EFFECTS OF INSULIN, RETINOIC ACID AND HYDROCORTISONE ON *IN VITRO* DERMAL WOUND-HEALING MODEL

DANIELA NEAGOŞ, VALENTINA MITRAN**, GEORGETA CHIRACU*, ANIŞOARA CÎMPEAN **, R. COCOŞ*, DANA IORDĂCHESCU***

*University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania
**Research Center for Biochemistry and Molecular Biology, University of Bucharest, 93 Spl. Independenţei, Bucharest, Romania

Abstract. Fibroblast culture systems are routinely used to investigate wound healing under a wide range of experimental conditions. To evaluate the effects of insulin, retinoic acid and hydrocortisone on wound healing we carried out the studies on a scraped cell monolayer and on a free floating fibroblast populated collagen lattice (FPCL) model. The extent of recovery of the scraped 2D surfaces and of the gel contraction was monitored by phase contrast microscopy, acquisition and analysis of images. Our data showed the positive effects of insulin on the wound healing behaviour of human dermal fibroblasts. Treatment with retinoic acid did not affect the healing process and hydrocortisone inhibited it.

Key words: human dermal fibroblast, free floating fibroblast populated collagen lattice, wound healing models, insulin, retinoic acid, hydrocortisone

INTRODUCTION

Wound healing is a complex series of interrelated events which combine to return the damaged tissue to as near normal function as possible. It follows a specific sequence of phases which may overlap. The process of wound healing depends on the type of tissue which has been damaged and the nature of the tissue disruption. For example, the closure of cutaneous wounds involves three well-known processes: epithelization which results in resurfacing of the wound, connective tissue deposition which replaces the damaged dermis, and contraction which brings the margins of open wounds together [4].

Obviously, the ability of cells to contract is important for wound closure. However, excessive cellular contraction can result in formation of scar tissue. Likewise, if cellular contraction is inhibited the wound healing may be impaired.

Received February 2006.
in final form March 2006.

ROMANIAN J. BIOPHYS., Vol. 16, No. 2, P. 93–102, BUCHAREST, 2006

Thus, cellular contraction during wound healing must be in an optimal range to facilitate wound closure and also reduce scar tissue formation [3, 10].

There are two main opinions concerning the mechanism of wound contraction: one is that myofibroblasts and special fibroblasts in wound granulation tissue, shrink and pull surrounding tissue to the centripetal [16], and the other is that myofibroblasts are unnecessary, and that the reorganization and compaction of collagen fibers occurs during fibroblast locomotion [2]. Since both opinions are based on appropriate experiments, it is very difficult to determine which is true. However, the majority of researchers support the idea that wound repair involves a phenotypic change of fibroblasts from quiescent to proliferating cells, and subsequently to migratory mediated through different chemotactic signals, and then to stationary matrix producing and contractile cells. Wound contraction is accomplished by myofibroblasts that contain α-smooth muscle actin and mediate contractile forces produced by granulation tissue in wounds. Granulation tissue is composed of a fibronectin-rich scaffold, fibroblasts, new blood vessels and increasing amounts of collagen types I/III. The apoptosis of myofibroblasts and vascular cells finally mediates the transition from a granulation tissue into a scar [5, 13].

Fibroblast culture systems are routinely used to investigate wound contraction under a wide range of experimental conditions. Since the introduction of a fibroblast-populated collagen lattice in the late 1970s, this type of *in-vitro* model has been extensively used to study fibroblast function [1]. Contraction of fibroblast/collagen gels has been used as *in-vitro* models for investigating the biological mechanisms of wound contraction [9, 14] and also the effects of various compounds aimed at stimulating (enhancing wound healing) or reducing (preventing scar formation) the rate of contraction [15]. The benefit of this model is that the fibroblasts are grown in a three-dimensional collagen gel culture, a matrix component native to the wound environment.

Diabetes is responsible for delayed or impaired wound healing, leading in many instances to chronic ulcer formation. Diabetic ulcers of the lower limbs and feet, in particular, are associated with high morbidity and often lead to amputation.

Although it is well known the general role of insulin in wound healing [12], the effect of insulin on protein metabolism in skin wound has not been assessed sufficiently. To repair skin defects, new proteins have to be deposited in the wounded area to restore skin integrity. Data indicate that high doses of insulin and glucose can be safely administered to massively burned patients to improve wound matrix formation and the insulin alone stimulated protein turnover in the skin wound [19].

Retinoic acid (RA) can help diabetics suffering from skin ulcers heal faster, or can stop them from forming in the first place [17]. It has been shown that RA reverses the inhibitory effects of glucocorticoids on wound healing and accelerates the formation of healthy granulation tissue [11]. However, RA has several contradictory effects on wound healing depending on the route or schedule of retinoid administration.

Corticosteroids are frequently used in the treatment of various illnesses due to their anti-inflammatory and immunosuppressive functions. Beside their therapeutic potential it is known that corticosteroids prevent scar tissue formation and have deleterious effects on wound healing [18].

In the present study, we evaluated the effects of insulin, retinoic acid or hydrocortisone on a scratched fibroblast monolayer and on a free floating fibroblast populated collagen lattice (FPCL) model of *in vitro* contraction in 48 h from the gel detachment.

MATERIALS AND METHODS

CELL CULTURE

The human dermal fibroblasts (HDF) were isolated by the explant culture method. Briefly, skin specimens were cut into small pieces, and they were placed in 35 mm Petri dishes and maintained in a viable state in DMEM media supplemented with 15% fetal bovine serum (FBS, Sigma) plates, 2 mM L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). The explants were incubated in a humidified atmosphere with 5% CO_2 at 37°C. After 2-3 days HDF began to proliferate around the explants. The culture medium was changed every 3 days. When culture surface was filled with HDF, the explants were removed and the primary culture was incubated for 24h. The culture medium (DMEM + 10% FBS + antibiotics) was changed and when HDF reached the confluence, meaning that contact inhibition blocked the proliferation process, we began to subculture the cells. Harvesting of HDF was made by trypsinization with 0.25% trypsin-0.1% EDTA solution which hydrolyzed the adhesion elements of the cells to the support and between them. After removing the trypsin the cells were mixed with 5 ml culture medium containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) 2 mM L-glutamine and 10% FBS. The culture medium is removed by aspiration and replaced with a new one every 2-3 days. Cultures were used between passages 3 and 7 for the experiments. The tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted.

CELL CONTRACTION

A free floating fibroblast populated collagen lattice (FPCL) model of *in vitro* contraction was used to study fibroblast mediated wound contraction. Rat tail type I collagen was dissolved in 0.5 M acetic acid at a concentration of 6 mg/ml and

stored at 4°C. The collagen solution was then mixed at 4°C with DMEM, FBS and 0.1 N NaOH. Fibroblasts were immediately mixed with the neutralized collagen solution (final concentration of 3 mg/ml) and 0.5 ml aliquots were then transferred to each well of a 24-well microtiter plate. The plates were incubated at 37°C in 5% CO_2 humidified atmosphere. Each collagen lattice contained 1.5 mg/ml of collagen, 5×10^5 cells/well and 10% FBS. After setting, the FPCL were fed with fresh DMEM/10% FBS, and the gels detached from the base of the well plate. Triplicate FPCL were cast for each test and control group. Cell morphology within the FPCL was monitored by phase contrast microscopy (Nikon Eclipse TS 100). The gels were photographed with a digital camera (Nikon CoolPix 4500). The ability of the fibroblasts to contract the gels was determined by quantifying the area and the perimeter of the gels using image analyzer software (SigmaScan).

EXPERIMENTAL WOUNDING AND TREATMENT

Confluent monolayers of HDFs or fibroblast-populated type I collagen lattices were washed once with calcium- and magnesium-free phosphate-buffered saline (PBS) and starved overnight in medium without FBS. The plates with monolayers were removed from the incubator the following morning, the medium was aspirated off and the monolayer was scraped with a trimmed hard rubber ebonite comb washed with 70 % ethanol and dried before use. In all cases the mechanical wounds with the same shape and sizes were practiced to have the same mechanical features in the repair process

To assess the effect of insulin, retinoic acid and hydrocortisone on the wound healing, the overlaying media of the gels received varying concentrations of insulin, retinoic acid or hydrocortisone:

- Insulin: 0.1 μg/ml; 1 μg/ml; 5 μg/ml
- Hydrocortisone: 0.01 mg/ml; 0.1 mg/ml; 1 mg/ml
- Retinoic acid: 0.5 μg/ml; 1 μg/ml; 5 μg/ml

The scraped monolayer and the FPCL model were incubated, for different periods of time, at 37°C in 5% CO_2 humidified atmosphere.

MONITORING OF 2-D AND 3-D CULTURES

Cell morphology was monitored by phase contrast microscopy (Nikon Eclipse TS 100). The gels were photographed with a digital camera (Nikon CoolPix 4500). The ability of the fibroblasts to proliferate in monolayer or to contract the gels was determined by quantifying the area of the gels using image analyzer software (SigmaScan).

RESULTS AND DISCUSSION

In a first step, the effects of these treatments on the behaviour of HDF grown in monolayer were studied. Confluent monolayers of HDF cells were deprived of FBS for 24 h prior to wounding. At the designated time, the remaining media were aspirated and the monolayer was scraped with a trimmed hard rubber ebonite comb washed with 70% ethanol and dried before use. Cultures with standard "wounds" were supplemented with DMEM medium (with or without 10% FBS) and incubated at 37°C. When mechanical wounded monolayers were incubated at 37°C in an atmosphere with 5 % CO_2 and controlled humidity, in DMEM medium with 10% SFB, the entire wounded area will totally recover in 31 hours. For some experiments, varying concentrations of insulin, RA or hydrocortisone were added to the conditioned media immediately after wounding.

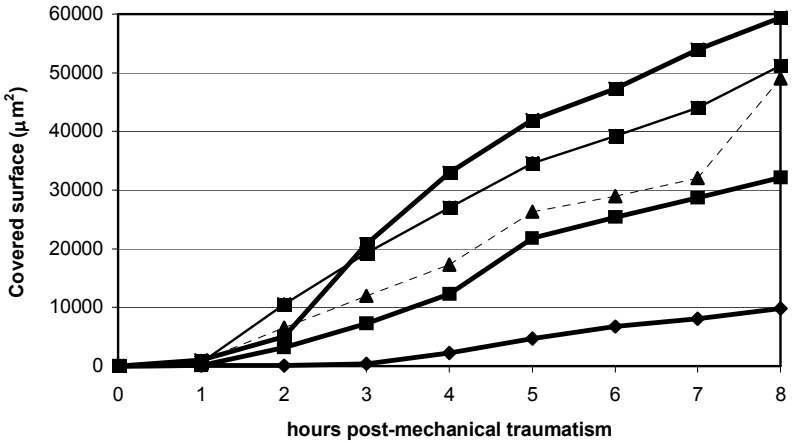

Fig. 1. Repair of a mechanical lesion practiced in a fibroblast monolayer, in DMEM medium (♦), DMEM + 10% FBS (■); insulin (■): 0.1 µg/ml (----), 1 µg/ml (▬▬) and 5 µg/ml (——).

In Figure 1 we present cover's kinetics of a wounded surface along 8 hours after wounding, in the case of fibroblast monolayer, incubated in DMEM medium without FBS (control sample A), in DMEM medium with 10% FBS (control sample B), as well as in DMEM medium without FBS but with added insulin in three different concentrations: 0.1, 1 and 5 µg/ml. The fastest cover of the wounded surface was observed in the case of human dermal fibroblasts cultured in DMEM medium with 1µg/ml insulin. If we consider that the surface of the wound is 59.400 μm^2, which represents the measured value in the case of the closed wound following the treatment with 1 µg/ml insulin, this means that in this case the

healing process takes place in a proportion of: 2%-after one hour, 35 %-after 3 hours, 55.6%-after 4 hours, 70.7%-after 5 hours, 79.6%-after 6 hours and 90.9%-after 7 hours.

After 8 hours, the healing process is realized in proportion of 16.5% if the cell monolayers are incubated in DMEM media without FBS, 54% in DMEM + 10% FBS, 82.5% when the cells are incubated in DMEM + 0.1μg/ml insulin and 86.2% in DMEM + 5 μg/ml insulin. The fastest healing process is obtained if the cells are incubated in DMEM media with 1 μg/ml insulin.

The data from Table 1 that represent the average of 2-3 experiments were considered in comparison with a control experiment that monitors the covering of the wounded surface in the case of the incubation of the cell monolayer with DMEM medium without FBS. The hydrocortisone, regardless of the tested concentration, leads to inhibition of the restoration process of the wound; after 8 hours was recorded a covering degree of the destroyed surface that represents 48.5 % (0.01 mg/ml), 45.2 % (0.1 mg/ml) and 29.4 % (1mg/ml) from the control value. Anyway, the hydrocortisone capacity to prevent the healing of the wounded area is proportional to the applied dose of steroid.

Also, the treatment with retinoic acid delays the restoration process of the fibroblast layer. In comparison with the control experiment, after 8 hours-concentrations of retinoic acid of 0.5 μg/ml and 1 μg/ml do not significantly affect the degree of restoration process of the wounded area. The treatment with the maximal concentration of retinoic acid (5 μg/ml) leads to a low increase of the speed of the healing process, in a manner which attenuates in time: after 6 hours (with 31.7%), 7 hours (with 30%) and 8 hours (with 17.3%).

The same types of experiments were made in the case of the incubation of monolayer human dermal fibroblasts, which were mechanically wounded and then treated with three concentrations of hydrocortisone (0.01-0.1 and 1 mg/ml) retinoic acid (0.5-1 and 5 μg/ml). Results of the experiments are presented in Table 1.

Table 1

Effects of incubation with hydrocortisone and retinoic acid on the healing speed of the mechanical wound in the bidimensional culture of fibroblasts

Hours Incubation	The lesion area covered with cells (μm^2)					
	Hydrocortisone			Retinoic acid		
	0.01 mg/ml	0.1 mg/ml	1 mg/ml	0.5 μg/ml	1 μg/ml	5 μg/ml
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	25	108	228
4	90	98	0	540	504	2056
5	450	504	18	840	1198	4295
6	1058	980	905	2385	3673	8923
7	3105	2845	1554	8005	7812	10405
8	4750	4430	2885	10980	10504	12876

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Cultured fibroblasts within an extracellular three-dimensional (3D) matrix such as collagen experience a richer, more complex physical environment and a markedly different geometry than the cells on 2D surfaces. Moreover, the FPCL model allows the visualization and quantification of cellular contraction and low differences between treatments.

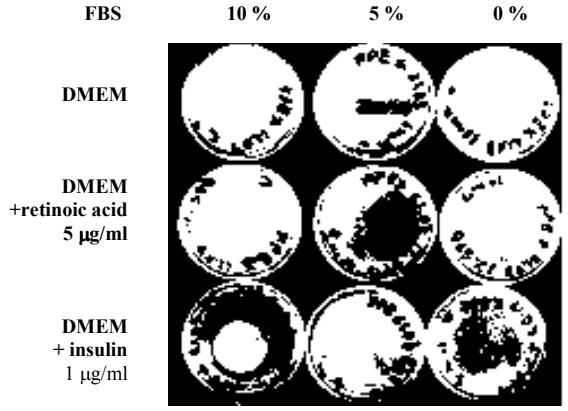

Fig. 2. Collagen gel contraction after 48 hours, in different variants of incubation post-lesion.

Contractility of fibroblasts was determined on FPCL model by evaluation of changes of collagen gel area over a 120 hours of incubation post-lesion, in different conditions: (i) with DMEM and without FBS, (ii) with DMEM and FBS, (iii) with DMEM and 5 μg/ml retinoic acid, (iv) with DMEM and FBS and 5 μg/ml retinoic

acid, (v-vii) with DMEM + 0.1-1-5 µg/ml insulin, (viii-x) with DMEM + FBS + 0.1-1-5 µg/ml insulin. Contraction of collagen gels was recorded through the photographic method using a digital camera (Nikon CoolPix 4500) and the transfer of the images on a computer with image analyzer software. Area changes (degree of contraction) were expressed as a ratio between gel area and initial area.

In Figure 2 we show micro photos taken from the gel's microtiter plate in which human dermal fibroblasts were cultured for 48 hours, after detachment of gels from the base of the well plate and in varying recovery conditions.

Results of this study had shown that: 1) lack of FBS leads to the absence of gels contraction, and the size of the contraction is proportional to the FBS concentration; 2) treatment with 5µg/ml retinoic acid inhibited gel contraction even in the presence of 10% FBS; 3) insulin presence (1 µg/ml) in medium leads to an increase of degree of gel contraction by the fibroblasts, which suggests that this hormone has a positive role in the skin wound healing process.

In Figure 3 we present micro photos which show the evolution of the gel contraction degree for a period of 72 hours, under the action of insulin, in the presence of 10 % FBS.

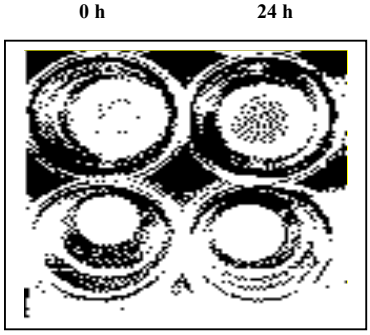

Fig. 3. Evolution of collagen gel contraction under the action of 1µg/ml insulin, for a period of 72 hours post-lesion.

Gel's area changes (degree of contraction) - evaluated in comparison with gel's initial area - were calculated across of 120 days. In this time the treatment with insulin was monitored (Figure 4) using a quantitative analyzer software (SigmaScan) and with the help of another software, MedCalc. This analysis confirms that insulin facilitates the reorganization of collagen lattice under the action of fibroblasts. Kelynack et al. [2000] showed that fibroblast's action over the collagen lattice contraction is a process mediated by the integrins, who are a group of receptors from the cell surface, with αβ heterodymeric structure, which are responsible for cell migration and communication with their extracellular matrix. Klein et al. [1991] noticed that when human dermal fibroblasts are cultured on a collagen lattice type I, they express in high quantity α2β1 integrin, known to be over-expressed as a reply to wound healing, while the granulation tissue is forming,

and is associated with periods of local proliferation and migration processes of the cells.

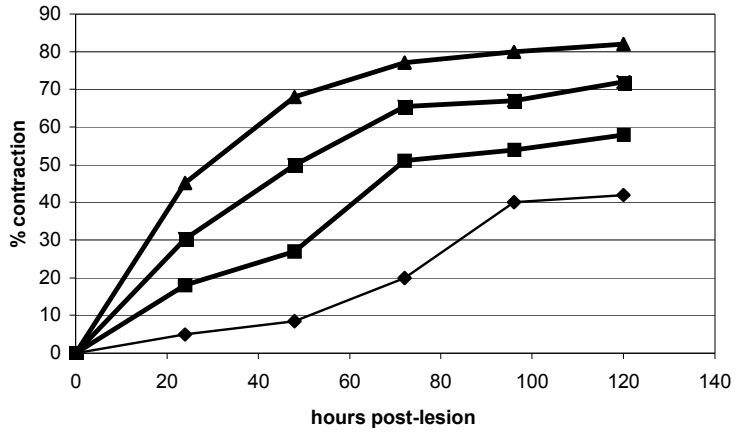

Fig. 4. Kinetics of the contraction of dermic equivalent after treatment with insulin + 10 % FBS of a mechanic lesion. Insulin concentration: 0 (♦); 0.1 μg/ml (■); 1 μg/ml (▲) si 5 μg/ml (■)

CONCLUSION

Studies on confluent monolayers of scraped HDF showed that the entire wounded area is totally recovered after 31 hours. Studies of the kinetics of covering of the wounded surface show that insulin (1 μg/ml) stimulates this process, after 7 hours 90.9% of the wounded surface is populated by the fibroblasts. Treatment with 5 μg/ml retinoic acid accelerates a little the process of wound healing but this process is not growing in time. Hydrocortisone, regardless of the tested concentration, inhibits the wound healing process.

There were realized studies on a free floating fibroblast populated collagen lattice (FPCL) model, which was used to evaluate the gel contraction in the presence or absence of the FBS and at different concentrations of insulin, retinoic acid or hydrocortisone. It was shown that without FBS, gel contraction does not take place, and the contractile properties of human dermal fibroblasts are proportional to the concentration of the FBS in the culture medium. Treatment with 5 μg/ml retinoic acid inhibits gel contraction even in the presence of 10% FBS. Addition of insulin (1 μg/ml) in the culture medium leads to an increased degree of gel contraction by the fibroblasts, suggesting the positive role of this hormone in the skin wound healing process.

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