Wound healing mediator production by human dermal fibroblasts grown within a collagen-GAG matrix for skin repair in humans

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ABSTRACT. Cell and tissue therapy applications in humans are being used increasingly, particularly for tissue repair. Several reconstructed skin models have been proposed. Wound healing involves overlapping steps of inflammation, cell migration and proliferation, neovascularisation, extracellular matrix production and remodelling. This is regulated by numerous cytokines and other soluble mediators. We have prepared dermal substitutes (DS) consisting of a collagen-GAG, three-dimensional matrix colonized by human dermal fibroblasts (HDF), isolated by skin explant or enzymatic digestion of the skin for potential therapeutic use in humans. To test the functionality of these DS, we measured (ELISA) the stimulatory effect on HDF in the matrix, of serial dilutions of human serum (HS) on the production of wound healing mediators: interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and tissue inhibitor of metalloproteinase-1 (TIMP-1). We observed: 1) a stimulatory effect of HS on HDF production of the different mediators tested, with a dose-dependent effect in the case of IL-8 and VEGF. 2) A matrix-potentiating effect on the production of the different mediators by HDF. 3) A decrease in the production of IL-8 and VEGF when HDF isolated by enzymatic digestion was used to colonize the matrix as compared with HDF isolated by skin explant. We conclude: 1) that the production by HDF, in a collagen-GAG matrix, of mediators involved in cutaneous wound healing is decreased when HDF are isolated by enzymatic skin digestion rather than by skin explant. 2) That measurement of the production of cytokines or other mediators could be a useful quality control to test the functionality of tissue-engineered DS for tissue repair therapy in humans and more generally of cells prepared for cell therapy.

Keywords: Collagen matrix, dermal fibroblasts, interleukin-8, keratinocyte growth factor, vascular endothelial growth factor, tissue inhibitor of metalloproteinase

INTRODUCTION

The use of cell and tissue therapy in humans is on the increase. In the case of burns which destroy both epidermis and dermis, the treatment of choice is to use an autologous, split-thickness skin graft. But in the case of extensive burns the area of healthy skin is not sufficient to achieve prompt wound coverage. Therefore, several reconstructed skin models have been proposed for permanent wound coverage. As collagen is a major component of connective tissue, dermal substitutes (DS) composed of fibroblasts within a collagen gel were developed by Bell *et al.* [1]. This model has been used *in vitro* [2], in animal models [3] and in humans [4,5] to analyse the role of dermal fibroblasts in skin physiology and cutaneous wound healing (CWH). The major drawbacks of this model are the low resistance of the collagen gel to collagenase degradation, and its fragility. For this reason collagen sponges have been developed [6] and validated *in vitro* [7] and *in vivo* [8]. To optimise the preparation of tissue-engineered DS for skin repair in humans, we prepared DS consisting of a collagen-GAG sponge [9] colonized by human dermal fibroblasts (HDF), isolated by skin explant or enzymatic digestion. Wound healing is a dynamic process that involves overlapping steps of inflammation, cell migration and proliferation, neovascularisation, extracellular matrix production and tissue remodeling, regulated by numerous cytokines and other soluble mediators [10]. Interleukin-8 (IL-8) [11], keratinocyte growth factor (KGF) [12], vascular endothelial growth factor (VEGF) [13], and tissue inhibitor of metalloproteinases (TIMP-1) [14] are involved in CWH, particularly in inflammation, keratinocyte migration and proliferation, angiogenesis and in extra-cellular matrix organization. As there is a need for parameters to test the functionality of cells prepared for cell therapy or tissue bioengineering, we have used the production of these CWH mediators to evaluate the function of HDF inside a

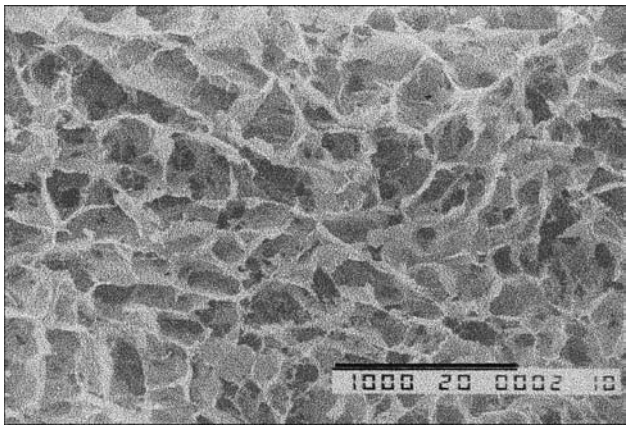

Figure 1
Scanning electron micrograph of a cross-section of the collagen-GAG sponge. Bar represents 1000µm.

collagen-GAG sponge or in monolayer. The results of this study may be useful for establishing the model of DS for use in human tissue therapy.

MATERIALS AND METHODS

Collagen-GAG sponge (CS)

CS were prepared as previously described [5] with minor modifications. Briefly, type I collagen, linked by low energy bonds to chitosan (95 % deacetylated chitin), and chondroitin 4-6 sulfate were dissolved in 0.1 % acetic acid and mixed. The final solution was poured onto lyophilization trays. After lyophilization, the CS was 2 mm thick and had an alveolar-like structure. The sponge was then sterilized by γ-irradiation. For scanning electron microscopy, dry samples of CS were sputter-coated with gold-palladium and observed under a Hitachi S800 microscope to control pore size. Observation of sponge samples showed a porous structure with pore sizes from 50 to 150 µm (Figure 1), which permit colonization by fibroblasts [9].

Dermal fibroblast isolation

Fibroblast cultures were established from normal skin harvested in the course of breast plastic surgery from three healthy women from 34 to 41 years old. Fibroblast populations were isolated from the three skin samples by both explant and enzymatic digestion.

Isolation by explant was performed as previously described [1]. Briefly, skin samples were trimmed of all subcutaneous fat, and cut into 2 mm^2 explants. Explants were placed in 60-mm diameter Petri dishes (Nunc, Life Technologies, Cergy Pontoise, France), and were incubated in a moist atmosphere of 5 % CO_2 at 37°C in complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Cergy Pontoise, France). This was supplemented with 10 % fetal calf serum (Fetalclone II, Perbio, Hyclone, Europe SA Industriezone III, 9320 Erembodegem, Aalst, Belgium), 4 mM glutamine (Unipex, Rueil Malmaison, France), 10 ng/ml epidermal growth factor (EGF) (Sigma, St Quentin Fallavier, France), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Panpharma, Fougères, France). Half of the medium was changed twice a week until subconfluence of fibroblasts was obtained after 21 to 28 days. Explants were then removed and the cells were subcultured by trypsin treatment (first passage, P1). HDF, free of keratinocytes, were frozen at P1.

Enzymatic fibroblast digestion was perfomed according to the manufacturer's instructions. Briefly, skin samples were incubated under agitation in 0.36 Wunsch units/ml collagenase and thermolysin solution (Liberase Blendzyme 3, Roche, Meylan, France), for two hours at 37°C. The solution obtained was filtered through a sterile nylon filter (Beckton-Dickinson, Le Pont de Claix, France) and centrifuged twice (700 g, 5 minutes) to remove any enzyme residue. Cells were then suspended in complete medium and propagated in tissue culture flasks until subconfluence was obtained after 5 days. These cells were also frozen at P1. HDF used for these experiments were free of keratinocytes.

CWH mediator production by fibroblasts in monolayer

The fibroblasts obtained either by explant or by enzymatic digestion were seeded into 12-well plates coated with collagen I (Beckton-Dickinson, Le Pont-de-Claix, France), at a density of 8.10^4 cells/well and grown in complete medium until day 3. Confluent cells were then left for 24 hours in DMEM containing 0.1 % calf serum. At day 4, the medium was replaced by DMEM supplemented with 0 %, 2.5 %, 5 % or 10 % human serum (HS) (Sigma, St Quentin Fallavier, France) or 10 % calf serum. After 24 hours incubation, supernatants were harvested, separated into aliquots and frozen (– 80°C) until assay of the different CWH mediators. The experiment was carried out in triplicate, with three strains of HDF at P3. The number of viable cells in each well was evaluated using the MTT test to be able to compare the CWH production of cells grown in monolayer or in CS.

CWH mediator production by fibroblasts in DS

The CS was cut into circular pieces (4 cm^2) and put into 12-well plates. For cell culture, CS were first washed with phosphate-buffered saline (PBS) (Life Technologies) and then equilibrated overnight with culture medium. HDF obtained either by explants or by enzymatic digestion at P3 were seeded on the CS at a density of 10^5 cells/cm^2 in complete medium. Medium was changed 3 times a week and 50 µg/ml of ascorbic acid (Sigma) solution was added every day. After 10 days of culture that corresponded to the steady state, the DS obtained were rinsed twice with 4 ml DMEM for 10 minutes before adding 4 ml DMEM supplemented with 0.1 % Fetalclone II. After 24 hours incubation (37°C, 5 % CO_2), the culture medium was removed, the DS were rinsed twice as described above, and then incubated for a further 24 hours with DMEM supplemented with 0 %, 2.5 %, 5 % or 10 % HS. The supernatants were then harvested and frozen under the same conditions until assay of the different CWH mediators. The experiment was carried out in triplicate with the same three strains of HDF (P3) used for monolayer cultures. The number of viable cells in each DS was evaluated using the MTT test.

Histology of the DS

At the end of the experiment (day 12), DS were fixed in Bouin fixative and embedded in paraffin. Five-µm sec-

tions were stained with hematoxylin, fuschine Ponceau, and Orange G Molybdic (Masson's trichrome).

MTT test

Because the DS cannot be enzymatically digested in conditions that permit a cell count, the number of viable HDF at the end of each experiment in monolayer or in DS was evaluated using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in thiazolyl blue (Sigma) as previously described [15].

CWH mediator assays

IL-8, KGF, TIMP-1, and VEGF were assayed by ELISA sandwich using, for the standard curves, dilutions of the respective recombinant proteins. IL-8 assay system (sensitivity 8 pg/ml) was from Immunotech (Marseille, France). KGF, VEGF and TIMP-1 assay systems were Quantikine Human from R&D Systems (Abington, UK) and had respective sensitivities of 15 pg/ml, 5 pg/ml and 0.08 ng/ml. When necessary, the level of each parameter in the test medium was subtracted from the level of the same parameter in the corresponding supernatant. Each assay was done in duplicate. The amount of IL-8, KGF, VEGF and TIMP-1 found in each well was related to the OD units obtained in the correspondind well using the MTT test.

Statistical analysis

Data are given as mean ± SEM. The effect of HS on CWH mediator production by HDF isolated by explant or by enzymatic digestion and grown in monolayer or CS were tested by two-way ANOVA. A p value < 0.05 was considered to be significant.

RESULTS

Histology of the DS

Histological observation of cross-sections of the DS showed homogeneous distribution of fibroblasts throughout the lattice with little stratification on the surface, whatever the culture conditions. A good tissue ingrowth was noted, consisting of a tissue rich in fibroblasts and dense in newly synthesized collagen as determined by Masson's trichrome. Morphologically, CS colonised by HDF isolated either by explant or by enzymatic digestion appeared similar (data not shown).

Production of factors involved in CWH

Figure 2 shows the production of KGF, VEGF, IL-8 and TIMP-1 by HDF isolated either by explant or by enzymatic digestion, grown in monolayer or within a CS and incubated with serial dilutions of HS. The concentration of the different mediators in the media were related to the OD value of the corresponding MTT test. The OD obtained after 24 hours in the presence of 0%, 2.5%, 5%, 10% HS where respectively 0.35 ± 0.03, 0.57 ± 0.02, 0.65 ± 0.02, 0.77 ± 0.03 for HDF isolated by explant in monolayer, 1.42 ± 0.10, 1.50 ± 0.10, 1.50 ± 0.20, 1.61 ± 0.13 for HDF isolated by explant in CS, 0.43 ± 0.03, 0.68 ± 0.02, 0.73 ± 0.01, 0.77 ± 0.02 for enzymatically isolated HDF grown in monolayer and 2.09 ± 0.12, 2.15 ± 0.12, 2.12 ± 0.11, 2.08 ± 0.9 for enzymatically isolated HDF in CS. The effect of treatment with HS and the effect of culture conditions were significant for the production of each mediator ($p < 0.0001$). When the interaction between the effect of treatment and culture conditions was considered, production of IL-8 and VEGF also reached statistical significance ($p < 0.0001$ and $p = 0.0026$ respectively), but KGF and TIMP-1 production did not ($p = 0.70$ and 0.97 respectively).

IL-8, KGF, VEGF and TIMP-1 were all produced by HDF in the presence of HS. For both HDF preparations, greater amounts of the different mediators were produced when cells were grown in CS rather than in monolayer. This phenomenon was more or less marked but constant. It was particularly clear when the production of IL-8 and VEGF was considered. For instance, IL-8 and VEGF production by explant HDF in the presence of 10% HS was respectively 7 and 3 times higher in DS than in monolayer. Under the same conditions, a dose-dependent stimulation of cytokine production by HS was particularly evident. KGF was constitutionally produced by HDF, even in the absence of serum, particularly when fibroblasts were grown in CS. The stimulation of KGF production by HS was weaker than the stimulation of IL-8 and VEGF production, even when HDF were grown in CS. TIMP-1 production by HDF was strongly induced by HS even in monolayers, but was between 2 and 3 times higher when grown in CS than in monolayer.

The production of the different mediators, and particularly IL-8 and VEGF production by HDF in CS, was higher (×2) for HDF prepared by explant than for HDF prepared by enzymatic digestion. In the different experiments, a control was also set up with cells in the presence of 10% fetal calf serum, corresponding to the conditions of preparation of DS for tissue therapy in humans. The production of the different mediators in the presence of 10% fetalclone II serum was not significantly different from their production in the presence of 10% HS (data not shown).

DISCUSSION

In this study, we have used the determination of IL-8, VEGF, KGF and TIMP-1 production by dermal fibroblast isolated by skin explant or enzymatic digestion and grown inside a CS, to test the functionality of the DS obtained. We have tested the stimulatory effect on fibroblasts of serial dilutions of HS because it contains numerous mediators, particularly growth factors and adhesive proteins that are in contact with skin cells at the initial steps of CWH [10]. Our goal was to mimic the response of the DS to blood contact that would occur during cell therapy with such a product. We observed a stimulatory effect of HS on HDF production of the different mediators tested, with a clear-cut, dose-dependent stimulation in the case of IL-8 and VEGF. Production of different mediators was the highest when HDF were isolated by explant and grown in a CS than in monolayer. Production of IL-8 and VEGF by HDF grown in a CS was significantly lower when the cells were isolated by enzymatic digestion.

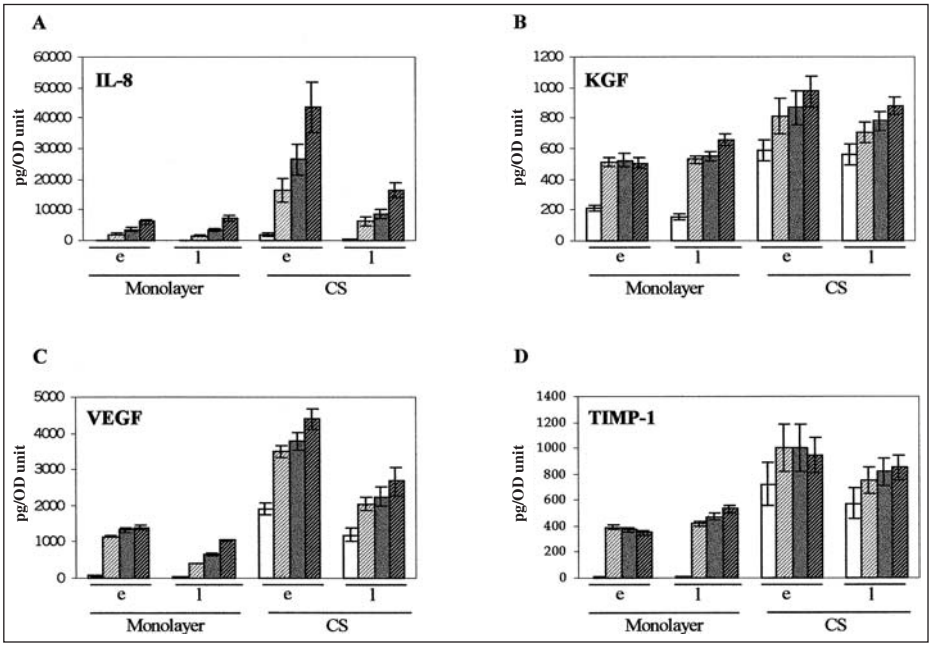

Figure 2

Production of IL-8 (A), KGF (B), VEGF (C) and TIMP-1 (D) by HDF isolated by explant (e) or by enzymatic digestion (l) and cultured in a monolayer or in CS in the presence of 0% (□), 2.5% (▨), 5% (▩), 10% (▨) of HS. Concentrations of the different mediators are related to the OD units obtained using the MTT test. Data are given as mean of the different values (n = 9), bars represent SEM. The effect of treatment with HS and of the different culture conditions were statistically significant for all parameters ($p < 0.0001$, ANOVA). When the interactions between HS treatment and the different culture conditions were considered, statistical significance was achieved for IL-8 ($p < 0.0001$) and VEGF ($p = 0.0026$).

It now appears that dermal fibroblasts play a central role in CWH by initiating inflammation *via* chemokine production and leucocyte recruitment [11], *via* VEGF production and granulation tissue formation [16, 17], and by their cross talk with keratinocytes [2]. IL-8 is the major polymorphonuclear chemoattractant produced in models of acute skin wounds in humans [18]. Therefore, the clear stimulatory effect observed for HS on IL-8 production by explant HDF grown in a CS represents important functional information. The activation of dermal fibroblasts by platelets appears to be the major step in granulation tissue induction [17], probably *via* the induction of VEGF production [16,19]. It has previously been reported that neonatal fibroblasts grown in a three-dimensional matrix in the presence of bovine serum display angiogenic activity, which is mainly due to VEGF [20]. In this study, the expression and production of VEGF was much more marked when fibroblasts were grown in a matrix than in monolayer [20]. Here we confirm a more marked VEGF production by adult dermal fibroblasts grown in a matrix rather than in monolayer. Moreover, HDF in a matrix have a basal production of VEGF that was stimulated by HS, which contains the platelet α granule growth factors [21] that are released when a skin wound occurs [10]. KGF and TIMP-1, which are important mediators for the migration and proliferation of keratinocytes, are both induced in the dermis during CWH [12,14]. In a tissue-engineered skin equivalent, TIMP-1 expression is confined to the dermal component [22]. It has previously been reported that fibroblast KGF expression and production is low under basal conditions but is induced in the presence of serum [23] in monolayer or in a collagen gel [24]. Here we show that the basal production of KGF by HDF is more marked when they are in a matrix than in monolayer, and that under these conditions the stimulatory effect of HS on KGF production is also greater. Similarly, production of TIMP-1 by HDF was also stimulated by HS as previously reported [25] and HDF showed a marked TIMP-1 basal production when grown in a CS. Our findings concerning the ability of a three-dimensional matrix to potentiate HDF functions and particularly cytokine and TIMP-1 production confirms previous reports [26-28], and emphasizes the potential therapeutic interest of this type of DS for skin repair.

Skin explants are the reference method for isolating pure populations of HDF within 3 to 4 weeks [1, 2, 4, 5, 29]. It could be useful to shorten the HDF production time by enzymatic digestion of the skin. However, in our hands HDF isolated after enzymatic digestion and grown in a CS produced IL-8 and VEGF levels significantly lower than when isolated by skin explant. The enzymatic digestion for HDF isolation has been described in the literature [30], but we found no paper concerning the functioning of enzymatically isolated HDF. However, regarding the recent therapeutic approaches to repair of bone defects, Voegele *et al.* have found differences in the functioning of osteoblasts depending on whether they were isolated after an enzymatic digestion or by spontaneous outgrowth [31]. We observed these HDF functional differences for cells from the same skin sample, studied simultaneously and at the same cell passage. A possible explanation could be that different cell populations are isolated by the two methods, the HDF population being probably more homogeneous after skin explant than after skin enzymatic digestion. These two different HDF populations were both morphologically free of keratinocytes.

The finding of these functional differences between the two HDF populations was very important for us from a practical point of view. Obviously we considered it was

more important to grow functional cells than to obtain them quickly. Since it appears important to colonize CS with functional fibroblasts we would prefer to use autologous DS to treat chronic wounds, where timing is not an issue, whereas allogeneic fibroblasts must be used if DS are required for deep burn coverage [5]. The procedures for conservation of these tissue-engineered DS colonized with allogeneic HDF have to be optimised to ensure their availability for these acute situations. The functions of these DS should be also tested after storage. In our study, IL-8 and VEGF appeared to be the cytokines involved in CWH that are most informative for testing the functionality of a DS. Cytokine production can easily be determined by ELISA and this represents a useful quality test of cell function for cell and tissue therapy.

ACKNOWLEDGEMENTS.

We thank M. Gasser for expert histological preparations and M. Laugier for SEM preparations.

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