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Identification of potential anti-photoageing algal compounds using an in-vitro model of photoageing

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Abstract

Stress-induced premature senescence (SIPS) has been proposed as an in-vitro model for testing the long-term effects of stressful events and to find molecules/natural extracts that protect against such stress. Premature senescence of human skin diploid fibroblasts (HDFs) can be induced by repeated subcytotoxic exposure to UVB, with the appearance of so-called biomarkers of senescence such as growth arrest, senescence-associated β-galactosidase activity, senescence-associated gene overexpression and the common 4977-bp mitochondrial DNA deletion. This model of UVB-induced premature senescence has been acknowledged as a robust in-vitro model in photoageing research. In this study, the potential anti-photoageing effects of a series of algal extracts were tested. The appearance of the biomarkers of UVB-induced premature senescence of HDFs was studied with or without algal extracts. One algal extract was shown to be particularly protective against UVB-induced SIPS. The results obtained here reinforce the notion that UVB-induced premature senescence of HDFs can be used to screen potential anti-photoageing compounds.

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Introduction

Exposure of human skin diploid fibroblasts (HDFs) to a variety of oxidative stresses and DNA damaging agents such as *tert*-butylhydroperoxide (*t*-BHP) (Dumont et al 2000), hydrogen peroxide (Frippiat et al 2001) or UVB (Debacq-Chainiaux et al 2005) induces stress-induced premature senescence (SIPS). Cells in SIPS display several features of replicative senescence such as irreversible growth arrest in G1, senescence-associated β-galactosidase activity (SA β-gal), the common 4977-bp mitochondrial DNA deletion, and change in expression level of several senescence-associated genes (for a review see Dierick et al 2003).

No in-vitro method exists for detecting the potential long-term effects of molecules at subcytotoxic concentrations in terms of days and weeks after (repeated) exposure to the molecule tested. SIPS can be used as an alternative in-vitro toxicological method for testing the long-term effects of molecules under development in the pharmaceutical, cosmetic and food industry (Toussaint et al 2000), either to test the potential deleterious effects of subcytotoxic exposure to molecules or to look for the potential protective effects of molecules/complex extracts against these deleterious effects (Toussaint et al 2000). For instance, previous studies determined that purified molecules (bilobalide) extracted from leaves of the *Gingko biloba* tree protected against SIPS induced by ethanol (Toussaint et al 1995).

Photoageing of the skin is mainly due to UVB-induced damage of the dermal connective tissue, resulting in qualitative and quantitative alterations of the dermal extracellular matrix (Jenkins 2002). UVB (290–320 nm), an essential component of sunlight, crosses the epidermis and reaches the upper dermis, which is composed mainly of fibroblasts and extracellular matrix (Rosette & Karin 1996). Photoageing is therefore considered as extrinsic ageing, in addition to intrinsic ageing (Wlaschek et al 2001). Following repeated exposure to subcytotoxic non-proapoptotic doses of UVB, HDFs undergo SIPS in-vitro (Debacq-Chainiaux et al 2005). This model can be used as an in-vitro model of photoageing since several common biomarkers are found in both skin photoageing and UVB-induced premature senescence, namely SA β-gal activity (Dimri et al 1995), the common 4977-bp mitochondrial

DNA deletion (Birch-Machin et al 1998), and an increase in the steady-state mRNA levels of *c-fos* and *c-jun* (Fisher et al 1998). In this study, we tested the potential anti-photoageing effects of a series of plant extracts. The level of appearance of the different biomarkers of senescence induced by UVB was studied in the presence or absence of the extracts.

Materials and Methods

Preparation of plant extracts

Extracts A1, A2, A3 and A4 were obtained by vacuum microwave hydrodistillation from the aerial parts of four seashore plants: A1, an extract of *Helichrysum italicum* (*Asteraceae*); A2, an extract of Rock rose (*Cistus monspeliensis*, *Cistaceae*); A3, an extract of Spanish lavender (*Lavandula stoechas L.*, *Lamiaceae*); and A4, an extract of Sea fennel or Rock samphire (*Crithmum maritimum L.*, *Apiaceae*). The extracts contained specific aromatic substances and some terpenes such as the cyclic monoterpenes fenchone (in A3) or dillapiol (in A4). Extracts A5 and A6 were obtained by a patented lixiviation method and concentrated by reverse osmosis from the brown seaweeds *Ascophyllum nodosum* (*Fucaceae*) and *Laminaria digitata* (*Laminariaceae*). The two macro algae were harvested from the North Brittany coast and immediately freeze-dried before storage. They contained algae-specific polyphenols (phloroglucinol derivates), polysaccharides (such as fucane, alginate, laminarine) and polyols (such as mannitol). Sample A7 was a natural earth marine water from Brittany. This water is rich in all minerals and trace elements, mainly calcium, magnesium and iron. The liquid samples A1–A7 were frozen and stored at –20°C. Sample A8 was a purified oligoalginate obtained by depolymerization from an alginate using an alginate lyase. Samples A9, A10 and A11 were obtained from the micro alga *Spirulina platensis*, after microfiltration through a membrane with 0.22-μm porosity (A9), then fractioned with Millipore membranes to select molecules less than 100 000 Da (A10) or 30 000 Da (A11). The extracts obtained (A8, A9, A10 and A11) were freeze-dried and stored at room temperature. They were diluted in culture medium just before incubation with the cell cultures.

Cell culture, algal extract incubation and cytotoxicity assay

AG04431 HDFs (Coriell Institute for Medical Research, USA) were classically grown in BME medium (Invitrogen, UK) + 10% (v/v) fetal calf serum (FCS) (Invitrogen, UK) and 2 mM L-glutamine and 100 μg mL^{-1} streptomycin. Cells at 55–60% of in-vitro proliferative lifespan were subcultivated at half confluence (10 000 cells cm^2) in BME + 1% FCS.

At 72 h after plating, the cells were incubated with increasing doses of algal extracts. Fresh culture medium was provided every day with or without the fresh algal extracts A1–A11 (blind study in respect of confidentiality imposed by a private company). After 1 h, 24 h or 7 days of incubation with the extract, the cellular protein content was assayed by the Folin method (Lowry et al 1951). The results (mean ± s.d. of triplicate experiments) are expressed as the percentage of surviving cells. This method was previously shown to give accurate estimates of the number of surviving cells under such conditions (Dumont et al 2000).

UVB: cytotoxicity, SA β-gal, morphotypes and [^{3}H]thymidine incorporation

At 72 h before the first stress, HDFs at 55–60% of in-vitro proliferative lifespan were subcultivated at half confluence (10 000 cells cm^2) in BME + 1% FCS. At 1 h before the stress, the cells were incubated with fresh BME + 1% FCS with or without algal extracts (for concentrations see Table 1). Before the stress, the cells were washed once with phosphate-buffered saline (pH 7.4, 10 mM, 0.9 g NaCl; PBS) and exposed to UVB radiation in a thin layer of PBS with or without algal extracts using three Philips TL 20W/01 lamps (Philips, The Netherlands) emitting UVB peaking at 311 nm and placed 30 cm above the flasks. The emitted radiation was checked under the flask lid using an UVR-radiometer with a UVB sensor (Bioblock Scientific, Belgium). After radiation, PBS was replaced by BME + 1% FCS with or without algal extracts. The stress was performed twice a day for 5 days. Control cells were exposed to the same culture conditions without UVB exposure.

At 48 h after the last stress, the cellular protein content was assayed by the Folin method. Alternatively, the cells were seeded at 48 h after the last stress in square 35-mm culture dishes (Falcon, UK) at 700 cells cm^2 in BME + 1% FCS. SA β-gal activity was determined 24 h later as described by Dimri et al (1995) and the proportion of mitotic and post-mitotic HDFs was recorded (Bayreuther et al 1988). The results are expressed as mean ± s.d. of three independent experiments in each of which 400 cells were considered.

In order to measure the incorporation of [^{3}H]thymidine into the DNA, the cells were seeded 48 h after the last stress in 24-well plates (Cell Cult, UK) at 10 000 cells/well in BME + 1% FCS. [^{3}H]Thymidine (specific activity 2 Ci mmol^{-1}, 1 μCi; Du Pont NEN, USA) was added to BME + 1% FCS for 48 h. Quantification of the radioactivity was performed by a scintillation counter (Packard Instrument Company, USA) as described by Dumont et al (2000). The results are expressed as mean ± s.d. of triplicate experiments.

Table 1 Cytotoxicity threshold (% v/v) of the different plant extracts

Algal extract	Cytotoxicity threshold (% v/v)
A1	10%
A2	10%
A3	10%
A4	10%
A5	0.5%
A6	5%
A7	20%
A8	0.05%
A9	0.01%
A10	0.01%
A11	0.05%

Real-time reverse transcription polymerase chain reaction (RT-PCR)

At 72 h after the last stress, total RNA was extracted from three independent cultures using the Total RNAgent extraction kit (Promega, USA). Total RNA (2 μg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, UK). Primers (Table 2) were designed using the Primer Express 1.5 software (Applied Biosystems, The Netherlands). Amplification reaction assays contained 1 × SYBR Green PCR Mastermix and primers (Applied Biosystems, The Netherlands) at optimal concentrations. A hot start at 95°C for 5 min was followed by 40 cycles at 95°C for 15 s and 65°C for 1 min, using the 7000 SDS thermal cycler (Applied Biosystems, The Netherlands). Melting curves were generated after amplification and data were analysed using the thermal cycler software. Each sample was tested in triplicate.

Detection of the common 4977-bp mitochondrial DNA deletion

Mitochondrial DNA was extracted as described by Filser et al (1997). The presence of mitochondrial DNA was assessed by PCR amplification of a 533-bp conservative region of the mitochondrial DNA using H1/L1 and the deletion was detected by nested PCR using primers H2/L2 and H3/L3 as described previously (Dumont et al 2000; Debacq-Chainiaux et al 2005), resulting in the amplification of a 404-bp fragment. The sequences and positions of the primers are given in Table 3.

Statistical analysis

The statistical analysis was performed using R (http://www.r-project.org) and SigmaStat (Systat Software, CA, USA) statistical software. The Kolmogorov–Smirnov test was used to check the normality of the distribution among the populations, and the homoscedasticity was also checked using the SigmaStat algorithm. When samples corresponded to normally distributed populations with homogeneous variance, a one-way analysis of variance was done followed by the post-hoc Dunnett's test. If the normality test or the homoscedasticity test failed, a non-parametric Kruskal–Wallis (analysis of variance on ranks) was realized followed by the post-hoc Dunn's test. $P > 0.05$ was considered significant.

Table 2 Real-time reverse transcription polymerase chain reaction primers

Genes	Positions (bp)	Sequences
apolipoprotein J	937–959	5′-gga tga agg acc agt gtg aca ag-3′
	1032–1050	5′-cag cga cct gga ggg att c-3′
fibronectin	5023–5041	5′-tgt ggt tgc ctt gca cga t-3′
	5111–5131	5′-gct tgt ggg tgt gac ctg agt-3′
gapdh	942–963	5′-acc cac tcc tcc acc ttt gac-3′
	1033–1053	5′-gtc cac cac cct gtt gct gta-3′

Table 3 Primers used for the detection of the common 4977-bp mitochondrial deletion

Primers	Positions (bp)	Sequences
H1	3817–3836	5′-ggg agg agt aat cag agg tg-3′
L1	3304–3323	5′-aac ata ccc atg gcc aac ct-3′
H2	8150–8169	5′-ccg ggg gta tac tac ggt ca-3′
L2	13 631–13 650	5′-ggg gaa gcg agg ttg acc tg-3′
H3	8197–8215	5′-cag ttt cat gcc cat cgt c-3′
L3	13 560–13 578	5′-gat gag agt aat aga tag g-3′

Results and Discussion

Determination of a subcytotoxic dose of 11 plant extracts

We determined the cytotoxicity threshold of 11 plant extracts on HDFs. HDFs were incubated with increasing concentrations of each algal extract for 1 h, 24 h or 7 days. Cell viability was then determined by cell counting, MTT assay (Mosmann 1983) and protein biomass assay (Lowry et al 1951). These methods were previously shown to give comparable results in these models (Dumont et al 2000). As an example, Figure 1A shows the results obtained with extract A8, the cytotoxicity of which was greatly enhanced when the cells were exposed for 7 days at concentrations of 0.10% or greater. Therefore the maximum concentration used for extract A8 throughout this study was 0.05%. The subcytotoxic concentrations of the 11 extracts were determined similarly and are given in Table 1.

Phototoxicity/photoprotection of the plant extracts after exposure to UVB

To determine if the extracts could protect against cytotoxic exposure to UVB, HDFs were exposed to doses of UVB ranging from 500 to 2000 mJ cm^2. Cell survival was determined by cell counting, MTT assay and protein biomass assay. Extracts A1, A2 and A5 provided 23%, 20% and 10% protection at cytotoxic doses of 500, 1000 and 2000 mJ cm^2 of UVB, respectively (data not shown).

Effect of the 11 plant extracts on UVB-induced SIPS

The main aim of this study was to determine the potential protective effect of the 11 extracts on UVB-induced premature senescence of HDFs. HDFs were exposed to UVB at a dose of 250 mJ cm^2, with two stresses per day for 5 days.

These stress conditions were shown to be subcytotoxic, non-proapoptotic and induced SIPS (Debacq-Chainiaux et al 2005). Under these conditions of repeated subcytotoxic exposure to UVB, the cells incubated with the extracts A3 and A5 displayed a slight pro-proliferative effect of 36% and 48%, respectively (Figure 1B), compared with the UVB-treated cells that were growth arrested. The biomass of the cells treated with extracts A4, A9 and A11 was lower (by <15%) in the UVB-treated cells compared with control cells. It has been shown that this must be interpreted as a decrease in

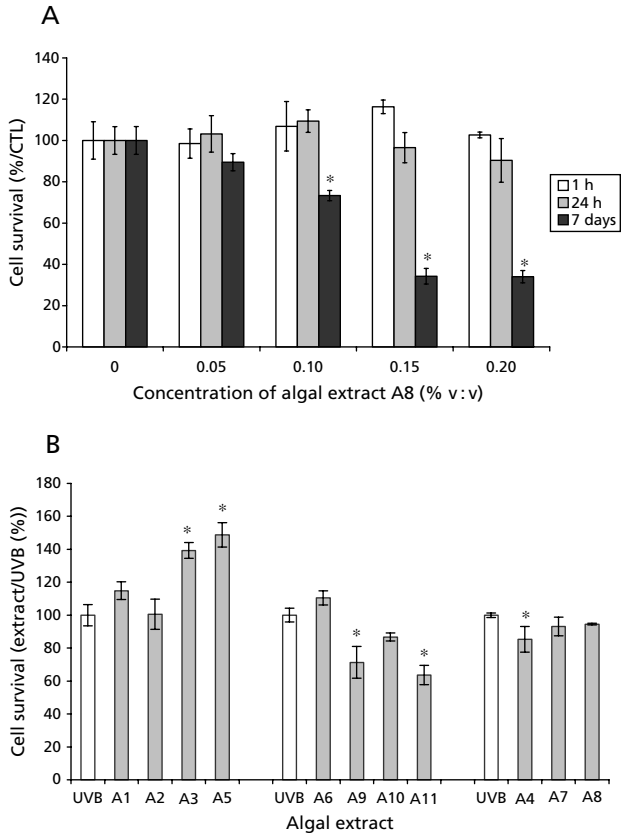

Figure 1 A. Example of a survival curve. Effect of the incubation of human skin diploid fibroblasts with plant extract A8 on cell survival. Cell survival after 1 h, 7 h or 7 days of incubation with extract A8 at 0.05, 0.10, 0.15 and 0.20%. Results are given as mean ± s.d. of three independent experiments. B. Effect of the incubation of human skin diploid fibroblasts with 11 algal extracts on the cell survival after 10 repeated exposures to UVB at 250 mJ cm^2. Cell survival was estimated at 24 h after 10 repeated exposures to UVB at 250 mJ cm^2 on cells incubated (A1–A11) or not (UVB) with algal extracts. Results are given as mean ± s.d. of three independent experiments. *$P > 0.05$ considered as significant.

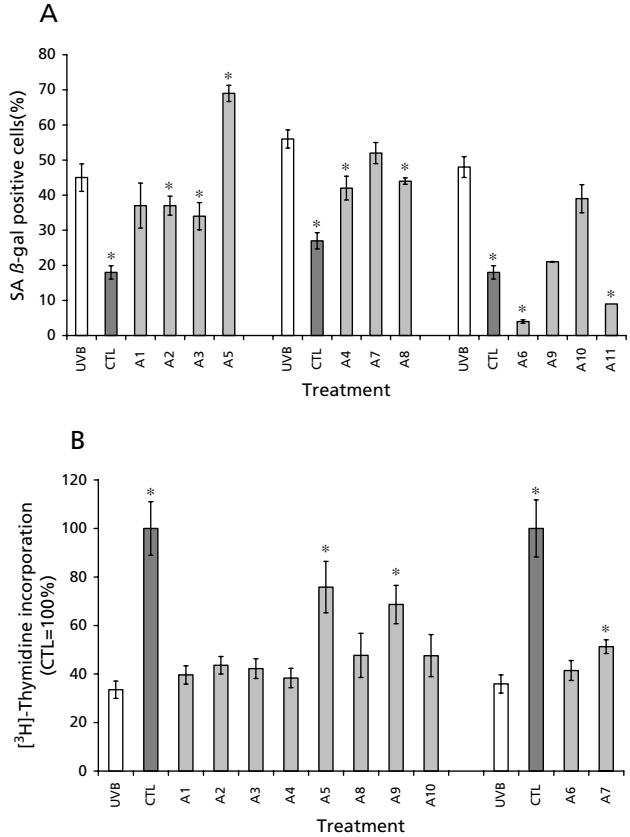

Figure 2 Effect of the incubation of human skin diploid fibroblasts with plant extracts on senescence-associated β-galactosidase activity and on the growth arrest induced after 10 repeated exposures to UVB at 250 mJ cm^2. A. Proportions of cells positive for the senescence-associated β-galactosidase activity at 72 h after 10 exposures to UVB at 250 mJ cm^2. B. Incorporation of [^{3}H]thymidine at 72 h after 10 exposures to UVB at 250 mJ cm^2. *$P > 0.05$ considered as significant.

proliferative potential and not as cell death. Indeed near-confluence control cells proliferated slightly during the 8 days of the experiment, explaining their 20% biomass increase, whereas the stressed cells did not (de Magalhaes et al 2002).

The percentage of HDFs positive for SA β-gal activity was determined at 72 h after the last stress. There was a 2.5-fold increase in the proportion of cells positive for SA β-gal activity in UVB-stressed cells compared with control cells, as previously reported (Debacq-Chainiaux et al 2005). Most extracts were protective against this UVB-induced increase in SA β-gal positive cells, with a dramatic 12-fold decrease with extract A6 (Figure 2A). Surprisingly, extract A5 increased the proportion of cells positive for the SA β-gal by 1.5-fold, the reason for which remains unexplained.

The level of [^{3}H]thymidine incorporated into DNA was determined at 72 h after the last UVB stress. As previously published (Debacq-Chainiaux et al 2005), there was a 65% decrease of [^{3}H]thymidine incorporation by HDFs in UVB-induced SIPS. Incubation with extracts A5, A7 and A9 before, during and after the UVB stress led to a significant increase of [^{3}H]thymidine incorporation when compared with the UVB-treated cells (Figure 2B).

It would be better not to develop anti-ageing compounds that increase the proliferation of UVB-damaged cells in order not to favour cell immortalization. Thus, three algal extracts (A6, A8 and A10) were selected as they protected against the induction of SA β-gal activity and did not level off the UVB-induced inhibition of growth arrest.

Morphotypes, steady-state mRNA expression of *fibronectin* and *apolipoprotein J* (*apo J*) and the common 4977-bp mitochondrial DNA deletion

Bayreuther et al (1988) showed that HDFs shift through seven different morphological stages ("morphotypes") during cell passaging and in-vivo skin ageing. The first three morphotypes are mitotic fibroblasts: MF I, MF II and MF III. Noteworthy, MF III divides very rarely. The last four morphotypes are post-mitotic fibroblasts (PMF IV, PMF V, PMF VI and PMF VII). The proportion of mitotic/post-mitotic morphotypes was previously used as an index of the senescence state of a cell

culture (Brammer et al 2004; Kampinga et al 2004; Salmon et al 2004). The HDFs in UVB-induced SIPS had a 65% decrease of MF in UVB-stressed cells compared with the control cells (Figure 3A). A 2-fold increase in the proportion of mitotic morphotypes was detected with the algal extract A6, which suggests the possibility of an uncoupling between the stress-induced changes of proliferative capability and the morphology of the cell.

The steady-state mRNA level of *apo J* and *fibronectin* was determined. It was previously found that *apo J* and *fibronectin* are overexpressed in replicative senescence and in SIPS induced by H_2O_2, *t*-BHP (Dumont et al 2000) or UVB (Debacq-Chainiaux et al 2005). Overexpression of *apo J* represents a protective mechanism against SIPS induced by *t*-BHP, ethanol and UVB (Dumont et al 2002; Debacq-Chainiaux et al 2005), which could be an interesting property of any anti-photoageing extract. Overexpression of *fibronectin* is characteristic of senescent morphogenesis (Kumazaki et al 1993). We studied the steady-state mRNA level of these genes with real-time RT-PCR using the housekeeping gene *gapdh* as a reference. The steady-state mRNA levels of *apo J* and *fibronectin* were increased by 1.9- and 1.7-fold, respectively, in the HDFs in UVB-induced SIPS as previously reported (Debacq-Chainiaux et al 2005). Extracts A6, A8 and A10 did not significantly decrease the level of *apo J* mRNA. The level of *fibronectin* mRNA was decreased when extract A6 was present, reaching the basal level, whereas extracts A8 and A10 further increased this level (Figure 3B).

The common 4977-bp mitochondrial DNA deletion can be detected in both replicative senescence and *t*-BHP- and UVB-induced SIPS (Dumont et al 2000; Debacq-Chainiaux et al 2005). After checking the integrity of the mitochondrial DNA (data not shown), nested PCR was performed. A 404-bp PCR product resulting from the deletion was found in the UVB-treated cells (Figure 3C). This product was not detected in HDFs incubated with algal extracts A6, A8 and A10, suggesting their potential protection against the UVB-induced deletion in mitochondrial DNA.

Extract A5 protected cells from the growth arrest induced by UVB-SIPS (Figure 2B) and could even be pro-proliferative (Figure 1B). These effects could be explained by the antioxidant property of algal polyphenols (Kang et al 2003).

After repeated exposure to UVB, extract A6 reduced the proportion of β-gal positive cells (Figure 2A), increased the proportion of mitotic morphotypes (Figure 3A), protected cells from the stress-induced increase of the steady-state mRNA level of *fibronectin* (Figure 3B), and protected mitochondrial DNA against deletion (Figure 3C). These characteristics make extract A6 an interesting extract. The protective effects could be linked to algal polyphenols but also to a stimulation of cellular metabolism by algal polyols, since extract A6 stimulated the cellular and mitochondrial respiration due to its high level of mannitol.

The sample A8 protected mitochondrial DNA against UVB-induced deletion (Figure 3C). As previously described, in presence of an oligoalginate, the apoptosis of epidermal Langerhans cells in human skin fragments exposed to UVB is reduced (Morvan & Vallee 2001). The sample A9 protected cells against repeated exposure to UVB as shown by a reduced expression of β-gal (Figure 2A) and an increase of

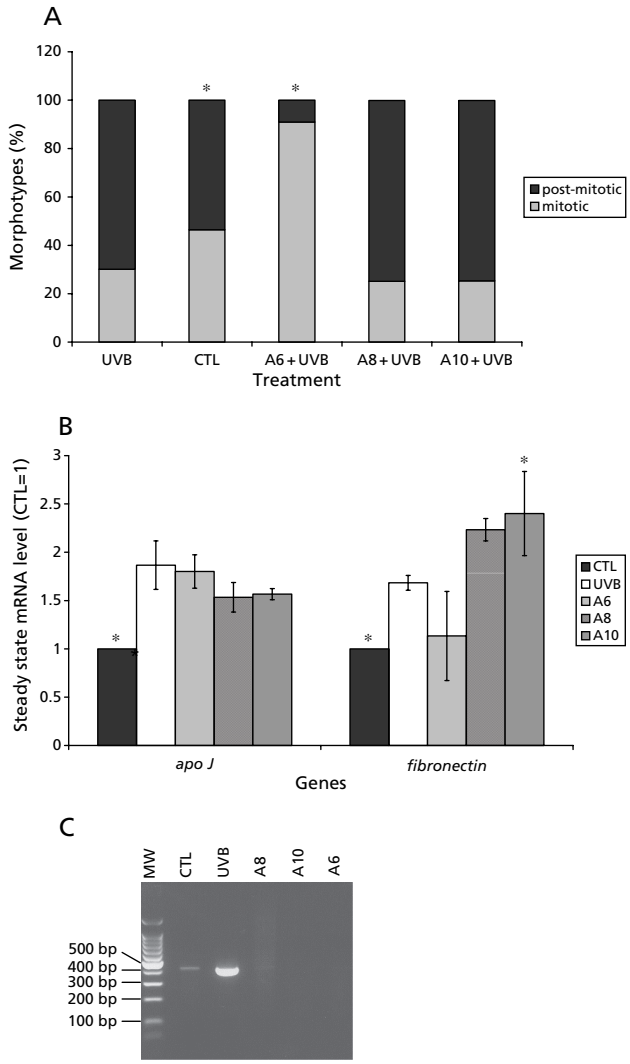

Figure 3 Effect of the incubation of human skin diploid fibroblasts with plant extracts A6, A8 or A10 on the proportion of morphotypes, the steady-state mRNA level of *apolipoprotein J* (*apo J*) or *fibronectin* and the common 4977-bp mitochondrial DNA deletion after 10 repeated exposures to UVB at 250 mJ cm^2. A. Proportions (%) of the mitotic and post-mitotic morphotypes at 72 h after 10 exposures to UVB at 250 mJ cm^2. Cells exposed to UVB were incubated (A6, A8 or A10) or not (UVB) with algal extracts. Cells submitted to the same culture conditions but without UVB exposure (CTL) were also studied. Results are given as mean ± s.d. of three independent experiments. B. Steady-state mRNA level of *apo J* and *fibronectin*. Total RNA was extracted at 72 h after 10 exposures to UVB at 250 mJ cm^2. The *gapdh* steady-state mRNA level was used as a housekeeping gene for real-time reverse transcription polymerase chain reaction. The results obtained from the UVB with or without algal extract (A6, A8 or A10) treated cells are expressed as percentages of the steady-state mRNA level of the respective mRNA species in control cells (CTL). Results are given as mean ± s.d. of three independent experiments. C. Detection of the common 4977-bp mitochondrial deletion by nested PCR at 72 h after 10 exposures to UVB at 250 mJ cm^2. The 4977-bp deletion was detected (404-bp fragment) in UVB-exposed cells (UVB) but not in UVB-exposed cells incubated with algal extracts (A6, A8 or A10) or in control cells not exposed to UVB (CTL). *$P > 0.05$ considered as significant.

thymidine incorporation (Figure 2B). The protection offered by the total *S. platensis* extract was owing to molecules with a molecular weight up to 100 000 Da because A10 had no significant protective effect (Figures 2B and 3A). By contrast, some low molecular weight substances were also protective because A11 (concentrated in molecules less than 30 000 Da) reduced β-gal expression in cells exposed to UVB (Figure 2A). The protection could be linked to small peptides produced by the algae under heat stress or saline stress as previously described (Dupré et al 2004).

Using this procedure of determination of toxicity, phototoxicity and biomarkers of SIPS, the extract A6 was selected as the most interesting anti-photoageing extract among the extracts tested.

Conclusion

The aim of this study was to determine the potential anti-photoageing effects of a series of plant extracts on a model of SIPS induced by repeated non-proapoptotic subcytotoxic exposure to UVB. This model does not induce apoptosis by using active caspase-3 detection and PARP cleavage as criteria (Debacq-Chainiaux et al 2005). This model represents a robust in-vitro model of dermal photoageing (Debacq-Chainiaux et al 2005). Concentrations of extracts ranging from 0.01% to 10% were tested. No phototoxicity was observed in any extract after HDFs were exposed to a single high UVB dose, whereas a phototoxic effect could be observed after repeated milder exposure (extract A11). An initial screening checked some easy-to-test biomarkers of senescence (SA β-gal activity, proliferation capability) before a secondary screening was carried out on morphology, overexpression of *apo J* and *fibronectin*, and detection of mitochondrial DNA deletion. These screenings selected the plant extracts A6, A8 and A10 as being the most protective against UVB-induced SIPS, with A6 offering the greatest protection.

The possibility of an uncoupling between the stress-induced morphological changes and the stress-induced growth arrest has already been observed from a mechanistic point of view (Chen et al 2001; Frippiat et al 2002; Debacq-Chainiaux et al 2005). The differences between the level of SA β-gal and post-mitotic morphotypes obtained with extract A6 compared with the control cells could be explained by a delaying effect of the protective extract on the stress-induced arrest of proliferation, with more proliferation for 48 h after the last stress in the presence of this extract.

In conclusion, SIPS represents an interesting in-vitro model allowing the pro- or anti-protective effects of molecules/complex extracts to be tested in the development of cosmetic products. The work performed here shows that this alternative testing is workable for the development of anti-photoageing compounds.

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