SKIN WOUND HEALING IN A FREE FLOATING FIBROBLAST POPULATED COLLAGEN LATTICE MODEL

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Abstract. Wound healing is a highly regulated process that involves reepithelization, granulation tissue formation and contraction. The aim of this paper is to study on a free floating fibroblast populated collagen lattice (FPCL) model the release of IL-1α and IL-1β in the medium and the pattern of the expressed matrix metalloproteinases MMP-2 and MMP-9 as a result of mechanical, oxidative, irritant and gamma irradiation induced lesions. Pro-inflammatory cytokines are clearly involved in the regulation of the wound healing. We studied the level of the extracellular IL-1α and IL-1β and found out a higher expression of the IL-1β in the first day of culture and a decrease of both IL-1 after 14 days of culture. Different phases of the wound healing involve some matrix metalloproteinase members and their tissue inhibitors. In our study, the zymographic analysis demonstrated the presence of MMP-2 and MMP-9 in the medium of fibroblasts grown in the three dimensional collagen network. The levels of MMP-2 and MMP-9 were gradually increasing in the first 5 days of culture, probably as a result of the action of IL-1β on the process of MMP activation. Our results recommend the three dimensional model of fibroblast culture in collagen gels as a useful model of *in vitro* study of wound healing.

Key words: experimental wounding, matrix metalloproteinase, soluble cytokines.

INTRODUCTION

Wound healing involves reepithelialization, granulation tissue formation and contraction. Reepithelialization and granulation tissue formation in turn involve proliferation and migration of cells from the wound edge to fill the wound site. Besides fibroblasts, granulation tissue consists of macrophages, blood vessels, and extracellular matrix (ECM) produced by fibroblasts. Once the wound is filled with granulation tissue and covered with a neoepidermis, a portion of the wound fibroblasts transforms into myofibroblasts, which contract the wound to reduce and strengthen the defect. Myofibroblasts are rich in F-actin bundles, which establish

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cell-cell and cell-ECM linkages and thus generate the force for wound contraction [2, 10].

Fibroblasts appear at the wound level 3 days after traumatism and reach a maximal level after 7 days. This rapid expansion of fibroblast population at the wound level is the result of some proliferation and migration processes. Proliferation and attraction of fibroblasts at wound site is induced by growth factors and cytokines initially produced by sanguine platelets and subsequently by macrophages and lymphocytes. Fibroblasts produce high quantities of collagen, the main component of the ECM which is responsible for tensile force of the bed-sore. Collagen is the first protein identified at the wound site and its level rises fast during the first post-traumatic 3 weeks. Collagen is initially deposited in an irregular way, after which the individual collagen fibrils are reorganized in bundles regularly aligned and orientated along stress lines [13].

Wound contraction is a centripetal displacement of the wound edges that facilitates its closing at 5–15 days after traumatism. This process is carried out by myofibroblasts that contain α-actin from smooth muscle and is mediated by contractile forces produced by granulation tissue from wound. Apoptosis of myofibroblasts and vascular cells mediates ultimately transition from granulation tissue to a scar – an avascular and acellular mass of collagen that allows the restoration of the continuity, resistance and tissue function. The final phase is remodeling in which the collagen is synthesized, degraded and reorganized [16].

ECM remodeling during transition from granulation tissue to scar is dependent both on the synthesis and degradation of the constituent proteins, especially of the collagen. Collagen degradation at the wound level is controlled by some proteolytic enzymes designated matrix metalloproteinases, that are secreted by macrophages, epidermal cells, endothelial cells and fibroblasts. Different phases of the wound repair process involve distinct combinations of matrix metalloproteinases (MMPs) and their tissue inhibitors [9].

Within MMP family there exists two members: MMP-2 (gelatinase A) and MMP-9 (gelatinase B) that contain fibronectin-like domain that interact with collagens, with similar substrate specificities, degrading type I, IV, V, VII and XI collagens and laminin. By their capacity to cleave type IV collagen, the two MMPs are involved in basement membrane remodeling, that regulates cell migration and proliferation during invasion of cancer cells and wound healing [5]. MMP-2 is secreted by tissue in a latent form, pro-MMP-2, that is activated mainly at cell surface, by cleavage of the N-terminal pro-domain through a process dependent of a membrane type MMP (MT1-MMP). MMP-9 is produced by a diversity of epithelial cells including keratinocytes, polymorphonuclear leucocytes and tumor cell [1]. In the MMP-9 gene promoter, Kobayashi *et al.* [7] was emphasized the presence of two regulatory elements involved in the induction of its expression. Actually, MMP-9 mRNA is slightly expressed in the adult human skin, but its expression increases following development of some invasive cancers. In the cell

lines is not detected a constitutive MMP-9 (mRNA and protein/secreted activity), expression being induced by cell treatment with phorbol esters or TGF-β [12,14].

The pattern of cytokines and growth factors involved in wound healing is complicated because: 1) the same factor is produced by more cell types concomitantly involved in different stages of the process; 2) these factors initiate signaling pathways that interact having complex effects at nuclear and cellular levels, and 3) many aspects are not yet elucidated. It was demonstrated activation of IL-1, TNF-α, TGF-β and VEGF during wound healing and that administration of PDGF, bFGF and TGF-β improves the parameters of wound healing [15].

In this paper, we show a study on a free floating fibroblast populated collagen lattice (FPCL) model concerning the release of interleukins IL-1α and IL-1β in the medium and the evolution of two matrix metalloproteinases MMP-2 and MMP-9 as a result of mechanical, oxidative, irritant and gamma irradiation induced lesions.

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 medium supplemented with 15% fetal bovine serum (FBS, Sigma), 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% CO2 at 37 ºC. 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 tripsynization with 0.25% tripsyn-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 culture medium containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), 2 mM L-glutamine and 10% FBS. The culture medium was 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.

EXPERIMENTAL WOUNDING AND TREATMENT

Confluent monolayers of HDF 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 cell monolayers were removed from the incubator the following morning, the medium was aspirated off and the cells were scraped with a trimmed hard rubber ebonite comb washed with 70% ethanol and dried before use.

There were realized the following lesions: *a*) mechanic – with the same shape and size; *b*) oxidative – cell treatment with 100 μM H_2O_2 for 20 minutes; *c*) as a result of skin irritation – fibroblast treatment with 35 μM SDS, for 20 minutes and *d*) as a result of gamma irradiation – source of Co^{60} with 4 Gy at a rate of dose of 0.0629 Gy/second, at room temperature. The cells were maintained on ice until they were returned to the laboratory from the radiation source. In the case of variants *b* and *c*, values that represent 1/3 from minimal toxic concentrations were selected, case in which the cells die after 48 hours of treatment. In variant *d*, it was selected the dose at which 50% from fibroblasts grown in monolayer die.

PREPARATION OF CELL-POPULATED THREE-DIMENSIONAL COLLAGEN MATRICES

Collagen matrices were prepared with rat tail type I collagen at 6 mg/ml in 0.5 M acetic acid solution. The collagen solution was then mixed on ice with DMEM containing 10% FBS and 0.1 N NaOH. Fibroblasts were immediately mixed with the neutralized collagen solution (final concentration of 3 mg/ml).

MONITORING OF 2-D AND 3-D CULTURES

Cell morphology was monitored by phase contrast microscopy (Nikon Eclipse TS 100). The cells were photographed with a digital camera (Nikon CoolPix 4500).

Cell viability was determined by dye exclusion test with Trypan blue. Living cells exclude the dye, whereas dead cells will take up the blue dye. The blue stain is easily visible, and cells could be counted using a phase contrast microscope by a haemocytometer.

MTT Assay measures the cell activity, proliferation rate and cell viability. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to the corresponding blue formazan. HDF were treated in 24-well plates and incubated with 500 μl/well of MTT (1mg/ml) in medium for 4 hours. After this time the formazan was released from the cells with DMSO. Absorbance of the supernatant was measured at 550 nm. Absorbance values that

are lower than the control cells indicate a reduction in the cell activity. Conversely, a higher absorbance rate indicates an increase in cell activity/proliferation.

CELL RELEASING FROM GEL

Fibroblasts included in collagen gels were recovered by gel treatment (~30 minutes at 37 °C) with 0.5 ml bacterial collagenase (2 mg/ml; Sigma Chemical Co.). Reaction was blocked with DMEM + 10%SFB, suspension being centrifuged (600 g, 10minutes) and cell pellet being resuspended in PBS and used in determination of cell number, viability and cell proliferation.

QUANTITATION OF LEVELS OF SOLUBLE CYTOKINES

IL-1α and IL-1β were quantitated by Chemicon kits: ChemiKine™ Interleukin-1alpha, sandwich ELISA and ChemiKine™ Interleukin-1beta, competitive ELISA. The operating procedure provided by the manufacturer was strictly followed, and the experimental results were expressed in pg cytokine/ml or pg cytokine/10^6 cells, by means of a calibration curve carried out with dilutions (0-400 pg/ml) of the human IL-1α and IL-1β standards. In both kits the assays were visualized using alkaline phosphatase and an ensuing chromogenic substrate reaction (p-nitrophenyl phosphate).

MATRIX METALLOPROTEINASE (MMP-2 AND MMP-9) ASSAY

The matrix metalloproteinase gene family is a class of metalloproteinases capable of ECM degradation. The present work focuses on MMP-2 and MMP-9 (gelatinases A and B), which are the main enzymes able to degrade type IV collagen and gelatine, the product of collagen degradation after lysis with collagenases. Unlike MMP-2, which is constitutively expressed by many cells, MMP-9 transcription can be highly induced by a wide range of agents. These agents include growth factors, cytokines, cell-cell and cell-ECM adhesion molecules, and agents altering cell shape [3, 11]. In MMP-9 gene promoter there are some responsive regions to transcription factors AP-1, NF-kB as well as to UVB radiations. These differences suggest that these two enzymes have different biological functions.

Gelatin zymography. Cell culture media were used for gelatin zymography according to the method of Kleiner and Stetler-Stevenson [6] with minor modifications. To detect gelatinase activity, culture-conditioned, serum-free media were collected, concentrated 10-fold using centrifugal filter units (Centricon YM-10) and analyzed by zymography. In order to normalize the MMP activity in the

different samples, the volume of samples used was adjusted to the same quantity of total protein in each lane. Samples were electrophoresed on SDS-7.5% polyacrylamide gels containing 0.1% gelatin, under nonreducing conditions, for MMP-2 and MMP-9 detection. For a semi-quantitative analysis of human matrix metalloproteinases, samples of recombinant human MMP-2 and MMP-9 (0.1 μg/μl, Sigma) were electrophoresed on the same gel. Following electrophoresis, gels were washed in 2.5% Triton X-100 in order to eliminate SDS and then incubated in 0.05 M Tris buffer (pH 7.6) containing 0.01 M $CaCl_2$, 0.05 M NaCl and 0.05% Brij 35 for 24 h at 37 ºC. Gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid to reveal zones of lysis within the gelatin matrix and destained in a mixture of ethanol:acetic acid: water (1:1:8). Molecular sizes of the bands displaying enzymatic activity were identified by comparison to prestained standard proteins (Sigma) and to human MMP-2 and MMP-9 standards (Sigma). The stained bands were further quantified by densitometry with the Bio1D software (Vilber-Lourmat, France). Linear range of intensity of the bands was assessed using recombinant MMP-2 or MMP-9 as standard. Linearity was between 10 and 200 ng of enzyme.

To confirm that the lytic bands correspond to the active forms of MMP, the samples were treated for one hour 1 mM APMA (p-aminophenylmercuric acetate) before electrophoresis. The obtained bands are compared with those obtained in the absence of APMA.

ELISA detection of MMP-2 and MMP-9. MMP-2 and MMP-9 were measured with Chemicon ELISA systems, with a sensitivity of 9.6 pg/ml. The system consists of *multi-wells* plates coated with anti-MMP antibodies. The enzyme will specifically bind to wells, the other components being removed by washing and aspiration. For example, to determine the total level of MMP-2 (zymogene and active forms) APMA together with detection reagent will be added in the medium. The endogenous level of active MMP-2 was detected in the absence of APMA. As standard, proMMP-2 was used that is activated in parallel with the sample. Resulted colour was read at 405 nm to a TECAN reader (Soft Magellan 3.0) and MMP-2 concentration was evaluated by extrapolation on the calibration curve. In these ELISA kits the enzyme used was alkaline phosphatase and the substrate was p-nitrophenyl phosphate.

RESULTS AND DISCUSSION

Regeneration of the FPCL model submitted to different types of lesions was monitored by phase contrast microscopy (Nikon Eclipse TS 100). We noticed that mechanical lesion of the 3-D support containing fibroblasts does not allow the normal growth of these cells in the areas with defects. Chemical treatments with 100 μM H_2O_2 or 35 μM SDS affects cell viability and also support structure in a

higher degree than in the first case. γ-irradiation (4 Gy) of the 3-D culture has as a result an increased degree of collagen gel contraction without an evident influence on cellular component of the dermal model (data unshown).

Study of the HDF viability after enzymatic digestion of the collagen gel shows that mechanical treatment has the most noxious effects, thus after 14 days the dermal equivalent being devoid of viable cells. The lowest effects are noticed in the case of the irritant treatment, when after 14 days the same viability with the control experiment is found (Table 1). Treatment with 100 μM H_2O_2 has as a result the death of 46.4% cells grown in collagen gel after one day. After 14 days, the same treatment leads to an increase of the viability to 68.3% suggesting the intervention of the defense antioxidant system from fibroblasts. After one hour from γ-irradiation, the cell viability was not affected significantly, but this treatment considerable decreased the number of viable fibroblasts from FPCL model after 14 days.

Table 1

Cell viability and proliferation in FPCL model subjected to a traumatic treatment

Lesions	Viability (%)		Proliferation (% from control)	
Time of relaxation	1 day	14 days	1 day	14 days
Control	97 ± 4.9	82 ± 1.6	100	100
Mechanic	24 ± 4.3	0	44	0
Oxidative	45 ± 4.3	56 ± 10.1	72	64
Irritating	88 ± 1.7	82 ± 4.2	98	71
Irradiation	93 ± 7.8	28 ± 4.6	93	14

Pro-inflammatory cytokines are clearly involved in the regulation of wound healing, especially at skin level. Taking into account the evolution of the wound healing and the cell types involved in this process, in the literature exist many studies, from which some have contradictory conclusions [15]. In fibroblast culture medium there were detected interleukins IL-1, IL-6, GM-CSF (*granulocyte macrophage colony-stimulating factor*), TGF-α and -β (*tumor growth factor*), NGF (*nerve growth factor*), PDGF (*platelet derived growth factor*), as well as members of FGF (*fibroblast growth factor*) family, considered to be mediators of the immune reactions, inflammatory processes and also of the skin wound healing [8].

Multifunctional cytokines IL-1 mediate the inflammatory response, having a central role in the regulation of the immune response. By its capacity to stimulate production of type I and II collagens by fibroblasts, IL-1 is involved in pathogenesis of different connective tissue diseases. In the skin wound healing process, IL-1 is produced mainly by epithelial cells and exogenous supply of IL-1 speeds up epidermal healing. However, until now is not known if IL-1 is overexpressed at skin lesion level and if this cytokine is involved in the processes

of normal reparation of skin lesions. There are 2 forms of IL-1: IL-1α and IL-1β, both with the same activities but different structures. IL-1α appears to be primarily membrane-associated while IL-1β can circulate. We studied the both forms of IL-1: -α and -β-codified by two different genes, that owing to sequence homology (30%) interact with the same receptors on the surface of the target cells. Both forms are synthesized in cytosol as precursors with a molecular weight of about 35 kDa (pro-IL-1), that following a proteolytic process generate two mature forms of 17 kDa. While IL-1α is also active in the precursor form, IL-1β become active only after proteolytic maturation under the action of interleukin 1β converting enzyme (ICE).

Taking into account cytomorphological observations, was measured the content of IL-1α and IL-1β in the culture medium only in the case of the control experiment, of the oxidative lesions and of those produced by irradiation, 1 and 14 days after treatment (Table 2). Results are expressed in pg cytokines/10^6 cells, being calculated by means of a calibration curve realized with dilutions (0–400 pg/ml) of the human IL-1α and IL-1β standards.

Table 2

The level of the extracellular cytokines IL-1 (expressed in pg/10^6 cells) at 1 and 14 day after oxidative and irradiation damage

	IL-1α	IL-1β
Control 1 day	77 ± 15	103 ± 14
14 days	21 ± 3	38 ± 6
H_2O_2 1 day	68 ± 12	3 900 ± 600
14 days	21 ± 1	52 ± 8
Co^{60} 1 day	73 ± 8	872 ± 44
14 days	21 ± 3	22 ± 4

In the case of the fibroblasts grown on a collagen gel, the level of the IL-1β is higher than that of the IL-1α – with 33.8% in the first day and with 80.9% in the 14^{th} day of culture. The level of both IL-1 decreases after 14 days of culture, with 72.7% in the case of IL-1α and with 63.1% in the case of IL-1β, showing a diminution of the signalization by pro-inflammatory cytokines, correlated with the decrease of the number of viable cells from the FPCL model subject to a traumatic treatment.

Incubation of the dermal equivalent with 100 μM H_2O_2 (20 minutes) was monitored after 1 day and after 14 days of relaxation from the date of the treatment. After 1 day, a significant increase (of about 38 times) of the extracellular level IL-1β was remarked and a lower decrease (of about 1.37 times) after 14 days. Gamma irradiation of the HDF 3-D culture has as a result a lower increase (of 8.5 times) of the IL-1β level and only after 1 day of relaxation.

Our results show that from the two forms of IL-1, IL-1β predominates extracellularly. The protein level of both forms of IL-1 increases after 1 day from the stress induction and decreases along the 14 days of culture of the dermal equivalent. It is known that to avoid the tissue lesions the activity the IL-1β is strictly controlled, the production of this form being regulated both at transcriptional and translational level [17].

Ford *et al*. [4] have characterized the pattern of the cytokines at the level of a sponge of polyuretan implanted in mouse and emphasized the increase of TNF-α (*tumor necrosis factor*), MCSF (*macrophage colony-stimulating factor*), IL-1 and IL-6, that promote tissue remodelling. The level of these cytokines decreases in the thirteenth day after generation of the lesion as a result of the site healing.

Study of matrix metalloproteinases allows the assessment of the way in which the ECM undergoes a process of remodelling during wound healing that facilitates especially cell migration at lesion site. The main objective of our researches has been the determination of a correlation between the evolution of the levels and activities of MMP-2 and MMP-9 and the increase of protein concentration of IL-1β, especially at 1 day after the traumatic treatment. This research objective was based on the ipothesis that IL-1β can affect the pro-MMP-2 activation, carried out by the cleavage of the N-terminal pro-domain by MT-MMP (*membrane type metalloproteinases*) [14]. We also based our research on the ipothesis that IL-1β triggers a signalling pathway that finally activate a transcription factor (AP-1 and NF-kB) that stimulates MMPs biosynthesis.

In Figure 1 we show the zymograms obtained after the electrophoretic separation of the proteins from the culture medium of fibroblasts grown in this FPCL model subjected to the oxidative and irritant treatments and irradiation. Our results confirm the presence of MMP-2 and MMP-9 in the cell culture supernatants. In the first 3 days post-oxidative lesion we found that the extracellular gelatinolytic activity is represented by MMP-9 induced, probably, by lesion. In the same period, the level of cytokine IL-1β was particularly high. MMP-9 was present in the active form, therefore, the activation process has already taken place.

Figure 2 shows the variation of the the active forms of MMP-2 and MMP-9 concentration during 14 days after the oxidative stress. After 5 days post-treatment, the extracellular gelatinolytic activity is represented by MMP-9 and MMP-2 (the active and latent forms). After 7 days of treatment, quantities of MMP-9, pro-MMP-2 and MMP-2 decrease, and in the 10^{th} day this decrease of the gelatinolytic activity become more prominent, being practically represented only by MMP-2. In the 14 days after trauma the presence of MMP-2 or MMP-9 was not detected any more, despite the fact that cell viability increases with 56% (Table 1). The results obtained by quantitative ELISA determinations confirm the semi-quantitative evaluations obtained by zymography, suggesting that at 24 h post-trauma a significant induction in the MMP-9 synthesis occurs, probably under the action of

the IL-1β, untill the 5th day when reaches a maximal value. The level of MMP-2 shows the constitutive profile of this gelatinase, has a maximal value in the 5th day post-treatment, perhaps owing to IL-1β that activates the latent proform.

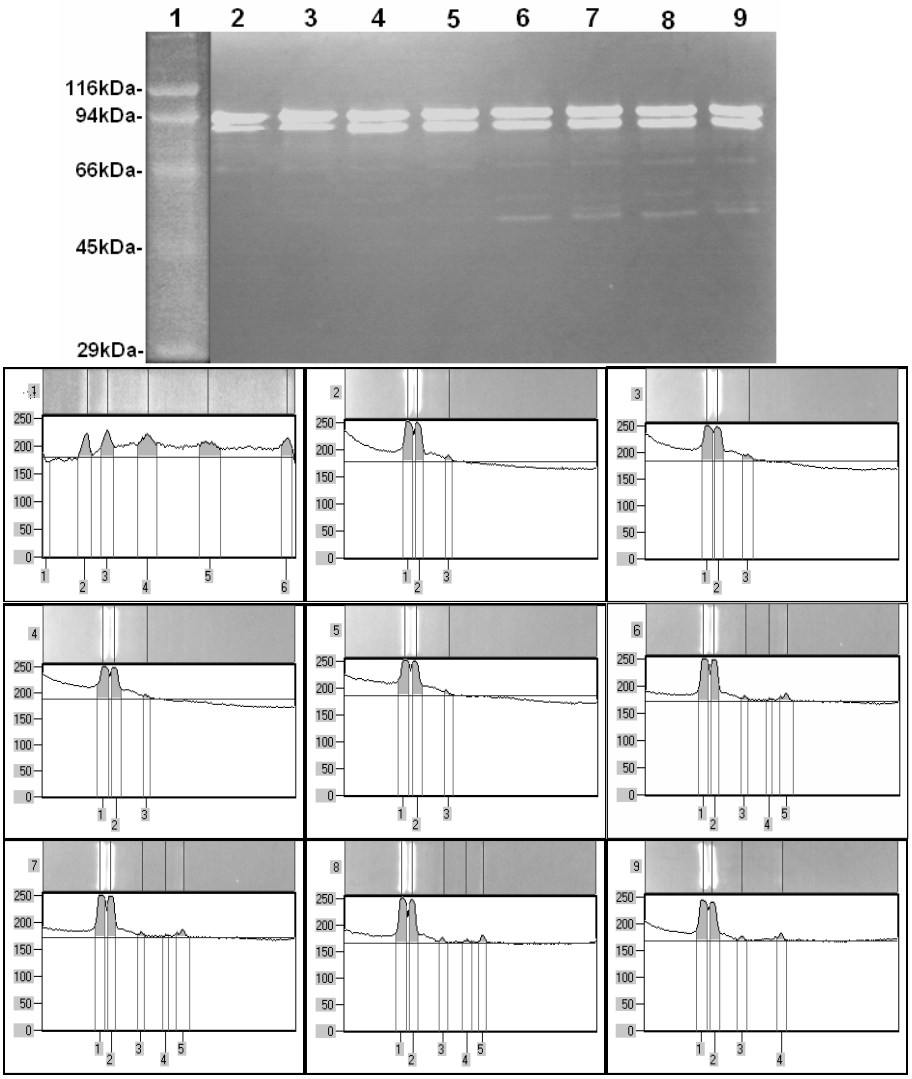

Fig. 1. Gelatin zymographic determination of the MMP secreted in the culture medium. A. Pattern of gelatinases on gelatin zymogram. B. The densitometric analysis of lytic MMP specific bands. 1. MW markers; 2. Control 1 day; 3. Control 4 days; 4. H_2O_2 1 day; 5. H_2O_2 14 days; 6. SDS 1 day; 7. SDS 14 days; 8. Co^{60} 1 day; 9. Co^{60} 14 days.

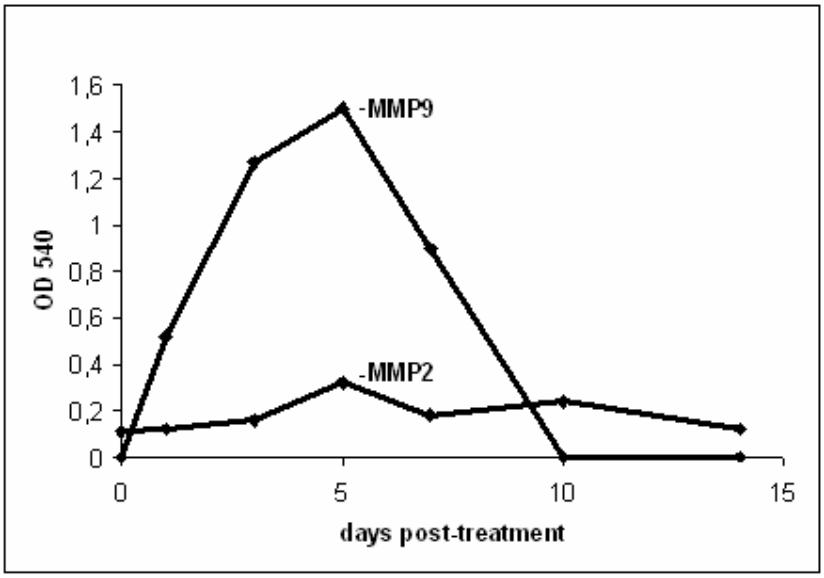

Fig. 2. The evolution of the proteic level of MMP-2 and MMP-9 post-oxidative treatment.

CONCLUSION

By studying the level of the extracellular IL-1α and IL-β in a free floating fibroblast populated collagen latice model we found out a higher expression of the IL-β in the first day of culture and a decrease of both IL-1 after 14 days of culture both in the case of control samples and of the oxidative lesions and those produced by irradiation. However, the decrease of IL-β level was higher in the lesional models than in the control samples suggesting that this cytokine is involved in the regulation of the wound healing.

Quantitative ELISA determinations of the MMP members secreted in the culture medium demonstrated a progressive increase of MMP-2 and MMP-9 specific activities in the first 5 days post-treatment, a decrease of both forms after 7 days of treatment and a more prominent decrease in the 10^{th} day post-trauma.

Our results concerning the pattern of interleukins IL-1α and IL-1β, and of MMP-2 and MMP-9 recommend the tridimensional model of fibroblast culture FPCL as a useful model of *in vitro* study of the wound healing process.

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