Biochimica et Biophysica Acta 1842 (2014) 116–125

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

# Decreased cell proliferation and higher oxidative stress in fibroblasts from Down Syndrome fetuses. Preliminary study

Amparo Gimeno [a,e], José Luis García-Giménez [b,e], Laura Audí [b,c], Nuria Toran [b,d], Pilar Andaluz [b,c], Francisco Dasí [e], José Viña [f], Federico V. Pallardó [b,e,f,*]

[a] Department of Anatomy and Human Embryology, Faculty of Medicine, University of Valencia, Av/Blasco Ibáñez, 15, E46010 Valencia, Spain
[b] CIBERER, The Biomedical Network Research Centre for Rare Diseases, Valencia, Spain
[c] Paediatric Endocrinology Research Unit, Hospital Vall d'Hebron, Autonomous University, Barcelona, Spain
[d] Pathology Department, Hospital Vall d'Hebron, Autonomous University, Barcelona, Spain
[e] Fundación Investigación Hospital Clínico Universitario de Valencia, Instituto de Investigación INCLIVA, Avda. Blasco Ibáñez 17, E46010 Valencia, Spain
[f] Department of Physiology, Faculty of Medicine, University of Valencia, Av/Blasco Ibáñez, 15, 46010 Valencia, Spain

ARTICLE INFO

*Article history:*
Received 6 May 2013
Received in revised form 25 October 2013
Accepted 27 October 2013
Available online 31 October 2013

*Keywords:*
Glutathione
Thioredoxin
Superoxide dismutase
Progeroid
Rcan1
Telomere length

ABSTRACT

Down Syndrome is the most common chromosomal disease and is also known for its decreased incidence of solid tumors and its progeroid phenotype. Cellular and systemic oxidative stress has been considered as one of the Down Syndrome phenotype causes. We correlated, in a preliminary study, the fibroblast proliferation rate and different cell proliferation key regulators, like Rcan1 and the telomere length from Down Syndrome fetuses, with their oxidative stress profile and the Ribonucleic acid and protein expression of the main antioxidant enzymes together with their activity. Increased oxidized glutathione/glutathione ratio and high peroxide production were found in our cell model. These results correlated with a distorted antioxidant shield. The messenger RNA (*SOD1*) and protein levels of copper/zinc superoxide dismutase were increased together with a decreased mRNA expression and protein levels of glutathione peroxidase (GPx). As a consequence the [Cu/ZnSOD / (catalase + GPx)] activity ratio increases which explains the oxidative stress generated in the cell model. In addition, the expression of thioredoxin 1 and glutaredoxin 1 is decreased. The results obtained show a decreased antioxidant phenotype that correlates with increased levels of Regulator of calcineurin 1 and attrition of telomeres, both related to oxidative stress and cell cycle impairment. Our preliminary results may explain the proneness to a progeroid phenotype.


## 1. Introduction

Down Syndrome (DS) is the most common chromosomal disease and one of the main causes of intellectual disability. The genes localized in chromosome 21 are linked to mitochondrial energy production, reactive oxygen species metabolism, brain development, neuronal loss, and Alzheimer's type neurodegeneration [1]. Another interesting feature is that individuals with DS have a reduced risk of solid tumors in all the age groups studied [2] and a progeria-like phenotype [3].

The presence of an oxidative stress status in DS patients has been well established [4–8]. The source of the systemic oxidative stress found in DS individuals is not clear, although it is known that Cu/Zn superoxide dismutase (Cu/ZnSOD) activity is increased in DS patients [9] and the ratio Cu/ZnSOD to catalase plus glutathione peroxidase [Cu/ZnSOD / (catalase + glutathione peroxidase)] is increased [10]. The activity of the selenoenzyme GPx could be hampered by the reported lack of selenium levels observed in DS [11]. Thus more hydrogen peroxide is generated by Cu/ZnSOD than catalase and glutathione peroxidase can catabolize, giving rise to an oxidative stress positive feed-back [12]. This process generates a positive feed-back inducing mitochondrial dysfunction [13] and impairing the respiratory complex enzymes [14], which in turn would keep increasing ROS production.

It was reported that cells from DS, or animal model of DS are prone to oxidative stress and apoptosis and have lower proliferation capability [15–19]. This finding could explain, at least in part, some of the progeria-like characteristics of the DS phenotype. Previous reports from our group have emphasized the importance of glutathione (GSH) in cell proliferation [20–22].

The purpose of our study was to clarify the cause of the progeroid-like phenotype and the reported impairment in cell proliferation. Here we provide new information showing that the high oxidative stress previously reported in DS is a very early event in development that is

*Abbreviations:* DS, Down Syndrome; Cu/ZnSOD, Cu/Zn superoxide dismutase; MnSOD, Mn superoxide dismutase; GPx, glutathione peroxidase; TL, telomere length; ROS, reactive oxygen species; DHR, dihydrorhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Trx1, thioredoxin 1; Grx1, glutaredoxin 1; BrdU, bromodeoxyuridine

* Corresponding author at: Department of Physiology, Faculty of Medicine, University of Valencia, Av/Blasco Ibáñez, 15, E46010 Valencia, Spain. Tel.: +34 963864646.
*E-mail addresses:* gimenoa@uv.es (A. Gimeno), j.luis.garcia@uv.es (J.L. García-Giménez), laura.audi@vhir.org (L. Audí), ntoran@vhebron.net (N. Toran), pilar.andaluz@vhir.org (P. Andaluz), francisco.dasi@uv.es (F. Dasí), jose.vina@uv.es (J. Viña), federico.v.pallardo@uv.es (F.V. Pallardó).

0925-4439/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbadis.2013.10.014

already present in fibroblasts from human fetuses, as reported previously in amniotic fluid form women carrying DS fetuses [23] and in brain fetuses [24,25]. In addition, our findings show a decreased antioxidant shield and an increased production of free radicals. These results partially explain the attrition of the telomere and the increased expression of Rcan1.

## 2. Material and methods

### 2.1. Human dermal fibroblast cultures from Down Syndrome (DS) and control (C) fetuses

Abdominal skin biopsies from six human fetuses, products of legal terminations, were collected with informed parental consent and approved by the Ethics Committee of the Hospital, within 2 h postmortem from the Fetal Tissue Bank at the Hospital Universitari Vall d'Hebron (fetaltissuepath@vhebron.net). Three fetuses were diagnosed as having Down Syndrome (DS): one 47, XY + 21, gestational age (GA) 20 weeks (w); one 47, XY + 21, GA 16 weeks; and one 47, XY + 21, t(21;21), GA 22 weeks. Three fetuses were considered as controls (C): one 46, XX, GA 22 weeks, diagnosed with complex cardiac malformation; one 46, XY, GA 22 weeks, diagnosed with anhydramnios with previous membrane rupture and the absence of congenital anomalies; and one 46, XX, GA 14 weeks, diagnosed with occipital encephalocele.

Primary fibroblasts were obtained from skin explants in a 25-$cm^2$ plastic culture flask with Eagle's Minimal Essential Medium (MEM) supplemented with nonessential amino acids, 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin). The flasks were maintained at 37 °C in a 95% humidified air–5% $CO_2$ atmosphere. The fibroblasts were released by enzymatic digestion with trypsin and sub-cultured in a 75-$cm^2$ plastic culture flask with 12 ml of medium. The cultures were fed by changing the medium every three days. After reaching confluence, the cells were washed and the pellet resuspended in 1 ml of MEM with 10% FCS and DMSO and frozen (24 h at −80 °C and then in liquid nitrogen). The cells were thawed and reincorporated into the cultures for ensuing experiments.

The cells were subsequently cultured in DMEM (Gibco, Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Sigma-Aldrich, MO, USA) in 5% $CO_2$ in air at 37 °C at a density of 20.000 cells/$cm^2$. The sub-cultured method used was digestion with trypsin. All experiments were performed with the same number of passages: 8–10. Studies were performed at different time points in cell culture.

### 2.2. Analysis of apoptotic cells by flow cytometry

Quantitative analysis of apoptotic cell death was performed by flow cytometry using Annexin V-Alexa Fluor 488 conjugate (Molecular Probes, OR, USA). Cells from culture supernatant and adherent cells collected after trypsinization, were stained with the reactive following the manufacturer's instructions, and 1 μg/ml of propidium iodide (PI) staining at room temperature for 15 min in the dark, followed by cytometric analysis in an Epics Elite cell sorter (Coulter Electronics, LA, USA). Fluorescence emissions were recorded at 515 ± 10 nm.

### 2.3. Cellular proliferation assay

Proliferation of DS and control fibroblasts was determined with the "Cell proliferation ELISA BdrU colorimetric" (Roche, NY, USA) as per the manufacturer's recommendations. The cells were cultured in a 96 well plate for 6 h, 24 h, 48 h, and 7 days. The colorimetric final reaction was measured with the spectrophotometer spectra MAXPLUS 384 (Molecular Devices, USA) at 370 nm using as reference wavelength 492 nm, during three consecutive five-minute periods. The variation of absorbance was proportional to the incorporation of BdrU.

### 2.4. Telomere length (TL)

DNA was extracted from approximately $5 \cdot 10^5$ cells using the DNeasy Blood and Tissue Handbook (Quiagen), and then quantified using the GeneQuant pro UV/Vis (GE Healthcare, Uppsala, Sweden). TL average in fibroblasts was measured with a validated quantitative (Q-PCR) based assay [26,27]. This method measures the average ratio of the telomere repeat copy numbers to a single gene (36B4) copy numbers (T/S ratio) in each sample. All samples were measured in duplicate, and their mean was used. Each of the experiments was performed in triplicate.

Briefly, the Q-PCR technique was performed using a LightCycler thermocycler (LightCycler 480II, Roche Diagnostics, Quebec, Canada) in a 384-well format. Duplicate DNA samples were amplified in parallel: 20 μl PCR reactions included 30 ng of sample DNA, the DNA master SYBR Green I kit (LightCycler® 480 Sybr Green I Master, Roche Diagnostics, USA) and 0.5 pmol/ml of specific primers for the telomere (forward: 5′CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3′; reverse 5′GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3′) and for the 36B4 (forward: 5′CAGCAAGTGGGAAGGTGTAATCC3′; reverse: 5′CCCATTCTATCATCAACGGGTACAA3′). The thermal cycling profile for both amplicons began with a cycle at 95 °C for 15 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 1 min and 72 °C for 10 s. The specificity of all reactions was determined by melting point curve analysis using one cycle at 95 °C for 5 s, 70 °C for 1 min, and one cycle at 40 °C for 30 s.

The technique was optimized by developing standard curves using serial dilutions from a reference DNA. This method measures the average ratio of telomere repeat copy number to a single gene (36B4) copy number (T/S ratio) in each sample.

The linear correlation coefficient ($r^2$) was >0.99 for the telomeric and 36B4 standard curves. The efficiency of the amplification was calculated from the slope of the linear curves. The average efficiency was 2.048 for telomeric amplification and 1.98 for 36B4 amplification. The T/S ratio was calculated using these efficiency values: T/S ratio = $\text{efficiency}^{Cp\ 36B4}/\text{efficiency}^{CpTel}$. The coefficients of variation (CV) within duplicates of the telomere length and 36B4 assays were 2.4% and 0.9%, respectively.

### 2.5. Peroxide levels: flow cytometry analysis

Acute intracellular production of reactive oxygen species (ROS) was assessed by incubation (30 min) of cell aliquots with dihydrorhodamine (DHR), 0.7 μM. These virtually non-fluorescent probes are oxidized to a fluorescent product. Fluorescence of a single cell was measured by a flow cytometer, Epics Elite cell sorter (Coulter Electronics, LA, USA). Fluorochrome was excited with an argon laser tuned at 488 nm and emission at 515 ± 10 nm. Forward angle and right angle light scattering were measured. Samples were acquired for 15,000 individual cells and ROS production was quantified by mean fluorescence intensities.

### 2.6. Determination of the GSSG/GSH ratio

The GSSG and GSH concentration was measured using DetectX® Glutathione Fluorescent Detection Kit (Arbors Assay, MI, USA) and the GSSG/GSH ratio was calculated. The cells were grown to confluence in seven days. The washed cell pellet was resuspended in 5% sulfosalicylic acid (w/v), at $10^6$ cells/ml, and was lysed and deproteinized by two freeze/thaw cycles in liquid nitrogen. The measurements were performed following the manufacturer's instructions. The fluorescent product was read at 510 nm with excitation at 390 nm in the fluorometer spectra MAX GEMINIS (Molecular Devices, Sunnyvale, USA). Subsequently, we incubated the plate with NADPH and glutathione reductase to convert all oxidized glutathione, GSSG, into GSH, which reacts with the excess ThioStar® to yield the signal related to total GSH content. GSSG was calculated as GSSG = [(GSHtotal − GSHfree) / 2].