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Expression profiling of senescent-associated genes in human dermis from young and old donors. Proof-of-concept study

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Abstract

It is often described that it is difficult to really discriminate the cause of intrinsic skin aging. The aim of this study was to compare the profiles of expression of senescence-associated genes in biopsies of dermis from young and old human donors. TGF-β1 was up-regulated in the dermis of old donors as well as the TGF-β1 -regulated genes. The anti-oxidant enzymes Selenium-dependent Glutathione peroxidase and Glutatione S-Transferase Theta 1 were also up-regulated in old dermis as well as Tumor Necrosis Factor Receptor Superfamily 1A. None of these genes had altered expression level in skin fibroblasts embedded in a collagen matrix and exposed to sublethal doses of UVB, suggesting their involvement in intrinsic aging. This study represents a proof-of-concept of larger whole transcriptome studies where all avenues should be used to subtract changes in gene expression due to extrinsic aging from changes potentially due to intrinsic aging.

Keywords : Skin fibroblasts ; Human dermis ; DNA arrays ; UVB ; Aging

Abbreviations

CDKi Cyclin kinase dependent inhibitor
FCS Fetal calf serum
GSH Glutathione
HDFs Human diploid fibroblasts
MMP Metalloproteinase
ROS Reactive oxygen species
RT-PCR Reverse transcription polymerase chain reaction SA β-gal Senescence-associated β-galactosidase activity SD Standard deviation
SIPS Stress-induced premature senescence
TGF-β1 Transforming growth factor-β1 UVB Ultraviolet B

INTRODUCTION

Aging of the skin, commonly associated with increased wrinkling, sagging and laxity, results from cumulative environmental insults, mainly UV-induced damage of the dermal connective tissue, in combination with intrinsic aging (Wlaschek et al. 2001). Intrinsic skin aging, largely genetically determined, resembles that seen in most internal organs and is thought to involve decreased proliferative capacity leading to cellular senescence, and altered biosynthetic activity of skin cells (Uitto 1986). Dermal fibroblasts are basic regulators of the synthesis and the degradation of extracellular matrix. Extrinsic aging, also called photoaging, is mainly due to UV-induced damage resulting in qualitative and quantitative alterations of the dermal extracellular matrix (Scharffetter-Kochanek et al. 2000). Ultraviolet B (UVB, 280-320 nm) and ultraviolet A (UVA, 320-400 nm) are essential components of sunlight. UVB radiation can reach the upper dermis and interact with cellular chromophores and photosensitizers, resulting in the generation of reactive oxygen species (ROS), induction of potentially mutagenic DNA damage, and activation of signaling pathways with induction of changes in the expression of many genes related to growth, differentiation, senescence and connective tissue degradation (Keyse 1993).

Aging-related transcriptomic profiling on human skin fibroblasts dealt with cultivated skin fibroblasts in replicative senescence or premature senescence induced by H_2O_2 or UVB (de Magalhaes et al. 2004; Debacq-Chainiaux et al. 2005; Ly et al. 2000; Yoon et al. 2004). Studies were also performed on human biopsies containing dermis and epidermis (Lener et al. 2006) and on human dermal fibroblasts cultivated from donors of various ages (Chondrogianni et al. 2004).

Since we could not ignore that repeated exposures to UVB can provoke many changes of gene expression and premature senescence in vitro on monolayers cultures of fibroblasts, we wished to substract changes due to UVB from the changes due to intrinsic aging. For doing so in conditions as close as possible to the in vivo conditions, we used equivalent dermises repeatedly exposed to UVB. Equivalent dermises are skin fibroblasts cultivated at low density in a collagen matrix.

A low density DNA array was generated to study the relative mRNA level of 240 genes involved in cellular senescence and stress response. The value of the technology of low density DNA arrays used in this study has been extensively demonstrated previously in a variety of models, as diverse as hepato-toxicology (de Longueville et al. 2002), breast cancer (Gillet et al. 2006), skin irritation (Borlon et al. 2007b), response to UVB stress (Borlon et al. 2007c; Debacq-Chainiaux et al. 2005) and cell senescence (de Magalhaes et al. 2004; Pascal et al. 2005; Zdanov et al. 2006). Numerous verifications with real time RT-PCR have been done on all these models. In addition, inter- and intra-platform reproducibility of gene expression measurements was demonstrated with this technology (Patterson et al. 2006; Shi et al. 2006). Nevertheless, a selected data set was verified herein using real time RT-PCR.

MATERIALS AND METHODS

Cell culture conditions for preparation of tissues and collection of human skin biopsies

AG04431 skin HDFs (Coriell Institute for Medical Research, USA) were grown in BME (basal medium Eagle) (Invitrogen, UK) + 10% (v:v) fetal calf serum (FCS) (Invitrogen, UK) and 2 mM L-glutamine. Cells at 55-60% of in vitro proliferative life span were subcultured in three-dimensional collagen lattices. Purified bovine skin type I collagen (Delvoye et al. 1991) was redissolved at 3 mg/ml in sterile 0.1% acetic acid. Three-dimensional collagen lattices were prepared as described in Lambert et al. (1992). Human dermal fibroblasts were seeded at $8*10^5$ cells/ml into a solution containing collagen I (0.5 mg collagen/ml) and grown for 72 h at 37°C 5% CO_2 in 100 mm-diameter bacterial dishes. Contraction of lattices was prevented by a braided inox steel thread (0.5 mm diameter) placed at the inner perimeter of the dish (Fig. 1a).

Human full-thickness skin biopsies and underlying adipose tissue (± 2 g/biopsy) were resected from Caucasian donors undergoing orthopedic surgery at the Namur Regional Hospital (Namur, Belgium). The ethical implications of using modern genomic technologies and biopsies of aged persons were considered (Matthews et al. 2005). Full ethical agreements were preliminarily obtained including informed consent. Skin samples were provided by a total of 49 donors (Table 1A), all without skin or inflammatory diseases. It was also assessed that the body areas where biopsies were resected from had been very little exposed to sun, largely reducing the effect of photo-aging compared with intrinsic aging.

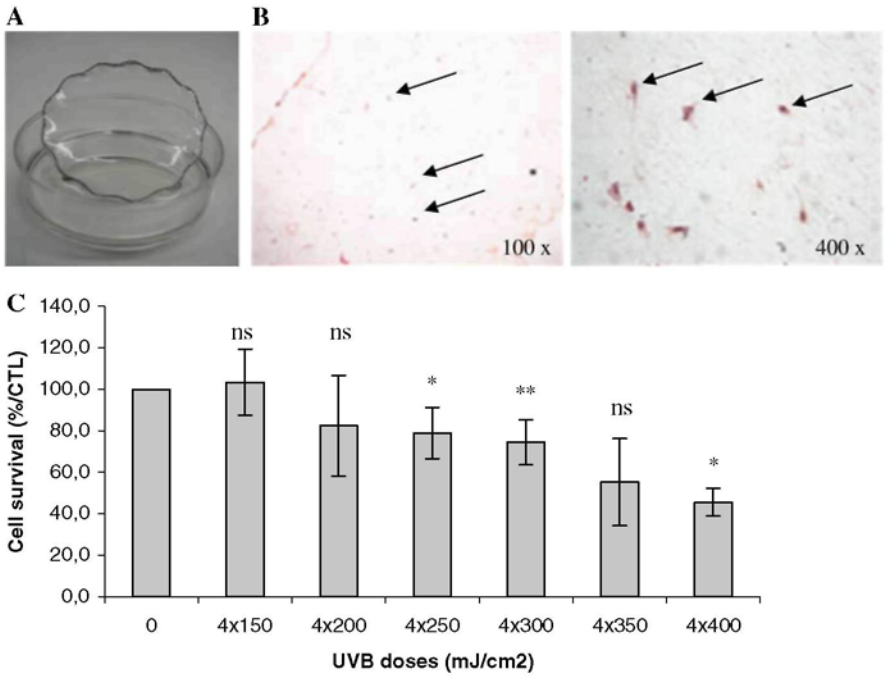
Immediately after harvest, skin samples were soaked in RNAlater RNA Stabilization Reagent (Qiagen, Germany) to prevent RNA degradation as well as artifactual changes in gene expression triggered by sample manipulation. Biopsies were then stored at 4°C for less than 5 days before isolation of dermis.

Histology

Reconstructed dermis was fixed in 10% formalin and embedded in paraffin. About 4 µm vertical sections were stained with hematoxylin/eosin.

Fig. 1 *Development of three-dimensional collagen lattices cultures with skin fibroblasts. (a) Three-dimensional collagen lattices. Contraction of lattices was prevented by a braided inox steel thread at the inner perimeter of the dish. (b) Histological sections of three-dimensional collagen lattices stained with hematoxilin/eosin. Some cells are pointed with arrows. (c) Survival of skin fibroblasts cultivated in lattices at 24 h after four exposures to UVB. Doses of UVB ranged from 0 to 400 mJ/cm^2 with two exposures per day for 2 days. Results are expressed as percentage of cell survival compared to cells non exposed to UVB (0 mJ/cm^2) considered as 100%. Results*

*are given as mean ± SD. of three independent experiments. Statistical analysis was carried out with Student's t-test. ns, Non-significant (P > 0.05); *, 0.05 > P > 0.01, **, P < 0.01*



UVB treatment and MTT assay

For UVB irradiations, three-dimensional collagen lattices were prepared with DMEM medium without phenol red (Invitrogen, UK). At 72 h after plating, lattices were washed once with phosphate-buffered saline pH 7.4 (10 mM phosphate, 0.9% NaCl) (PBS) and exposed to UVB radiation in a thin layer of PBS. Irradiation with UVB was performed using a closely spaced array of three Philips TL 20W/01 lamps (Philips, The Netherlands), emitting UVB peaking at 311 nm which deliver uniform irradiation at a distance of 30 cm. The energy output was measured using a UVR radiometer with a UVB sensor (Bioblock Scientific, Belgium) under the flask lid. The output of Philips TL 20 W/01 under the flask was 10.99 ± 0.9 W/m^2, irradiation time was 234 ± 8 s. After irradiation, PBS was replaced by DMEM + 10% FCS. The exposure was repeated twice a day for 2 days. Control cells were kept in the same culture conditions without UVB exposure.

The viability of the fibroblasts cultivated in collagen matrix after UVB irradiations was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltet-razolium bromide (MTT, Sigma) by quantifying living metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye (Green et al. 1984). Cytotoxicity was calculated as percentage of formazan formation in cells exposed to UVB compared to control cells.

Table 1 *(A) Skin biopsies: age of subjects and tissular origin; (B) primers used for quantitative real time RT-PCR*

A

Age group	"Young" subjects	"Old" subjects
Number of biopsies (sex ratio)	12 (7 men, 5 women)	37 (22 men, 15 women)
Age range (years)	20-30	60-90
Mean age (years)	24.8 ± 2.8	76.0 ± 7.7
Origin of biopsies	knee (10) and hip (2)	knee (12), hip (7), back (5), shoulder (3)

B

Genes	Positions (bp)	Sequences (forward and reverse)
GAPDH	942-963	5'-acc cac tcc tcc acc ttt gac-3'

	1033-1053	5'-gtc cac cac cct gtt gtc gta-3'
$p21^{WAF-1}$	495-515	5'-ctg gag act ctc agg gtc gaa-3'
	599-617	5'-cca gga ctg cag gct tcc t-3'
CTGF	242-258	5'-caa gct gcc cgg gaa at-3'
	365-384	5'-gga cca ggc agt tgg ctc ta-3'
COL1A1	4071-4090	5'-ccc aag gac aag agg cat gt-3'
	4125-4142	5'-cat cgg cag ggt cgg ag-3'
MMP1	977-997	5'-cat gcg cac aaa tcc ctt cta-3'
	1101-1125	5'-gaa cag ccc agt act tat tcc ctt t-3'
Osteo	868-889	5'-gag acc tgt gac ctg gac aat g-3'
	957-982	5'-gga agg agt gga ttt aga tca caa ga-3'

Low density DNA-array

Microarray design, RNA extraction, cDNA labeling and hybridization

The DualChip™ human aging is a low-density DNA array developed in collaboration with Eppendorf (Germany). This array, representing a range of 240 genes involved in senescence of HDFs, contains two arrays per glass slide (a control and a test) with three identical subarrays per array. The sequences of the DNA covalently linked to the glass slide were carefully chosen by sequence comparison. Checks were made by realizing single hybridization of each cDNA separately to ensure that no cross-hybridization takes place with other spot than the corresponding one. To evaluate the reliability of the experimental data, several positive and negative hybridization and detection controls are included on the microarray. For normalization, internal standard controls and housekeeping genes were arrayed on the slides.

Skin biopsies, stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany), were dissected and the upper-dermis, mainly composed of fibroblasts and extracellular matrix, was isolated from the other skin layers (epidermis and hypoder-mis). Total RNA was extracted using TRIZOL Reagent (Invitrogen, UK) from pieces of upper dermis of skin biopsies and from three independent cultures of fibroblasts in collagen matrix at 72 h after UVB treatment. Quality control of total RNA was realized on agarose gels. The amount of RNA was determined spectrophotometrically at 260 nm. For skin biopsies, total RNA (15 µg) was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, UK). For reconstituted dermis, total RNA (250 ng) was amplified and retrotranscribed according manufacturer's instruction (MessageAmp TM II-Biotin Enhanced kit, Ambion, UK). Three independent experiments were performed. Hybridization on DualChip human aging was carried out as described by the manufacturer. Detection was performed using a Cy3-conjugated IgG anti-biotin (Jackson Immuno Research Laboratories, USA).

Imaging, data normalization and analysis

Fluorescence of the hybridized arrays was scanned (Packard ScanArray, PerkinElmer, USA) at a resolution of 10 µm. To maximize the dynamic range of detection, the same arrays were scanned at three photomultiplier gains (50, 70, 100). The scanned 16-bit images were imported into the ImaGene 4.1 software (BioDiscovery, USA). The fluorescence intensity of each DNA spot (average intensity of each pixel present within the spot) was calculated using local mean background subtraction. A signal was accepted when the average intensity after background subtraction was at least 2.5-fold higher than its local background. The three intensity values of the triplicate DNA spots were averaged and used to calculate the intensity ratio between the reference and the test samples.

The ratios were normalized in two steps. First, the values were corrected using a factor calculated from the intensity ratios of the six internal standards in the reference and test samples. These internal standards were located at two different locations on the array allowing to consider the local background and array homogeneity in the normalization process. However, as the internal standard control does not take into account the purity and quality of the mRNA, a second step of normalization was performed.

We performed the second step of normalization with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level and with the average intensity obtained for eight housekeeping genes. This choice was first based on several published studies related to skin ageing and in vitro senescence of skin fibroblasts where GAPDH was

selected as housekeeper (Basu-Modak et al. 2003; Brink et al. 2000; Debacq-Chainiaux et al. 2005; Seo et al. 2003). However, we checked the relevance of GAPDH as the reference housekeeping gene in this part of the work by real time RT-PCR. The variance of the normalized GAPDH was used to generate an estimate of expected variance, leading to a predicted confidence interval for testing the significance of the ratios obtained. Ratios outside the 95% confidence interval were determined to be significantly different using a specifically designed statistical test (de Longueville et al. 2002; de Magalhaes et al. 2004).

Statistical test

As for microarray analyses: Significance Analysis of Microarray (SAM) test was performed using the TIGR Multi-Experiment Viewer (Saeed et al. 2003). The other statistical analyses were carried out with the Student's *t*-test. *, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$.

Validation of relative gene expression by real time RT-PCR

Total RNA (2 µg), extracted from reconstituted tissues treated with UVB or not, was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, UK). Primers (Table 1B) were designed using the Primer express 1.5 software (Applied Biosystems, The Netherlands). These specific primers correspond to the gene sequence present on the DualChip™ human aging (Eppendorf, Hamburg, Germany). Amplification reaction assays contained 1X SYBER Green PCR Mastermix (Applied Biosystems, The Netherlands), and primers at optimal concentration. PCR reactions without cDNA were performed as template-free negative controls. All PCR reactions were made in duplicates with a hot start at 95°C for 5 min, 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. The abundance of RPL13A mRNA was used as reference for semi-quantification with the "ΔΔCT method" (Pfaffl 2001).

More than 40 verifications with real-time RT PCR of the relative gene expression level obtained with this microarray have already been done in several senescence-related models (Borlon et al. 2007a; de Magalhaes et al. 2004; Debacq-Chainiaux et al. 2005; Zdanov et al. 2006). Nevertheless, further verifications were done in the studies. In order to spare mRNA obtained from the biopsies, the verifications were done with RNA from equivalent dermis.

RESULTS

Changes of mRNA profiles in the in vivo dermis from old versus young donors

Collection of skin biopsies

A total of 49 skin biopsies were collected from patients undergoing orthopedic surgery at the Namur Regional Hospital (Belgium) following the ethical rules set by its ethical committee, and were sorted into two age groups: 12 biopsies from young people (mean age: 24.8 ± 2.8 years) and 37 biopsies from old people (mean age: 76.0 ± 7.7 years).

The existing protocols for sample collection, storage, dermis isolation and RNA extraction had to be optimized in order to avoid changes in gene expression due to manipulation, increase the yields of transcripts and preserve them from degradation. Once ameliorated, these procedures allowed to select 10 RNA extracts using the 28S/18S ratio (value > 1.5) for further transcriptomic analysis: four extracts from young individuals and six from old individuals. Before reverse transcription and labelling, total RNA integrity and quality was controlled by agarose gels.

To ensure these extracts could be normalized by GAPDH, 10 real-time RT-PCR reactions were performed with RNA from young (four samples) and old (six samples) donors. Mean Ct values from the two age groups were compared with a student *t*-test, as shown by Pfaffl et al. (2002), allowing to assess that the levels of GAPDH transcripts were not significantly different between old and young. Thus GAPDH was relevant as for normalizing the results.

Low density cDNA microarray experiments and data analysis

Pairwise RNA profiles comparisons were performed for each of the six dermis from old subjects versus each of the four of the dermis of the young donors, generating a set of 24 "fold change" ratios for every 240 genes

represented on the arrays. The matrix of results (24 × 240) obtained was submitted to statistical analysis by SAM (False positive < 0.01) and *t*-tests (with *P*-value < 0.01) in order to select a panel of transcripts with a significant differential expression between old and young subjects. This increased drastically the stringency of the analysis in order to minimize the risk of false-positive transcripts. Increased stringency also allowed to select transcripts with a higher probability to have biological implications in skin ageing. Indeed, the number of biopsies per group was small and the intra-group variability of RNA profiles had to be considered since we expected a greater variability than when working with in vitro cell cultures. Eight mRNA species were significantly differentially expressed as found by both the SAM and the *t*-tests (Table 2A). Among these eight transcripts, five transcripts were up-regulated in dermis from old donors and were of considerable interest.

Firstly, TGF-β1 (*Transforming Growth Factor-beta1*), a multi-functional cytokine involved in cell growth, differentiation and biosynthesis of extracellular connective tissue (Derynck and Zhang 2003), was found up-regulated in dermis from old donors. TGF-β1 is overexpressed in skin in vivo after UVB exposure (Quan et al. 2002b), as well in human skin diploid fibroblasts (HDFs) in replicative senescence (Pascal et al. 2005) or in stress-induced premature senescence induced by UVB, H_2O_2, ethanol or *tert*-butylhydroperoxide (*t*-BHP) (Debacq-Chainiaux et al. 2005; Frippiat et al. 2001, 2002; Pascal et al. 2005). The fact that the dermis biopsies were from regions of the body not intensely exposed to UVB suggest that TGF-β1 over-expression found herein could be due to intrinsic aging only.

Abundance of SM22 (*Transgelin*) transcript was also increased in the dermis from old donors. SM22 encodes a putative calcium binding protein involved in senescence-induced morphological changes (Lecka-Czernik et al. 1996). Interestingly, SM22 mRNA abundance increases in in vitro replicative senescence and in premature senescence induced by *t*-BHP, H_2O_2 (Dumont et al. 2000), or UVB (Chainiaux et al. 2002). SM22 overexpression in premature senescence induced by H_2O_2 or UVB was shown to depend on TGF-β1 (Debacq-Chainiaux et al. 2005; Frippiat et al. 2001).

DPT (*Dermatopontin*) is a component of the extracellular matrix with possible functions in cell-matrix interactions and matrix assembly. This up-regulation of DPT found herein is not in agreement with data published on cultivated replicatively senescent human dermal fibroblasts (Yoon et al. 2004). Interestingly, DPT mRNA and protein levels are up-regulated by TGF-β1 in non senescent fibroblasts (Kuroda et al. 1999). Worth to note, DPT was also found to augment the biological activity of TGF-β1 (Okamoto et al. 1999).

Table 2

a

Gene symbol	Gene name	Function	Genbank	Old/young
DPT	Dermatopontin	Extracellular matrix/cell structure	XM_001897	↑ 4.3
SM22	Transgellin	Extracellular matrix/cell structure	M95787	↑ 1.8
TNFRSF1A	Tumor necrosis factor receptor superfamily 1A	Cell signaling/receptor	X55313	↑ 1.8
TGFB1	Transforming growth factor beta 1	Growth factor and cytokine	NM_000660	↑ 1.5
GPX	Glutatione peroxidase	Antioxidant enzyme	M21304	↑ 1.4
GSTT1	Glutatione S-transferase theta 1	Antioxidant enzyme	NM_000853	↑↑*
CST6	Cystatin M	Metabolism/structural protein	U62800	↓ 6.3
EGR2	Early growth response 2	Cell signaling/receptor	NM_000399	↓ 6.9

b

Gene symbol	Gene name	Function	Genbank	UVB/CTL
ID1	Inhibitor of DNA binding	Cell signaling	X77956	↑↑*
MMP3	Metalloproteinase 3	Extracellular matrix/cell structure	NM_002422	↑↑↑*
COL3A1	Collagen III-alpha	Extracellular matrix/cell structure	NM_000090	↓4.5
FMOD	Fibromodulin	Extracellular matrix/cell structure	NM_002023	↓*
COL1A1	Collagen I-alpha	Extracellular matrix/cell structure	NM_000088	↓ 2.7
CTGF	Connective tissue growth factor	Extracellular matrix/cell structure	U14750	↓ 2.3

c

	Real time RT-PCR	cDNA array
$P21^{WAF-1}$	1.3	1.4
CTGF	0.3	0.4

Osteo	0.7	0.6
MMP1	2.8	2.1
COL1A1	0.1	0.4

[a] Genes differentially expressed in skin biopsies from old donors compared to biopsies from young donors. The SAM test (false positive <0.01) combined to *t*-tests (*P*-value < 0.001) selected 8 genes on 146 detected as significantly differentially expressed. For each couple of experiments and for each gene, the value $\log_2\left(\frac{E_o}{E_y}\right)$ was determined (where E_0 is the expression of the gene for biopsies from old donor, and E_y its expression for biopsies fromyoung donors). Genes are sorted according the up-regulation and down-regulation in old versus young. * qualitative result, significant up-regulation but no quantitative ratio could be calculated due for instance to very low expression in one of the experimental conditions

[b]Genes differentially expressed in reconstituted dermis exposed to UVB compared to control reconstituted tissues not exposed to UVB. The SAM test selected 6 genes on 120 detected as significantly differentiallyexpressed (false positive <0.05). For each couple of experiments and for each gene, the value $\log_2\left(\frac{E_{uvb}}{E_{ctl}}\right)$was determined (where E_{uvb} is the expression of the gene reconstituted tissuesexposed to UVB, and E_{ctl} its expression for controltissues). Genes are sorted according the up-regulation and down-regulation in UVB versus CTL reconstituted tissues. * qualitative result, significant up-regulation but no quantitative ratio could be calculated due for instance to very low expression in one of the experimental conditions

[c] Comparison between the data obtained with real-time RT-PCR and the DualChip human aging for AG04431 fibroblasts in reconstructed dermis for five genes at 72 h after the last UVB exposure

The abundance of the mRNA of Se-dependent GPX (*Glutathione* (*GSH*) *peroxidase*) and GSTT1 (*GSH S-transferase thetal*), both antioxidant enzymes, was increased in old dermis. GPX uses glutathione as a co-factor and catalyses both the conversion of UV-induced H_2O_2 production into H_2O and O_2, and the detoxification of toxic lipid peroxides (Afaq and Mukhtar 2001). GPX is up-regulated in human skin diploid fibroblasts in UVB - induced premature senescence (Debacq-Chainiaux et al. 2005). Se-dependent GPX is not strongly affected by UV and is considered to be most important basal antioxidant defence system of the skin (Fuchs et al. 1989; Shindo et al. 1994). GSTT1 also protects against oxidative stress through export of oxidized GSH outside of the cell (Ketterer et al. 1993). Heritable GSTT1 deficiency may be a genetic determinant of individual skin sensitivity towards UV irradiation (Kerb et al. 1997) and GSTT1 gene deletion is much more frequent in young subjects than in centenarians (Gaspari et al. 2003).

A possible explanation of the over-expression of these two anti oxidant enzymes could be due to the increase of the concentration of ROS, with age likely subsequent to the accumulation of defective mitochondria (Meewes et al. 2000; Shigenaga et al. 1994).

We observed also an increased mRNA level of TNFRSF1 A, encoding *tumor necrosis factor receptor superfamily 1A*. TNFRSF1A is widely expressed and appears to be the major receptor for soluble TNF-induced signaling (Grell et al. 1995). The intracellular domain of TNFRSF1A contains a death domain motif, involved in TNF-induced apoptosis via activation of caspases. TNF-alpha induces apoptosis much more efficiently in human senescent than in non senescent fibroblasts (Baud and Karin 2001). Although TNFRSF1A is not reported in other gene profiling studies related to skin aging, its up-regula-tion in aged skin might be consistent with a higher sensitivity of senescent cells to apoptosis induced by TNF-alpha.

Changes of mRNA profiles in the in vitro equivalent dermis after exposure to UVB

Skin fibroblasts were cultivated in collagen lattices as described in "Materials and methods" section in order to mimic skin structure. Contraction of lattices was prevented by a braided inox steel thread placed at the inner perimeter of the dish (Fig. 1a). H&E-stained paraffin sections show a typical morphology of dermal tissue composed essentially of extracellular collagen with embedded fibroblasts (Fig. 1b).

A dose of UVB with maximum 20% mortality was determined in order to allow repeated exposures of these lattices to UVB. We set up a protocol for four exposures, with two exposures per day. Cell viability was evaluated by MTT test at 24 h after the last exposure to UVB (Fig. 1c). The dose used throughout this study was 300 mJ/cm^2 twice a day for 2 days.

Low density cDNA microarray experiments and data analysis

Total RNA was extracted at 72 h after UVB treatment to avoid early changes in gene expression. About 250 ng RNA were amplified and retrotranscribed as described in the Materials and Methods. All cDNA labeled samples were hybridized in triplicates. Experiments were performed in triplicates. Reproducible hybridization patterns were obtained. Scanning, normalization, statistical treatment and data mining followed previously established procedures.

All test conditions were compared with the corresponding control and each of the triplicate test condition was compared with each of the triplicate controls. Thus, for each couple of experiments and for each gene, three values $\log_2 \left(\frac{E_{uvb}}{E_{ctl}}\right)$ were determined (where E_{uvb} is the expressionof the gene reconstituted tissues exposed to UVB, and E_{ctl} its expression for control tissues). Out of the 240 genes represented on the arrays, we could only take into account 120 genes. Indeed, only 120 genes showed a higher expression than background for at least one of the experimental conditions.

To verify the expression data generated with the microarray, we used quantitative real time RT-PCR. The data obtained with the DualChip Human Aging and real time RT-PCR were compared for five transcripts chosen among the transcripts displaying differential expression but not necessarily passing the statistical test: $p21^{WAF-1}$ CTGF, Osteo, MMP1 and COL1A1. The level of up- or down-regulation was comparable with both techniques for all tested genes (Table 2C).

We performed a significance analysis of micro-arrays (SAM) test statistic in order to select genes that were differentially expressed in a significant manner in tissues exposed to UVB compared to control tissues. Only 6 transcripts passed the SAM tests (false positive < 0.05) and are listed in Table 2B. The high variability observed between all the triplicates can partially be explained by the higher variability of the cells response in vivo.

Interestingly, five of the six transcripts passing the statistical test were transcripts playing a role in the remodeling of extracellular matrix: *metalloproteinase 3* (*MMP3*) was up-regulated and *collagen III-alpha* (*COL3A1*), *collagen I-alpha* (*COLIAI*), *Connective tissue growth factor* (*CTGF*) and *fibromodulin* (*FMOD*) were down-regulated after exposures to UVB.

FMOD is an extracellular matrix protein normally produced by collagen rich tissues and is involved in a variety of adhesion processes of connective tissue, and can bind to collagen. It was already shown as highly down-regulated in post-mitotic fibroblasts (Petri et al. 1999), although induced by pro-lethal doses of UV (Bevilacqua et al. 2005). FMOD is decreased in premature senescence induced by repeated sublethal exposure to UVB of skin fibroblasts cultivated in monolayers (Debacq-Chainiaux et al. 2005).

UVB-induced senescence was also shown to stimulate the expression of matrix degrading metallo-proteinases (Debacq-Chainiaux et al. 2005). Here, we found a marked up-regulation of MMP3, already known as a major contributor to skin photoaging process (Brenneisen et al. 1996; Fisher et al. 1996).

Type I and type III collagens (COL) are the major collagens of skin connective tissue and were down-regulated after exposures to UVB. UV irradiations inhibit collagen synthesis (Brenneisen et al. 2002; Rittie and Fisher 2002). Both collagens COLIAI and COL3A1 were already found as down-regulated after psoralen plus UVA-induced premature senescence of skin fibroblasts (Borlon et al. 2007a).

Connective tissue growth factor (*CTGF*) is a proadhesive matricellular protein that plays an essential role in the formation of blood vessels, bone, and connective tissue (Leask and Abraham 2003). This mitogenic factor is potently induced by TGF-β1. It has been hypothesized that CTGF mediates several of the downstream actions of TGF-β1 (Kim et al. 2004). CTGF has been reported to be inhibited after UVB irradiations (Quan et al. 2002a). Moreover, UV-induced reduction of CTGF expression may contribute to reduced procollagen synthesis observed in UV-irradiated human skin fibroblasts.

Lastly, *Inhibitor of DNA binding I* (*IDI*), was up-regulated after UVB exposures, and can also be linked to the TGF signaling pathway. In fact, ID proteins are important parts of signaling pathways in senescence-related growth arrest (Norton 2000). ID1 prevents basic helix-loop-helix transcription factors from binding to DNA (Norton 2000). ID1 is induced after exposure to UVB (Zhang and Rosdahl 2003) in early melanomas (Polsky et al. 2001).

None of these genes were commonly overexpressed by UVB treatment and in dermis of old donors, which suggests in part that the age-specific changes were not induced by long term response to UVB.

DISCUSSION

In this work, we wished to know whether in vitro senescence-related genes were also differentially induced in skin biopsies from old human dermis. For this purpose, we used a low density senescence-specific DNA-array and compared, on one hand, dermis from old versus young donors, and on the other hand, skin fibroblasts

cultivated in collagen lattices 3 days after exposures to UVB or not. This double comparison aimed at excluding or discriming cases where gene expression level would change similarly in both approaches.

A high density microarray analysis recently performed on human full-skin biopsies (epidermis and dermis) revealed that the aging process in the human skin is connected with the deregulation of various cellular process like the cell cycle control, cytoskeletal changes, inflammatory response, signaling and metabolism (Lener et al. 2006). This work on dermis from young and old human donors, suggests an up-regulation of SM22 (transgelin) and DPT (dermato-pontin) in old tissues. Aging affects a variety of genes involved in cell adhesion or the extracellular matrix, as flat and large cell shape is a well known characteristic of replicatively senescent cells. The up-regulation of GPX and GSTT1 confirms an alteration of oxidative level in skin cells with aging, that might be due to an increase of oxidized substrates. Anyway, among the genes with increased mRNA levels in old tissue, TGF-β1 is probably the most interesting: it induces the appearance of biomarkers of senescence in prematurely senescent fibroblasts like the over-expression of SM22. GPX is up-regulated in prematurely senescent human fibroblasts after exposure to sublethal oxidant stress (Debacq-Chainiaux et al. 2005; Frippiat et al. 2001, 2002; Pascal et al. 2005). In this context, the most interesting genes are TGF-β1, SM22 and GPX which are upregulated in replicative senescence, premature senescence and dermis biopsies of aged donors. These results seem to reinforce the hypothesis that oxidative stress plays a significant role in in vivo aging of the skin, and suggest that the presence of ROS in the skin might not only result from exposure to UV, but maybe also from intrinsic causes like accumulation of defective mitochondria with aging or other UV-independent environmental stress (e.g. food, pollution).

On the other hand, photo-aging of the skin in vivo is a complex biological process affecting the dermis (Ma et al. 2001; Scharffetter-Kochanek et al. 2000; Wlaschek et al. 2001). Skin fibroblasts cultivated in collagen matrix before exposure to UVB, mimic these major changes of the dermis with a down-regulation of COL1A1 and COL3A1, CTGF and FMOD and up regulation of MMP3. None of the changes found in vivo in the dermis were found after the exposures of equivalent dermis to UVB, excluding at least an obvious connection between these changes and exposure to UVB. Since the skin biopsies were obtained from unexposed regions of the body, this absence of overlap is a good sign in favor of this work.

UVB and UVA generate oxidative stress in skin via interaction with cellular chromophores and photosensitizers, resulting in transient and permanent genetic damage and in the activation of cytoplasmic signal transduction pathways, and thus changes in gene expression related to growth, differentiation, replicative senescence and connective tissue degradation. ROS play a substantial role in the collagen metabolism. They not only directly destroy interstitial collagen, but also inactivate tissue inhibitors of metalloproteases and induce the synthesis and activation of metalloproteases, confirmed here by an up-regulation of the MMP3.

In conclusion, this preliminary study constitutes a proof-of-concept of larger studies not only with senescence-related genes, but also addressing the whole transcriptome, where any avenues should be used for subtracting changes in gene expression due to various types of exposures (pollution, tobacco smoke, UVA, UVB, etc.) in different in vitro or animals models from the changes found when comparing a much greater number of dermis from young and old human subjects.

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