Evaluation of Senescence Induced Prematurely by Stress. Application for cosmetic active ingredients

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Summary

Living cells are continuously subject to all sorts of stress such as ultraviolet rays on skin cells. Tests made in various laboratories show that when young fibroblasts (i.e. at the beginning of their proliferate life) were repeatedly put under stress at subletal doses, they acquired a phenotype similar to Senescence Induced Prematurely by Stress (SIPS). The work presented hereafter was made on a new model of senescence induced prematurely by stress from ultraviolet B rays (UVB). The human fibroblast model was put under repeated UVB stress, causing SIPS. Several ageing biomarkers were used in order to characterise the cells that underwent stress:

- an increase in the proportion of positive cells with senescence associated β-galactosidase activity (SA β-gal) measured by a specific coloration,
- the proportion in the different morphological stages that fibroblasts undergo during culture visualised by microscopic observation,
- the expression of genes known for overexpressing during senescence, particularly fibronectin and apolipoprotein J, measured by Real Time-PCR,
- the common deletion of 4,977 bp in mitochondrial DNA, evaluated by nested PCR.

Studying the variation of these 4 biomarkers, we have evaluated the protective effect of a Laminaria digitata extract (LDE) that can be used as a natural active ingredient for anti-ageing cosmetics.

Introduction

Cellular ageing is under a genetically programmed process. Each cell type has a limited number of mitoses (approximately 50 cycles for fibroblasts in *in vitro* culture). But living cells are continuously subject to all sorts of stress that stimulate ageing, such as ultraviolet rays on skin cells, atmospheric pollution in the lungs, but also, the derivatives of radical oxygen species (ROS) during the inflammation process in blood vessels and tissues. Cells contain specific systems for protection and reparation, but when repair does not take place immediately, some modifications may become irreversible and accumulate without immediate visible alteration.

When normal skin cells were exposed to various types of subcytotoxic repeated stress generating ROS, without inducing cellular death, in radical molecules such as tert-butylhydroperoxyde, at repeated exposure to hydrogen peroxide, or even repeated prolonged exposure to normobaric hyperoxia with 40% partial pressure in oxygen, they display a senescence-like phenotype. As stress increases, the proportion of cells in senescence phenotype should increase when a culture reaches growth arrest. These cells express biomarkers of senescence: senescence-like cell morphology, decrease of DNA synthesis, lack of response to mitogenic stimuli, irreversible growth arrest triggered by telomere shortening counting cell generations [1] and increase in the proportion of cells positive for senescence-associated beta-galactosidase (SA beta-gal). Senescent cells express a high level of SA beta-gal activity (detected by a blue coloration) compared to young cells, linked to an increase in the size of lysosomes [2, 3]. Moreover, numerous genes are known for having an increase in there relative mRNAs during senescence, particularly the extra-cellular matrix protein fibronectin. More recently, the stimulation of apolipoprotein J (apo J) was also considered as a ageing marker [4]. This chaperone protein may provide better survival for cells exposed to multiple stress. During senescence the mitochondrial genome undergoes alterations. Amongst these, an increase in the 4,977 bp deletion frequency is without a doubt the most spectacular. This is not observed in cells from young individuals,

this type of deletion is considered as a marker of cellular ageing. Lastly, the morphotypes of cultured fibroblasts go from a fusiform "young" morphotype to a "senescent" morphotype. During cellular ageing, a drifting of pre-mitotic morphotypes to post-mitotic morphotypes can be observed [5]. Seven different morphotypes (MF) were determined: MF1, MF2 and MF3 morphotypes correspond to still mitotic states. MF4, MF5 and MF6 morphotypes characterise post-mitotic states. A 7th morphotype established as corresponding to a deterioration stage is rarely detectable. All these biomarkers characterise an acquired phenotype called Stress-Induced Premature Senescence (SIPS).

We have used an original SIPS model using human skin fibroblasts in culture under consecutive stress by UV rays. This SIPS model and these markers allowed us to study the effect of several natural extracts. The effects observed with a liquid extract of the brown seaweed *Laminaria digitata* (LDE) was described here as an example.

Material and methods

Cells and cell culture

Human skin fibroblasts were obtained from the Coriell Institute for Medical Research (USA). They were routinely cultivated in 75 cm² flasks containing 15 ml of basal medium Eagle (BME) supplemented with 10 % (v/v) fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. Culture were grown at 37°C in an atmosphere containing 5% CO2. They are seeded in squared 35 mm culture dishes (Falcon, UK) 3 days before radiation.

Laminaria digitata extract (LDE) preparation

Laminaria digitata extract (LDE) was obtained by a short-time extraction made at room temperature with demineralised water using freeze-dried seaweed, followed by a filtration with 0.22 µm filter and a concentration by reverse osmosis in order to obtain a concentration of approximately 5 % (w/w) dry mater.

Exposure to UVB

Cells cultures at half-confluence were submitted to ten repeated subcytotoxic exposures to UVB stress with two stress per day for 5 days. Cells were washed once with phosphate buffer saline pH 7.4 (PBS) and exposed to 125 mJ/cm² UVB radiation in a thin layer of PBS using a Philips TL 20W/01 lamps (Philips, The Netherlands). The emitted radiation was checked using a UVR-radiometer with an UVB-sensor (Bioblock Scientific). Control cultures at the same population doubling followed the same schedule of medium changes without UVB treatment. Some cultures were made with addition of 5 % (v/v) LDE between each UVB exposure.

SA beta-galactosidase activity

At 48 hours after the last stress, cells were seeded at the density of 700 cells/cm². After 24 hours, the beta-gal activity on cells was measured according to the method developed by J. Campisi [2]. Briefly, cells were fixed by 2 % formaldehyde and 0.2 % glutaraldehyde, washed twice in PBS and treated for 16 hours with 5-bromo-4-chloro-3-indolyl-beta-D-galactoside in 40 mM citic acid / sodium hydrogenophosphate, 5 mM potassium ferrocyanure, 150 mM sodium chloride and 2 mM magnesium chloride. Then cells were washed twice in PBS and twice in methanol before to be dried at room temperature. The proportion of cells positive for SA beta-Gal activity was determined by counting 400 cells per dish. Triplicate were performed. Results are expressed as mean values +/- SD.

Morphotype analysis

At 48 hours after the last stress, cells were seeded at the density of 700 cells/cm². After 24 hours, cells were fixed for an immediate microscopical observation for classification of their morphotype according to the parameters previously described by Bayreuther [5].

RNA isolation and semi-quantitative RT-PCR

At 72 hours after the last stress, cells were harvested and pelleted. Total RNA was extracted using a total RNA isolation kit (Promega, USA). Semi-quantitative RT-PCRs were performed with an incorporation of a fluorescent molecule (SYBR-Green) during amplification step; the fluorescent signal is proportional to the quantity of double-stranded DNA present. This method enables to quantify the relative level of mRNA to apolipoprotein J (Apo J) and fibronectin in cells that have undergone, or not, successive stress. The primers for Apo J, fibronectin or GAPDH were obtained from Applied Biosystems. The PCR conditions were the following: denaturation, 95°C, 5 min; annealing, 94°C, 15 s; elongation, 65°C, 60 s; 40 cycles. Fluorescence was measured using a fluorimeter directly linked to the thermocycler (ABI Prim 7000, Applied Biosystems, CA, USA). Level of expression of Apo J and fibronectin mRNAs were expressed in function of GAPDH mRNA expression.

Deletion of mitochondrial DNA

At 72 hours after the last exposure, the cells were lysed in 0.2 M NaOH / 1% SDS, to extract cellular DNA. The nested PCR method was used to study the extracted DNA in order to demonstrate mDNA deletion. The primers specific for the deleted fragment (H3-L3) and an area never deleted (H1-L1) were obtained from Eurogentec. RT-PCR products were electrophoresed on a polyacrylamide gel and analysed with an instant imager. Molecular weight DNA was provided from Promega.

Results

LDE decreased the up-regulation of beta-gal positive cells induced by stress

At the beginning of the culture (the day of the seeding), the proportion of SA beta-gal positive cells is relatively low (18 +/- 2 %). After the culture without UVB (day 10), the proportion slightly increased (29 +/- 2 %). After 10 successive UVB stress, the frequency of SA beta-gal cells highly increased: 48 +/- 3 % instead of 29 % (figure 1). Treatment by 5 % LDE significantly diminished the number of senescent cells that express a strong beta-galactosidase activity (5 +/- 1 %). The proportion of positive cells having undergone UV stress was inferior to the proportion of cells that did not undergo stress.

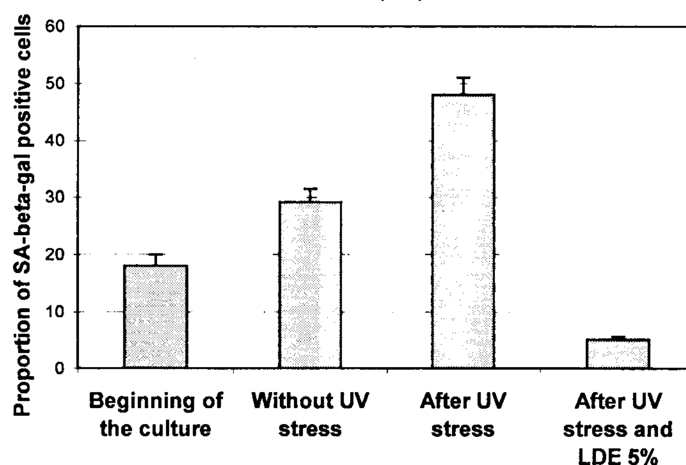

Figure 1: Effect of LDE on UVB-induced SA beta-gal expression

LDE prevented modification of cellular morphotype induced by stress

Cells cultured without exposure to UV mainly expressed the MF2, MF3, MF4 and MF5, and very few MF6 and no MF7 (figure 2). After treatment by UVB, the proportion of cells with MF2 and MF3 decreased, and the proportion of cells with MF5 dramatically increased. The proportion of MF4 was not significantly modified but some cells expressed the MF7. Cells exposed to UVB but treated with 5 % LDE delayed this movement from mitotic morphotypes to post-mitotic morphotypes. Proportion of MF2 and MF3 were more important than control without UV stress, and proportion of MF5 highly decreased. MF6 and MF7 were not expressed.

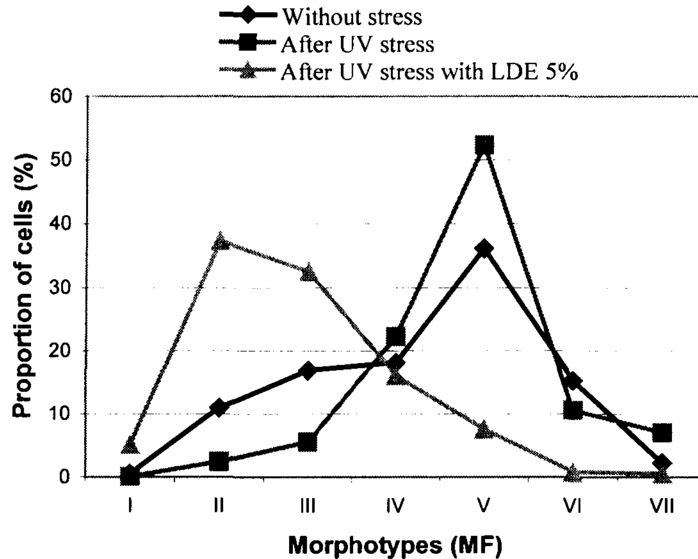

Figure 2: Effect of LDE on UVB-induced cellular modifications

LDE decreased the up-expression of Apo J and fibronectin induced by stress

The relative levels of fibronectin gene mRNA increased by a factor 1.7 between UVB stressed cells and unstressed controlled cells (figure 3, left). Cell treatment with 5 % LDE provides protection in the order of 35 %. In absence of stress, there is a very weak expression of chaperone proteins. However, stress can activate their appearance. The relative level of mRNA of apolipoprotein J gene increased by a factor 1.9 between UVB stressed cells and unstressed controlled cells (figure 3, right). By contrast with fibronectin, the level of apo J expression was not significantly modified by the treatment with 5 % LDE.

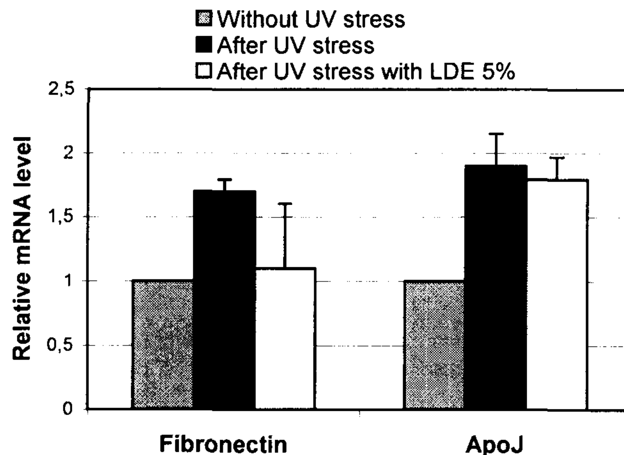

Figure 3: Effect of LDE on UVB-induced fibronectin and Apo J expression

LDE prevented the mitochondrial DNA deletion induced by stress

Mitochondrial DNA deletion frequency increased in stressed cell DNA compared to unstressed cells (figure 4). According to the decrease of the quantity of deleted DNA in the gel (figure 4, right), treatment with 5 % LDE protected the mitochondrial DNA against its fragmentation.

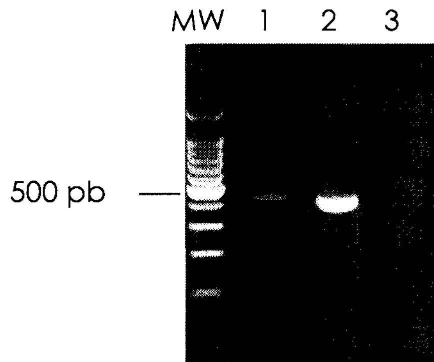

Figure 4: Effect of LDE on UVB-induced mitochondrial DNA deletion.
1: Unstressed cells ; 2: Stressed cells ; 3: Stressed cells + 5% LDE

Discussion

Numerous papers have already presented effect of UVB on skin cells after *in vivo* and *in vitro* studies. But only few studies have been made in order to study the long-term effect of UVB on cells, and particularly about the cellular modifications during ageing. It is well known now that senescent cells express specific biomarkers such as morphological changes and increased SA beta-gal activity.
Our results confirm that UVB exposure increases the appearance of these biomarkers as previously described [6]. The beta-gal expression in stressed cells increases in function of UVB doses [7]. These results are well correlates with stress induced by hydrogen peroxide [1].

In this work, we have studied the effect of successive UVB stress on expression of senescence-associated genes, such as fibronectin and apolipoprotein J (Apo J). The increase of fibronectin expression has been previously described [3]. The up-regulation of this extra-cellular matrix protein is linked to aging process, and could be linked to an elevation of transforming growth factor beta 1 (TGF-beta 1) produced by human fibroblasts after subcytotoxic hydroperoxide stress [8]. Addition of Laminaria digitata extract (LDE) in the culture medium decreased the UVB-induced overexpression of fibronectin confirming that LDE acts on another biomarkers of senescence. Curiously, it did not modify the Apo J expression. Since its overexpression in human fibroblasts protects them against premature senescence, LDE gives good cell protection. Apo J is now considered as a chaperone molecule [4].

A protection was shown after addition of LDE in the culture medium. It could be explained by the disposability of some substrate for the energy metabolim. Indeed, the availability of substrate for mitochondrial metabolism is known to modulate the appearance of the stress-induced premature senescence (SIPS). It has been showed that SA beta-gal activity is lower in cells stressed by hydrogen peroxide in a medium containing D-glucose than in cells stressed in glucose-free medium [9]. The effect on mitochondrial metabolism could be directly linked to the protection of mitochondrial DNA by incubation with LDE before and after UVB exposure. This result is confirmed by some other results obtained with LDE in absence of stress. Indeed, we have also demonstrated that LDE at 5% significantly stimulated the protein synthesis by human dermal fibroblasts after 7 days of culture, and stimulated the mitochondrial enzymatic activity (measured with the MTT method) of human dermal fibroblasts after 24 hours of culture. Moreover, LDE at only 0.1% stimulated the oxygen consumption of human dermal fibroblasts.

Conclusion

We have studied the expression of some biomarkers in human skin cells exposed to successive stress. These biomarkers characterise the SIPS, and are directly linked to cellular ageing. According to these studied biomarkers, LDE decreased the appearance of cellular ageing. The cells keeps a young form morphotypes, continue to express a low level of SA beta-galactosidase, did not expressed high level of fibronectin but maintain its potential to express a high level of chaperone proteins. Moreover, it reduces mitochondrial DNA deletions and allows to maintain a good cellular energetic metabolism. This natural extract could be used as an active ingredient for anti-ageing range.

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