibrin as a scaffold for transferring keratinocytes onto the wound. They can be applied in a solid form as a thin flexible transparent film (Ronfard et al., 2000; Pellegrini et al., 1999) or they can be sprayed onto the wound as a keratinocyte–fibrin glue suspension (Horch et al., 1998). The fibrin matrix can be prepared from commercial fibrin glue, which is produced from pooled human plasma, or from the patient's own autologous blood. The commercial fibrin glue is a two-component tissue sealant based on fibrinogen/factor XIII/fibronectin concentrate and human thrombin concentrate. Normally it is used for achieving hemostasis, to seal tissues and to support the healing of the wound. It also improves the adherence and engraftment of skin substitutes, it may have a protective effect against infection and is a suitable delivery vehicle for exogenous growth factors (Currie et al., 2001).

Fibrin-based cultured skin substitutes, using a feeder layer of murine fibroblasts for isolation and cultivation of keratinocytes (Rheinwald and Green, 1975), have been successfully grafted onto a mouse (Meana et al., 1998) and human full-thickness burns (Ronfard et al., 1991, 2000; Kaiser et al., 1994; Pellegrini et al., 1999). Keratinocytes cultured without the feeder layer in a serum-free medium supplemented with bovine pituitary proteins were also efficiently transplanted as keratinocyte–fibrin glue suspensions onto full-thickness wounds of athymic mice (Horch et al., 1998), onto chronic leg ulcers (Horch et al., 2001; Drukala et al., 2001) and with the addition of calf serum to the culture medium also onto full-thickness burns (Stark et al., 1995). The growth of keratinocytes cultured on a feeder layer and fibrin glue has already been studied (Ronfard et al., 2000; Pellegrini et al., 1999). These studies have shown that fibrin does not affect the clonogenicity of keratinocytes nor does it induce their clonal conversion with the consequent loss of epidermal stem cells. We have not found a single study that would evaluate the growth of keratinocytes on a fibrin matrix without using a feeder layer in the defined serum-free conditions. During recent years defined keratinocyte culture media without bovine pituitary proteins have become commercially available. The growth of keratinocytes in such conditions is possible, thus enabling a production of safer and more defined skin substitutes.

The aim of our study was to evaluate the growth of keratinocytes on a fibrin glue matrix using such a defined keratinocyte medium. Since, the presence and persistence of clonogenic keratinocytes in skin substitutes are one of the essential factors for successful engraftment, we also decided to study the colony-forming efficiency (CFE) of keratinocytes released from subconfluent and confluent cultures grown on a fibrin matrix in order to demonstrate the preservation of clonogenic keratinocytes. A culture of proliferating keratinocytes rapidly degrades the fibrin glue, therefore a fibrin-based skin substitute must be cultured in a medium that contains aprotinin, which is an inhibitor of serine protease and prevents plasmin from cleaving fibrin (Ronfard and Barrandon, 2001). For this reason, we also investigated the influence of aprotinin on the growth of keratinocytes. We used a fibrin matrix prepared from commercial fibrin glue (Tissucol), which according to the manufacturer contains the following substances: fibrinogen (70–110 mg/ml), fibronectin (2–9 mg/ml), factor XIII (10–50 U[1]/ml), plasminogen (40–120 μg/ml), bovine aprotinin (3000 KIU/ml) and thrombin.

2. Materials and methods

2.1. Materials

Amphotericin B (Fungizone), gentamicin, defined keratinocyte serum-free medium (defined keratinocyte-SFM medium) without bovine pituitary extract and Trypan blue stain all by Invitrogen Corporation (Paisley, UK). Tissucol Kit™ by Baxter-Hyland Immuno (Vienna, Austria). Trypsin-EDTA solution (0.05% trypsin/0.02% EDTA) and penicillin–streptomycin solution by Sigma-Aldrich Chemie (Taufkirchen, Germany). Soybean Trypsin inhibitor, HBSS (Hanks' balanced salt solution without Mg^{2+} and Ca^{2+}), MTT (thiazolyl blue), isopropanol, dispase all purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Aprotinin (Antagosan) was supplied by Hoechst Marion Roussel (Frankfurt am Main, Germany). Our experiments were carried out in cell culture dishes made by Corning (NY, USA).

[1] One unit corresponds to the amount of Factor XIII contained in 1 ml of normal fresh plasma.

2.2. Isolation and culture of keratinocyes

With the approval of the National Ethical Committee we obtained normal adult human skin samples from breast reductions and abdominoplasties. Keratinocytes were prepared according to the GibcoBRL (Life Technologies) protocol with the exception of minor modifications. Firstly, each skin biopsy was decontaminated for 5 s in 70% ethanol and then it was re-hydrated in the sterile Hanks' balanced saline solution without Ca^{2+} and Mg^{2+}. The excess of connective tissue was trimmed off and the remaining skin was cut into 5 mm × 5 mm pieces, which were incubated overnight at 4 °C in a solution of dispase (25 U/ml), supplemented with gentamicin (50 μg/ml), amphotericin B (2 μg/ml), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Subsequently, the epidermis was peeled off and keratinocytes were extracted from it during a 15 min digestion in 0.05% trypsin/0.02% EDTA solution at 37 °C. The suspension of keratinocytes was filtered through a 100 μm cell strainer, washed in a solution of soybean inhibitor (1 mg/ml), centrifuged at 200 × *g* for 5 min and plated into a plastic cell culture dish in a defined keratinocyte-SFM medium supplemented with amphotericin B (2 μg/ml), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. They were fed with fresh medium every 3–4 days. The cells were serially sub-cultured at a split ratio of 1:3 and the keratinocytes at passages 1–4 were used for all experiments. Before each experiment the cells were stained with trypan blue solution to asses their viability and were microscopically counted in a haemocytometer chamber.

2.3. Preparation of fibrin glue-coated plates

Cell culture plates were coated with fibrin glue (Tissucol^{TM}, Baxter-Hyland Immuno) as described previously by Pellegrini et al. (1999). The original stock solution of thrombin was diluted to 3 IU/ml with 1.1% NaCl and 1 ml $CaCl_2$ solution; 5.8 ml of the same saline solution was added to the original 5 ml of the fibrinogen stock solution. Each well (9.2 cm^2) of a six-well plate was coated with a mixture of 230 μl thrombin and 230 μl fibrinogen solution. The plates were then left for 10 min at 37 °C for complete solidification. When the fibrin matrix needed to be detached from the plate, as is the case in the process of skin substitute preparation, plates made by Golias (Ljubljana, Slovenia) or plates not treated for cell culture made by Greiner Bio-One (Kremsmünster, Austria) were used (Pellegrini et al., 1999), since in Corning's plates fibrin stuck to the bottom and therefore could not be manually detached.

2.4. Influence of fibrin glue on the colony-forming efficiency of keratinocytes

Colony-forming assay was performed in triplicate on fibrin-coated and bare plastic six-well plates. Keratinocytes were plated with 1000, 2000 or 4000 viable cells per well and incubated in keratinocyte-SFM medium supplemented with amphotericin B (2 μg/ml), gentamicin (50 μg/ml) and aprotinin (150 KIU/ml). The cell culture medium was changed twice per week, 7 days later the colonies were stained with MTT. The colonies were incubated in the culture medium with 0.5 mg/ml of MTT for 1 h. Intracellular dehydrogenases convert MTT to blue formazan, which colours the cells blue. The colonies with a diameter exceeding 0.5 mm were counted and the colony-forming efficiency was calculated according to the following formula (Boyce and Ham, 1983): (number of colonies × 100/number of live cells plated) = CFE.

2.5. Colony-forming efficiency of keratinocytes released from the fibrin glue

Keratinocytes were plated at a density of 5000 cells/cm^2 in six fibrin coated plastic dishes and incubated in a keratinocyte-SFM medium supplemented with amphotericin B (2 μg/ml), gentamicin (50 μg/ml) and aprotinin (150 KIU/ml). When subconfluent growth was reached – approximately at 70% confluence – three cultures were immediately tested for CFE, while the remaining ones were further incubated until confluence was achieved and were only then processed for CFE. The cultures were trypsinized and 1000 or 2000 viable cells were plated in fibrin coated six-well plates, incubated for 7 days and processed in order to determine the percentage of clonogenic cells. All experiments were performed in a triplicate.

2.6. Influence of fibrin glue on cell growth

Two thousand keratinocytes per well were plated in four fibrin-coated and four non-coated wells within a 24-well plate. The cells were incubated in a defined keratinocyte-SFM medium supplemented with amphotericin B (2 μg/ml), gentamicin (50 μg/ml) and 150 KIU/ml of aprotinin. After 6 days the MTT assay was performed: cells were incubated for 1 h in a culture medium with 0.5 mg/ml of MTT. The stain was then eluted with acidified isopropanol (0.04N HCl in isopropanol) and the optical density was measured at 570 nm and subtracted for the optical density at 620 nm. For negative controls the same protocol was applied but the cells were excluded. Their optical densities were subtracted from the optical densities of cell culture supernatants. The cell growth was measured in four replicates by MTT assay.

2.7. Influence of aprotinin on cell growth

Eight thousand keratinocytes per well were plated in 12-well plates and incubated in a defined keratinocyte-SFM medium supplemented with antibiotics, antimycotic and various concentrations of aprotinin (0, 75, 150, 300 and 450 KIU/ml). Following 7 days of cultivation, the MTT assay was performed in order to measure the growth of keratinocytes. For negative controls the same protocol was applied but the cells were excluded. The influence of aprotinin was studied in triplicate cultures.

2.8. Statistics

Data were expressed as means ± standard deviation. The differences between means were assessed by the Student's *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Influence of fibrin glue on colony-forming efficiency and cell growth

Four independent experiments were performed with keratinocyte subcultures derived from skin samples from four different donors. The graph in Fig. 1 demonstrates the results of a representative experiment performed with passage 1 keratinocytes: colonies were formed by 19.8 ± 3.2% of cells plated on the fibrin compared to 9.6 ± 1.0% of cells plated on bare culture plastic. Keratinocytes grown on fibrin achieved significantly higher CFE values than those cultured on plastic ($n = 3$; $P = 0.006$). We performed the experiment four times and on each occasion we found significantly higher numbers of colonies on fibrin surfaces then on plastic surfaces ($P < 0.01$). Hence: 19.8% versus 9.6%; 1.2% versus 0.6%; 1.9% versus 0.2%; 1.8% versus 0.2%.

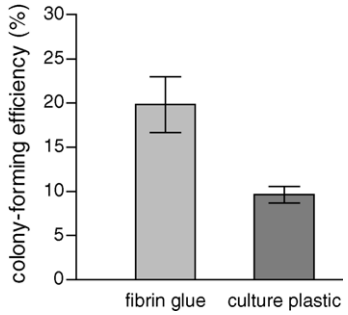

Fig. 1. Colony-forming efficiency of keratinocytes grown on fibrin glue and on cell culture plastic. Results shown are the means of three parallel cultures ± S.D. ($P < 0.05$; Student's *t*-test).

Growth of keratinocytes on fibrin glue or on bare culture plastic was evaluated by MTT assay. Results represented in Fig. 2 show that fibrin glue significantly enhances the growth of keratinocytes ($n = 4$; $P = 0.0003$). Four parallel cultures were used to perform this experiment.

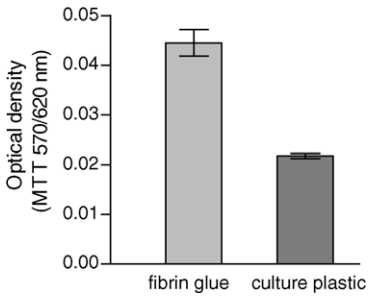

Fig. 2. Growth of keratinocytes on fibrin glue and on cell culture plastic. Results shown are the means of four parallel cultures ± S.D. ($P < 0.05$; Student's *t*-test).

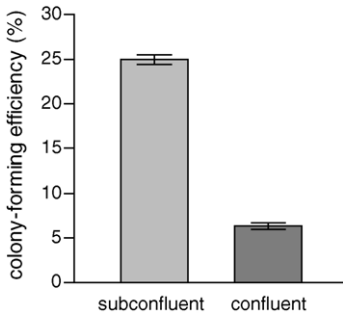

Fig. 3. Colony-forming efficiency of keratinocytes released from subconfluent and confluent cultures grown on fibrin glue. Results shown are the means of three parallel cultures ± S.D. ($P < 0.05$; Student's *t*-test).

3.2. Clonogenic potential of keratinocytes released from fibrin glue

We measured the clonogenic potential of keratinocytes released from subconfluent and confluent cultures grown on fibrin glue. Keratinocytes plated at 5000 cells/cm^2 on a fibrin matrix reached about 70% confluence within 3 days. A further 2 days of incubation were necessary in order to reach a confluent state. Cells released from subconfluent cultures grown on fibrin attained significantly higher CFE values than those released from confluent cultures. The results of this representative experiment are presented in Fig. 3: colonies formed in 25 ± 1.0% of cells released from subconfluent secondary cultures compared to 6.3 ± 0.6% of cells released from the confluent cultures ($n = 3$; $P = 0.00001$). Experiments with cells at higher passages displayed similar results although they contained less clonogenic cells. Keratinocytes released from the subconfluent passage four cultures contained 3.8 ± 0.3% of clonogenic cells, while the ones released from confluent cultures contained 1.1 ± 0.1% of clonogenic cells ($n = 3$; $P = 0.00005$).

3.3. Influence of aprotinin on cell growth

At first we tried to produce a skin substitute without adding aprotinin to the culture medium. However, proliferating keratinocytes dissolved the fibrin matrix, therefore the skin substitute lost its elasticity and could not be detached from the plate. After that we prevented fibrinolysis by using aprotinin at 150 KIU/ml (Ronfard et al., 2000). The resulting skin substitute was transparent and delicate but retained sufficient elasticity for potential clinical application (Fig. 4). Results in Fig. 5 show that aprotinin at 75, 150, 300 and 450 KIU/ml does not significantly affect the growth of keratinocytes compared to cultures that were incubated without it ($n = 3$; $P > 0.2$). The presence of aprotinin in the culture medium (negative control) did not change the optical density of the culture supernatant.

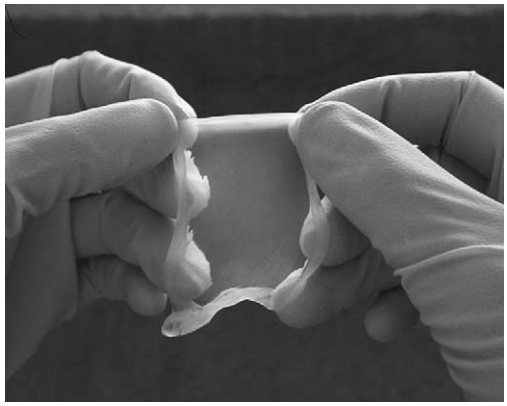

Fig. 4. Photography of fibrin-based skin substitute.

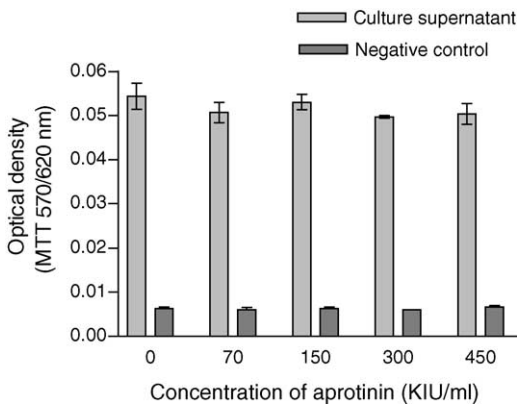

Fig. 5. Influence of aprotinin on the growth of keratinocytes. Results shown are the means of three parallel cultures ± S.D. ($P > 0.2$; Student's *t*-test).

4. Discussion

Fibrin glue has been successfully used in fibrin-based skin substitutes (Ronfard et al., 1991; Kaiser et

al., 1994; Pellegrini et al., 1999; Horch et al., 2001). Earlier studies demonstrated the preservation of clonogenic keratinocytes within a fibrin matrix. They were able to isolate 16–66% (Ronfard et al., 2000) and 12.9–45.2% (Pellegrini et al., 1999) clonogenic cells from graftable cultures prepared with a feeder layer of murine 3T3 cells on a fibrin matrix. Additionally, it has been shown that the migration of keratinocytes into the fibrin matrix does not impair their colony-forming efficiency (Ronfard and Barrandon, 2001). Our results also demonstrate that subconfluent secondary cultures grown without a feeder layer on fibrin glue using the defined medium contained about 25% clonogenic cells. Furthermore, we clearly demonstrated that subconfluent cultures grown on fibrin contained more clonogenic keratinocytes than confluent cultures, our results supported by previous studies on a cell culture plastic surface (Boyce and Hansbrough, 1988; Poumay and Pittelkow, 1995). We also demonstrated that in comparison to a plastic surface the fibrin glue increases the clonogenic potential of keratinocytes and enhances their growth, which is in contrast to findings showing that compared to cell culture plastic the fibrin matrix does not affect the growth of clonogenic keratinocytes (Ronfard et al., 2000; Pellegrini et al., 1999). The explanation to this discrepancy is because they used a feeder layer, thereby masking the enhancing effect of the fibrin matrix. In support of our findings are published results, that under suboptimal growth conditions, extracelullar matrix components can modulate keratinocyte growth (Woodley et al., 1990). We could also show that the fibrin matrix promotes the growth of keratinocytes at later passages, when low seeding densities were applied (108 cells/cm^2), while it was impossible to culture such low number of cells on the bare plastic surface. The addition of aprotinin to the culture medium was essential for the maintenance of the elastic structure of the fibrin-based skin substitute. Ronfard and Barrandon demonstrated that keratinocytes could dissolve the fibrin matrix by migrating into it with a rate up to 2.1 mm/day. 150 KIU/ml of aprotinin stopped 90% of migrating cells, while 500 KIU/ml completely suppressed the migration (Ronfard and Barrandon, 2001). We showed that aprotinin at 75, 150, 300 and 450 KIU/ml does not influence the growth of keratinocytes. Hence, it can be used in production of skin substitutes.

The fibrin matrix, which was used in our study, was prepared from diluted commercial fibrin glue (Tissucol). It contained fibrinogen (16.2–25.5 mg/ml), fibronectin (0.46–2.08 mg/ml), thrombin (1.5 IU/ml), factor XIII (2.3–11.6 U[1]/ml), plasminogen (9.3–27.8 μg/ml) and aprotinin (694 KIU/ml), as calculated from the manufacturer's specification. Some substances present in the fibrin glue were proven to influence the growth of keratinocytes. It has been shown that fibrin inhibits keratinocyte spreading and replication in the absence of fibronectin and cross-linking with factor XIII (Weiss et al., 1998). The mechanism of fibronectin action could be explained by inhibition of terminal differentiation of human keratinocytes (Adams and Watt, 1989) and by enhancing outgrowth of clonogenic keratinocytes through its main receptor $\alpha_5\beta_1$ integrin (Bata-Csorgo et al., 1998). One of the biological markers of keratinocyte stem cells is a high surface expression of β_1 integrins (Watt, 1998), which was also found in keratinocytes grown in the fibrin matrix prepared from Tissucol^{TM} (Reissig and Schmidt, 1998). Furthermore, human thrombin, which is also present in small quantities in the fibrin matrix, has been shown to enhance the growth of keratinocytes in a dose-dependent manner in the absence of other growth factors (Derian et al., 1997).

In conclusion, we have shown that the fibrin matrix prepared from commercial fibrin glue enhances the colony-forming efficiency of keratinocytes and enables the cultivation of cells at low seeding densities within the defined serum-free medium. We also showed that a fibrin-based skin substitute with a subconfluent epithelium preserves more clonogenic cells than a confluent one. Finally, a fibrin-based skin substitute produced in the defined keratinocyte medium could be safely used to treat various skin defects.

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