BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 712–716 (1997)
ARTICLE NO. RC977371

Down-Regulation of Ku Autoantigen, DNA-Dependent Protein Kinase, and Poly(ADP-ribose) Polymerase during Cellular Senescence

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Received August 25, 1997

During aging and cellular senescence mutations accumulate in genomic and mitochondrial DNA. Ku autoantigens, DNA-dependent protein kinase, and poly (ADP-ribose) polymerase have an essential role in DNA damage recognition. Our purpose was to find out whether cellular senescence of fibroblasts affects the protein components that recognize DNA damage and induce the repair process. We compared presenescent and replicatively senescent human WI-38 fibroblasts with each other and with SV-40 immortalized and serum-deficient quiescent WI-38 cells. Our results showed that replicative senescence significantly decreased the nuclear level of both p70 and p86 components of Ku autoantigen. SV-40 immortalization and cellular quiescence did not affect the level of the p86 component but slightly increased that of p70. Both replicative senescence and cellular quiescence decreased the activity of DNA-dependent protein kinase in WI-38 fibroblasts. On the other hand, SV-40 immortalization increased the activity of DNA-dependent protein kinase. The protein level of poly(ADP-ribose) polymerase (PARP) was strongly decreased in replicatively senescent fibroblasts. Quiescence of early-passage fibroblasts also slightly reduced the protein level of PARP. Apoptosis was not observed in replicatively senescent fibroblasts. Our results show that replicative senescence and to some extent cellular quiescence down-regulate the recognition system of DNA damage involving Ku autoantigens, DNA-dependent protein kinase, and PARP and hence could enhance the accumulation of DNA damage during aging.

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Mutations in DNA accumulate during the aging of somatic tissues, as well as in cultured fibroblasts during replicative senescence (1, 2). The mitochondrial genome is particularly prone to mutations (3, 4). Prominent accumulation of mutations does not, however, appear until old age. Calculations of the number of DNA rearrangements and point mutations have shown that cellular and organismal aging correlates with the accumulation of DNA damage (1, 2, 4). It seems that aging decreases the inherent capacity of DNA in somatic cells to remain stabile and reduces the efficiency of the DNA repair systems, although it is not known whether the accumulation of DNA damage is a cause of aging or only a consequence of reduced metabolism.

Ku autoantigen and DNA-dependent protein kinase both have an important role in the recognition of DNA damage and in the activation of DNA repair machinery (5, 6). Several studies have shown that Ku autoantigen, a well-characterized heterodimer of 70 and 86 kDa proteins, recognizes and binds to double strand DNA breaks (7-9). Interestingly, Ku autoantigens, after binding to DNA breaks, recruits DNA-dependent protein kinase to bind to and form a complex with Ku proteins (9). DNA-dependent protein kinase can be activated by double-strand DNA-breaks (7) and by nicks and larger single-stranded gaps (10). DNA-dependent protein kinase is a serine/threonine type protein kinase and phosphorylates several important proteins, such as p53, Sp-1, SV-40 T-antigen, replication factor A, and RNA polymerases I and II (*see* 6, 11). It has been suggested that the activation of DNA-dependent protein kinase triggers a signal transduction cascade which activates DNA repair machinery, induces the expression of DNA repair genes, and arrests the cell-cycle progression (5, 6).

Poly(ADP-ribose) polymerase (PARP) is another enzyme which is involved in DNA damage recognition

0006-291X/97 $25.00

and repair (12). PARP is one of the death substrates during apoptosis and a target for ICE family of caspases (13). Interestingly, Grube and Burkle (14) have shown that the PARP activity in leukocytes of several mammalian species strongly correlates with species-specific life span. Higher PARP activity could efficiently maintain genome integrity and stability during a longer life span.

The purpose of this study was to find out whether the replicative senescence of human WI-38 fibroblasts affects the expression of Ku autoantigens, PARP and DNA-dependent protein kinase. In addition, we compared the effects of SV-40 immortalization and serum deficiency -induced quiescence to those of replicative senescence. We found that replicative senescence significantly down-regulated the expression of Ku autoantigens and PARP and the activity of DNA-dependent protein kinase. These results suggest that cellular senescence reduces the efficiency of DNA repair initiation and hence could be responsible for the increase in the accumulation of DNA mutations.

MATERIALS AND METHODS

Human diploid WI-38 lung fibroblasts were obtained from the American Type Culture Collection. Fibroblasts were cultured and replicatively senesced as described earlier (15). Mitotic quiescence of WI-38 fibroblasts was induced in both low-passage and late-passage as well as in SV-40 immortalized WI-38 fibroblasts by culturing the cells without fetal calf serum for 72 h (15, 16). Nuclear proteins were isolated by the method of Dignam et al. (16) using modifications described in detail earlier (15).

The protein levels of p70 and p86 Ku autoantigens were assayed using Western blot technique. Primary antibodies for p70 (M19) and for p86 (J15) were from Santa Cruz company. Polyclonal primary antibody against poly(ADP-ribose) polymerase was obtained from Boehringer Mannheim. Twenty μg of each protein sample was used to run 10% SDS-PAGE. Low molecular weight stardard mixture from Pharmacia was used as a molecular weight standard. Proteins were transferred from gel to Hybond nitrocellulose filters (Amersham) using Bio-Rad Semi-dry Transfer Blotter. Membranes were blocked with 2% BSA in PBS containing 0.05% Tween-20. Membranes were incubated with primary antibodies (1:2000 dilution) and then with secondary antibodies conjugated with HRP (1:4000 dilution). Results were visualized using Chemiluminescence Reagent from Pierce and Hyperfilm-ECL from Amersham.

The activity of DNA-dependent protein kinase was assayed as described in detail by Anderson and Lees-Miller (11). Briefly, the specific SQE-peptide (EPPLSQEAFADLWKK) of human p53 was used as a phosphorylation target. This peptide sequence contains only human p53 Ser 15 motif for phosphorylation, which is highly specific for the DNA-activated protein kinase (11). The linearity of the reaction for protein content and incubation time was tested. Six μg of nuclear protein in a total volume of 20 μl were incubated for 15 min at 30°C. Ten ng of poly (dI-dC) (Pharmacia) was used as an activator.

The effect of cellular senescence on the integrity of genomic DNA was studied using conventional agarose gel electrophoresis for internucleosomal fragmentation and field inversion gel electrophoresis (FIGE) for high molecular weight fragments. "In-drop-agarose-embedding" procedure used for sample preparation has been recently described elsewhere (18). Gels were stained with ethidium bromide and viewed under UV-transilluminator.

Immunocytochemical staining of p70 and p86 Ku autoantigens was done according to the protocol described earlier for myogenin (19). Antibodies against Ku autoantigens were the same as used for the Western assays. Cultured fibroblasts were stained with nuclear dye Hoechst 33258 (Sigma) to show possible apoptotic changes.

RESULTS AND DISCUSSION

Ku autoantigens play a central role in the recognition of DNA damage, because they bind to double-strand DNA breaks and initiate the DNA repair process (5, 6). Interestingly, we observed that cellular senescence *in vitro* considerably reduced the nuclear protein level of both p86 and p70 Ku autoantigens in WI-38 fibroblasts (Fig. 1). SV-40 immortalization and cellular quiescence did not affect the level of p86 Ku autoantigen but slightly increased the protein level of p70 (Fig. 1). Immunocytochemical stainings showed that both the protein p70 and p86 were mainly localized in the nuclei (Fig. 2), as observed earlier in several other cell types (20). Cytochemical study did not show any changes in the localization of these proteins during cellular senescence, immortalization or quiescence (Fig. 2). Western blot assays from the cytoplasm of WI-38 fibroblasts showed a very similar down-regulation in the cytosolic levels of p86 and p70 Ku proteins as in nuclei during

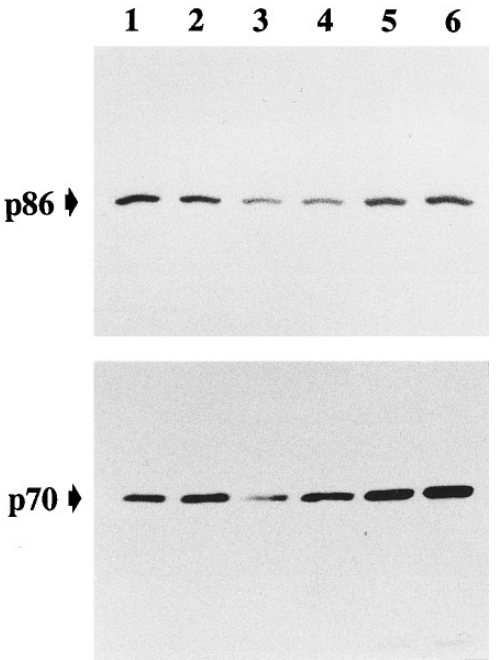

FIG. 1. Nuclear level of p70 and p86 Ku autoantigens in replicatively senescent, quiescent and SV-40 immortalized WI-38 fibroblasts. Nuclear protein samples were prepared and Western assays done as described in Methods. Separate lanes show proteins prepared from proliferating early-passage (lane 1), early-passage quiescent (lane 2), replicatively senescent late-passage (lane 3), late-passage quiescent (lane 4), proliferating SV-40 immortalized (lane 5), and SV-40 immortalized quiescent (lane 6) WI-38 fibroblasts. Upper part of the figure shows a filter stained with anti-p86 and lower part a filter stained with anti-p70 antibody.

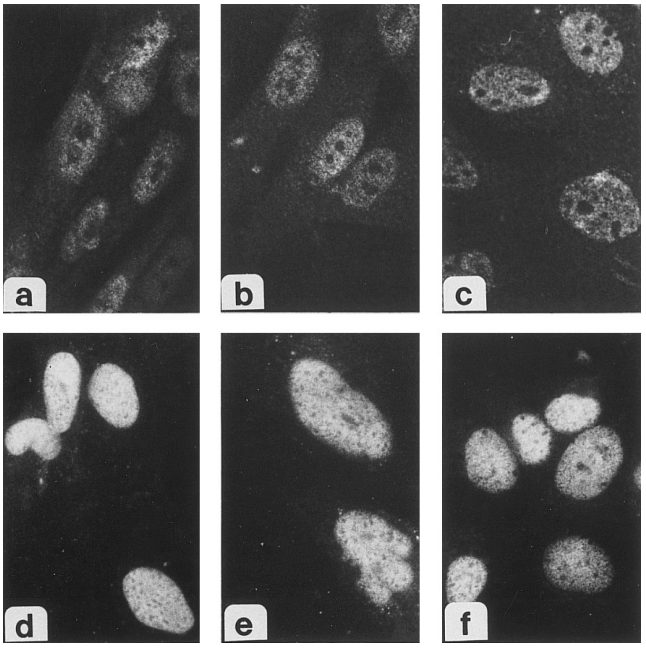

FIG. 2. Immunostaining of p86 and p70 Ku autoantigens in proliferating, replicatively senescent and SV-40 immortalized WI-38 fibroblasts. (**a-c**) Staining with anti-p86 antibody and (**d-f**) staining with anti-p70 antibody. (**a** and **d**) Proliferating early-passage; (**b** and **e**) senescent late-passage; and (**c** and **f**) SV-40 immortalized fibroblasts. Antibodies and staining conditions were as described in Materials and Methods.

cellular senescence (data not shown). These observations show that the level of Ku proteins decreases during cellular senescence that could retard the recognization phase of DNA damage and thus impair the DNA repair.

Our pilot experiments showed that the enzyme activity of DNA-dependent protein kinase was almost all located in the nuclear fraction (data not shown). SQE-peptide used in all experiments is a very specific substrate for the DNA-dependent protein kinase. The phosphorylation activity with or without poly(dI/dC) was very low in the cytoplasm, even though there is a wide variety of different type of protein kinases.

Replicative senescence of WI-38 fibroblasts caused a strong decrease in the activity of DNA-dependent protein kinase. Its activity fell to 30-40% of that found in proliferating early-passage fibroblasts (Fig. 3). On the contrary, the enzyme activity was significantly higher in SV-40 immortalized WI-38 fibroblasts than in early-passage fibroblasts (Fig. 3A). Since the proliferation capacity decreases during replicative senescence, it seems that the proliferation intensity of fibroblasts may affect the activity of the DNA-dependent protein kinase. Therefore, we studied whether mitotic quiescence would down-regulate the enzyme activity. Interestingly, the activity of DNA-dependent protein kinase in early-passage quiescent fibroblasts fell to the level found in slowly dividing senescent fibroblasts (Fig. 3B). The quiescence of senescent fibroblasts did not affect the enzyme activity (Fig. 3B). Our results thus suggest that the activity of DNA-dependent protein kinase is dependent on the proliferation, rather than the senescence, of fibroblasts.

The function of the DNA-dependent protein kinase is still largely unknown. Reduced enzyme activity during senescence could retard the activation of the DNA repair machinery and thus lead to the accumulation of DNA damages, as has been observed during senescence (1). Furthermore, the reduction in the activity of DNA-dependent protein kinase could reduce the phosphorylation of transcription factors, such as Sp-1, p53, and AP-1 (11), which could specifically regulate transcription and direct gene expression. We have recently observed that the DNA-binding activities of Sp-1 and AP-1 are strongly reduced in replicatively senescent human WI-38 fibroblasts (15). However, it seems that the decrease in the enzyme activity of DNA-dependent protein kinase is rather a consequence of the cell cycle arrest than its cause.

The activation of PARP enzyme is an immediate cellular response to DNA damage (12). PARP induces poly-ADP ribosylation of some chromatin proteins and also the enzyme itself upon binding to DNA strand breaks. However, several observations, *e.g.* transgenic animal models, suggest that PARP is not a crucial component in DNA repair itself (5) but may be valuable in the recognition phase of DNA damage. Interestingly, our Western analysis showed that PARP protein is nearly absent in the nuclei of senescent fibroblasts (Fig.

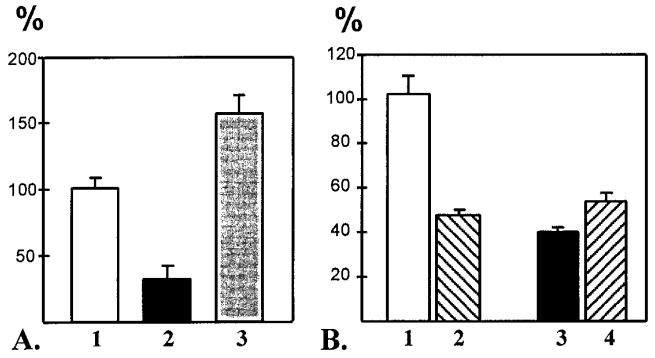

FIG. 3. Activity of DNA-dependent protein kinase in replicatively senescent, quiescent and SV-40 immortalized WI-38 fibroblasts. (**A**) Effects of replicative senescence and SV-40 immortalization: early-passage proliferating (column 1), late-passage senescent (column 2), and SV-40 immortalized (column 3) WI-38 fibroblasts. (**B**) Effect of serum deficiency -induced quiescence: early-passage proliferating (column 1) and quiescent (column 2) fibroblasts, late-passage high-serum (column 3) and serum-free quiescent (column 4) fibroblasts. Values of proliferating early-passage fibroblasts are calculated to 100%. Values are ± S.E. (n=4). Differences between early- and late-passage fibroblasts (A) and between proliferating and quiescent early-passage fibroblasts (B) are statistically significant ($p<0.01$, *t* test).

4). Quiescence of early-passage WI-38 fibroblasts also slightly reduced the nuclear level of PARP protein (Fig. 4). PARP is one of the first proteins broken down by proteolysis during apoptosis (13). CPP32 cleaves a full-length 116 kD PARP protein into 85 and 25 kD apoptotic fragments. In our study, we could not find this apoptotic fragment in replicatively senescent cells but a faint apoptotic fragment could be seen in SV-40 immortalized cells after three days of serum deficiency (Fig. 4). This observation was also verified by staining fibroblasts with a nuclear dye Hoechst 33258 to show apoptotic nuclei. Replicatively senescent fibroblasts did not show apoptotic nuclei but the quiescent SV-40 immortalized fibroblasts showed a few apoptotic nuclei (data not shown). This observation is in agreement with earlier studies which show that senescent human fibroblasts are more resistant to apoptosis than early-passage fibroblasts (21).

Next, we studied whether the reduced recognition capacity of nick and double-strand breaks would appear as an increased DNA fragmentation. Disintegration of genomic DNA to oligonucleosomal size of 180-200 bp is a typical marker of apoptosis (22). However, ordered higher molecular weight DNA cleavage into 50 to 300 kb fragments may precede the oligonucleosomal fragmentation and induce chromatin condensation (23). In some cell types, high molecular weight DNA fragmentation is also associated with cellular differentiation (24). Although replicative senescence involves profound changes in both chromatin and nucleolar organization, we could not find any 50 kb fragments in replicatively senescent fibroblasts, and 300 kb fragments showed nearly equal level in early- and late-passage WI-38 fibroblasts (Fig. 5). Instead, cellular quiescence induced by serum withdrawal caused the appearance of 50 kb fragments both in early- and late-passage fibroblasts and especially in SV-40-immortalized fibroblasts (Fig. 5). These results show that high molecular weight DNA fragmentation is not related to replicative senescence but to early phases in serum deficiency -induced cellular apoptosis. As noted earlier, PARP cleavage was also present in the quiescent SV-40 immortalized fibroblasts (Fig. 4). Oligonucleosomal DNA ladder could not be found in any of our fibroblast samples (data not shown).

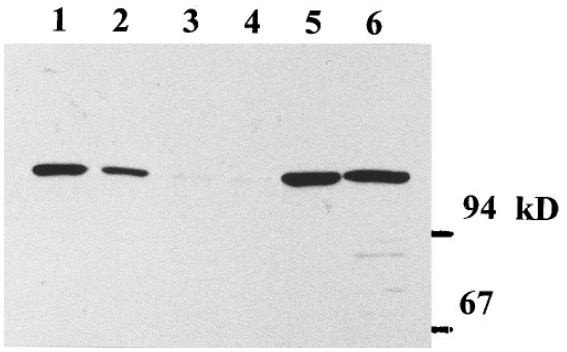

FIG. 4. Nuclear protein level of poly(ADP-ribose) polymerase in replicatively senescent, quiescent and SV-40 immortalized WI-38 fibroblasts. Separate lanes show proteins prepared from proliferating early-passage (lane 1), early-passage quiescent (lane 2), replicatively senescent (lane 3), senescent quiescent (lane 4), proliferating SV-40 immortalized (lane 5), and SV-40 immortalized quiescent (lane 6) WI-38 fibroblasts. Note the apoptotic p85 fragment on lane 6. Western assay was done as described in Materials and Methods.

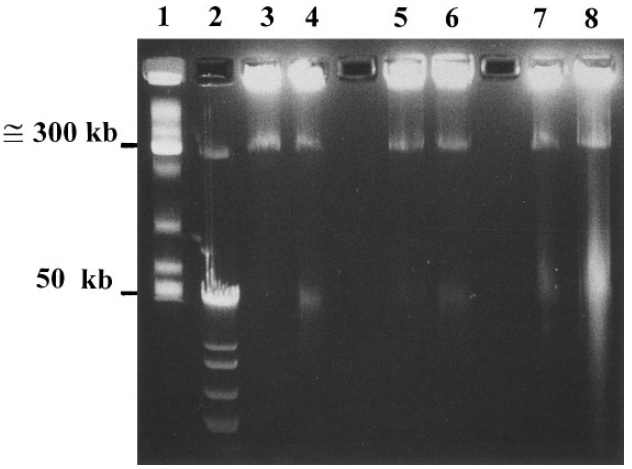

FIG. 5. Pattern of high molecular weight DNA disintegration in replicatively senescent, quiescent and SV-40 immortalized WI-38 fibroblasts. Lanes: λ-DNA ladder molecular weight marker (lane 1), 1 kb-DNA ladder and λ-DNA (lane 2), early-passage proliferating (lane 3), early-passage quiescent (lane 4), late-passage high-serum (lane 5), late-passage quiescent (lane 6), SV-40 immortalized proliferating (lane 7) and SV-40 immortalized quiescent (lane 8) fibroblasts. Figure shows the pattern of DNA cleavage revealed by field inversion gel electrophoresis (FIGE). Molecular markers were from New England Biolabs.

Several studies have shown that the accumulation of DNA mutations is due to the age-dependent decline in DNA repair capacity, both in tissues (25) and cultured fibroblasts (26, 27). In particular, DNA repair capacity is reduced in progeric cells (27). Our results suggest that the down-regulation of Ku autoantigens, DNA-dependent protein kinase, and PARP enzyme could retard the recognition of DNA damage and thus impair the DNA repair during cellular senescence.

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