A MARINE NUTRACEUTICAL PROMOTES FIBROBLASTS PROLIFERATION, MIGRATION AND COLLAGEN SYNTHESIS IN DAMAGED CELLS: AN IN VITRO STUDY REVISITING THE CONCEPT OF “CELL THERAPY”?


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ABSTRACT
The aim of the present study was to establish if Celergen, a novel marine protein-hydrolysate nutraceutical, would qualitatively implement the natural cell recovery in a non-toxic wound healing in vitro model through its inner characteristics as cell nourishment. A single stripe was
scraped on the external surface of the glass coverslips and the wound was allowed to heal for 24 hours to allow quantitative analysis such as: cell viability, cell proliferation, cell motility, collagen synthesis, collagen I quantification and collagen gene expression by specific PCR.

Cultures were treated with 5, 10, 25, 50 and 100 μg/ml of Celergen, incubated for 24 hours. As compared to untreated control, Celergen-treated fibroblasts significantly induce a whole range of biochemical events involved in the wound healing process, including cell proliferation and secretion of collagen, and increase in cell viability. This is likely to be due to the upregulation of proteins associated with fundamental cellular proliferation and anti-apoptosis function. In addition, Celergen may have anti-aging property in terms of induction of procollagen gene expression.

**Introduction**

Proteins are natural polymers and comprise almost 15% of the human body. Collagen is the most important protein of the extracellular matrix (ECM) and is the most prominent protein found in mammals, being 25% of the total protein mass and around 80% of skin when calculated as dry weight. Overall, types I, II, and III are the major types of collagen present in connective tissue and represent 90% of all collagen in our body. Collagen molecules act as a structural scaffold in tissues and its main characteristic is represented by their stiff, triple-stranded helical configuration (1). Quantitative and qualitative changes in collagen content play an important role in the destruction of organ architecture and function. For instance, type I collagen is the main collagenous component also in vital locations such as in the renal interstitium and blood vessels (2). However, collagen matrix functions also as a scaffold for regeneration (3, 4) and when applied to a tissue defect, the sprouting of capillaries and the migration of fibroblasts into the collagen brings about the induction of angiogenesis and fibroplasia. As a matter of fact, the promotion of angiogenesis in a injured tissue represents a crucial issue for tissue regeneration and recent studies in tissue engineering suggest that fibroblasts play a significant role in triggering and guiding the angiogenic process. This is because fibroblasts preserve the structural integrity of connective tissue by constantly secreting
growth factors and extracellular matrix precursors, which are necessary for endothelial cells adhesion and spreading. Interestingly, quite recently it has been proven in vivo, that at the site of injury fibroblasts actively interplay with myogenic cells so to stabilize extracellular matrix (5) and help developing the basement membrane of the muscular tissue. Moreover, the presence in vitro of fibroblasts, by paracrine signalling, may enable cardiomyocytes to shift towards a phenotypic change with also objective functional benefit such as an enhanced contractility (6).

We have recently shown that Celergen, a GMP-controlled marine nutraceutical, could significantly inhibit ultraviolet (UV)-induced matrix-metalloproteinases (MMP) transcription and exerts an antioxidant protecting effect on skin fibroblasts (7). However, the aim of the present study was not to directly apply a noxious injury model but a more physiological wound healing in vitro model (8). Indeed, while the prototype “cell therapy” practiced in the past by alternative medicine has raised several concerns and even caused fatalities, the nowadays stem cell research stream had entirely revolutionised the field and provided new avenues. In between, we focussed to establish if Celergen, per sè, could qualitatively implement the natural cell recovery through its inner characteristics as cell nourishment.

Materials and Methods

Celergen obtained from Swisscap company (100 mg composition: DNA extract from fish milt 46 mg, fish collagen hydrolysate plus fish elastin 35 mg, whole fish protein hydrolysate 6 mg, lutein-coenzyme Q10-selenium 11 mg). Beforehand, samples were blindly sent to an official Good Manufacturing Practice- and Good Laboratory Practice- registered toxicology laboratory which found no traceable amounts of heavy metals, yeast or microbiological pollutants (Redox Lab, Monza, Italy, report n. 2013001054/LAB).

Cell culture
Human dermal fibroblasts (passage 6-12; Cambrex, Walkersville, USA) cells were cultured in Dulbecco's modified (high glucose) Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum, 2 mM glutamine and 100 μg/ml each of penicillin and streptomycin and incubated under a fully humidified atmosphere containing 5% CO2 at 37°C. For experiments, cells were collected from subconfluent monolayers with trypsin/EDTA. Recombinant human epidermal growth factor (EGF) used as positive control in the chemotaxis migration assay was obtained from Sigma (see above).

**Preparation for cell studies**

The cells were incubated for 24 hours with Celergen dissolved in media at final concentrations of 5, 10, 25, 50 and 100 μg/ml for the cell viability evaluation and 10 and 100 μg/ml to assess the cell cycle assay, using equivalent volumes of media as the control vehicle in both cases.

**Cell Viability**

Cell viability was assessed using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye mitochondria reduction in living fibroblasts according to Sladowski et al. (9). Briefly, 5 × 10^5 fibroblasts were incubated with 1 mg/mL of MTT prepared in supplemented EMEM culture medium, for 2 h at 37°C and 5% CO2; fibroblasts were washed three times with 0.2 M Phosphate Buffer Saline (PBS) pH 7.4. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl)-2-, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, partly by the action of dehydrogenase enzymes to generate reducing equivalents of NADH and NADHP. Then, the reduced MTT formazan crystals were solubilised with dimethylsulfoxide (DMSO) and quantified by spectroscopic means. After 48 hours treatment with each Celergen concentration, the medium in each well was aspirated and 100 μl of MTT solution (1 mg/ml) was supplemented. After incubation for 4 hours at 37°C, the medium containing MTT solution was removed and the formazan crystals present in the viable cells were solubilised with 100 μl of
DMSO. Test medium was used as background control as well as an empty liposome group as a treatment control. Three independent sets of experiments performed in triplicates were evaluated. The absorbance of each well was read at 540 nm using a microplate reader.

**Cell proliferation response**

For cell proliferation assays, the serum content was decreased to 2% and the assays were carried out in a 96-well culture plate, as elsewhere described (10). Briefly, cells were serum starved for 24 hours and then were seeded at 2,500 cells/well and allocated in reduced serum (2%) for 24–48 hours before treatment. Treatments were then added and cells were left to incubate for 4–5 days. Cell cultures were then halted by trichloroacetic acid and cell number was assessed by using sulforhodamine B (Sigma-Aldrich). The absorbance of sulforhodamine B staining at 570nm (which is related to cell number) was finally calculated in a microplate reader. The total number of cells was also counted using an inverted microscope.

**Wound healing-migration assay**

Cells were grown to confluence on a 24-well dish, the medium was aspirated, and serum starved for 24 hours. Fresh medium with or without Celergen or (asic fibroblast growth factor (bFGF) was added. A single stripe (500 μm wide) was perpendicularly scraped on the external surface of the glass coverslips with a disposable pipette tip and the wound was allowed to heal for 24 hours to allow quantitative analysis. The average size of wound closure was assessed by calculating the width of the wound. Repopulation of the wounded area based on the average distance migrated by the cells as the difference of the cell front relative to the 0 h time point was observed under phase contrast microscopy at 0 and 24 h after scraping. The speed of migrating cells into the wounded area was observed by microphotography and measured using ImageJ software (http://rsbweb.nih.gov/ij).

In separate parallel dishes replicating the same wound model, fibroblasts were treated with varying concentrations of Celergen and examined for 24 hours afterwards. Cultures treated with empty
liposomes only served as the negative control. Fibroblasts were treated with 5, 10, 25, 50 and 100 μg/ml of Celergen, incubated for 24 hours. The relative migration rate of cells with various treatments of Celergen was determined as compared with the control. In particular, migration rate of cells in 0.1% DMSO was used as control, and the migration rate was defined as 100%. Each experiment was performed in triplicate.

**Total collagen synthesis and type I collagen quantification**

Overall collagen synthesis was measured by immuno-fluorescence using monoclonal anti-collagen antibodies, tagged with a fluorescent marker, which were obtained by the reaction of mice splenocytes immunized with human collagen cells. The test results were evaluated by measuring the intensity of fluorescence in the cells treated with Celergen as compared to untreated control.

As for the measurement of type I collagen, fibroblasts were seeded in 96-well plates (Corning, Corning, NY, USA) at 6,000 cells/well, and were grown for 2 days in 2% serum to late subconfluence. Then, treatments were added and the cultures were maintained for 4 more days. At the end of the study, cell media were collected and type I collagen was quantified by a sandwich enzyme-linked immunoabsorbent test. Briefly, 96-well plates were coated with goat antitype I collagen antibody (1:40, Southern Biotechnology, Inc., USA) overnight at 4°C and incubated with conditioned media for 1 hour. After rinsing, the sandwich was added a biotinylated anti-type I collagen antibody for 1 hour and then by horseradish peroxidase labelled streptavidin for 30 minutes. The assay was developed with peroxidise substrate 2,20-azino-bis-3-ethylbenziazoline-6-sulfonic acid (ABTS single reagent, Chemicon International) and read at 405 nm.

**Procollagen I gene expression: RNA extraction and quantitative real time polymerase chain reaction**

The cDNA was amplified to estimate the gene expression level by quantitative real time polymerase
chain reaction (PCR). PCR reactions were performed using a SYBR premix Ex Taq kit and conducted using a Takara dice real time system. The region amplified were as follows: human α1(I) procollagen nucleotides 312–607, 5′ - AAC GGC AAG GTG TTG TGC GAT G, 3′ - AGC TGG GGA GCA AAG TTT CCT C and GAPDH nucleotide 331 to 1038, 5′ - ACC ACC ATG GAG AAG GCT GG, 3′ - GGT TTC TTA CTC CTT GGA GG as an internal control. The PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining. Gels were scanned using Master Scan (Scanalytics, Billerica, MA, USA) and the signal intensity of bands was calculated. Each experiment was carried out by amplification of GAPDH which served as an internal control and the intensities of the cDNA bands for type I collagen were normalized to the GAPDH band intensities.

**Statistical analysis**

For each parameter, average values with standard deviations were calculated and presented as the mean±SD. The unpaired t-test was used to compare the data. Differences were considered significant at $P<0.05$. Migration assay data were analysed by a two-way ANOVA with the independent factors experiment and treatment followed by Bonferroni post-hoc tests. Monolayer wound healing assay data were analysed by a two-way ANOVA with the independent factors experiment and treatment followed by Bonferroni post-hoc tests. Statistical analysis was performed with Statistica 6.0 (Statsoft Inc., Tulsa, USA).

**Results**

**Cell Viability**

In order to rule out any possible interference of Celergen with another tetrazolium salt MTT, the direct reductive potential of it in a cell-free system was initially tested and found that its value was
comparable to the blank (medium only). Cell survival was estimated after 24 h and 48 h treatment and cultures with more than 90% viable cells were considered to be unaffected, 80 - 90% as moderately affected while values of less than 80% viable cells were regarded to represent a cytotoxic effects of the compound. Considering the above parameters, no cytotoxicity or “moderate interference” of Celergen was observed at any of the dosage employed.

As shown in fig 1, cell viability, when observed at 48h, significantly increased from 10% to 19% after treatment with Celergen at concentrations ranging from as low as 10 to 100 μg/ml (p<0.05 vs control) and without a significant dose-response pattern.

**Fibroblast proliferation assay**

As compared to untreated cultures (using empty liposomes only), a significant dose-related increase in proliferation of fibroblast in response to treatment with Celergen was observed (p<0.01, figure 2). On the whole, more stimulation of cell growth was observed with Celergen at 50μg/ml concentration and higher concentrations did not further enhanced the proliferation rate.

**Cell migration assay**

Celergen treatment significantly enhanced cell migration of human dermal fibroblasts when tested by scratch wound assay. After 12hr of treatment with 10μg of Celergen, the migratory activity of the fibroblast could already be observed by microscopic observation (fig. 3). Complete covering of the wound was seen within 24 hours of treatment and this appeared to be comparable, if not quantitatively more overtly, than what observed with bFGF (no statistical analysis could be applied). This response was not recorded in the untreated cell culture plate. Moreover, when checking the quantitative analysis of migration distance, it appeared that Celergen co-culture significantly enhanced the repairing migratory activity of fibroblasts from doses as low as 10μg/ml and without any significant dose-response effect (fig.4).
**Total collagen synthesis**

Cells treated with Celergen showed a significant increase in fluorescence representing total collagen production (Fig. 5) as compared to the control. This appeared starting at dosages of 10mg/ml (p<0.01) and further increased in the other higher dosages (p<0.05 vs 10mg/ml and p<0.001 vs control) at a comparable extent among them. Indeed, the fluorescence method showed a multiplication by factor up to 18.6 of the optical density of the cell culture caused by collagen synthesis of a culture of 10^5 cells/ml in 48 hours. The results of the fluorescence assessment enabled an estimation of about 43 ng collagens for the control culture and 800 ng collagen for the culture enriched with 100μg/ml of Celergen. Control with empty liposomes showed only a marginal, not significant increase of collagen synthesis. The same effect appeared when carrying out the quantitative estimation of collagen I (fig 6), where dosages as low as 10μg/ml significantly (p<0.05 vs control) enhanced its production.

**Procollagen gene expression**

As compared to untreated control (empty liposomes), the cultures treated with Celergen from dosages of 10μg/ml upward showed a significant upregulation of pro-collagen I (p<0.01, fig. 7). This effect didn’t show a significant dose-relationship, although a mild trend appeared. This effect already took place at 12h (p<0.01 vs control, data not shown) and were more quantitatively evident when observed at 24h (fig. 7).

**Conclusion**

The wound repair process implies a great deal of complexity and involves dynamic interactions of extracellular matrix moieties, several growth factors and a range of resident cells. In this context, collagen plays an essential role in wound healing process and it is indeed largely used for wound
repair treatments due to its biocompatibility and low immunoreactivity (11). Interestingly, quite recently fish collagen has been used to shape up novel nanofibrous tridimensional scaffolds (12).

Type I and type III collagen are present in the highest levels in the skin, forming 80% and 15% of the total collagen present respectively. Moreover, type I collagen is a major extracellular matrix component in the dermis and it synthesized and secreted in a soluble form by skin fibroblasts and deposited extracellularly. The regulation of cell proliferation is central to tissue morphogenesis during the development of multicellular organisms and, as for skin, it is known that during ageing, fibroblasts decrease their production of adequate amounts of collagen (13).

The present study clearly suggests that Celergen has a definite effect in promoting migration and proliferation of fibroblasts and has been shown to stimulate the proliferation of human fibroblasts, quickening functional cell recovery. Indeed, cell proliferation and cell migration are two important events necessary for wound healing in particular but for tissue regeneration overall (14) together with their latest ability to beneficially modify angyogenetic mechanisms (15).

Our data also implicate that Celergen could increase procollagen production although further studies are needed to unfold more detailed mechanisms and figure out whether this is a direct effect or indirect by suppressing MMP-1 gene expression in fibroblasts. Nonetheless, in our previous study (7) we had shown that the same marine compound enabled a significant inhibition of UV-induced MMP transcription and a decrease in the release of MMP-1 in the medium.

Whatever, the deeper mechanisms to be unfolded as yet, from the present data it would appear that the microenvironment created by these stromal cells in the scaffold modulates a number of fundamental molecular activiations and can potentially be used in regenerative medicine as overall.

References


Author disclosure. No competing financial interests exist.

Legends

Fig 1

Fibroblasts Viability test: effect of graded concentrations of Celergen (at 48h observation).

Three independent sets of experiments performed in triplicates were evaluated at 24h. Test medium (M) was used as background control and an empty liposome group as a treatment control (C). * p<0.05 vs control group.

Fig 2
Cell Proliferation response: effect of time and concentrations of Celergen. Cell number was assessed by using sulforhodamine B and expressed as optical density (OD) 570X 1000. Empty liposome group was used as a treatment control (C). * p<0.01 vs control group

Fig 3

Wound healing-migration assay. The speed of migrating cells into the wounded area under Celergen or bFGF (basic fibroblast growth factor) effect was monitored by microphotography and calculated using ImageJ software (http://rsbweb.nih.gov/ij).

Fig 4

Quantitative analysis of cell migration. effect of time and concentrations of Celergen The values are the media of three independent experiments. Empty liposome group was used as a treatment control (C). * p<0.05 vs control group

Fig 5

Total Collagen synthesis in a wound-healing model under graded doses of Celergen. Collagen synthesis was measured by comparing the intensity of immuno-fluorescence versus untreated control (empty liposome). * p<0.01 vs control group, ** p<0.05 vs Celergen 10μg/ml and p<0.01 vs control group.

Fig 6

Collagen content into extracellular matrix in a wound-healing model: effect of graded doses of Celergen

Empty liposome group was used as a treatment control (C). * p<0.05 vs control group
Fig 7. Effects of Celergen on the procollagen 1 gene expression in wound damaged dermal fibroblasts model (24h observation).

To analyze the effects of Celergen on the gene expression of procollagen by a real time-polymerase chain reaction analysis, fibroblasts were pretreated with the indicated concentration of Celergen and underwent wound damage model. Data represent the mean±SD of triplicate experiments. * p<0.01 vs control group.