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Down-regulation of Asymmetric Arginine Methylation During Replicative and H_2O_2-induced Premature Senescence in WI-38 Human Diploid Fibroblasts

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Protein arginine methylation is one of the post-translational modifications which yield monomethyl and dimethyl (asymmetric or symmetric) arginines in proteins. In the present study, we investigated the status of protein arginine methylation during human diploid fibroblast senescence. When the expression of protein arginine methyltransferases (PRMTs), namely PRMT1, PRMT4, PRMT5 and PRMT6 was examined, a significant reduction was found in replicatively senescent cells as well as their catalytic activities against histone mixtures compared with the young cells. Furthermore, when the endogenous level of arginine-dimethylated proteins was determined, asymmetric modification (the product of type I PRMTs including PRMT1, PRMT4 and PRMT6) was markedly down-regulated. In contrast, both up- and down-regulations of symmetrically arginine-methylated proteins (the product of type II PRMTs including PRMT5) during replicative senescence were found. Furthermore, when young fibroblasts were induced to premature senescence by sub-cytotoxic H_2O_2 treatment, results similar to replicative senescence were obtained. Finally, we found that SV40-mediated immortalized WI-38 and HeLa cell lines maintained a higher level of asymmetrically modified proteins as well as type I PRMTs than young fibroblasts. These results suggest that the maintenance of asymmetric modification in the expressed target proteins of type I PRMTs might be critical for cellular proliferation.

Key words: dimethylarginines, human diploid fibroblasts, H_2O_2-induced premature senescence, protein arginine methyltransferases, replicative senescence.

Abbreviations: aDMA, asymmetric N^G, N^G-dimethyl arginine; CPDL, cumulative population doubling level; HDFs, human diploid fibroblasts; MMA, N^G-monomethyl-arginine; PRMT, protein arginine methyltransferase; RS, replicative senescence; SA-β-gal, senescence-associated β-galactosidase; sDMA, symmetric N^G,N'^G-dimethyl arginine.

Protein arginine methylation is one of the common post-translational modifications that occur in eukaryotes. Although the presence of methyl-arginine in proteins was first identified in calf thymus in 1967 (*1*), nine subclasses of mammalian protein arginine methyltransferases (PRMTs) have now been cloned (*2*). Based on their reaction products, PRMTs are classified into two types: type I enzymes catalyse the formation of N^G-monomethyl-arginine (MMA) and asymmetric N^G, N^G-dimethyl arginine (aDMA), and type II enzymes form MMA and symmetric N^G,N'^G-dimethyl arginine (sDMA). The type I methyltransferases include PRMT1, PRMT3, PRMT4/CARM1, PRMT6 and PRMT8, and the type II enzymes contain PRMT5, PRMT7 and PRMT9 (*3*).

To identify the biological significance of PRMTs, gene ablation experiments were performed for PRMT1 and CARM1/PRMT4, which are the two best-characterized members of the PRMT family (*4*, *5*). However, PRMT1 null embryos died at approximately embryonic day 6.5, and PRMT4 null mice died at birth, indicating that these enzymes have an essential role in cellular metabolism. In contrast to organismal level, recent progress has revealed that cellular processes modulated by this modification include transcriptional regulation, RNA processing, translation, signal transduction, proteins trafficking and DNA repair (*6*). Consistent with these findings, earlier studies demonstrated that PRMTs' activities were highly elevated in proliferating tissues and cancer cell lines (*7–9*). In addition, Lim *et al*. (*10*) reported that the expression and activities of PRMT1 and PRMT5 are highly up-regulated within 3 days of regeneration after 70% partial hepatectomy in rat, indicating that cellular proliferation and the activities of PRMTs are correlated.

To investigate the interrelation between the cellular proliferation and level of protein arginine methylation, we chose the normal human diploid fibroblasts (HDFs) that have commonly been used as a useful model for studying the molecular mechanisms of cellular proliferation, tumourigenesis and ageing. During the proliferative lifespan of the HDFs in culture, there is an initial rapid proliferative phase with progression to a state of irreversible growth arrest, a process termed as replicative

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senescence (RS) (*11*). In addition to RS by serial passage in cultures, cellular senescence can be induced prematurely in the early passage of cells (termed premature senescence) by exposure to subcytotoxic concentration of stressors, such as hydrogen peroxide, ethanol, UV light and γ-irradiation (*12*). The common senescent phenotypes of the two ageing processes are characterized by the overexpression of CDK inhibitors such as p16 and p21, the increased activity of checkpoint inhibitors, p53 and Rb, cellular enlargement and flattening and increased senescence-associated β-galactosidase (SA-β-gal) activity (*13*).

In the present study, we investigated the patterns of expression of PRMT subfamily members, as well as their enzymatic activities, and arginine dimethylated proteins in WI-38 fibroblasts during RS and H_2O_2-induced premature senescence.

MATERIALS AND METHODS

Cells, Reagents and Antibodies—WI-38 normal human diploid fibroblasts, SV40 large T antigen-mediated immortalized WI-38 (WI-38 VA13) and HeLa cell lines were purchased from the American Type Culture Collection (ATCC). *S*-adenosyl-L-[methyl-^{14}C]methionine (specific activity; 57 μCi/mmol) and histone mixtures (type II-AS) were from Amersham Biosciences (UK) and Sigma (MO, USA), respectively. Primary antibodies against PRMT1, PRMT4 and Sam68 were obtained from Upstate (NY, USA), and anti-PRMT6 antibody was from Imgenex (CA, USA). Anti-p21 and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

Cell Culture—ATCC provided cumulative population doubling level (CPDL) 25 for the WI-38 fibroblasts. Both normal WI-38 and WI-38 VA13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, and maintained at 37°C in 5% CO_2. HeLa cells were cultured under the same condition, except RPMI-1640 in place of DMEM.

Preparation of Cell Extracts—To prepare whole-cell lysate, cells were lysed on ice in a lysis buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2% NP-40) and protease inhibitor cocktail (Roche, Mannheim, Germany) for 20 min, and the cell extracts were collected after centrifugation of the lysate at 12,000*g* for 15 min. The extracts were used for methylation reaction and western immunoblot analysis. For isolation of the cytosolic fraction, the cells were homogenized in 4 vols of 5 mM sodium phosphate (pH 7.4) containing 5 mM EDTA and 0.25 M sucrose. The homogenate was then centrifuged at 100,000*g* for 60 min. The supernatant was filtered through Centriprep-10 (Millipore, MA, USA) to remove endogenous transmethylation inhibitors (*14*, *15*) and the concentrated top fraction was used for methylation reaction.

Western Blotting Analysis—An equal amount of protein (10 μg) was resolved on a 13% acrylamide gel. The fractionated proteins were transferred to an Immobilon-P polyvinylidenedifluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with blocking buffer (3% non-fat milk in 20 mM Tris–HCl, pH 7.4, 3 mM KCl, and 140 mM NaCl) for 1 h at room temperature (RT) and subsequently probed with a relevant primary antibody for 15 h at 4°C. After extensive washing with Tris-buffered saline (TBS), the immunoblot was incubated with peroxidase-conjugated secondary antibody for 1 h at RT. Then, it was washed with TBS/0.05% Tween-20 and water, and the bound antibodies were visualized by the western blotting detection reagents (Millipore, MA, USA). The relative intensities of specific signals were quantified using Kodak MI imaging system (Rochester, NY, USA). Because a recent study showed that actin and tubulin (frequently used as internal controls) were overexpressed during replicative senescence (*16*); GAPDH was used as loading control in this study.

Senescence-associated β-Galactosidase Assay—For senescence-associated β-galactosidase (SA-β-gal) activity assay, cells were seeded at a density of 5×10^3 cells per well in a 12-well plate, and the activity was determined 1 day (for RS) and 3 days (for H_2O_2-induced premature senescence) after seeding using a SA-β-gal staining kit from Sigma, according to the manufacturer's instructions. Senescent cells were identified as blue-stained cells by inverted microscope, and a total of 300 cells were counted in 10 random fields to determine the percentage of SA-β-gal positive cells.

Induction of H_2O_2-induced Premature Senescence of WI-38 Fibroblasts—For induction of premature senescence, the exponentially growing fibroblasts at CPDL 28 were inoculated at a cell density of 5×10^5 in 100 mm dishes. On 3 days after seeding, the cells were incubated with the culture medium containing indicated concentrations of H_2O_2 for 2 h. For a second H_2O_2 treatment, the cells were subcultured after being cultured for 4 days following the first treatment and treated again for 2 h with the same concentrations of H_2O_2 on 3 days after seeding. After the second H_2O_2 treatment, the fibroblasts were washed twice with PBS and incubated in fresh complete medium for another 3 days, and then the cells were harvested for western blot analysis or subjected to SA-β-gal activity assay.

Assay for Protein Arginine Methyltransferses—The activities of the PRMTs were determined as previously described (*9*). The incubation mixture contained 0.1 M potassium phosphate (pH 7.6), 0.04 mM Ado[methyl-^{14}C]Met (126 dpm/pmol), a whole-cell lysate or cytosolic fraction (50 μg of protein), histone mixtures (150 μg) in a total volume of 0.125 ml. The reaction was initiated by the addition of Ado[methyl-^{14}C]Met after a 5 min preincubation at 37°C. The incubation was carried out for 60 min at 37°C and the reaction was terminated by the addition of 5 ml of 15% trichloroacetic acid (TCA), followed by 0.2 ml (4 mg) of γ-globulin as a carrier. After centrifugation at 2,100*g*, the pellet was further treated with 0.5 M potassium borate (pH 11.0) to hydrolyse any carboxyl methylesters formed by protein carboxyl methyltransferase activity. Then, the pellet was washed with 15% TCA and 95% ethanol. The pellet was finally suspended in 5 ml of scintillation solution and counted for incorporated radioactivity in a Tri-Carb 3100TR liquid scintillation analyser (PerkinElmer, CT, USA). The enzyme specific activity is defined as picomoles of [methyl-^{14}C] group transferred per minute microgram of enzyme.

RESULTS AND DISCUSSION

Down-regulation of Expression and Enzyme Activities of PRMTs During Replicative Senescence of WI-38 Fibroblasts—Under our culture conditions, WI-38 fibroblasts reached senescence state at CPDL of late 50 to early 60 times (Fig. 1A, filled circle). When WI-38 fibroblasts reached the end of their life span, the proliferative activity was significantly decreased and the replicatively senescent fibroblasts displayed a typical enlarged morphology that was distinctly different from that of young cells (Fig. 1B and C). The senescence biomarker, SA-β-gal

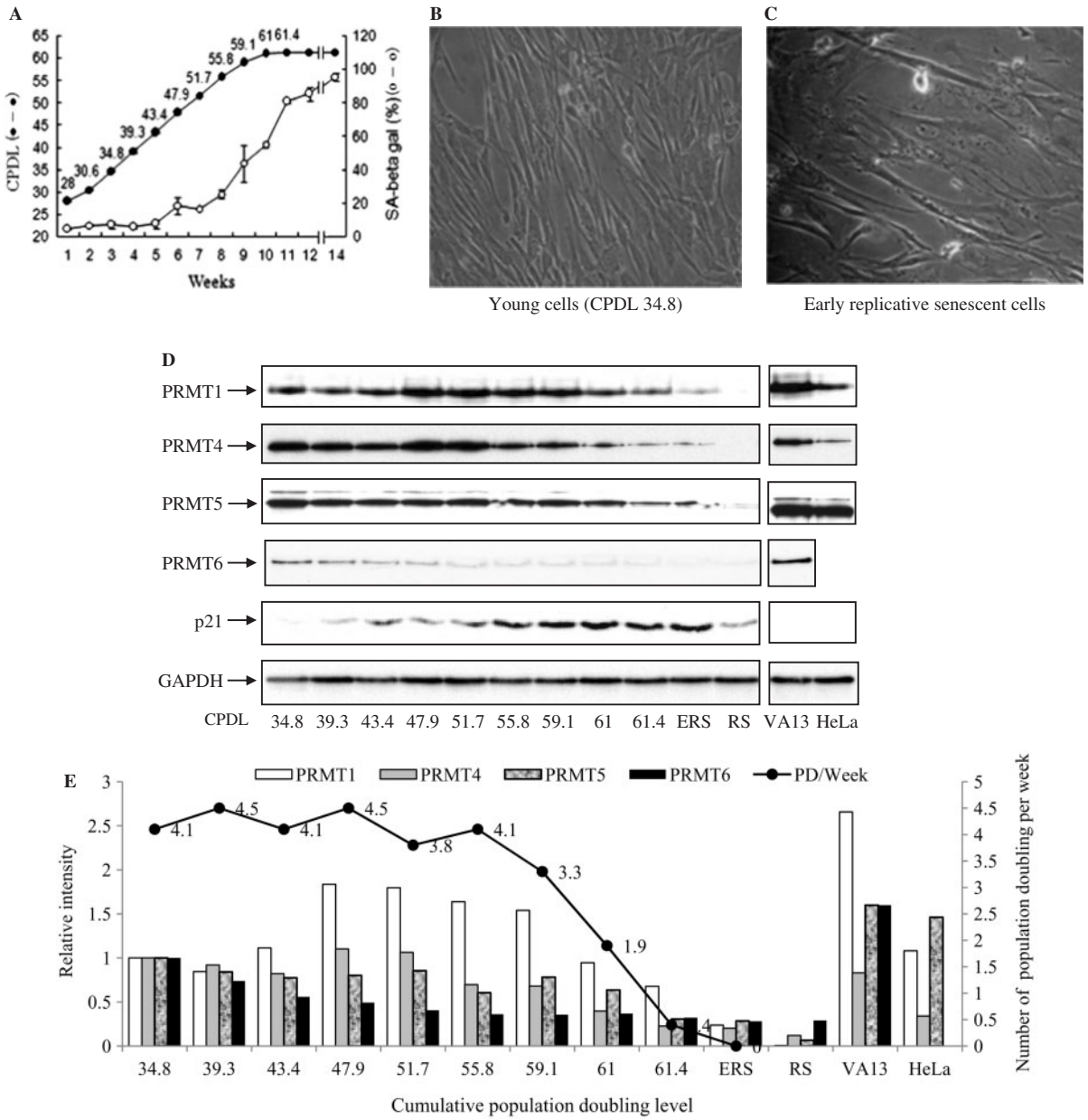

Fig. 1. **Age-dependent changes of biomarkers and PRMTs expression during the life span of WI-38 fibroblasts.** After inoculation of WI-38 fibroblasts (CPDL 25), the cells were routinely subcultured every seventh day for calculation of CPDL and various biochemical experiments were carried out. Under these culture conditions, WI-38 fibroblasts reached a senescence state at CPDL of late 50 to early 60 times, and one of their life spans is shown. Population doubling was stopped by serial passage in the 12th week after starting the culture. Replicative senescence was obtained by maintaining the ERS (12 weeks) for 2 weeks without subcultivation. (A) At every subcultivation, the cumulative population doublings were calculated (closed circles) and the percentage of SA-β-gal positively stained cells were quantified (open circles) as described in the MATERIALS AND METHODS section. (B and C) Representative photographs of young proliferating and ERS cells at 5 days after seeding, respectively. (D) Whole-cell lysates (10 μg) from age-dependent fibroblasts and two immortalized cell lines (VA13 and HeLa) were subjected to western immunoblot analysis with respective antibodies. VA13 indicates SV40-mediated immortalized WI-38 cell line. (E) Densitometric analysis of PRMTs in (D) was carried out. Closed circles indicate the number of population doubling per week during the life span of WI-38 fibroblasts.

activity was detectable in 85.6% of the early replicative senescent (ERS) cells at 12 weeks when only 5% of young cells showed SA-β-gal activity (Fig. 1A, open circle). Furthermore, p21 expression was gradually up-regulated during RS (Fig. 1D). In more fully senescent cells at 14 weeks, however, p21 expression showed a drastic decrease, compared with the ERS cells. This observation is consistent with previous reports that p53 senses senescence-inducing stimuli, which then activates the expression of p21 and results in cell cycle arrest, whereas p16 is responsible for the maintenance of senescent state (*17*, *18*).

To investigate the protein expression of PRMTs during replicative senescence, we chose four best characterized PRMT members, including three type I PRMTs (PRMT1, PRMT4 and PRMT6) and one type II PRMT (PRMT5). As seen in Fig. 1D and E, while all the PRMTs tested showed different expression profiles during RS, a remarkable reduction of the PRMT members was observed in replicatively senescent cells. In particular, significant reduction of PRMT6 was detectable at CPDL 47.9, even though the fibroblasts showed relatively constant doubling level to CDPL 55.8. In contrast, the PRMT members except for PRMT4 showed higher expressions in WI-38 VA13 and HeLa cell lines than in young fibroblasts (Fig. 1E).

In previous studies, histones H3 and H4 are known to be arginine methylated by PRMT1 (*19*), PRMT4 (*20*), PRMT5 (*21*) and PRMT6 (*22*). To confirm whether the decreased expression of the PRMTs leads to lower enzymatic activity, age-dependent cell extracts were prepared and endogenous activity of PRMTs was assayed with histone mixtures from calf thymus as previously reported (*9*). As shown in Table 1, specific activities of ERS and RS cells were reduced to 80.8% and 55.8%, respectively, compared with that of the young cells. Since it is known that histone lysine methyltransferases are present in nuclei fraction (*23*), the cytosolic fractions from young, ERS and WI-38 VA13 cells were prepared to exclude lysine methyltransferase activity. When enzyme activity was determined with the cytosolic fractions, the activity from ERS cells was further reduced to 68.3%. In contrast, the activity of WI-38 VA13 cells showed 11.6% increase compared with that of the young cells (Table 1). The significant elevation in the cytosolic activity might have been due to the removal of endogenous transmethylation inhibitors such as *S*-adenosyl-L-homocysteine and peptide inhibitors (*14*, *15*) during the preparation of the fraction with a 10-kDa cut-off membrane. It should be pointed out that the expressed PRMTs are not routinely correlated with the activity of the PRMTs (*10*, *24*); however, the present data clearly indicated that not only was the expressed PRMTs reduced in RS cells, but also the enzymatic activities were down-regulated.

Down-regulation of the Expression of PRMTs in Oxidatively Premature Senescent WI-38 Fibroblasts—Among the stressors which can induce premature senescence in HDFs, oxidative stress is one of the most commonly used inducers of premature senescence. Therefore, we examined whether the four PRMT members are reduced in H_2O_2-induced premature senescent WI-38 fibroblasts. When the fibroblasts at CPDL 28 were

Table 1. **Enzyme activities of PRMTs using histone mixtures (type II-AS) as methyl acceptors.**

Cell stage	Whole-cell lysate		Cytosolic fraction	
	Specific activity	Relative (%)	Specific activity	Relative (%)
Y	1.71	100	3.97	100
NRS	1.41	82	–	–
ERS	1.38	80.8	2.71	68.3
RS	0.95	55.8	–	–
VA13	1.72	100	4.43	111.6

The reaction conditions and experimental procedure are described in 'MATERIALS AND METHODS'. Specific activity is defined as picomoles of [methyl-^{14}C] group transferred per minute per milligram. The activity assay was carried out duplicate.

treated with increasing concentrations of H_2O_2, the population of prematurely senescent fibroblasts was markedly elevated at 300 and 500 μM H_2O_2, confirmed by SA-β-gal activity (Fig. 2A and C), as well as p21 expression (Fig. 2D). When the expressions of four PRMT members were determined by western blot analysis, type I PRMTs (PRMT1, PRMT4 and PRMT6) were more significantly down-regulated in H_2O_2 concentration-dependent manner than type II PRMT5 (Fig. 2D and E).

Down-regulation of Asymmetric Arginine Methylation During Cellular Senescence—Methylarginine-specific antibodies SYM10, SYM11 and ASYM24 are rabbit polyclonal antibodies against alternative arginine–glycine (RG) sequences containing sDMA, SmD3 RG sequences containing sDMA, and alternative RG sequences containing aDMA in all the arginyl positions, respectively (*25*). Thus, we determined the level of methylarginine-containing proteins in the selected age-dependent and premature senescent cell extracts. As shown in Fig. 3A, the extracts from young fibroblasts contained higher aDMA-containing polypeptides at a molecular weight region between 60 and 100 kDa, which were significantly reduced in both replicative (Fig. 3A, left) and H_2O_2-induced premature senescent cells (Fig. 3A, right). In addition, overall level of the modification in immortalized cell lines was higher than in young fibroblasts (Fig. 3A, VA13 and HeLa). This phenomenon was paralleled with the expression of type I PRMTs (Fig. 1E), and the results suggest that the maintenance of asymmetric modification in the expressed target proteins of type I PRMTs might be critical for cellular proliferation.

Next, we determined the level of total sDMA-containing proteins in the extracts by SYM10/11 antibodies. In replicatively senescent cells, while symmetric dimethylation of two proteins between 50 and 75 kDa (Fig. 3B, indicated by arrows) decreased, the modification of them in two immortalized cells was highly elevated in parallel with the expression of PRMT5 (Figs 1E and 2E), indicating that PRMT5 might catalyse the symmetric modification of the proteins. On the contrary, a clear increase in symmetric dimethylation of 100 and 140 kDa polypeptides (Fig. 3B, indicated by asterisks) was observed in replicatively and oxidatively senescent cells. A possible explanation of this phenomenon is the role of other type II PRMTs such as PRMT7 and PRMT9.

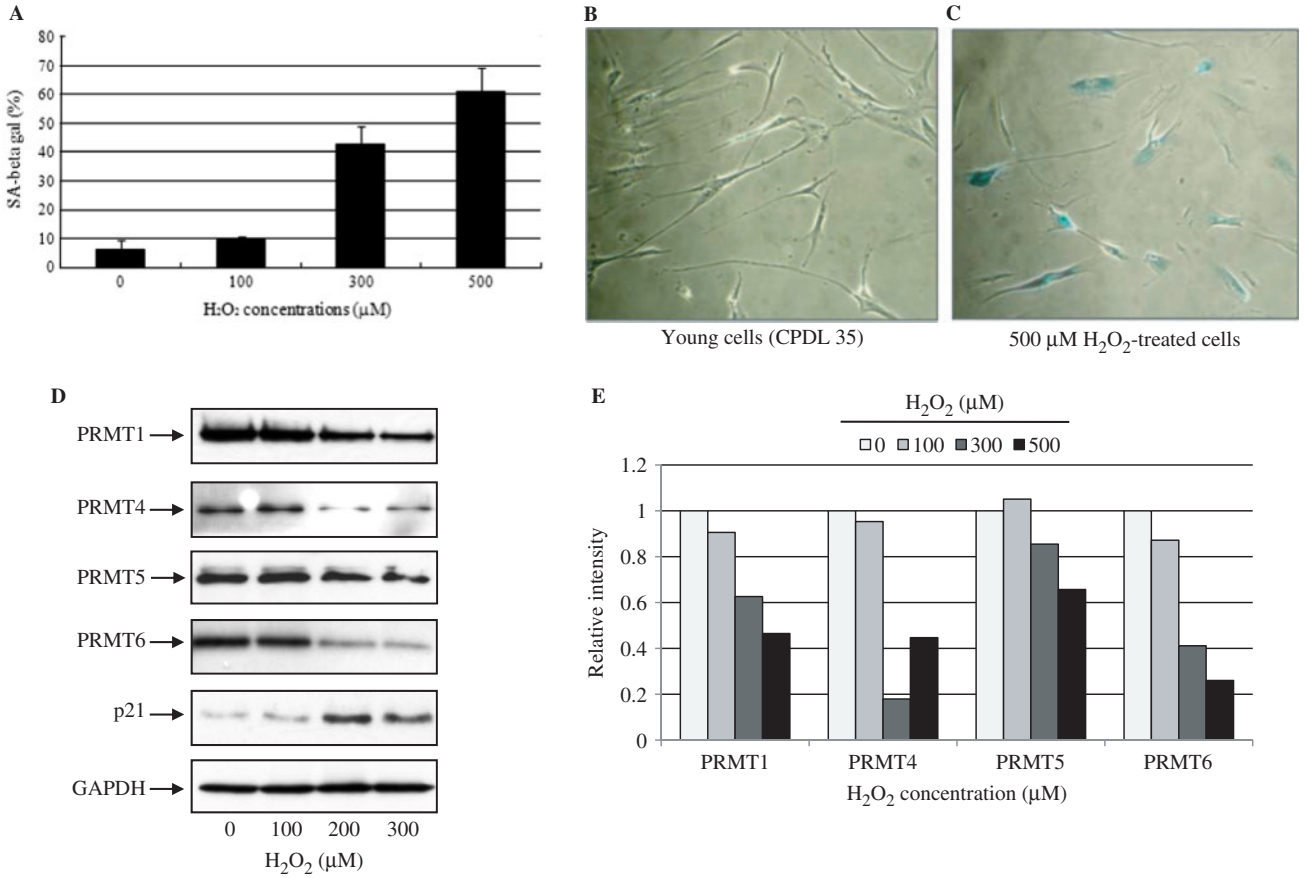

Fig. 2. **Down-regulation of PRMTs expression in H_2O_2-induced premature senescent WI-38 fibroblasts.** Premature senescence of WI-38 cells at PD 28 was induced by increasing concentrations of H_2O_2 treatment as described in the MATERIALS AND METHODS section. (A) The percentage of SA-β-gal positively stained cells were calculated by the same method as in Fig. 1A. (B and C) Representative photographs of untreated control (CPDL 35) and 500 μM H_2O_2-induced premature senescent cells, respectively, after SA-β-gal staining. (D) The PRMT subfamily members and p21 protein level were analysed by western blot analysis. (E) Relative intensities of the PRMT members in (D) were quantified by densitometric analysis.

Protein Arginine Methylation and Cellular Proliferation—In the present study, we have shown the significant down-regulation of type I PRMT members and subsequent aDMA-containing proteins in both replicatively and oxidatively senescent cells compared to the young cells. In contrast, the levels of the aDMA-containing proteins and type I PRMTs were highly elevated in immortalized cell lines compared to normal young fibroblasts (Figs 2E and 3A). Consistent with our findings, recent studies showed that asymmetric modification has an essential function in oncogenesis (*26*, *27*). In particular, among the PRMTs tested in this study, down-regulation of PRMT6 showed the earliest reduction with replicatively and oxidatively premature senescent progresses (Figs 1E and 2E). In previous studies, methylation of arginine residues 25, 57 and 59 in HMGA1a protein by PRMT6 has been reported (*28*). Interestingly, the modification of Arg25 and Arg57 has been suggested to correlate with apoptotic process and tumour progression (*29*, *30*). Therefore, it is interesting to identify PRMT6-mediated change(s) of arginine modifications in HMGA1a proteins from immortalized, young and senescent cells.

Recently, Polotskaia *et al*. (*31*) showed that endothelial cells resulted in significant reduction of asymmetric dimethylation after treatment with peroxynitrite (a powerful oxidant) or a culture on surfaces coated with glycated collagen, which induces formation of peroxynitrite and premature senescence of endothelial cells. In the present study, we also found the reduction of asymmetric modification in H_2O_2-induced senescent cells. However, no decrease of asymmetric dimethylation was observed as well as the expression of type I PRMTs in subcytotoxic concentrations of H_2O_2-treated WI-38 VA13 cells (data not shown). Together, these data suggest that down-regulation of asymmetric dimethylation by oxidative stress might serve as a distinct process in cellular senescence. The mechanism by which oxidative stress causes the reduction of the modification and its subsequent effect on cellular ageing must further be identified.

FUNDING

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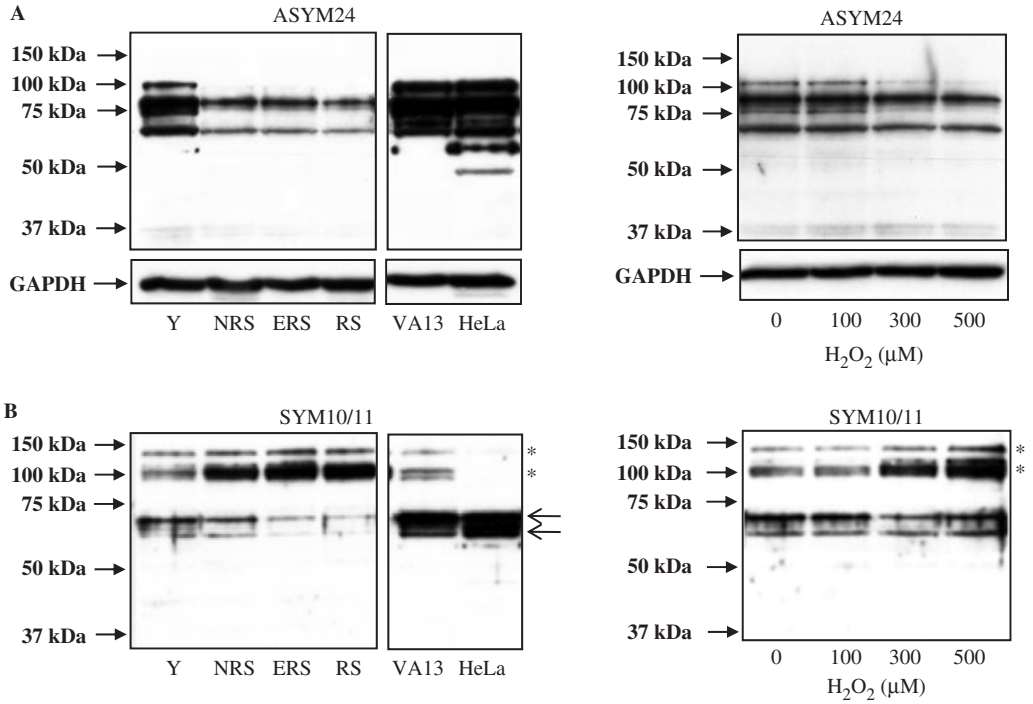

Fig. 3. **A comparison of the level of methylarginine-containing polypeptides between actively proliferating and senescing cells.** Whole-cell lysates (10 μg) of the indicated cell extracts were subjected to western blot analysis. To examine symmetric arginine methylation, SYM10 and SYM11 antibodies were mixed in the same blocking buffer at optimum dilution ratios after preliminary assays. (A) The levels of asymmetric arginine methylation in the selected age-dependent WI-38 as well as two immortalized cells (left) and increasing concentrations of H_2O_2-treated young fibroblasts (right) were determined by ASYM24 antibodies. (B) The levels of symmetric arginine methylation of the same cell extracts in (A) were analysed by SYM10/11 antibodies. Asterisks and arrows in (B) indicate gradually increased and decreased signals detected by SYM10/11 antibodies, respectively. Y and NRS indicate young cells at CPDL 34.8 and near senescent cells at CPDL 61.4 after starting the culture in Fig. 1, respectively.

CONFLICT OF INTEREST

None declared.

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