Cell Death and Differentiation (2004) 11, 962–973

www.nature.com/cdd

The different apoptotic potential of the p53 codon 72 alleles increases with age and modulates *in vivo* ischaemia-induced cell death

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Received 09.10.03; revised 29.12.03; accepted 15.1.04; published online 07.5.04
Edited by V De Laurenzi

Abstract

A common arginine to proline polymorphism is harboured at codon 72 of the human p53 gene. In this investigation, we found that fibroblasts and lymphocytes isolated from arginine allele homozygote centenarians and sexagenarians (Arg+) undergo an oxidative-stress-induced apoptosis at a higher extent than cells obtained from proline allele carriers (Pro+). At variance, the difference in apoptosis susceptibility between Arg+ and Pro+ is not significant when cells from 30-year-old people are studied. Further, we found that Arg+ and Pro+ cells from centenarians differ in the constitutive levels of p53 protein and p53/MDM2 complex, as well as in the levels of oxidative stress-induced p53/Bcl-xL complex and mitochondria-localised p53. Consistently, all these differences are less evident in cells from 30-year-old people. Finally, we investigated the *in vivo* functional relevance of the p53 codon 72 genotype in a group of old patients (66–99 years of age) affected by acute myocardial ischaemia, a clinical condition in which *in vivo* cell death occurs. We found that Arg+ patients show increased levels of Troponin I and CK-MB, two serum markers that correlate with the extent of the ischaemic damage in comparison to Pro+ patients. In conclusion, these data suggest that p53 codon 72 polymorphism contributes to a genetically determined variability in apoptotic susceptibility among old people, which has a potentially relevant role in the context of an age-related pathologic condition, such as myocardial ischaemia.

Cell Death and Differentiation (2004) **11,** 962–973.
doi:10.1038/sj.cdd.4401415
Published online 7 May 2004

Keywords: ageing; p53 codon 72 locus; oxidative stress; cell death; myocardial ischaemia

Abbreviations: <2c cells, cells with hypodiploic DNA content; ACS, acute coronary syndrome; Arg+, arginine/arginine carriers; CCU, Coronary Care Unit; CK-MB, creatine kinase, MB fraction; CRP, C-reactive protein; dc, detached cells; DFs, dermal fibroblasts; dRib, 2-deoxy-D-ribose; e.s.l., equal sample loading; HDL, high-density lipoprotein; LCLs, lymphoblastoid cells lines; NQw-MI, non-Q wave myocardial infarction; p53Arg, p53 codon 72 arginine allele; p53Pro, p53 codon 72 proline allele; PBMCs, peripheral blood mononuclear cells; PI, propidium iodide; P-p53ser 15, serine 15-phosphorylated p53; Pro+, proline/proline or proline/arginine carriers; pTet-On, tetracycline inducible plasmid; pTRE, tetracycline responsive element plasmid; Qw-MI, Q-wave myocardial infarction; S.D., standard deviation; S.E.M., standard error of the mean; UA, unstable angina; $\Delta\Psi^{dim}$ cells, cells with depolarised mitochondria

Introduction

In vitro cell death is mediated by a variety of mechanisms, either p53-dependent or independent, that are elicited by a wide range of stimuli, among which are stresses, like the exposure to free radicals, reducing sugars, and low oxygen tension.[1–3] *In vivo,* at the systemic level, these same agents play a major role in the basic mechanisms of ageing and age-related patho-physiologic processes, such as atherosclerosis.[4–7]

At the genetic levels, the data recently obtained on p66Shc null mice confirmed the existence of profound ties among the mechanisms involved in cell death, ageing, and age-related diseases. Indeed, this mouse strain is characterised by a 30% extension of lifespan,[8] a decrease in the levels of *in vivo* systemic and tissue oxidative stress and vascular apoptosis,[9] and by an abrogation of oxidative stress-induced, p53-dependent apoptosis.[8–10] Together with other literature data, these results suggest that p53-dependent mechanisms are candidates to play a crucial role in the pathogenesis of age-related diseases, such as atherosclerosis and myocardial dysfunction, that is, the major causes of morbidity and mortality among elderly people.[11–14]

In humans, the age of the donor affects the *in vitro* susceptibility to oxidative stress-induced apoptosis, but a wide interstudy and interindividual variability has been reported.[15,16] A major uncontrolled factor in these investigations is the genetic diversity among individuals. In this regard, it is known that p53 gene harbours a common sequence

variation, which yields an arginine to proline aminoacidic substitution at codon 72. When transfected in p53-null cells, the two alleles differ in the capacity to modulate apoptosis, to be targeted to the mitochondria, to be degraded by the proteasome, and to bind MDM2.[17–20]

Here we investigated the relationship between the susceptibility to undergo apoptosis and p53 codon 72 genotype in cells (blood leucocytes, dermal fibroblasts (DFs), lymphoblastoid cell lines (LCLs)) obtained from people of different ages (30-year-old, sexagenarians, and centenarians), all carefully checked for their healthy status.[21] The choice of including cells from healthy centenarians is due to the fact that, at variance with healthy sexagenarians, these people *de facto* escaped from the detrimental effects of age-related diseases. Consequently, data obtained from their cells offer the possibility to study the mechanisms of physiological (successful) *in vivo* ageing, disentangling these latter from those of age-related diseases.[21–22] 2-Deoxy-D-Ribose (dRib) was chosen as an oxidative stress-inducing agent.[23,24] This molecule belongs to a group of reducing sugars that trigger apoptosis, generating an increase in the levels of intracellular peroxide and carbonil radicals, as well as a decrease in intracellular GSH, all phenomena that can be abrogated by *N*-Acetyl-Cysteine.[25–28] dRib is in fact a relevant source for *in vivo* oxidative stress[29,30] being synthesised by thymidine phosphorylase, an enzyme whose expression is induced by hypoxia and chronic inflammation, two conditions that share in the pathogenesis of atherosclerosis and its complications.[31,32]

Since we noted that the difference in *in vitro* apoptosis susceptibility of the p53 codon 72 genotypes was evident predominately in old people (sexagenarians and centenarians), we investigated the *in vivo* relevance of the phenomenon in old subjects. Purposely, we correlated p53 codon 72 genotype with serum Troponin I and CK-MB levels (which are specific quantitative markers for the extent of the ischaemic injury) in a group of aged patients (65–99 years of age) affected by acute coronary syndrome (ACS), a clinically relevant condition determined by ischaemic cell death of the myocardial tissue.

Results

The impact of p53 codon 72 polymorphism on oxidative stress-induced apoptosis increases with age

Peripheral blood mononuclear cells (PBMCs), DFs, and LCLs were obtained from subjects previously assessed for the p53 codon 72 genotype.[33,34] Subjects were considered as follows: Pro+ (proline/proline or proline/arginine carriers) and Arg+ (arginine/arginine carriers); see Materials and methods.

To evaluate the impact of p53 codon 72 genotype on the response to dRib, we exposed PBMCs from 20 young people, 16 aged people, and 8 centenarians to 10 mM dRib treatment for 48 and 72 h. The percentage of cells with hypodiploid DNA content (<2c cells) was taken as an index of apoptosis, the percentage of cells with depolarised mitochondria ($\Delta\Psi^{dim}$ cells) was taken as an index of the mitochondrial involvement in dRib response. We found that in centenarians' PBMCs exposed to dRib, the percentages of <2c and $\Delta\Psi^{dim}$ cells were higher in Arg+ than in Pro+ samples (Figure 1a and b, right panels). Similarly, in dRib-treated PBMCs from aged people, we found higher percentages of <2c cells, but similar percentages of $\Delta\Psi^{dim}$ cells in Arg+ with respect to Pro+ samples (Figure 1a and b, middle panels). At variance, we found no significant difference in the percentages of <2c and $\Delta\Psi^{dim}$ cells between Arg+ and Pro+ in dRib-treated PBMCs from young people (Figure 1a and b, left panels).

To rule out a possible tissue-specificity of the above phenomenon, we exposed DFs from eight young people, four aged people, and six centenarians to 20 mM dRib treatment for 48 and 72 h. As an index of cell death, the percentage of <2c cells (Figure 1c) and the number of cells detached (dc) from the plastic substrate (Figure 1d) were assessed. We found higher levels of <2c cells and dc in dRib-treated Arg+ DFs with respect to Pro+ DFs from centenarians (Figure 1c and d, right panels) and aged people (Figure 1c and d, middle panels). At variance, no difference was detected in DFs from young people (Figure 1c and d, left panels).

We then assessed the apoptotic features of DFs from young people and centenarians exposed to 20 mM dRib for 48 and 72 h. We found that 20 mM dRib treatment elicited nuclear condensation in all DF cultures (Figure 2a). Arg+ and Pro+ DFs from young people showed similar levels of cleaved PARP 85 kDa fragment, which was detectable only after 72 h of dRib exposure. At variance, high levels of PARP 85 kDa fragment were detectable at both 48 and 72 h of dRib exposure in Arg+, but not in Pro+ DFs from centenarians, being detectable in the latter only after 72 h of dRib exposure (Figure 2b). The levels of cleaved 20/17 kDa Caspase 3 fragments were barely detectable in dRib-exposed DFs from young subjects, while they were markedly increased in dRib-exposed DFs from centenarians (Figure 2b). However, only Arg+ DFs from centenarians displayed a detectable decrease of full-length 37 kDa caspase 3 fragment, as a consequence of dRib exposure (Figure 2b).

The assays of apoptotic features were somewhat difficult in DFs, likely because they are substrate-attached, slowly replicating cells which undergo apoptosis only after a long-term dRib exposure (48–72 h). We then performed the above measurements on LCLs established from Arg+ and Pro+ centenarians, which are rapidly replicating cells and undergo measurable levels of apoptosis within 24 h of 10 mM dRib treatment. Similar to what we had observed in DFs, we found that the percentage of <2c cells (Figure 2c), the cleavage of caspase 3 and PARP was higher in Arg+ with respect to Pro+ LCLs exposed to 10 mM dRib treatment (Figure 2d). Interestingly, these differences were evident even in the absence of dRib treatment (Figure 2c and d), suggesting that the functional difference between p53 codon 72 alleles plays a role on constitutive apoptosis. We reasoned that the evidences obtained in the above experiments were in favour of the hypothesis that a functional difference between p53 codon 72 alleles, recently described in an *in vitro* model employing an exogenous p53,[17] is likely to play a role when endogenous p53 is activated by stimuli that induce cell death.

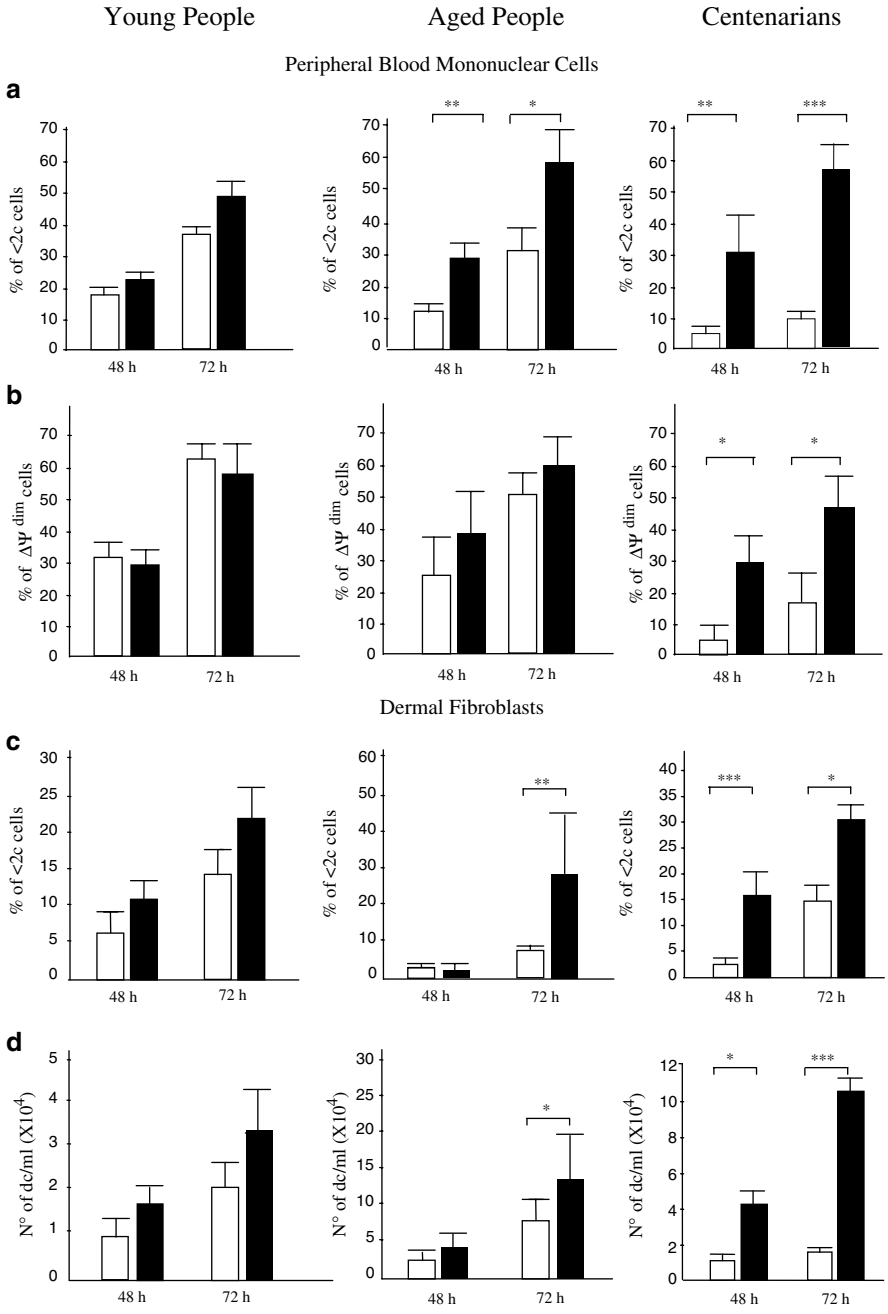

Figure 1 Susceptibility to dRib-induced apoptosis in PBMCs and DFs from subjects of different ages. PBMCs: 20 young people (eleven Pro + *vs* nine Arg +), 16 aged people (nine Pro + *vs* seven Arg +), and eight centenarians (four Pro + *vs* four Arg +). DFs: eight young people (four Pro + *vs* four Arg +), four aged people (two Pro + *vs* two Arg +), and six centenarians (three Pro + *vs* three Arg +). Centenarians (right panel), Aged people (middle panel), young people (left panel). (**a**) Percentage of PBMCs with hypodiploid DNA content (<2c cells); (**b**) percentage of PBMCs with depolarised mitochondria ($\Delta\Psi^{dim}$ cells); (**c**) percentage of DFs with hypodiploid DNA content (<2c cells); and (**d**) number of detached DFs per ml of culture medium (dc). *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P < 0.001$, by GLM ANOVA test. Data are expressed as percentage ± S.E.M. White bars: Pro + subjects, black bars: Arg + subjects

Biochemical differences between p53 codon 72 alleles in the absence and in presence of exogenous oxidative stress

We then assessed the activation of p53, that is, the levels of total p53 and of serine 15 phosphorylated p53 (P-p53ser15) protein, in Arg + and Pro + DFs whole-cell lysates, obtained from young people and centenarians. We found that dRib exposure elicited an increase in total p53 and P-p53ser15 protein in DFs, which was higher in Arg + compared to Pro + cells, both in cells from young people and centenarians (Figure 3a). To assess whether the increased levels of P-p53ser15 in Arg + were entirely due to the higher levels of total p53 in these cells, we compared the amounts of P-p53ser15 in SaOs2 p53-null cells, transiently transfected with either or pCMS-p53Arg or pCMS-p53Pro expression

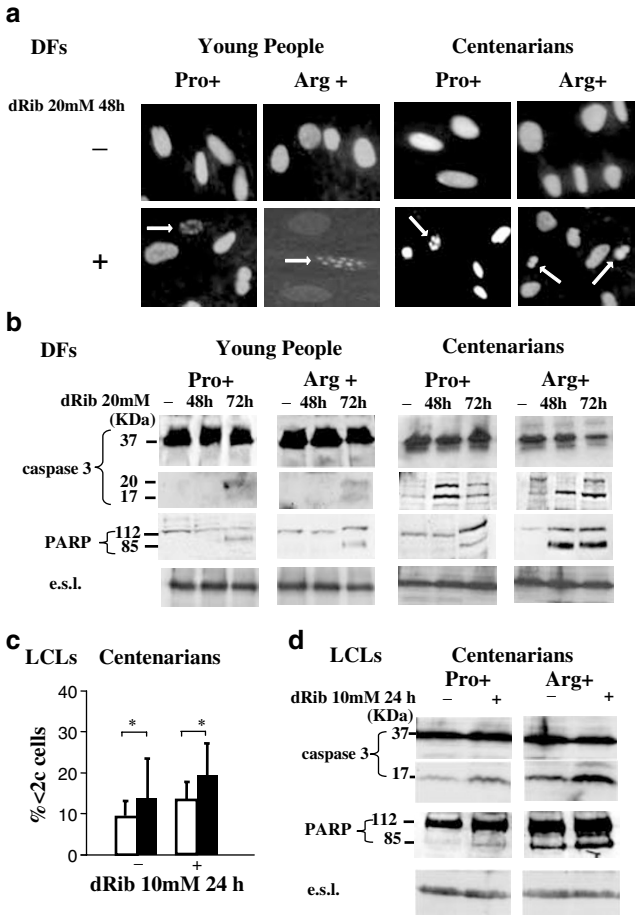

Figure 2 Apoptotic features of Arg+ and Pro+ DFs and LCLs after dRib treatment. (**a**) Fluorescence microscopy analysis of nuclear morphology by Hoechst 33258 staining of DFs after 48 h of 20 mM dRib treatment. Arrows indicate the condensed/fragmented nuclei; (**b**) Western blot analysis of Caspase 3 and PARP cleavage on DFs in absence (−) or after 48 or 72 h of 20 mM dRib treatment. Equal sample loading (e.s.l.) was assessed by Tubulin immunoreactivity; (**c**) cytofluorimetric analysis of hypodiploid DNA content (<2c cells) on LCLs from centenarians in the absence (−) or presence (+) of 24 h 10 mM dRib treatment. Data are expressed as percentage ± S.E.M. and are referred to eight different LCLs (four Pro+ *vs* four Arg+). White bars: Pro+ subjects, black bars: Arg+ subjects. *: $P = 0.044$, GLM ANOVA test; (**d**) Western blot analysis of Caspase 3 and PARP cleavage in LCLs from centenarians in the absence (−) or presence (+) of 24 h 10 mM dRib treatment. E.s.l. was assessed by Actin immunoreactivity

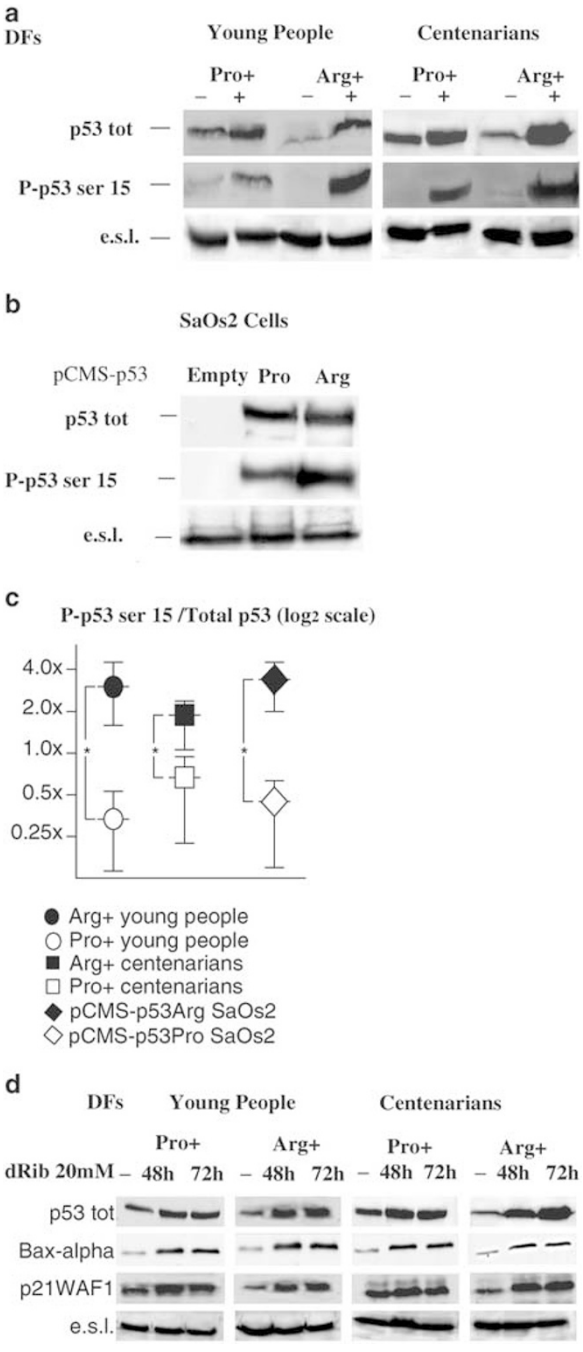

Figure 3 Western blot analysis of total p53 and Serine 15 phosphorylated p53 (P-p53 ser 15). (**a**) DFs from young people and centenarians, in the absence (−) or presence (+) of a 48 h 20 mM dRib treatment e.s.l. was assessed by Tubulin and (**b**) SaOs2 cells transiently transfected for 24 h with pCMS-p53Arg/p53Pro plasmids. E.s.l. and equal transfection efficiency were assessed by EGFP. (**c**) Densitometric analysis of Western blot for total p53 and P-p53 ser 15 in dRib-treated DFs ($n = 3$, including the one showed in Figure 3a) and pCMS-transfected SaOs2 cells ($n = 3$, including the one showed in Figure 3b). Ordinate Log_2 scale represents P-p53 ser 15 over Total p53 ratio. Data are expressed as fold ± e.s.m. *$P < 0.05$, GLM ANOVA Test. (**d**) Kinetic analysis of total p53 and p53 downstream genes (Bax-alpha and p21WAF1) in DFs from young people and centenarians, analysed by Western blot, after 24 and 48 h of 20 mM dRib treatment. E.s.l. was assessed by Tubulin immunoreactivity

vector (Figure 3b). Densitometric analysis showed that, after normalising the cell extracts for GFP protein and loading equal amounts of total p53, pCMS-p53Arg transfected cells had higher levels (about six-fold) of P-p53ser15 with respect to pCMS-p53Pro ones (Figure 3c). p53 protein activation was further demonstrated by Western blot analysis of two p53 downstream genes, namely Bax-alpha and p21WAF1 (Figure 3d). We found that both Arg+ and Pro+ DFs from young people and centenarians had similar levels of expression of such genes. Furthermore, p53AIP1 induction was also investigated, but no detectable levels of such gene were found (data not shown).

By comparing the levels of total p53 between Arg+ and Pro+ DFs, we observed that even in the absence of dRib

treatment, the levels of total p53 protein were reproducibly higher in Pro+ than Arg+ DFs (Figure 4a). This phenomenon could not be observed in pCMS-p53Arg/p53Pro transfected SaOs2 cells, and we reasoned that this inconsistency was due to the high rate of CMV promoter-driven gene transcription. In an effort to mimic a more physiologic rate of synthesis of the p53 protein, we stably transfected SaOs2 cells with a tetracycline-inducible (pTet-On) plasmid. These cells were then transiently co-transfected with pTRE-p53Arg/p53Pro response plasmids, and they were exposed to low (0.1 μg/ml) and high (1 μg/ml) doses of doxycycline (Figure 4b). By means of this experimental approach, we confirmed that the levels of total p53 protein were higher in pTRE-p53Pro than in pTRE-p53Arg-transfected cells, but only in the presence of a low dose of doxycycline, which causes a low induction of gene transcription. On the contrary, in the presence of a high dose of doxycycline, the amount of p53 protein in pTRE-p53Pro/p53Arg transfected cells was similar. As the level of the p53 protein is tightly regulated by its degradation rate, we hypothesised that the above findings could be due to the previously described higher resistance of the p53Pro to the degradation by the proteasome, which yields increased steady-state levels of the p53 protein, particularly at low levels of gene transcription.[18]

The degradation of the p53 protein by the proteasome requires the physical interaction with MDM2, mediated by the polyproline domain, in which the codon 72 is harboured.[35] We therefore assessed the amounts of p53/MDM2 complex in DFs (in the absence of oxidative stress) as well as in pCMS-p53Arg/p53Pro transiently transfected SaOs2 cells. Consistent with the data on total p53 protein levels, we found that higher amounts of MDM2 were co-immunoprecipitated together with p53 from Arg+ DFs whole-cell lysates, compared to Pro+ ones (Figure 4c). The difference was clearcut and reproducible in cells from centenarians, whereas it was present but less evident and consistent in cells from young people. Interestingly, we found that whole-cell lysates from centenarians contained increased levels of MDM2 with respect to those from young individuals (Figure 4c). In agreement with earlier reports,[17] we found that MDM2 co-immunoprecipitated at a higher extent with p53Arg than p53Pro allele in pCMS-transfected SaOs2 cells (Figure 4d).

Independent studies recently found that MDM2 targets p53 to the mitochondria to induce apoptosis,[17] and that the

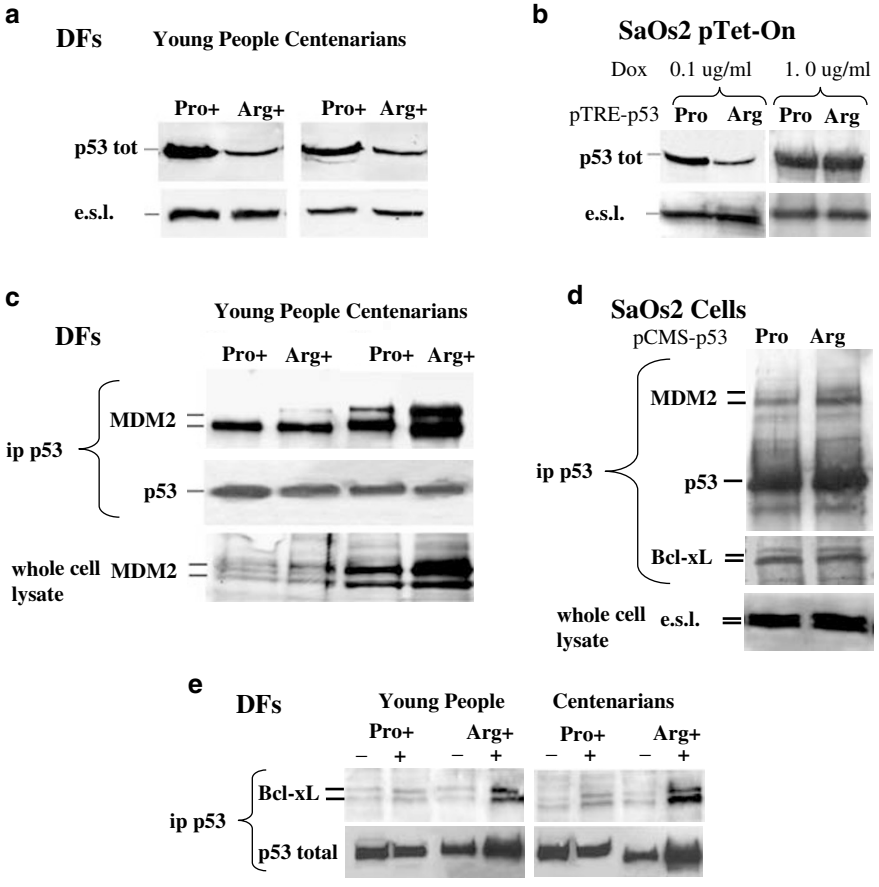

Figure 4 Biochemical differences between p53 Arg and Pro codon 72 alleles. (**a** and **b**) Western blot analysis of constitutive p53: e.s.l. was assessed by Tubulin immunoreactivity. (**a**) DFs from young people and centenarians; (**b**) pTet-On SaOs2 cells transiently co-transfected with pTRE-p53Arg/p53Pro plasmids, exposed to 0.1 and 1 μg/ml doxycycline (Dox) for 48 h; (**c** and **d**) Western blot analysis of immunoprecipitated p53. (**c**) DFs from young people and centenarians, probed with antibody for MDM2 and p53; e.s.l. was assessed by densitometric scanning of Red Ponceau staining (data not shown). (**d**) SaOs2 whole-cell lysate transiently transfected with pCMS-p53Arg/p53Pro plasmids, probed with antibody for MDM2, Bcl-xL and p53. e.s.l. and transfection efficiency were assessed by EGFP; (**e**) DFs from young people and centenarians in the absence (–) or presence (+) of a 48 h of 20 mM dRib treatment, probed with an anti-Bcl-xL. E.s.l. was assessed by densitometric scanning of Red Ponceau staining (data not shown)

interaction of mitochondrially localised p53 with Bcl-xL induces apoptosis.[36] To test whether these phenomena could play a role in the different extent of cell death occurring in Arg+ and Pro+ DFs, we assessed the immunoprecipitated p53 for Bcl-xL immunoreactivity. We found that, after 48 h of 20 mM dRib treatment, p53/Bcl-xL complex increased substantially in Arg+ in DFs from young people and centenarians, whereas only a slight increase of p53/Bcl-xL complex was evident in Pro+ DFs (Figure 4e). Interestingly, similar amounts of p53/Bcl-xL complex were present in pCMS-p53Arg/p53Pro transfected SaOs2 cells (Figure 4d).

To assess whether the different mitochondrial localisation of p53 was correlated with the different apoptotic susceptibility observed in Arg+ and Pro+ DFs from centenarians, we assessed the localisation of endogenous p53 in the mitochondria of DFs by fluorescence microscopy. We found that, as a consequence of dRib exposure, different patterns of p53 localisation could be observed: nuclear, mitochondrial-cytoplasmic, and nuclear/mitochondrial-cytoplasmic (Figure 5 a–d). The prevalent pattern of p53 localisation in Pro+ DFs was the nuclear one (Figure 5b), whereas mitochondrial-cytoplasmic and nuclear/mitochondrial-cytoplasmic ones were prevalent in Arg+ DFs from centenarians (Figure 5c and d). At variance, p53 positive cells in Arg+ and Pro+ DFs cultures from young people displayed a similar p53 localisation (Figure 5e–f). In fact, the absolute percentage of p53 positive cells in dRib-treated DF cultures was quite low (about 2–3%). We hypothesised that this finding was due to the long-term exposure time required by dRib to elicit cell death in DFs (48 h). In fact, the apoptogenic localisation of p53 to mitochondria occurs in a short temporal interval (1–2 h) and few cells at a given time are expected to show a mitochondrially localised p53.[2] We then conceived that a stimulus that elicits massive cell death in few hours could enhance the difference between Arg+ and Pro+ DFs in p53 localisation. Purposely, we evaluated p53 localisation on DFs exposed to hydrogen peroxide (H_2O_2 500 μM). As expected, a larger number of DFs displayed p53 immunoreactivity (about 50%), and consistent with the data on dRib, we found that most of the p53 positive cells showed a nuclear localisation of p53 in Pro+, and a mitochondrial-cytoplasmic p53 in Arg+ DFs cultures from centenarians (Figure 5g and h). This difference was not evident in DFs from young people (Figure 5i and l).

Remarkably, no difference on p53 mitochondrial localisation was found in pCMS-p53Arg/p53Pro transfected SaOs2 cells (data not shown), which did not differ in apoptotic rate (p53Arg *vs* p53Pro: 57 ± 15 *vs* 63 ± 18, GLM ANOVA $P = 0.34$, and Dumont *et al.*[17]), as well as in the levels of p53/Bcl-xL complex (see Figure 4d), further suggesting that the targeting of p53 to Bcl-xL and then to the mitochondria is a prerequisite for the disclosure of their different apoptotic potential.

p53 codon 72 polymorphism modulates *in vivo* cell death

The above data suggest that one of the causes of the difference in the apoptotic response to dRib between Arg+ and Pro+ DFs could be their involvement in a recently described mitochondrial-dependent, transcription-independent, apoptotic mechanism.[17,36] Interestingly, a similar pathway was described in p53-transfected ventricular myocytes,[37] and plays a major role in hypoxic cell death.[2] As the hypoxic death of myocardial tissue is one of the most prevalent pathologic conditions in old people, we then tested the hypothesis that the myocardial cell death *in vivo* is modulated by p53 codon 72 polymorphism. Purposely, we collected DNA from 130 patients aged more than 65 years (age range 65–99, median age: 80 ± 9), affected by ACS. We then correlated p53 codon 72 polymorphism to a number of clinical and biochemical parameters.[38] Multiple regression analysis showed that the serum mean levels of CK-MB and Troponin I (two markers related to the extension of the ischaemic damage) were significantly higher in Arg+ patients than in Pro+ ones. In particular, Arg+ showed about a two-fold increase in Troponin I levels with respect to Pro+ ACS patients. The other clinical, biochemical, and prognostic parameters were not significantly different between Arg+ and Pro+ ACS patients (Table 1).

Discussion

In this study, we found that blood leucocytes and DFs obtained from p53 codon 72 arginine allele homozygote (Arg+) healthy sexagenarians and centenarians undergo an oxidative stress-induced apoptosis to a higher extent than proline carriers (Pro+). At variance, we found no significant difference in the apoptosis rate between Arg+ and Pro+ cells obtained from young people.

So far, the increased capacity of the arginine allele (p53Arg) with respect to the proline one (p53Pro) to elicit apoptosis has been observed in p53-stably transfected cells, melanoma cell lines,[17] virus-transformed cell lines,[39] as well as in blood leucocytes from lung carcinoma patients, but not in blood leucocytes from healthy people,[40] in embryonic human fibroblasts[17] and transiently transfected cell lines (Dumont *et al.*,[17] and this investigation). These data, on one side suggest that p53 codon 72 alleles have different biochemical properties, and on the other, suggest that their functional difference, yet intrinsic, it is not always overt, being likely dependent on the intracellular environment in which it is harboured.

In an attempt to shed light on the mechanism(s) responsible for the above phenomenon, we found that, paralleling the results of apoptosis, Arg+ and Pro+ DFs differ in a number of features, particularly when they are obtained from centenarians. In particular, we found that, in the absence of exogenous oxidative stress, Arg+ cells have lower levels of endogenous total p53 (we were able to reproduce this phenomenon by means of a p53Arg/p53Pro Tet-On system developed in SaOs2 cells), and higher amounts of p53/MDM2 complex (a phenomenon reproducible in SaOs2 p53Arg/p53Pro transfected cells). Moreover, at least in LCLs cells in which this phenomenon is measurable, Arg+ cells show higher amounts of spontaneous apoptosis, accompanied by increased levels of cleaved caspase 3 and PARP.

Interestingly, these findings are in agreement with two previous pieces of literature data, the first indicating that the

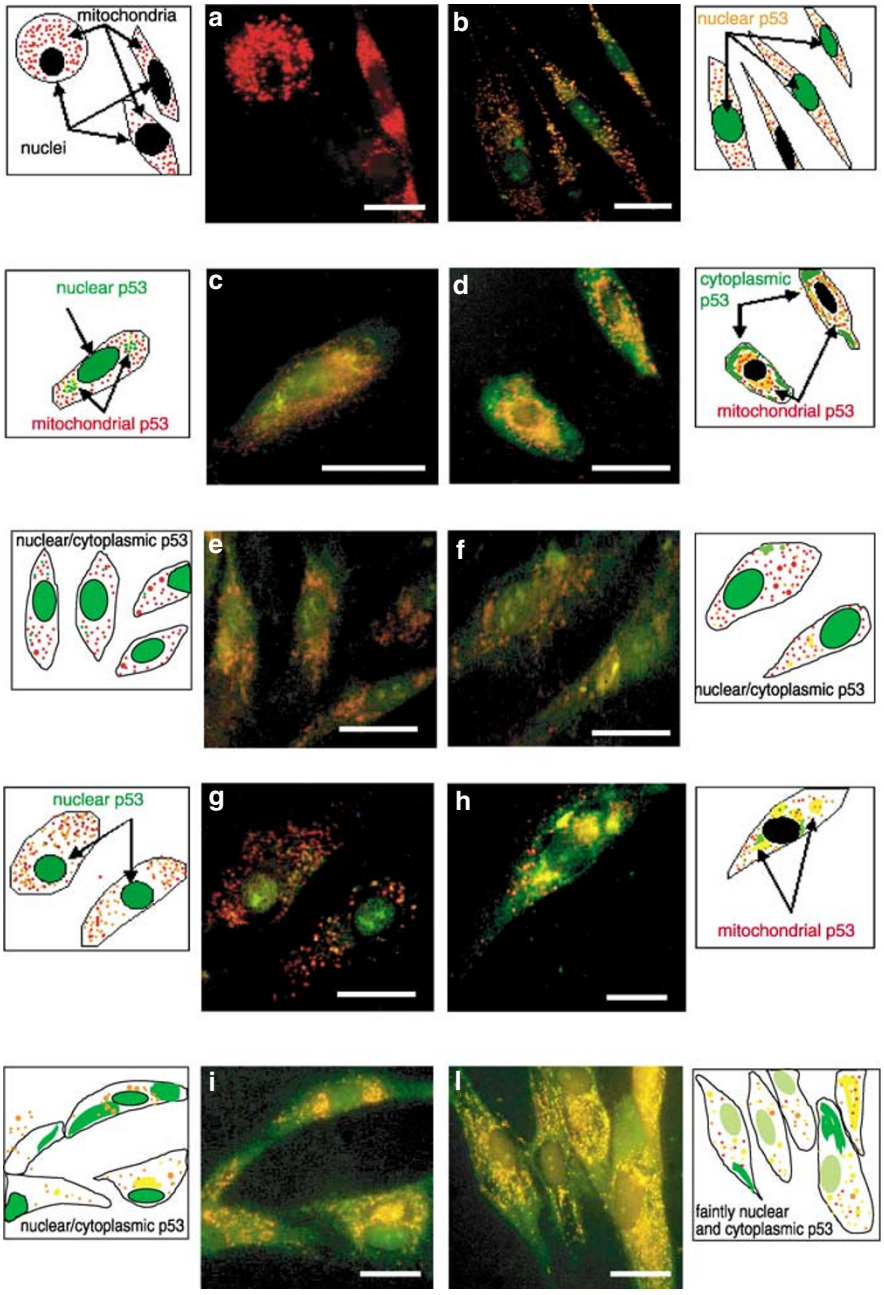

Figure 5 p53 localisation in DFs. Staining was performed by using FITC-conjugated anti-p53 mouse monoclonal antibody (green fluorescence) and the $\Delta\psi$-sensitive dye MitoTracker Red CMX-Ros™ (red fluorescence). Digital zooming was used to better highlight p53 localisation. (**a** and **d**) Patterns of p53 expression in DFs from centenarians after 48 h of 20 mM dRib treatment: (**a**) negative, (**b**) nuclear, (**c**) nuclear/mitochondrial, (**d**) cytoplasmic/mitochondrial, (**e** and **f**) representative example of a Pro+ (**e**) and Arg+ (**f**) DFs culture from young subjects after 48 h of 20 mM dRib treatment, (**g**–**l**) p53 expression in DFs from Pro+ (**g**) and Arg+ (**h**) centenarians and from Pro+ (**i**) and Arg+ (**l**) young people after 2 h of 500 μM H_2O_2 treatment. Ruler: 20 μm. Cartoons schematically reproduce the pictures

p53Arg allele is endowed with an enhanced capacity to bind to MDM2 protein and to elicit apoptosis in cells stably transfected with temperature-sensitive p53Arg/p53Pro alleles,[17] the second pointing out that the p53Arg allele is susceptible to a higher extent to Human Papilloma Virus E6 protein-mediated proteasomal degradation in p53/E6 doubly transfected cell lines.[19] Consequently, in this paper we found that both these mechanisms could play a role in the regulation of constitutive levels of endogenous p53 in DFs.

When we challenged Arg+ and Pro+ cells with exogenous oxidative stress, we found that, although the activation of p53 protein (increase in total p53 and P-p53ser15 protein levels)[41] is higher in Arg+ DFs, no difference in the activation of proapoptotic genes, such as Bax-alpha or p53AIP1, with respect to Pro+ cells occurs. Rather, we found that Arg+ DFs exposed to oxidative stress contain higher levels of p53 bound to the mitochondrial protein Bcl-xL, compared to Pro+ DFs.[36] Moreover, only in DFs from centenarians, Arg+ but

Table 1 Baseline characteristics of 130 aged ACS affected patients

	P53 codon 72 genotype		
	Arg+ ($n=77$)	**Pro+ ($n=53$)**	***P***
Age (years ± S.D.)	80 ± 7 (range: 65–99)	79 ± 7 (range: 66–95)	0.68
Sex (M/F)	45/32	31/22	0.89
Risk factors for ACS			
Smokers *n* (%)	12 (15.6)	5 (9.4)	0.30
Hypertension *n* (%)	54 (70.1)	35 (66.0)	0.62
Diabetes mellitus *n* (%)	23 (29.9)	17 (32.1)	0.78
Hypercholesterolaemia *n* (%)	53 (68.8)	33 (62.3)	0.44
CRP (mg/dl ± S.D.)	4.8 ± 5.5	4.1 ± 5.6	0.46
Vitamin B_{12} (pg/ml ± S.D.)	434 ± 245	435 ± 345	0.95
Folate (ng/ml ± S.D.)	5.8 ± 2.6	5.8 ± 2.7	0.85
Homocysteine (μmol/l ± S.D.)	4.8 ± 5.5	4.1 ± 5.6	0.54
ACS diagnosis *n* (%)			0.99
UA	18 (23.4%)	14 (26.4%)	
Qw-MI,	34 (44.2%)	23 (43.4%)	
NQw-MI	25 (32.4%)	16 (30.2%)	
Ischaemia markers			
Troponin I (ng/ml ± S.D.)	42 ± 73	24 ± 33	0.002*
CK-MB (ng/ml ± S.D.)	84 ± 141	78 ± 123	0.047*

Qw-MI: Q-wave-myocardial infarction; NQw-MI: non-Q wave-myocardial infarction; UA: unstable angina; CRP: C-reactive protein. **P* refers to multiple regression analysis of ln transformed CK-MB and Troponin I values. Age, sex, smoking habits, and risk factors for ACS were included as covariates.

not Pro+ DFs show a mitochondrial localisation of p53. At variance, we found no difference in p53 intracellular localisation between Arg+ and Pro+ DFs from young people. Finally, again in keeping with previous data,[17] we found that p53Arg and p53Pro alleles show a similar apoptotic potential in p53-transiently transfected SaOs2 cells, in which similar amounts of p53/Bcl-xL complex and mitochondria-localised p53 were detected (Dumont *et al*.,[17] and data not shown).

Overall, the available data on p53 codon 72 suggest that the differential targeting to the mitochondria of p53Arg and p53Pro is required to disclose their functional difference in apoptotic potential.[17] Consequently, in the same cell strain or lineage, there may be permissive or nonpermissive conditions for this phenomenon. This consideration is valid both for explaining the findings obtained in cells harbouring exogenous p53 (i.e. p53 codon 72 alleles differ in their apoptotic potential in stably, but not in transient transfected SaOs2 cells; see Dumont *et al*.[17] and this investigation), as well as endogenous p53 (p53 codon 72 alleles differ in their apoptotic potential in cells from old people and centenarians, but not young individuals). A likely candidate to play a role in this pathway is MDM2 protein, which differentially binds to the p53Arg and p53Pro alleles. In this regard, we found that MDM2 protein increases in DFs from centenarians, but at present, its causative role on the phenomenon here described remains speculative.

The *in vivo* relevance of such a genetically determined difference in apoptosis susceptibility between aged individuals was then examined in a group of elderly patients affected by acute ischaemic damage of the myocardial tissue, in which massive hypoxic cell death occur. We found that Arg+ subjects have higher serum levels of cardiac Troponin I and CK-MB, two qualitative/quantitative markers for the extent of the ischaemic damage at the myocardial level.[38] This finding can be better understood by taking into account that hypoxia-induced cell death mediates an oxidative stress-induced damage at the mitochondrial level in cardiomyocytes,[42] and it induces the targeting of p53 to the mitochondrion.[2] Moreover, it is known that p53 activates mitochondrial-dependent death pathway in ventricular myocytes.[37] Thus, it is reasonable to conceive that if dRib (*in vitro*) and hypoxia (*in vivo*) elicit similar pathways of cell death, a different apoptotic susceptibility between Arg+ and Pro+ old individuals in both situations will ensue. In this regard, although the contribution of apoptosis to ischaemic heart is less known than that of necrosis,[43] there is evidence that apoptosis occurs *in vivo* in ischaemic myocardium,[43] and that both forms of cell death share common mechanisms, especially at the mitochondrial level.[44] At present, the data on our patient's series are not sufficient to disentangle the various causes of the different extents of the ischaemic damages between Arg+ and Pro+, such as a different extent of the infarct size, a different extension of the atherosclerotic damage, and a different capacity of tissue repairing. All these questions are worth addressing in future studies aimed at elucidating the role of p53 in the regulation of the homeostasis of the myocardial tissue in the context of ageing and age-related diseases. The available data on mice indeed reveal an important role of p53 in the phenomena related to the ageing of cardiac tissue.[45]

In conclusion, in this paper we found that the difference between p53 codon 72 alleles in the capacity to modulate cell death is substantial only when cells from old people are assessed. This functional difference *in vitro* has relevance for cell death occurring *in vivo* as a consequence of myocardial ischaemia in aged people. These data suggest that mechanisms determining the susceptibility to *in vitro* apoptosis are affected by the age of the cell donor, and they can provide insights into the individual susceptibility towards major age-related diseases, such as cardiovascular diseases.

Materials and Methods

Cells from healthy subjects with known p53 codon 72 genotype

PBMCs, DFs, and EBV-transformed B cell lines (LCLs) were obtained from subjects previously assessed for the p53 codon 72 genotype.[33] Namely, 28 healthy young people (aged 30 ± 3 years), 20 healthy aged people (aged 69 ± 1 years) selected from a group of actively exercising aged people, in a good physical performance status, 22 healthy centenarians, categorised 'A' for their healthy status, as previously described.[21,22] All the subjects were devoid of any clinical or biochemical abnormalities at the moment of blood collection or skin biopsy. As the p53 codon 72 proline/proline genotype is quite rare in the Italian population (about 8–10%),[33] and that the proline allele is likely to exert a dominant effect on the arginine one (Wu *et al.*,[39] Biros *et al.*[40] and preliminary data, data not shown), two groups of subjects were considered for this study: Pro+ (proline/proline and proline/arginine genotypes) and Arg+ (arginine/arginine genotype) subjects. In detail, PBMCs were separated by discontinuous gradient centrifugation from the whole blood of 20 young people (mean age: 30 ± 3 years): 11 Pro+ (one proline/proline and 10 proline/arginine) and nine Arg+ subjects; 16 aged people (mean age: 69 ± 1 years): nine Pro+ (one proline/proline and eight proline/arginine) and seven Arg+ subjects; eight centenarians (mean age: 101 ± 1 years): four Pro+ (four proline/arginine) and four Arg+ subjects. PBMCs were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine. DFs long-term cultures were established from eight young people (mean age: 28 ± 6 years): four Pro+ (two proline/proline and two arginine/proline) and four Arg+ subjects; four aged people (mean age: 60 ± 3 years): two Pro+ (two arginine/proline) and two Arg+; six centenarians (mean age: 101 ± 1 years): three Pro+ (two proline/proline and one proline/arginine) and three Arg+ subjects. DFs were cultured in DMEM supplemented with 10% FCS. DFs from the 6th to the 14th passage were used for all the experiments. A negligible percentage of β-galactosidase positive cells were present in the DFs cultures assayed (data not shown). LCLs were established according to standard procedures from eight centenarians: four Pro+ (two proline/proline and two proline/arginine genotype) and four Arg+ subjects. Cells were cultured in RPMI supplemented with 10% FCS and 2 mM L-glutamine. In no case PBMCs, DFs, or LCLs were obtained from the same individual.

ACS affected patients

In all, 130 elderly consecutive patients (76 males and 54 females), aged from 65 to 99 years (males: mean age 80 ± 7 years; females: mean age 79 ± 7 years), were diagnosed for ACS. According to ACS diagnostic guidelines,[46] this syndrome comprehends Q-wave-Myocardial Infarction (Qw-MI), non-Q wave-Myocardial Infarction (NQw-MI), and Unstable Angina (UA). ACS is diagnosed when two of the following criteria are satisfied: (1) clinical presentation (chest pain, epigastric pain, or nontypical symptoms (persistent shortness of breath, unexplained weakness, syncope, or a combination of these); (2) specific ECG modifications: ST-segment elevation $\geqslant 0.1$ mV and > 0.2 mV in other leads; ST-segment depression or T-wave inversion $\geqslant 0.1$ mV; and (3) alterations in biochemical markers: myocardium-specific Troponin > 0.05 ng/ml, MB fraction of creatine kinase (CK-MB) > 10 ng/ml. In particular, patients are classified as NQw-MI and Qw-MI, if they present ECG and biochemical markers alterations (CK-MB > 2 times the upper limit of normality), and UA if they present clinical symptoms and alterations in ECG but not in biochemical markers. At the time of Coronary Care Unit (CCU) admission, we evaluated total and high-density lipoprotein (HDL) cholesterol levels in all patients. The determination of ischaemia markers, such as CK-MB fraction of creatine kinase and Troponin I, was documented on CCU admission and after 6, 12, and 24 h. Data about risk factors for ACS were collected (presence of diabetes, smoking habits, hypertension, hypercholesterolaemia). Standard biochemical parameters, as well as serum folate, vitamin B_{12}, homocysteine, C-reactive protein were also available for all the patients (evaluated 24 h after CCU admission) and were used as covariates in Multiple Regression analysis. All subjects gave their informed consent after admission to the CCU (INRCA, Ancona, Italy).

P53 codon 72 genotype assessment

PBMCs, DFs, and LCLs were obtained from subjects whose p53 codon 72 genotype had been previously assessed on DNA extracted according to standard procedures from frozen whole leucocytes aliquots.[33] The p53 codon 72 genotype was confirmed in all the cultured cells (DFs and LCLs) by PCR amplification of the p53 exon 4 (5′-GCAGAGACCTGTGGGAAGCGA-3′ and 5′-ACCGTAGCTGCCCTGGTAGGT-3′) followed by Automatic sequencing in a CEQ2000 Automatic Sequencer (Beckman, Fullerton, CA, USA). The p53 codon 72 polymorphism in ACS patients was assessed on DNA extracted according to standard procedures from frozen whole leucocytes aliquots. p53 codon 72 alleles were amplified using the primers pair indicated above. PCR cycling conditions were as follows: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, for 28 cycles. The protocol was also carried out for 30 cycles. In total, 15 μl of PCR products were digested with *Bst*UI restriction enzyme (New England Biolabs, Inc., Beverly, MA, USA) as recommended by the supplier, and fragments were separated on 2% agarose gel. The arginine allele was identified by the presence of the restriction BstUI enzyme recognition sites.

Treatment of DFs, PBMCs, and LCLs with dRib

The reducing sugar dRib (Sigma, St Louis, MO, USA) was used to elicit oxidative stress-induced apoptosis.[23,24] dRib exposure time length, and dRib concentrations (20 mM for DFs cultures, 10 mM for PBMCs, 10 mM for LCLs) were chosen on the basis of previous data (Kletsas *et al.*,[23] Barbieri *et al.*,[24] and preliminary experiments, data not shown). Under the above-described culture conditions, PBMCs are in a quiescent state.[24] With respect to DFs, cultures were allowed to reach confluence, medium was changed, and cells were cultivated for at least 3 days in medium containing 0.5% FCS, prior to being exposed to 20 mM dRib treatment. This procedure does not elicit apoptosis in DFs but rather it leads to an accumulation of cells in the late G1 phase.[23] DFs were then reseeded at low density (60 000/cm^2) in FCS-supplemented medium in the presence or absence of 20 mM dRib and assayed for apoptosis at 48 and 72 h. Note that cells were allowed to attach to the plastic substrate before dRib exposure. LCLs were plated at a density of 2×10^5 cells/ml in RPMI 10% FCS, and incubated with dRib for 24 h.

Expression vectors

The amplified p53 full-length cDNA fragment was cloned in TOPO TA™ vector (Invitrogen). The *Eco*RI-excised fragment was subcloned into a pCMS-EGFP or in a pTRE2Pur plasmid (Clontech, Palo Alto, CA, USA). The entire insert was sequenced to assess the exact orientation and the absence of any sequence variation from the p53 wild-type sequence, by using the following primers pairs: F1:5′-GCCATGGAGGAGCCGCAGTC-3′, R1:5′-AAGGGACAGAAG ATGACAGG-3′; F2:5′-GCACCAG-CAGCTCCTACACC-3′, R2:5′-CCACTCGGATAAGAT GCTGA-3′;

F3:5′-GCCCCTCCTCAGCATCTTAT-3′, R3:5′-AGGAGCTGGTGTTGTTGGGC-3′, in a Beckman CEQ2000 automatic sequencer. The arginine/proline variants were also obtained by site-directed mutagenesis (Gene Editor™, Promega, Madison, CA, USA) using primers ARG-MT: 5′-GAGGCTGCTCCCCGCGTGGCCCCTGCACC-3′ or PRO-MT: 5′-GAGGCTGCTCC CCCCGTGGCCCCTGCACC-3′. As expected, no difference was found between experiments performed with p53 codon 72 alleles obtained from homozygous subjects and those obtained by *in vitro* mutagenesis (data not shown).

Transfection assays

p53-null SaOs2 cells were cultured in DMEM 10% FCS. SaOs2 cells were seeded in six-well plates at a concentration of (2×10^5 per plate), and after 24 h were transiently transfected with 1 μg of pCMS-EGFP, either empty, or carrying the p53 arginine (pCMS-p53Arg) or proline (pCMS-p53Pro) allele, using the Effectene system, according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). After 24 h, cells were collected and lysed to be assayed by Western blot.

SaOs2 pTet-On cells were obtained by transfecting cells with 1 μg of pTet-On™ plasmid (Clontech) using the Effectene system (Qiagen) and kept in the presence of increasing concentrations (from 100 to 600 μg/ml) of G418 (Sigma, St Louis, MO, USA) for 4 weeks. G418 resistant stably transfected cells were then employed for transient transfection assay, using pTRE2Pur plasmid, either empty, or carrying the p53 arginine (pTRE-p53Arg) or proline (pTRE-p53Pro) allele. A total of 1×10^5 pTet-On stably transfected cells were seeded in six wells plated and were transfected with 1 μg of either pTRE plasmid for 24 h and exposed to increasing doses of doxycycline (0.1–1 μg/ml) for 48 and then lysed for Western blot assay.

Cytofluorimetric analysis of cell death and mitochondrial potential

All the cytofluorimetric analyses were performed using a FACScalibur cytometer (BD, San José, CA, USA) equipped with an Argon ion laser tuned at 488 nm. In all analyses, a minimum of 10 000 cells per sample were acquired in the list mode and analysed with Cell Quest software.

The DNA content in DFs, PBMCs, LCLs, and p53CMS-p53Arg/Pro transfected SaOs2 cells was assessed using propidium iodide (PI) staining. The appearance of a hypodiploid peak of PI fluorescence was taken as an index of apoptosis in DFs, PBMCs, and LCLs. Briefly, cells were resuspended in hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml PI (Sigma), and kept for at least 1 h at 4°C. Therefore, cells underwent FACS analysis, and those with low PI fluorescence were considered apoptotic. With respect to SaOs2 cells, cells with low PI fluorescence were considered apoptotic only when also positive for green fluorescence due to GFP-expression. Inasmuch as it is known that cells detached from the culture substrate display the characteristics of cells dying by apoptosis, the number of DFs was taken as an additional index of apoptosis. Culture supernatants were centrifuged at $350 \times g$ for 10 min, and cell pellets were resuspended in 300 μl of PBS, then passed through the flow cytometer at low speed. Each sample was allowed to run for 30 s after flow stabilisation. Considering that FACScalibur sips 12 μl per minute at low speed, it is easy to calculate the number of cells that was present in the sample applying the formula: (number of cells counted in 30 s/6μl) $\times$ 300. Mitochondrial membrane potential loss ($\Delta\Psi^{dim}$) was evaluated by using the potentiometric probe JC-1 (Molecular Probes, Eugene, OR, USA), which changes reversibly its fluorescent emission from red/orange to green as $\Delta\psi$ decreases (over values of about 80–100 mV). Briefly, PBMCs were stained with 2.5 μg/ml JC-1 and kept at room temperature for 20 min, washed twice with PBS, resuspended in a total volume of 400 μl PBS, and examined by FACS analysis.[47]

Western blot assay and co-immunoprecipitation assay

Whole-cell lysates for Western blot were obtained from DFs cultured in the presence or absence of 20 mM dRib for 48 h, from LCLs cultured in the presence or absence of 10 mM dRib for 24 h, and from SaOs2 transfected cells. Cells were lysed in RIPA Buffer (1% Triton x-100, 50 mM Tris-HCl (pH 8.0), 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), added of protease inhibitor cocktail (Sigma) and 1 mM $NaVO_4$. To obtain whole-cell lysate for co-immunoprecipitation assay, DFs and SaOs2 were lysed in CO-IP Buffer (0.5% NP-40, 50 mM Tris-HCl (pH 8.0), 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), with addition of protease inhibitor cocktail (Sigma), and 1 mM $NaVO_4$. Co-immunoprecipitation assay was performed as follows: after triplicate quantisation with Bradford assay, 200 μg of proteins were washed with Protein A Sepharose-conjugated beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA), incubated with anti p53 antibodies (1 μg DO-1 and 1 μg of pAb421) at 4°C overnight in extensive shaking, washed four times with cold CO-IP buffer, and incubated for 2 h with Protein A Sepharose-conjugated beads. The obtained pellet was resuspended in Laemly buffer and directly loaded onto polyacrylamide gel. For Western blot analysis, 30 μg of whole-cell lysates were loaded for each lane. The antibodies used were: anti-Poly(ADP-ribose) polymerase (PARP) (BIOMOL Research Laboratories, Plymouth, PA, USA), anti-p53 (DO-l), anti-β tubulin (H-2359), anti-MDM2 (SMP-14), anti-Bcl-xL (S-18), anti-Bax alpha (clone B-9), anti-p21WAF1 (C-19), anti-actin, anti-tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-Serine 15 phosphorylated p53 (Oncogene Research Products, San Diego, CA, USA), anti-Caspase 3 (Cell Signalling Technology, MA, USA and Biomol, Plymouth Meeting, PA, USA), and anti-EGFP (Clontech, CA, USA). All primary antibodies were probed by a secondary Horse Radish Peroxidase (HRP) conjugated antibody (Bio-Rad). Chemiluminescent assay was used for detection (Santa Cruz). Gel imaging was performed using a GelDoc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) acquisition system and Quantity One 4.1.1 software (Bio-Rad).

Fluorescence microscopy

DFs were cultured on plastic chamber slides Lab Tek II (Nalge Nunc, Naperville, IL, USA) and treated with or without 20 mM dRib for 48 h, then fixed with 4% paraformaldehyde in PBS for 10 min, then permeabilised with 0.1% Triton X-100 and stained for nucleic acid with 5 μg/ml Hoechst 33258 stain (Sigma), and mounted with ProLong antifade (Molecular Probes). Slides were then observed with an Orthoplan fluorescent microscope (Leitz, Bielefeld, Germany) with $\times$ 60 oil-immersion magnification. Images were collected with a Kodak image analysis system and analysed with Thumbs Plus 5 software (Cerious Software, Charlotte, NC, USA). For experiments of p53 localisation, DFs were incubated with 20 mM dRib for 48 h or with 500 μM H_2O_2 for 2 h, then stained with 80 nM MitoTracker Red CMXRos (Molecular Probes) 45 min before the end of dRib incubation, fixed as described above and stained according to standard protocols for intracellular antigens with monoclonal antibodies against p53 (clone DO-1, Santa Cruz) revealed by

a FITC-conjugated secondary antibody (BD). Exposure time and fluorescence enhancement were fixed at a value by which green and red fluorescences were undetectable in unstained samples.

Statistical analysis

GLM ANOVA, Multiple Regression analysis, and Fisher's exact test were used to test for differences in quantitative variables, which were log transformed when required. Fisher's Exact test was performed in categorical variables. Data analysis was performed by SPSS 10 Package (SPSS, Chicago, IL, USA).

Acknowledgements

This work was supported by AIRC (Associazione Italiana Ricerca sul Cancro) Grant, EU (European Union) Grants 'FUNCTIONAGE', 'ECHA', 'T-CIA', and ex 40% MIUR, (Italian Ministry of University) Grant, and FIRB (Fondi Istituzionali per la Ricerca di Base) and Ministero della Salute (Italian Ministry of Health) Finalizzate 2003 Grant to CF.

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