ELSEVIER

Biochemical Pharmacology 64 (2002) 1011–1017

Biochemical Pharmacology

Stress-induced premature senescence and replicative senescence are different phenotypes, proteomic evidence

Jean-François Dierick[a,b], François Eliaers[a], José Remacle[a], Martine Raes[a], Stephen J. Fey[b], Peter Mose Larsen[b], Olivier Toussaint[a,*]

[a]*Laboratoire de Biochimie et Biologie Cellulaire, University of Namur (FUNDP)-URBC, Rue de Bruxelles, 61 B-5000 Namur, Belgium*
[b]*Center for Proteome Analysis, Forskerparken 10B, DK-5230 Odense M, Denmark*

Received 31 January 2002; accepted 22 April 2002

Abstract

In this paper, we illustrate how a proteomic analysis can be useful to approach complex biological problems, in this case the concept of stress-induced premature senescence (SIPS). According to the stochastic theories of ageing, damage that accumulate with time in the cellular components are responsible for cellular ageing. As a corollary, some sort of premature senescence should appear if the damage level is artificially increased due to the presence of stressing agents at subcytotoxic level. It has been shown, in several different models, that at a long-term after subcytotoxic stresses of many different natures, human diploid fibroblasts (HDFs) display biomarkers of replicative senescence (RS), which led to the concept of SIPS as compared to telomere-dependent RS. We compared RS and SIPS of HDFs by proteome analysis. SIPS was induced by two very different stressors: *tert*-butyhydroperoxide or ethanol. First, only a part of the protein expression changes observed in RS were also observed in SIPS. Second, HDFs in SIPS show changes specific either to the long-term effects of *t*-BHP or ethanol or independent of the nature of the stress. These changes have been termed ''molecular scars'' of subcytotoxic stresses. This work is also an excellent opportunity to discuss on important methodological issue in proteomics: the absolute requirement to start from reliable and reproducible models, which was the case in this study. We also focus on the data handling and statistical analysis allowing to use two-dimensional gel electrophoresis patterns in a semi-quantitative analysis.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Ageing; SIPS; Proteomics; Stress; 2DGE; Theories; Fibroblasts

1. Introduction

A proteome is the entire protein complement expressed by a genome at a given time and under given conditions. Today, the analysis of proteomes generally involves two major technologies: two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS). 2DGE is, up to now, the best technique to analyse the protein expression of a cell or tissue type since it allows to detect and quantify, not all but at least a representative part of the proteins expressed by a cell or tissue type at a given time. 2DGE is already effective since 1970s and had always been improved since then [1]. A major breakthrough has been achieved when mass spectrometric identification of 2DGE separated proteins became possible [2,3]. Today, thanks to improvements in MS, automation and bioinformatics, massive identification of proteins from 2DGE is routinely performed. This technical revolution gave the biologists access to the proteome, which led to the emergence of the concept and definition of proteome first used at the Siena 2D electrophoresis meeting in 1994 and later published in 1996 [4].

Since this definition was established, the number of studies using proteome analysis has increased exponentially over years. Most of proteomic studies are aimed at identifying and/or characterising proteins involved in many different biological problems such as pathologies, cellular differentiation, toxicology, etc. In this study we present an example where 2DGE alone is used to gather valuable information about a complex biological process without posterior mass spectrometric identification of proteins. We will present a study where 2DGE was used to compare the protein expression changes observed in RS and in SIPS, which were commonly thought to be very similar phenotypes. The results will be also discussed in regard to the theories of ageing, to stimulate thoughts about RS and SIPS.

* Corresponding author. Tel.: +32-81-72-41-32; fax: +32-81-72-41-35.
E-mail address: olivier.toussaint@fundp.ac.be (O. Toussaint).*URL:* http://www.fundp.ac.be/urbc/.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0006-2952(02)01171-1

2. From replicative senescence to stress-induced premature senescence

The concept of RS comes from the observations by Hayflick and Moorhead that normal HDFs cultivated *in vitro* irreversibly stop dividing after a certain number of cumulative population doublings (CPDs) [5]. This limited mitotic life span has been observed in many other eukaryotic cell types and has been interpreted as a manifestation of cellular ageing. In addition to the irreversible growth arrest, senescent HDFs present several other typical features when compared to "young" HDFs: a typical senescent morphology, lipofuscin accumulation, changes in the activity of several enzymes [6,7], presence of a senescence-associated β-galactosidase activity (SA β-gal) [8], changes in the expression level of many genes [9], etc. (For a review see [10]).

Several theories were developed to explain the occurrence of RS. The most successful concepts were the "generalised error," [11] "the critical threshold of error accumulation" [12] and the "global failure of maintenance." [13] According to these very similar concepts, random modifications appear in the cellular environment with time leading to random damages in the cellular components. These damaged cellular components are not completely eliminated or repaired and therefore accumulate with time and progressively impair the cellular functions. Once a critical level of damage is reached the cells die. Another very popular theory, the theory of free radicals, was proposed at the end of the 1950s and postulated that reactive oxygen species (ROS), at that time "oxygen free radicals," were the major agents responsible for the oxidation of cellular components [14], and were responsible for cellular ageing.

As a corollary of this theory, cells should undergo premature senescence in conditions of artificially increased ROS level. Experimental models have been set up to test this theory. HDFs at early CPDs where exposed to subcytotoxic doses of different stressing agents such as H_2O_2, *tert*-butylhydroperoxide (*t*-BHP), ethanol, UV irradiations, strong electromagnetic fields, hyperoxia, etc. [15]. At a long-term (2–3 days) after subcytotoxic stress, the HDFs display a typical senescent morphology and are postmitotic. Several other biomarkers of RS have been found at a long-term after subcytotoxic stress: irreversible growth arrest at the G1/S phase of the cell cycle due to overexpression of several cyclin-dependent kinase inhibitors such as $p21^{waf\text{-}1}$ and hypophosphorylation of the retinoblastoma protein, telomere shortening, appearance of the SA β-gal activity, mitochondrial DNA deletions, etc. [16–21]. Changes in expression level of a limited number of genes found in RS were also observed at a long-term after subcytotoxic stresses [18,21,22]. This long-term response to subcytotoxic stress was named SIPS [23]. This phrase is based on a definition of RS that encompasses the notion that oxidative stress participates in senescence. This phrase could be "stress-induced senescence-like phenotype" if one reduces the definition of senescence to a phenomenon due to telomere shortening only. Different cell types such as foetal lung and adult skin HDFs, endothelial cells and melanocytes have been reported to undergo SIPS (for reviews see [15,23]).

3. Is SIPS identical to RS?

From the observations cited above, the simplest hypothesis was that long-term effects of subcytotoxic stresses included induction of RS. However, this hypothesis was biased since all the initial studies were aimed at checking whether biomarkers of RS would also appear in SIPS. To get rid of this hypothesis it was necessary to compare HDFs in RS or in SIPS more globally. 2DGE was an obvious way to do so at the functional level of proteins, even if all cellular proteins species would not be visualised on 2D gels. SIPS was induced in WI-38 HDFs by two different stressors at subcytotoxic concentration: ethanol and *t*-BHP.

3.1. *t*-BHP and ethanol-induced SIPS

t-BHP is an organic hydroperoxide that generates ROS [24]. In order to induce SIPS, HDFs at early CPDs were exposed every day to 30 μM *t*-BHP for 1 hr, and this for 5 days, representing subcytotoxic conditions. Stresses were performed in culture medium plus serum to remain close to physiological conditions. After each stress, the HDFs were rinsed and given fresh culture medium plus serum. At 72 hr after the five subcytotoxic *t*-BHP stresses, many biomarkers of RS were induced such as a 4-fold increase in the proportions of HDFs positive for SA β-gal activity [18].

Ethanol fluidifies biological membranes [25], and thereby modifies many biological parameters such as ion fluxes and induces conformational changes in macromolecules. It can also provoke a peroxidative stress in different cell types such as hepatocytes and gastric epithelial cells, which express ROS-generating ethanol detoxification systems [26]. To our knowledge, no data were published on peroxidation induced by ethanol in HDFs. SIPS was induced by a daily 2 hr exposure to 5% ethanol, for 5 days. Cell stress, cell rinsing and culture conditions were the same as those used to obtain *t*-BHP-induced SIPS. Previous studies have shown that repeated subcytotoxic exposures to ethanol induce the appearance of biomarkers of RS at a long-term [27].

The models of SIPS analysed are reliable since reproducible results are obtained from experiment to experiment achieved under monitoring of a few biomarkers of senescence. This is of crucial importance when starting a proteome analysis. In order to fulfil these reproducibility requirements, six protein extracts have been produced for each of the five experimental conditions tested. Moreover the six samples per experimental condition have been

produced from two independent labelling experiments performed at 1 year of interval. Different batches of serum and media and different vials of frozen HDFs were used in these two labelling experiments.

4. Strategy for proteome analysis

We analysed the proteomes of HDFs in five *experimental conditions*. HDFs under 60% of their proliferative life span (young HDFs). HDFs over 95% of their proliferative life span (senescent HDFs). HDFs under 60% of their proliferative life span exposed to five stresses under *t*-BHP or ethanol, in the conditions previously described as inducing SIPS. Lastly, control HDFs under 60% of their proliferative life span were treated like the stressed HDFs but the stressing molecules were omitted.

[^{35}S]-Methionine protein labelling was chosen first in order to get the most quantitative results when comparing the protein expression between the different experimental conditions. Indeed, the use of radioactive isotopes avoids a lot of quantification bias encountered when chemical protein labelling or protein staining is used. Moreover combined with phosphorimaging, the use of radioisotopes allows quantification along a very wide linear dynamic range compared to other detection methods and is also less easy to saturate. Second, protein labelling was started 48 hr after the last stress or trypsination and lasted for 20 hr. It was hypothesised on a reasonable basis that, at 48 hr after the last stress, the immediate stress response is over and that only the long-term expression changes participating in the establishment and/or the maintenance of the SIPS phenotype would be observed.

Table 1
Design of a proteomic analysis aimed at comparing RS and SIPS

Experimental conditions	Labelling experiment A (sample)	Labelling experiment B (sample)	Sample
Young	3	3	6
RS	3	3	6
SIPS–ethanol	3	3	6
SIPS–*t*-BHP	3	3	6
Control	3	3	6
Total number of samples			30

Two gels per sample (acidic and basic domain). Total number of gels: 60.

The 2DGE procedure used is based on two 2D gel systems: isoelectric focusing (IEF) and non-equilibrium pH gradient electrophoresis (NEpHGE) in order to separate proteins with the broadest isoelectric point range as possible. IEF separates the proteins in a pH gradient going from 3.5 to 7.0 and NEpHGE from 6.5 to 11.0 [28]. For each of the six protein extracts produced for each of the five experimental conditions studied, one IEF and one NEpGHE 2DGE were run, thus representing a total of 60 2DGE (Table 1). The images obtained after digitalisation of the 2D patterns where analysed using the Bio-Image system. An example of the images obtained is presented in Fig. 1.

4.1. Analysis of the 2DGE patterns

Using this procedure for 2DGE, 1122 spots have been located on the IEF side and 697 on the NEpHGE side, representing a total of 1819 spots located and quantified for each sample. For each spot the integrated intensity (I.I.) was classically calculated as the intensity of this particular

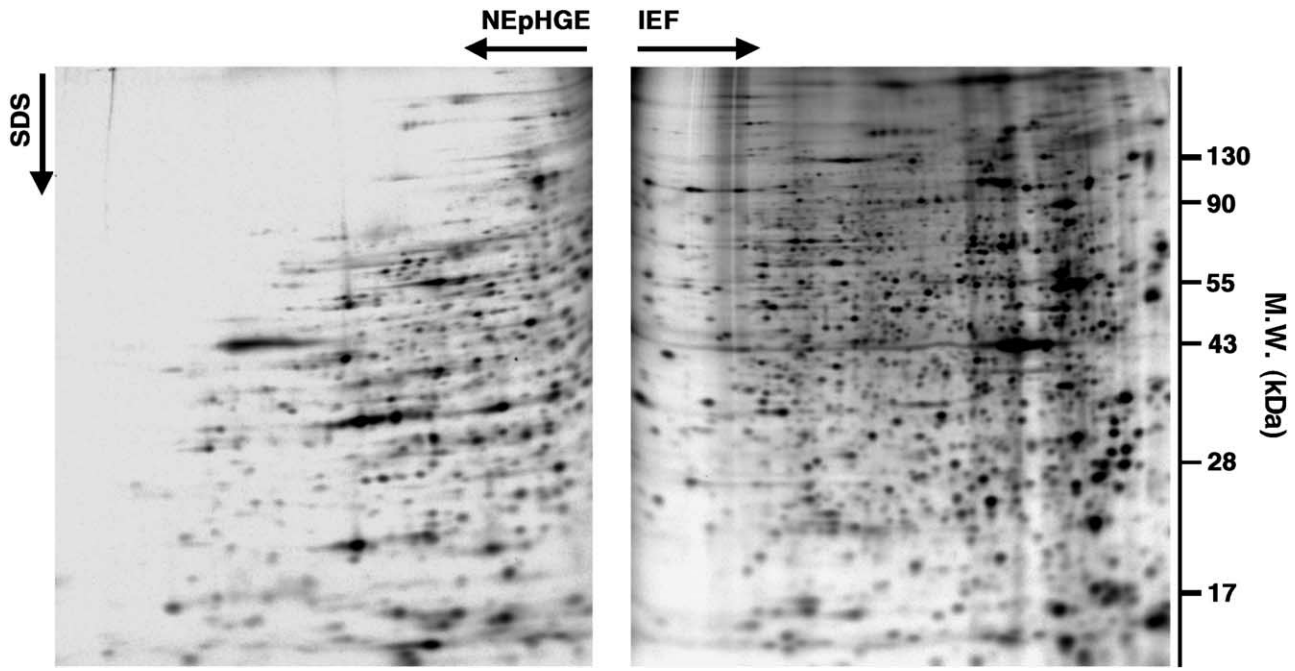

Fig. 1. Typical 2DGE pattern obtained from young WI-38 HDFs.

spot divided by the sum of the intensities of all the spots located on the same gel, expressed in percents. The three steps of the procedure for the selection of the spots presenting a significantly different I.I. between two experimental conditions is recapitulated in Fig. 2. First step, the selection of spots presenting a significant variation of I.I. between two situations was performed by a Student's

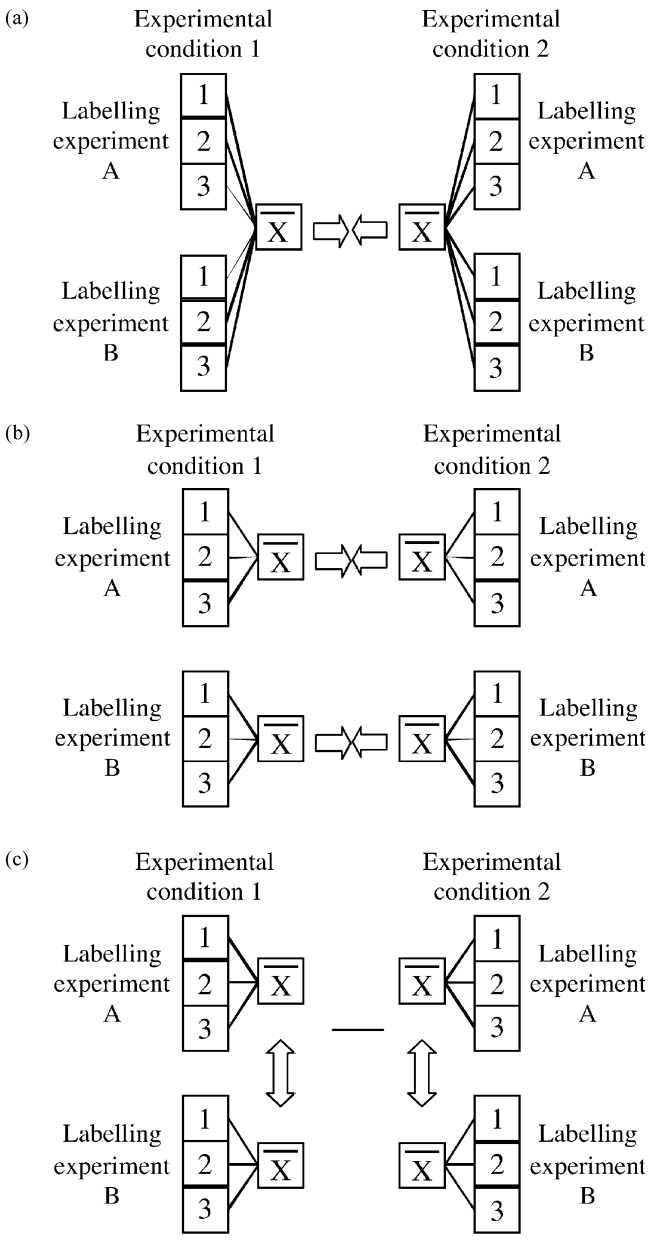

Fig. 2. Representation of the procedure used for the selection of spots presenting a significantly different I.I. between two experimental conditions (1 and 2). The six values of I.I. for each spot in each experimental condition are grouped by triplicates produced in two independent labelling experiments (A and B). According to the steps of the selection procedure, $\overline{X}$ represents mean values either of three values of I.I. for each spot as obtained after labelling experiment A or B, or of the six values of I.I. (labelling experiments A + B). According to the class of data compared, ⇨⇦ and ⇔ indicate that these values must be respectively significantly different or not according to the Student 's *t*-test, for a spot to be selected as presenting a significant difference in I.I.

t-test ($P < 0.05$ and $N = 6$). Among the *t*-test selected spots we kept those presenting a minimum of 30% of variation of I.I. between the two experimental conditions (Fig. 2a). Second step, a complementary control was performed in order to check the reproducibility of the selected changes: the variation of I.I. observed between two experimental conditions for each set of triplicates coming from the two independent labelling experiments had to be significant in each independent labelling experiment (Fig. 2b). Third step, a spot was dismissed if there was a significant difference in I.I. between the samples of the same experimental condition but coming from one of the two independent experiments (Fig. 2c). Moreover, in the case of the *t*-BHP- or ethanol-treated HDFs, we selected a spot when its I.I. was significantly different when compared both to "young" HDFs *and* to control HDFs. This double comparison was used to ensure that the variation in I.I. was due to the treatment and not to the multiple medium replacements taking place during the period of repeated stress.

5. Materials and methods for 2DGE

5.1. Preparation and labelling of the protein extracts

At 50 hr of recovery in DMEM culture medium + 10% FCS after the end of the last stress (stressed HDFs and control HDFs) or the last subculture (HDFs at early or late CPDs), the HDFs were incubated for 20 hr with 150 μCi [^{35}S]-methionine (Amersham) per well in 300 μL DMEM/F12 (methionine-free, Gibco) culture medium supplemented with 10% dialysed FCS and 1 μg/mL of cold methionine (Sigma, St. Louis, MO, USA). They were washed twice with HBSS buffer and lysed with a 9.5 mol/L urea buffer (Pharmacia), 2% (w/v) Nonidet P-40 (Sigma), 5% (v/v) β-mercaptoethanol (Fluka) and 2% (v/v) ampholytes pH 7–9 buffer (Pharmacia).

5.2. High resolution two-dimensional gel electrophoresis (2DGE)

We used a modified version of the procedure described by Byrjalsen *et al*. [28]. First dimension electrophoreses were carried out in capillary glass containing 4% (w/v) acrylamide (Pharmacia), 2% Nonidet P-40, 9.5 mol/L urea and ampholytes creating a continuous pH gradient from 3.5 to 7.0 for IEF gels and from 6.5 to 11.0 for non-equilibrium pH gradient electrophoresis (NEpHGE) gels. The 2D SDS PAGE (12.5% acrylamide) were performed in slab gels. Analytical gels were performed with an amount of lysate corresponding to 2×10^6 cpm loaded per gel.

After electrophoresis, the gels were fixed in 45% methanol and 7.5% acetic acid for 45 min, soaked for 45 min in Amplify (Amersham International), dried and exposed to ADC-cassettes (Agfa-Gevaert NV) at room temperature

for 4 days before image acquisition (Agfa Diagnostic Centre, Agfa-Gevaert NV). The images were analysed using the Bio-Image system (B.I. Systems Corporation).

6. Results of the analysis

The spots selected as presenting a significantly different I.I. between the different experimental conditions studied were sorted according to their pattern of expression change. Six classes of spots, used throughout this paper, were defined (Fig. 3).

Among the 1819 analysed spots:

- 50 spots presented a significantly different I.I. between young and senescent HDFs;
- 25 between HDFs in ethanol-induced SIPS and young + control HDFs;
- 13 between *t*-BHP-induced SIPS and young + control HDFs.

This represents a total of 88 variations corresponding to 64 different spots, since some spots presented variations in I.I. in more than one condition. A representative example of spots variation is presented in Fig. 4.

Six spots where selected in all three situations studied (RS and SIPS induced by ethanol or *t*-BHP). These were called "class I" spots. Six other spots presented variations in I.I. in RS and in SIPS induced by ethanol (but not by *t*-BHP): these are the "class II" spots. Six spots showed variation in I.I. in ethanol- and *t*-BHP-induced SIPS (but not in RS). These are the "class III" spots. No changes were common to RS and SIPS induced by *t*-BHP. The remaining changes were specific to only one situation: these are the "class IV," "class V" and "class VI" spots (Fig. 3).

7. What can be concluded from such a proteomic analysis

The main question raised by this work was to know whether all the changes observed in RS would be also observed in SIPS.

As shown in Fig. 3, only a part of the 50 changes in I.I. observed in RS were also present in SIPS. Indeed, out of these 50 changes selected in RS, only 12 changes (classes I and II) were present in RS and ethanol-induced SIPS while only 6 changes (class I) were present in *t*-BHP-induced SIPS (these 6 changes were also observed in ethanol-induced SIPS).

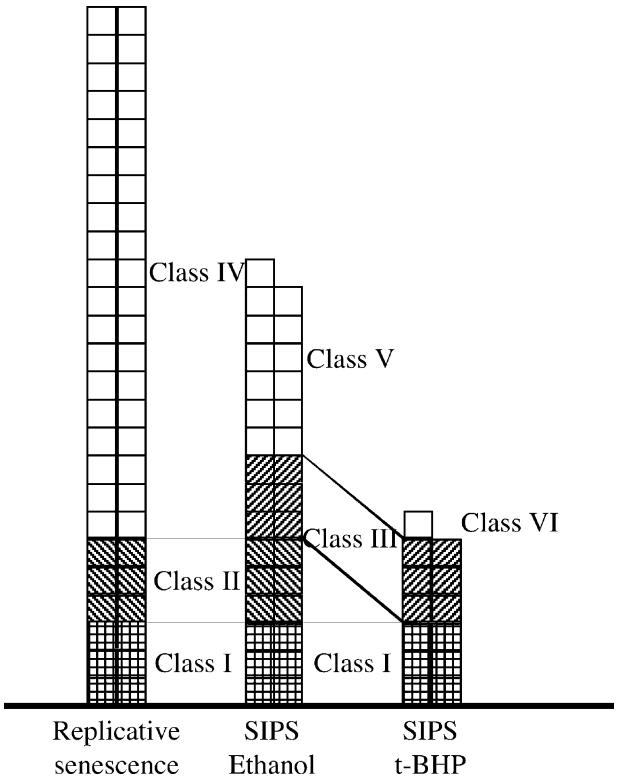

Fig. 3. Representation of the pattern of variation of the spots presenting a significantly different I.I. between the different experimental conditions. Each square represents a variation observed in the indicated situation. Class I, the spots which underwent I.I. change in all three situations; class II, the spots with I.I. change in RS and in ethanol-induced SIPS; class III, the spots with I.I. change in ethanol- and *t*-BHP-induced SIPS; class IV–VI concern spots with I.I. change only in, respectively, RS, ethanol-induced SIPS and *t*-BHP-induced SIPS.

These results mean at least that RS and SIPS are different phenotypes at the level of protein expression, although they share a limited number of other biomarkers like morphology, SA β-gal activity, etc.

7.1. Molecular scars

The changes specific to one type of stress ("class V" and "class VI" spots) represent long-term biomarkers of the exposure to this typical stress, here ethanol or *t*-BHP. They

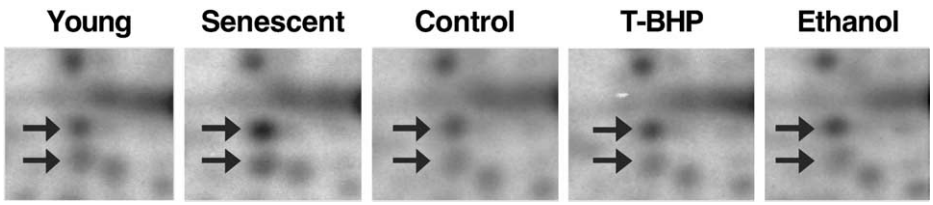

Fig. 4. Example of variation observed on a representative gel in each condition studied. The two spots indicated by the arrows: I500 (top) and I502 (below) present significative change in I.I. in senescent HDFs when compared to young HDFs but not in *t*-BHP or ethanol-induced SIPS.

can be considered as ''molecular scars'' of the long-term effects of these stresses [23]. Class II spots suggest there exist biomarkers common to the long-term effects of different stresses. Such pioneering studies on molecular scars, i.e. stable changes in protein expression occurring on the long-term after subcytotoxic stress, could be extended to other kinds of agents at subcytotoxic concentration, in order to know if these molecules have potential long-term harmful effects.

7.2. Theoretical interpretation of the proteomic results

The theory of stability of far from equilibrium open systems applied to cellular ageing allowed to predict how the random modifications in the cellular components could affect cellular ageing, depending on a balance between the level of damage and the kinetics of generation of damage, on one hand, and the cellular potential for efficient stress response, on the other hand [29]. With time passing and damage accumulation, the cell pass through different steady states, each characterised by a certain stability which is expressed in thermodynamic terms as a minimum of entropy production. In response to stress, cells try to eliminate the cause of the stress and to repair the damage generated. If the stress is of very high intensity, cells die. If the stress is of low intensity, cells perfectly cope with it. There will not be any long-term modification when the immediate response is over. However, if the stress is of higher intensity, remaining in the subcytotoxic range, cells will not be able to repair all the damage caused by the stress. According to the theory of stability of far from equilibrium open systems, cells will either drop to a lower steady state of internal entropy production (=lower metabolic activity) than before the stress and will undergo some sort of accelerated ageing, or will die.

All the genes which undergo senescence-induced changes in their level of expression must not necessarily undergo similar changes in SIPS or *vice versa*. Only a subset of genes which undergo senescence-specific expression changes, as observed in this study, could be sufficient to trigger SIPS. Other subsets of genes could undergo long-term stress-specific changes, as also observed in this work. Therefore, it could be that a global sum of changes in gene expression determines the fate of a cell towards normal or stress-induced senescence, and not individual changes. Cellular senescence would not occur as long as adaptation in the expression of defence systems maintains a young phenotype in spite of a moderate accumulation of damage. This might explain why the expression level of some defence systems may still be high in aged biological systems, as recently shown *in vivo* for the ageing mice where DNA repair systems and antioxidant systems were shown to be highly expressed [30]. These defence systems could also remain overexpressed due to an acceleration in the kinetics of damage generation in senescent cells.

8. Conclusions and perspectives

The lessons from this multi-comparative 2DGE study are numerous. It confirms the importance of several methodological aspects. First, the necessity to start from reliable biological models, as it was the case in this study. Second, the choice of the protein detection and quantification method in 2DGE is also important as well as the of the statistical analysis of the 2DGE patterns in order to get semi-quantitative results.

It shows also that in some cases and when used correctly 2DGE allow to gather valuable information about complex biological processes, even without posterior mass spectrometric identification of proteins.

At a more fundamental point of view, we showed for the first time that RS and SIPS are different at the level of protein expression, even if they share similar phenotypic features. Moreover, HDFs in SIPS display protein expression changes which can be classified as follows: first, the changes common with RS, second the changes specific to each kind of stress and, third the changes related to SIPS independently of the nature of the stress applied. The last two types of protein expression changes have been defined as ''molecular scars'' of subcytotoxic stresses.

As perspectives, it would be interesting to perform similar studies with a system allowing to analyse more protein species. This is possible today by the combination of multiple narrow pH ranges for the first dimension gels and/or by cell fractionation prior to protein expression analysis. Mass spectrometric protein identification is the next step in such analysis in order to understand the roles of the proteins undergoing expression changes in the establishment of RS and/or SIPS. Furthermore, similar studies could be undertaken at the mRNA level using differential display RT-PCR, SAGE expression analysis or DNA arrays.

Acknowledgments

J.-F. Dierick is a FRIA Fellow, Belgium. O. Toussaint is a Research Associate of the FNRS, Belgium. We thank the European Union, 5th Framework Programme, Quality of Life, R&D, 'Functionage' Project (QLK6-CT-2001-00310), and the Région Wallonne 'Modelage' Project.

References

[1] O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250:4007–21.
[2] Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci USA 1993;90:5011–5.
[3] Mann M, Wilm M. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal Chem 1994;66:4390–9.

[4] Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez J-C, Jan JX, Gouley AA, Humphrey-Smith I, Williams KL, Hochstrasser EF. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. BioTechnology 1996;14:61–5.
[5] Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strain. Exp Cell Res 1961;25:585–621.
[6] Houben A, Raes M, Houbion A, Remacle J. Alteration of enzymes in ageing human fibroblasts in culture. I. Conditions for the appearance of an alteration in glucose 6-phosphate dehydrogenase. Mech Ageing Dev 1984;25:23–34.
[7] Houben A, Raes M, Houbion A, Remacle J. Alteration of enzymes in ageing human fibroblasts in culture. II. Conditions for the reversibility and the mechanism of the alteration of glucose 6-phosphate dehydrogenase. Mech Ageing Dev 1984;25:35–45.
[8] Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubeli I, Pereira-Smith O, Peacocke M, Campisi J. A biomarker that identifies senescent human cells in culture and in ageing skin *in vivo*. Proc Natl Acad Sci USA 1995;92:9363–7.
[9] Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. Curr Biol 1999;9:939–45.
[10] Campisi J. Replicative senescence and immortalization. In: Stein GS, Baserga A, Giordano A, Denherdt DT, editors. The molecular basis of cell cycle and growth control. New York: Wiley, 1999. p. 348–73.
[11] Orgel LE. Ageing of clones of mammalian cells. Nature 1973;243:441–5.
[12] Remacle J, Lambert D, Raes M, Pigeolet E, Michiels C, Toussaint O. Importance of various antioxidant enzymes for cell stability. Confrontation between theoretical and experimental data. Biochem J 1992;286:41–6.
[13] Holliday R. Toward a biological understanding of the ageing process. Perspect Biol Med 1988;32:109–23.
[14] Adamson GM, Harman AW. A role for the glutathione peroxidase/reductase enzyme system in the protection from paracetamol toxicity in isolated mouse hepatocytes. Biochem Pharmacol 1989;38:3323–30.
[15] Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol 2000;35:927–45.
[16] Chen QM, Ames BN. Senescent-like growth arrest induced by hydrogen peroxide in human diploid fibroblasts F65 cells. Proc Natl Acad Sci USA 1994;91:4130–4.
[17] Chen QM, Tu VC, Catania J, Burton M, Toussaint O, Dilley T. Involvement of Rb family proteins, focal adhesion proteins and protein synthesis in senescent morphogenesis induced by hydrogen peroxide. J Cell Sci 2000;116:4087–97.
[18] Dumont P, Burton M, Chen QM, Gonos ES, Frippiat C, Mazarati J-B, Eliaers F, Remacle J, Toussaint O. Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. Free Rad Biol Med 2000;28:361–73.
[19] Frippiat C, Chen QM, Zdanov S, Magalhaes J, Remacle J, Toussaint O. Subcytotoxic H_2O_2 stress triggers a release of TGF-β 1 which induces biomarkers of cellular senescence of human diploid fibroblasts. J Biol Chem 2001;276:2531–7.
[20] Sitte N, Merker K, Von Zglinicki T, Grune T, Davies K. Protein oxidation and degradation during cellular senescence of human BJ fibroblasts. Part I. Effects of proliferative senescence. FASEB J 2000;14:2495–502.
[21] von Zglinicki T, Saretzki G, Docke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Exp Cell Res 1995;220:186–93.
[22] Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN. Molecular analysis of H_2O_2-induced senescence-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. Biochem J 1998;332:43–50.
[23] Brack C, Lithgow GJ, Osiewacz HD, Toussaint O. Molecular and Cellular Gerontology. EMBO J 2000;19:1929–34.
[24] Ochi T, Miyaura S. Cytotoxicity of an organic hydroperoxide and cellular antioxidant defense system against hydroperoxides in cultured mammalian cells. Toxicology 1989;55:69–82.
[25] Sanchez-Amate MC, Carrasco MP, Zurera JM, Segovia JL, Marco C. Persistence of the effects of ethanol *in vitro* on the lipid order and enzyme activities of chick-liver membranes. Eur J Pharmacol 1995;292:215–21.
[26] Mantle D, Preedy VR. Free radicals as mediators of alcohol toxicity. Adverse Drug React Toxicol Rev 1999;18:235–52.
[27] Toussaint O, Michiels C, Raes M, Remacle J. Cellular ageing and the importance of energetic factors. Exp Gerontol 1995;30:1–22.
[28] Byrjalsen I, Larsen PM, Fey SJ, Thormann L, Pedersen BJ, Christiansen C. Two-dimensional gel analysis of human endometrial proteins: cyclic changes in the expression of specific proteins during the normal menstrual cycle. Human Reprod 1995;10:13–8.
[29] Toussaint O, Raes M, Remacle J. Ageing as a multi-step process characterized by a lowering of entropy production leading the cell to a sequence of defined stages. Mech Ageing Dev 1991;61:45–64.
[30] Lee CL, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of ageing and its retardation by caloric restriction. Science 1999;285:1390–3.