Matrigel increases the rate of split wound healing and promotes keratinocyte 'take' in deep wounds in rats

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Abstract

The influence of matrigel, a mixture of the components of the basement membrane, on the wound healing was studied in a model of experimental wounds in rats. Matrigel was found to increase the rate of epithelization of split-thickness wounds. The model of deep wound was developed in which the host animal could not provide enough migrating and proliferating keratinocytes to cover the wound area. The model is relevant to severe burns and injuries in humans. When rat keratinocyte suspension was transplanted into deep wounds, cell retention in the wound bed was only observed if matrigel was added together with the cells. Increasing matrigel concentration in the wound was seen to enhance the rate of wound area coverage by the cells. Although the process of healing seemed macroscopically normal, after histological screening of the biopsies cell in the wound appeared as amorphous aggregates and tubules rather than stratified epidermis.

Introduction

After skin damage the process of wound healing begins which includes several stages and involves cooperation between different cell types, fibroblasts and keratinocytes being the most important players. Cells in the wound bed interact either through cell contacts or by secreting biologically active substances. The components of the extracellular matrix, synthesized by the wound cells have a very important role in the process of wound healing (Gailit and Clark, 1994). The very crucial extracellular matrix structure in the skin is the basement membrane, underlying basal layer of epidermis and thus separating epidermis from dermis. Between the proteins in the skin basement membrane are collagen IV (Kefalides, 1972), laminin-1 (Timpl et al., 1983), laminin-2 (Sewry et al., 1986), laminin-5 (Marinkovich et al., 1992), entactin (Carlin et al., 1981), proteoglycans (Hassel et al., 1985), nidogen (Timpl et al., 1983). Indeed, various components of basement membrane were shown to influence keratinocyte migration and proliferation both

in culture and during the course of re-epithelization. Collagen IV was found to enhance keratinocyte adhesion and proliferation in culture. (Murrey et al., 1979; Woodley et al., 1990). Laminin-1 serves rather as an anti-migratory agent and helps to stabilize stratified epidermis once it is established (Sudbeck et al., 1997).

One of the most studied biochemical preparations consisting of basement membrane elements is the extract of mouse EHS-sarcoma, known as matrigel. Laminin-1, entactin/nidogen, collagen IV, proteoglycans are found in matrigel (Kleinman et al., 1986). As it has been isolated from the tumor, matrigel usually contains growth factors, such as TGF β , bFGF, PDGF, as well as a number of unidentified factors (Vukicevic et al., 1992). In some preparations proteases are found (Salo et al., 1991). It is well established that matrigel contributes to the maintenance of differentiation of epithelial cells in culture; amongst them are hepatocytes (Kleinman et al., 1986), mammary epithelial cells (Barcellos-Hoff et al., 1989), and gastric mucosal cells (Mikami et al., 1994). In some culture systems matrigel was shown to promote cell

proliferation (Shintani et al., 1993), though it was reported to decrease growth of other cell types (Yoshimo et al., 1990). It is interesting that matrigel exhibits its influence on cells both *in vitro* and *in vivo*. In the *in vivo* study, Kleinman et al. (1986) showed that matrigel supports neuron expansion.

Since basal layer of epidermal cells is in close contact with the basement membrane, it is very probable that matrigel could influence their behavior. Surprisingly, although the effect of matrigel on many different types of cells was extensively investigated, only limited data are available on the behavior of keratinocyte both *in vitro* and *in vivo* in the presence of matrigel. It was observed *in vitro*, that matrigel enhanced initial attachment as well as proliferation of keratinocytes (Dawson et al., 1996). Having used an acellular non-viable dermis, which served as a model of a homograft allodermis, the same authors showed that cultivation of keratinocytes on matrigel prior to grafting them to the dermis did not appear to confer any substantial benefit to the attachment and retention of these cells (Dawson et al., 1996).

The aim of the present work was to examine matrigel influence on wound healing in an animal model. The rate of split-thickness rat wounds healing was measured after the addition of matrigel. In a model of deep rat wounds matrigel was added together with allogeneous keratinocytes in order to study on the retention of the grafted cell in the wound site.

Materials and methods

Keratinocyte isolation

Rat epidermal keratinocytes were obtained from the skin of newborn rats (2–4 days old). Keratinocytes were selected according to a modified Rheinwald's method (Rheinwald, 1980). The skin was washed in Hank's solution containing 2000 U/ml gentamycin, made free from under-laying fat tissue and cut into small pieces (5–10 mm in size). The fragments were incubated in 0.5% dispase solution in PBS (Boehringer Mannheim GmbH) for 1 h at 37 °C. Then the pieces were transferred into Dulbecco's PBS and the epidermis was separated from the dermis along the line of the basal membrane by using a forceps. The epidermis was incubated in 0.125% trypsin for 10–15 min, under shaking at 50 rotations per minute. At the end of trypsinisation the trypsin was inactiv-

ated by addition of 5% fetal calf serum. Then the cell suspension was obtained after 10 cycles of pipetting using a pipette with a broad aperture. The suspension was filtered through a nylon gaze followed by centrifugation at 1000 rpm and resuspended in PBS buffer.

Matrigel preparation

Matrigel was isolated from the mouse EHS-sarcoma as previously described (Kleinman et al., 1986). The composition of matrigel was determined by SDS-PAGE in 6% gel. In addition, collagen IV content was estimated by measuring the oxyproline concentration (Bergman and Loxley, 1963).

Grafting keratinocytes on full-thickness wound

All experiments were conducted under anaesthesia by intramuscular injection of callipsol (70 mg/kg) and droperidol (1 mg/kg). Full-thickness skin discs were excised from the back of the adult rat down to the muscle. A plastic ring of 2.5 cm in diameter was sewed to the wound bed (Figure 1). The second ring was sewed to the wound edge in order to prevent skin contraction. Keratinocyte suspension was grafted onto the prepared wound, 4×10^5 cells in 0.3 ml of PBS buffer per wound. Matrigel solution (0.2 mg/ml) was added together with the cell suspension. In the experiments on the influence of matrigel concentrations used were 8, 4 and 0.2 mg/ml. The cell suspension was grafted onto the wound either with matrigel or without. The wound was covered with three layers of waxed wound dressing 'Paranett' and then by a sterile cotton wool balls fixed by banding the ligature ends over it. The bandages were removed on the 4th day under anaesthesia done with a mixture of callipsol and droperidol. After examining and estimating the wound condition, taking biopsies and photographs, a multilayer gauze covering the wound defect was sewed to the skin. The following examinations of the wound condition were done on the 7th and 14th day. Biopsy taking was performed on day 4 day 7 and day 14 after grafting. After that the biopsies were processed for light and electron microscopic examination.

The experiments were conducted in three repetitions, each including minimally 8 animals per group. Formation of a thin layer of neo-epidermis was regarded as the indicator of successful keratinocyte attachment on the wound.

Figure 1. Diagramme of full-thickness wound model, presenting the position of the two plastic rings.

Processing the bioptates for light and electron microscopic examination

The bioptates were fixed in the 0.25% glutaric aldehyd (BDH) solution for 4 h then rinsed in 0.3 M sucrose solution and again fixed in 1% OsO_4 solution. After that the material was rinsed twice in distilled water. Then the bioptates were dehydrated with ethanol according to the following scheme: 70% – for 10 min, 90% – for 10 min, and finally 100% – twice, for 15 min for each repetition. After that the bioptates were placed in araldit (BDH) for 10 h at room temperature, followed by an incubation for 24 h at 60 °C. The sections were stained with methylene blue. The sections for electron microscopy were stained with a aqueous solution of uranyl acetate followed by lead citrate treatment (Glauert, 1965).

Experiments with split-thickness skin wounds

Experiments with split-thickness skin wounds were conducted on rats under anaesthesia. Split skin sheets of a thickness of 0.2 mm were excised with a dermatome after depilation. The wound square was 2–3 cm^2 . The wound surfaces were treated with matrigel and covered with bandages. The time of the epithelization of split wound was registrated for each animal individually. The experiments were conducted in three repetitions, each including minimally 8 animals per a group. From the third day onwards bioptates were taken for histological examinations. Complete restoration of skin was regarded as criteria for epithelization of the split wounds.

All experimental results were statistically treated. Results were compared using a standard T-Test, differences were considered to be significant if the p value was smaller than 0.05. The results shown in each table are the means of 3 experiments.

Results

Experiments with split-thickness wounds

In the experiments concerning split-thickness wounds, matrigel was used for healing without adding keratinocytes. We observed the time of wound epithelization. Matrigel strongly enhanced the rate of epithelization (Table 1). The wounds were covered with new epidermis in 3–4 days when matrigel had been applied versus 8–10 days in the group of rats without matrigel addition. Histological examinations confirmed the appearance of the neo-epidermis (Figure 2). The re-epithelized skin consisted of three main layers. The bottom layer was the muscle fascia, followed by thick dermis in which glands and hair follicles are located. On top of these two layers of old undamaged skin thin reconstructed epidermis was formed, consisting only of two to three cell layers.

Modeling a full-thickness wound

In order to construct a deep wound bed free from granulation tissue, we have developed the following method for preparing wounds (Figure 3). The wound bed was formed of muscle tissue. Wound edges were delimited by two polyethylene rings, one adjusted to

Figure 2. Histological section of a split-thickness wound after healing ($\times 120$).

Table 1. Influence of matrigel on the rate of epidermis regeneration in the split-thickness wounds

Variant	Number of animals in group	Rate of regeneration (days)
Control	46	9.1 ± 0.9
Matrigel	24	3.5 ± 0.4
		$p < 0.01$

the wound bottom and the other to the level of the skin surface. Thus, both keratinocyte migration from the edges and contraction of the skin were prevented. The experimental wound before cell application is shown in Figure 3A. Figure 3B displays the addition of the keratinocyte suspension onto the fresh wound. The wound was covered by a waxed bandage cut as a disc of a diameter smaller than that of the plastic rings. The wound after grafting of the cells and covering by a cotton wool ball is presented in Figure 3C. The ball was fixed with the ends of the same ligatures that fixed the rings.

Table 2. Influence of matrigel on rat keratinocyte 'take' in the full-thickness wounds

Variant	Number of animals in group	Number of animals with keratinocyte 'take' on day 14 (the percentage is given in brackets)
REK without matrigel	18	3 (16.7 ± 0.0)
REK + matrigel 0.2 mg/ml	24	22 (91.7 ± 3.9)
		$p < 0.05$

The method also allows to obtain a granulated wound if desired. For this purpose wound modeling was carried out as shown in Figure 3A but without transferring cells. Five days after wound modeling, the cotton wool ball was removed, the bandage taken off. Granulated wound so formed can serve as a model of the granulation tissue.

Grafting keratinocytes on full-thickness wound

We studied the influence of matrigel on the retention of keratinocyte suspension in freshly made deep wounds. Matrigel was applied to the wound together with a keratinocyte suspension. On the 4th day after having undone bandages and examined the wound, we could see wounds in two different states depending on whether matrigel had been added or not. In the wound without matrigel the site consisted of muscle surface with little granulation covered with exsudate and no signs of wound closure appeared. Often the wound became infected. We could not find traces of grafted cells in the place. In histological sections of the biopsies taken from these wounds only muscle tissue was seen with sharp edge opened toward the wound site, tiny layer of granulation tissue occasionally covered it (not shown).

While examining on the 4th day the wounds to which matrigel had been added, we observed that some kind of wound coverage definitively emerged. The wound surface appeared mate, pale, and relatively dry. We concluded that the layer covering the injured site was extremely fragile and it seemed difficult to collect is for sample. Table 2 summarizes the results of final examination of the wounds on day 14. While successful wound healing was observed in all animals treated with matrigel together with a keratinocyte

Figure 3. Modeling full-thickness wound. (A) Design of the wound bed. (B) Transplantation of the cell suspension. (C) Appearance of wound after the operation.

Figure 4. Influence of matrigel concentration on the take of keratinocytes in the full-thickness wound model.

Figure 5. Section of the biopsy obtained from wounded skin, showing transplanted keratinocytes ($\times 160$).

suspension, this was negligible in the control animals treated without matrigel. As shown in Figure 4, increasing the concentration of added matrigel enhanced the take of keratinocytes. It was more obvious on day 4, whilst on day 7 transplantation keratinocyte take was observed in almost all rats treated with matrigel irrespectively of the concentration. Figure 5 displays the results of histological investigation of the biopsy material taken from the wound for which keratinocyte take had been observed. In contrast to the macroscopic observation, which showed wound coverage by an apparently well developed epithelium, an amorphous cell mass was found. The cells forming the mass were round, with large nuclei and bright cytoplasm, which is a characteristic of poorly differentiated cells. Hence, the structure of the tissue formed after transferring cell suspension in matrigel solution was atypical. No

multi-layer epithelia was formed. Nevertheless, electron microscopy of the cells from the material retained in the full-thickness wound revealed that the cells were keratinocytes, as they had keratin tonofilaments and desmosomes (Figure 6).

Discussion

There is a major difference between the two types of wounds used in the study. Indeed, undep skin wound in rat is naturally well repairing even without any treatment. In general it is known that undep burn wounds as well as wounds of other origin can be healed without applying intensive methods which involve, for instance, cultivating cells, because dermis remains undamaged. Epidermal cells originated from

Figure 6. Morphology of keratinocytes in a full-thickness wound (done by electron microscopy $\times 20\,000$). Keratin tonofilaments are marked with an arrow. Desmosomes are marked with an asterisk.

below the damaged skin surface are able to migrate and proliferate enough to cover wounded area. Even so, we observed acceleration of healing when matrigel was applied to the wound. Matrigel addition lead to a nearly 3-fold increase of the epithelization rate. Such an increase may be attributed to the enhanced keratinocyte migration from the wound edges as well as higher proliferation rate of the cells. As we could observe any increase in the wound healing rate only when the very high concentration of matrigel was added, we suggested the role of its minor components in the promotion of keratinocyte migration and proliferation. Good candidates for such a role could be the growth factors recently found in matrigel, namely TGF β , EGF, b FGF, PDGF (Vukicevic et al., 1992).

On the contrary, in a deep wound cells belonging to the damaged skin are prevented from taking part in the wound repair. Therefore, it is possible to observe the effect of allogeneous cell transplantation on the healing process. Cells transplanted into a deep

wound ought to interact with the wound bed formed by the muscle fascia. Hence, addition of extracellular matrix components could be beneficial in this model.

Our previous experience showed that sheets of epidermis made from newborn rat keratinocyte are composed of three layers when compared with eight layers for human cultivated epidermis (Gorelik et al., 1997). Since epidermis so formed is very fragile and not easy to graft we used cell suspension in our experiments. In agreement with our experience Khalfan and coworkers had noticed that cultivated epidermis lost its shape after enzymatic cell dissociation from the culture vessel, which led the transplantation to fail. They suggested the use of keratinocyte suspension rather than epidermal sheets as a grafting material in clinical practice to prevent these failures (Khalfan et al., 1989).

Dealing with newborn rat keratinocytes we were able to make only allotransplantations. Although it is not resolved yet whether transplanted allogeneous keratinocytes are finally present in the newly formed epidermis or whether they are rejected, their positive effect on wound healing had been proven by clinical practice. Hefton et al. (1983) were the first who successfully used allogeneous epithelia grown *in vitro* for healing 3 patients with a second-degree burns. Even when allogeneous keratinocytes were shed from epidermis during epithelization several studies proved that they somehow promoted wound healing (Bell and Rosenberg, 1990). Our results also demonstrated the positive role of allogeneous cells on the healing of deep wounds.

In a deep wound model both epidermis and dermal layer were damaged, and the basement membrane was also destroyed. Thus, when transplanting keratinocyte suspension it was necessary to add either substances which enhance basement membrane regeneration or the basement membrane elements themselves to the suspension. We showed here that keratinocyte take into deep wound did not occur until matrigel was added. Apparently, the wound provided specific conditions ($t = 37^\circ\text{C}$, pH, ionic strength) for matrigel to turn into gel even at low concentration. Matrigel might stimulate keratinocyte adhesion and growth and might prevent occasional cell loss from the wound.

However, some negative aspects of using matrigel together with cell suspension had been observed. Since matrigel seems to form instantly a gel, keratinocytes appear to remain entrapped in it. This might have contributed to the prevention of epidermis formation and maturation observed in this study. Interestingly, there is evidence for spheroid formation inside the

matrigel gel which closely resembles our findings (Limat et al., 1994). The authors observed hair follicle cells as well as epidermal cells to form spheroids while culturing them entrapped in matrigel gel. The cells were reported to differentiate toward the center of the spheroid. We have not seen any degree of differentiation in the structures formed by the transplanted keratinocytes (histological examination). One of the reasons might be the short term character of our experiments. Nevertheless we could observe tonofilaments in the cells suggesting some degree of differentiation of these cells (electron microscopic examination). Prevention of keratinocyte differentiation by matrigel was reported in a study concerning skin equivalent consisting of dermis covered by matrigel (Maruguchi et al., 1994). They supposed that matrigel does not promote differentiation of human keratinocytes. Our data seem to be in agreement with this statement. However, in our case the whole picture is compromised by the use of matrigel as a three-dimensional matrix rather than an attachment substrate.

Judging the results of full-thickness wound study we concluded that perhaps, in spite of the fact, that keratinocytes did not differentiate they could provide temporary wound coverage and in the same time they could activate self-healing of the wound by an yet unknown mechanism. Taken together our results suggest that matrigel is a positive regulator of the process of wound healing.

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