Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-κB

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Both the aging of animals and the senescence of cultured cells involve an altered pattern of gene expression, suggesting changes in transcription factor regulation. We studied age-related changes in transcription factors nuclear factor (NF)-κB, activator protein factor-1 (AP-1) and Sp-1 by using electrophoretic mobility shift binding assays; we also analysed changes in the protein components of NF-κB complex with Western blot assays. Nuclear and cytoplasmic extracts were prepared from heart, liver, kidney and brain of young adult and old NMRI mice and Wistar rats as well as from presenescent, senescent and simian virus 40-immortalized human WI-38 fibroblasts. Aging of both mice and rats induced a strong and consistent increase in the nuclear binding activity of NF-κB factor in all tissues studied, whereas those of AP-1 and Sp-1 decreased, e.g. in liver. Protein levels of p50, p52 and p65 components of the NF-κB complex did not show any age-associated changes in the cytoplasmic fraction but in the nuclear fraction the level of p52 strongly increased in heart and liver during aging. The protein levels of inhibitory IκB-α and Bcl-3 components were not affected by aging in any of the tissues studied. Replicative cellular senescence of human WI-38 fibroblasts induced a strong decrease in nuclear NF-κB, AP-1 and Sp-1 binding activities. Protein levels of p50 and p52 components of NF-κB complex were decreased in the nuclear fraction of senescent WI-38 fibroblasts but in the cytoplasm of senescent fibroblasts the level of p65 protein was increased. Cellular senescence also slightly decreased the protein levels of IκB-α and Bcl-3. Transfection assays with NF-κB-enhancer-driven chloramphenicol acetyltransferase reporter gene showed a significant down-regulation of NF-κB promoter activity in senescent WI-38 fibroblasts. Results suggest that the aging process might be regulated differently in tissues and cultured fibroblasts, perhaps reflecting differences between mitotic and post-mitotic cells. In tissues, aging seems to involve specific changes in the regulation of NF-κB components and perhaps also changes in the DNA-binding affinities of the NF-κB complex.

INTRODUCTION

Aging involves a progressive loss of physiological capacities at both the cellular and tissue levels [1,2]. There are a number of theories of the aging mechanism, which can be roughly divided into error accumulation theories and active programming theories [1]. Research is increasingly focusing on the identification of the genetic basis of age-related repressive gene regulation, which could lead to aging deficiencies in biochemical processes. During development, gene expression is regulated at the level of transcription [3]. The transcription of genes is determined by the combined action of activating and repressive transcription factors that bind to specific sequences in promoter and enhancer regions [4]. The rate of transcription is selectively affected both in senescent cells in culture and in aging tissues *in vivo* [1,5,6].

First evidence now suggests that age-related alterations in the transcription level of various genes could be due to selective changes in transcription factor binding properties and levels of expression during aging [7–10]. Ammendola et al. [7] observed that the binding activity of Sp-1 factor was greatly decreased in the brain and several other tissues in aged rats. The expression of Sp-1 at the protein level, however, was not affected by aging [7]. Dimri and Campisi [8] observed that the binding activities of activator protein factor-1 (AP-1), cAMP-response-element-binding protein (CREB) and CAAT-binding transcription factor complexes to their cognate sequences were decreased in senescent human WI-38 fibroblasts. Senescence-associated changes at the transcription factor binding level turned out to be very selective because the binding activities of octamer-binding protein-B and transcription factor IID were increased and those of nuclear factor (NF)-κB and Sp-1 were unaffected [8]. These observations show examples of the discrepancy in the results between cellular aging *in vivo* and the frequently used *in vitro* aging model, which is based on the Hayflick limit of division capacity of diploid fibroblasts in culture [11].

Several lines of evidence show that aging is associated with increased levels of oxidative stress [12–14]. We were interested to study whether aging also affects the regulation of the oxidative-stress-responsive transcription factor NF-κB [15]. Here we report that aging strongly increased the nuclear binding activity of NF-κB factor but not those of Sp-1 and AP-1, which mostly decreased or were unaffected during aging in rat and mouse tissues. By contrast, in cultured senescent fibroblasts the nuclear binding activities of all transcription factors studied consistently decreased. Further studies on the regulation of the NF-κB complex showed that, in animal tissues, aging selectively increased the nuclear level of the p52 component of the NF-κB complex. These results show that aging specifically modifies transcription factor binding activities and that aging is regulated differently in tissues and cultured fibroblasts. However, the aging response could be repressive to the function of the NF-κB signalling system in both tissues and cultured fibroblasts.

Abbreviations used: AP-1, activator protein factor-1; CAT, chloramphenicol acetyltransferase; CREB, cAMP-response-element-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift binding assay; NF, nuclear factor; SV, simian virus.

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MATERIALS AND METHODS

Animals and materials

Wistar Hannover rats and NMRI mice were obtained from the National Laboratory Animal Center (Kuopio, Finland). Male ($n = 10$) and female ($n = 6$) mice, half of them young adults (4 months old) and the other half old (2 years old) were used in the study. Female rats were of three age categories, with six animals in each group: young adult (7 months), middle-aged (18 months) and old (30 months). Animals were killed with carbon dioxide. Tissues were removed, washed in ice-cold 0.9 % NaCl, frozen in liquid nitrogen and stored at −80 °C.

Double-stranded oligonucleotide-binding sites to NF-κB, AP-1 and Sp-1, as well as the T4 polynucleotide kinase, were from Promega. Poly(dI-dC)·poly(dI-dC) was obtained from Pharmacia Biotech. PAGE chemicals and protein assay reagent were from BioRad. Primary antibodies against p50 (NLS), p52 (K-27, D-32), IkB-α/MAD-3 (C-15) and Bcl-3 (C-14) were from Santa Cruz and that against p65 (14631A) was from Pharmingen.

Cell culture

Human lung WI-38 fibroblasts were obtained from the American Type Culture Collection. The passage number was 15 when we obtained the fibroblasts. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and the antibiotics penicillin and streptomycin. Fibroblasts were replicatively senesced by culturing up to passage 35 before being used in the assays.

Isolation of proteins

Nuclear proteins were isolated by the modified method of Dignam [16], described earlier in detail [17,18]. Tissues prepared for experiments were the cortices of brain, medial lobe of liver, right kidney and cardiac ventricles. Frozen tissues were weighed, transferred to Corex tubes and homogenized with an Ultra-Turrax homogenizer in ice-cold hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1.0 mM dithiothreitol (DTT), 10 mM Hepes, pH 7.9]. Homogenates were incubated for 10 min on ice and centrifuged (25000 ***g***, 15 min, 4 °C; RC5C Sorvall Instruments, Du Pont). Cytoplasmic proteins were collected from the supernatants and nuclear proteins from the pellets. Pellets were washed once and centrifuged at 10000 ***g*** for 15 min at 4 °C after which pellets were suspended in ice-cold low-salt buffer [25 % (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 20 mM KCl, 20 mM Hepes, pH 7.9]. Nuclear proteins were released by adding a high-salt buffer (25 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 1.2 M KCl, 20 mM Hepes, pH 7.9) drop by drop to a final concentration of 0.4 M KCl [16,17]. Samples were incubated on ice for 30 min, with smooth shaking. Soluble nuclear proteins were recovered by centrifugation (25000 ***g***, 30 min, 4 °C) and proteins were stored at −80 °C. The concentration of total protein in samples was measured with the Protein Assay Reagent kit of Bio-Rad.

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of NF-κB, Sp-1 and AP-1 transcription factors in nuclear extracts [19]. The binding activity of NF-κB was also analysed in cytoplasmic extracts after the activation of inactive cytoplasmic NF-κB with deoxycholate [20].

Consensus and mutated double-stranded oligonucleotides of NF-κB, AP-1 and Sp-1 were labelled with T4 polynucleotide kinase by using the protocol of Promega. Labelled oligonucleotides were purified with native 4 % PAGE and dissolved from the gel to distilled water by overnight incubation at 4 °C.

Protein–DNA binding assays were performed with 10 μg of nuclear protein and 25 μg of cytoplasmic protein in each reaction. Because salt can affect the binding activity [19], the concentration of salt was adjusted to the same level in all samples by adding low-salt and high-salt buffer. Unspecific binding was blocked by using 1 μg of poly(dI-dC)·poly(dI-dC). The binding medium contained 4 % glycerol, 1.0 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 1.0 mM DTT and 10 mM Tris/HCl, pH 7.5. In each reaction 20000 c.p.m. of radiolabelled probe was included. In NF-κB binding assays, 1.0 % (v/v) Nonidet P40 was included. To activate the inactive cytoplasmic NF-κB complex, 2.0 % (w/v) deoxycholic acid was included in the binding assay. Samples were incubated at room temperature for 15 min. Bound and unbound probes were separated in a native 4 % polyacrylamide gel with a running buffer of 50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5. Binding results were revealed on an autoradiography film (Reflection Nef-496, Du Pont) exposing for 1–2 days at −80 °C.

Western blot assay

NF-κB protein components were assayed in cell extracts with Western blot assays by using primary antibodies from Santa Cruz against p50 (NLS), p52 (K-27 and D-32), IκB-α/MAD-3 (C-15) and Bcl-3 (C-14) and against p65 (14631A) from Pharmingen. A 15 μg portion of each protein sample was separated in SDS/PAGE (12 % gel) with a running buffer of 25 mM Tris, 192 mM glycine and 0.1 % SDS. Prestained SDS/PAGE standard mixture (Bio-Rad) was used as a molecular mass standard.

Proteins were transferred from the gel to membrane (Immobilon-P; Millipore) with a Pharmacia LKB Multiphor II blotting unit and a buffer of 50 mM Tris, 40 mM glycine, 0.038 % SDS and 10 % (v/v) methanol. Membranes were blocked [5 % (w/v) fat-free milk in PBS containing 0.1 % Tween-20 for 1 h at room temperature] and incubated with the primary antibody [1:200 dilution in PBS containing 1 % (v/v) BSA and 0.1 % Tween-20] for 2 h at room temperature. The secondary antibody used was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000 dilution in PBS containing 1 % BSA and 0.1 % Tween-20) for 1 h at room temperature. Results were revealed on autoradiography film by using Renaissance Western Blot Chemiluminescence Reagent (Du Pont) in accordance with the manufacturer's protocol.

Transfections

Young (passage 20) and senescent (passage 33) human WI-38 fibroblasts were transiently transfected with plasmid NFκB-TK10-CAT, which contains a mouse TNF-α κB enhancer and chloramphenicol acetyltransferase (CAT) reporter gene [21]. Fibroblasts were co-transfected with pCH110 (Amersham) containing the *lacZ* reporter gene to exclude differences in transfection efficiences and translation capacities between young and senescent WI-38 fibroblasts. Transfections were made with the calcium phosphate precipitation method and proteins were harvested after 48 h. The expression of CAT was analysed with a CAT-ELISA kit (Boehringer) and that of β-galactosidase by using chlorophenol red-fA-D-galactopyranoside substrate (Boehringer). Assays were made in accordance with the manufacturer's protocol.

Statistics

The autoradiographs were analysed with an Image Grabber 2.1 (Neotech Ltd.) and Image 1.43 programs to measure the mean densities of binding bands. Results were analysed with a univariate analysis of variance and the non-paired Student's t test.

RESULTS

Up-regulation of nuclear NF-κB binding activity in tissues during aging

Transcription factors AP-1, Sp-1 and NF-κB showed a variable binding level in different tissues and specific changes during aging. Most interestingly, a strong and consistent up-regulation of nuclear NF-κB binding activity was observed during aging in all tissues studied, in both rats and mice (Figure 1, and results not shown). The up-regulation of this oxidative-stress-responsive transcription factor was most marked in liver and heart. The increase in the nuclear binding activity of NF-κB seemed to coincide with the aging process, because an increased level of nuclear NF-κB binding was already observable in middle-aged rats (Figure 1). The increase in binding activity from 4- to 24-month-old mice varied from 45% in brain to 120% in liver. In rat, the highest age-related increase occurred in heart, where the change was 3-fold (Figure 1B). All these changes were statistically significant ($P < 0.01$). However, the total binding activity of the cytoplasmic NF-κB complex, activated by deoxycholate, did not show any statistically significant changes during aging in any of the tissues studied. Figure 1D shows the binding activities in mouse liver.

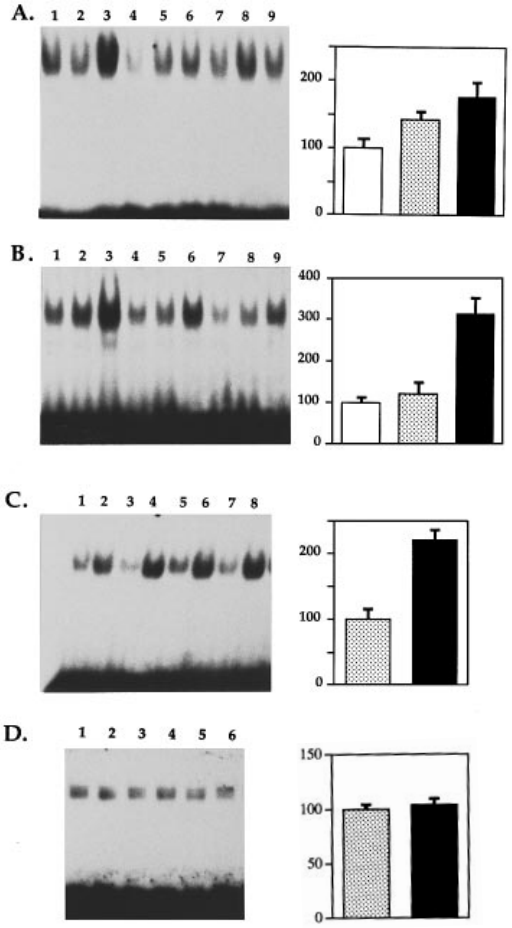

Figure 1 Aging-induced up-regulation of NF-κB binding activity in rat and mouse tissues

The EMSA method was used to compare nuclear NF-κB-binding activities in different tissues of rats and mice. Rat liver (**A**) and heart (**B**) samples of young adult (lanes 1, 4 and 7), middle-aged (lanes 2, 5 and 8) and old (lanes 3, 6 and 9) rats are shown on the left and the corresponding densitometric values from all animals in each group ($n = 6$) are presented on the right. (**C**) Mouse liver nuclear samples of young adult (lanes 1, 3, 5 and 7) and old (lanes 2, 4, 6 and 8) mice. (**D**) Mouse liver cytoplasmic samples of young adult (lanes 1, 3 and 5) and old (lanes 2, 4 and 6) mice. NF-κB-binding activity was activated by deoxycholate (see the Materials and methods section). On the right are densitometric results of eight young (grey column) and eight old (black column) mice. The values for young adult rats and mice are calculated to 100%; variations shown are S.D. All nuclear NF-κB differences between young adult and old animals are statistically significant ($P < 0.01$).

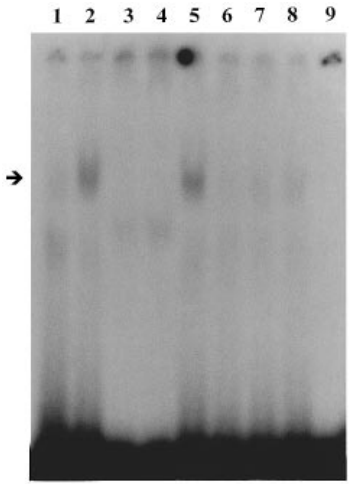

Figure 2 Effects of mutation of the NF-κB binding site and the addition of competing unlabelled consensus oligonucleotides on NF-κB binding in rat liver

The EMSA method was used to compare the nuclear NF-κB-binding activity of liver to consensus oligonucleotides in young (lane 1) and old (lane 2) rats, as well as to mutated oligonucleotides in young (lane 3) and old (lane 4) rats. The addition of competing unlabelled consensus oligonucleotides to the NF-κB-binding reaction of old rat liver: no addition (lane 5); 100-fold (lane 6), 50-fold (lane 7) and 10-fold (lane 8) excess. Lane 9, probe without protein sample. The arrow shows the NF-κB binding.

All age-related changes in the nuclear NF-κB binding activity were quantitative because any new binding complexes could not be observed with the PAGE retardation method used (Figure 1). Furthermore we studied the specificity of NF-κB binding by using the mutated binding site (Santa Cruz) and competing unlabelled oligonucleotides. These experiments showed that NF-κB-binding activity is specific because the mutated NF-κB-binding site does not bind the complex, and the addition of 10-fold unlabelled oligonucleotides to the binding reaction strongly decreased the NF-κB binding activity (Figure 2).

To compare the highly increased nuclear NF-κB binding activity with other transcription factors we also analysed the binding activities of AP-1 and Sp-1 from the same nuclear extracts. In agreement with the observations of Ammendola et al. [7], the binding activity of Sp-1 was strongly down-regulated during aging in the brain and heart of mice and in the liver and heart of rats. In rat brain, however, there was no age-related difference in the binding activity of Sp-1 (results not shown). The binding activity of AP-1 showed a high inter-individual variance and the only statistically significant age-related alteration observed was the 60% reduction in mouse liver (results not shown).

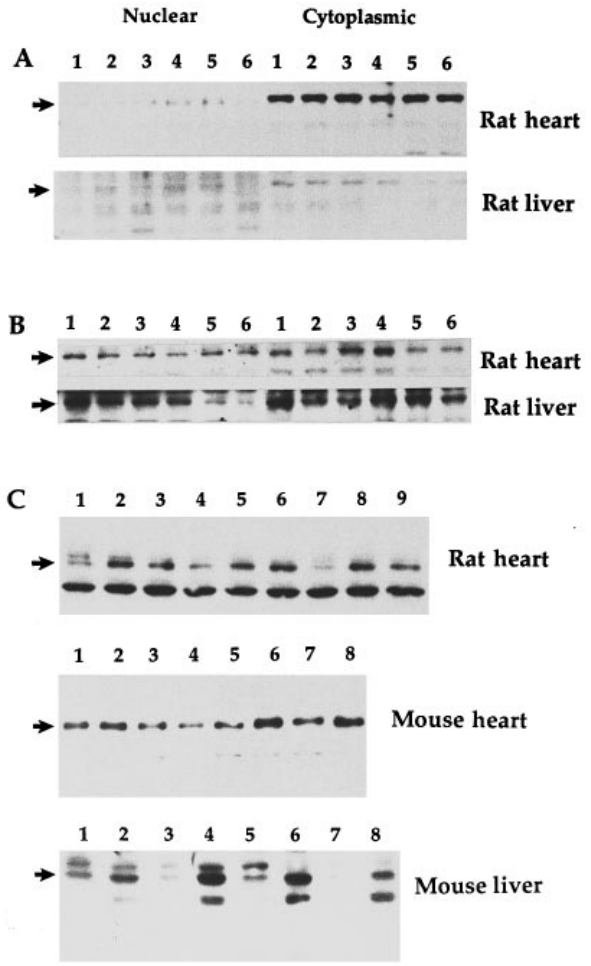

Figure 3 Protein levels of NF-κB components in tissues of mice and rats of different ages

Western blot assays of p65 (**A**), p50 (**B**) and p52 (**C**) protein components of the NF-κB complex in nuclear and cytoplasmic fractions of rat and mouse heart and liver. (**A**) Nuclear and cytoplasmic samples of rat liver and heart of young adult (lanes 1 and 4), middle-aged (lanes 2 and 5) and old (lanes 3 and 6) animals. (**B**) Nuclear and cytoplasmic fractions of rat heart and liver of young adult (lanes 1 and 4), middle-aged (lanes 2 and 5) and old (lanes 3 and 6) animals. (**C**) Nuclear fractions of the heart of young adult (lanes 1, 4 and 7), middle-aged (lanes 2, 5 and 8) and old (lanes 3, 6 and 9) rats. Below are the nuclear fractions of mouse heart and liver of young adult (lanes 1, 3, 5 and 7) and old (lanes 2, 4, 6 and 8) animals. Arrows indicate the positions of the specific proteins.

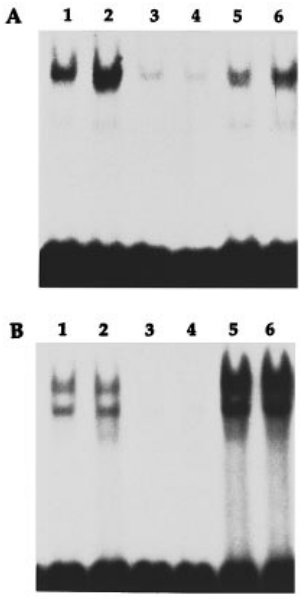

Figure 4 Effects of replicative senescence, SV-40 transformation and quiescence on NF-κB and Sp-1 binding activities

The EMSA method was used to compare nuclear NF-κB (**A**) and Sp-1 (**B**) binding activities in proliferating (lane 1), senescent (lane 3) and SV-40-transformed (lane 5) human WI-38 fibroblasts and corresponding quiescent fibroblasts (lanes 2, 4 and 6). The quiescence of young, senescent and SV-40-transformed fibroblasts was induced by culturing cells in 0.2% fetal calf serum for 72 h [8].

Changes in protein components of NF-κB complex during aging

The increase in the binding activity of nuclear NF-κB observed during aging could be due to changes in constitutive protein levels of NF-κB components, changes in the binding affinity of the components or changes in the distribution of NF-κB components between cytoplasm and nuclei. We analysed the protein levels of p50, p52 and p65 as well as inhibitory IκB-α and Bcl-3 by Western blot immunostaining in nuclear and cytoplasmic samples of liver and heart tissues from mice and rats. Figure 3(A) shows that the p65 component of NF-κB complex is present mostly in cytoplasm, and age-related differences were not observed in either the total protein levels or the distribution. The protein level of p50 was more abundant and more evenly distributed between nuclei and cytoplasm than the p65 component (Figure 3B). Age-related alterations were not observed in any tissue.

The amount of the p52 component of NF-κB complex in the nuclear fraction increased strongly during aging both in the heart and liver of mice and rats (Figure 3C). The protein level of p52 in the cytoplasm was low and was not affected by aging (results not shown). In the liver of old animals, there frequently appeared a low-molecular-mass immunopositive band, which could be a proteolytic fragment of p52 protein (Figure 3C).

The protein levels of inhibitory IκB-α and Bcl-3 were not affected by aging in any tissue studied (results not shown). In heart, IκB-α was nearly evenly distributed between the nuclear and cytoplasmic fractions but in liver it was located mainly in the cytoplasm. The inhibitory Bcl-3 was located totally in the nuclear fraction both in liver and heart.

Replicative senescence of human WI-38 fibroblasts in culture

Replicative senescence of WI-38 fibroblasts strongly decreased the proliferative capacity of cells and induced morphological changes, such as flatness of fibroblasts. In the cellular senescence experiments we compared the results of aging with those observed in simian virus (SV)-40-immortalized WI-38 fibroblasts. In contrast with the aging-induced up-regulation of NF-κB binding activity in tissue, in senescent fibroblasts the nuclear binding activity of NF-κB was strongly decreased (Figure 4). The quiescence of the growth potential of WI-38 fibroblasts by serum deficiency surprisingly showed an up-regulation of NF-κB-binding activity in both young proliferative and SV-40 immortalized fibroblasts, but not in quiescent senescent WI-38 fibroblasts

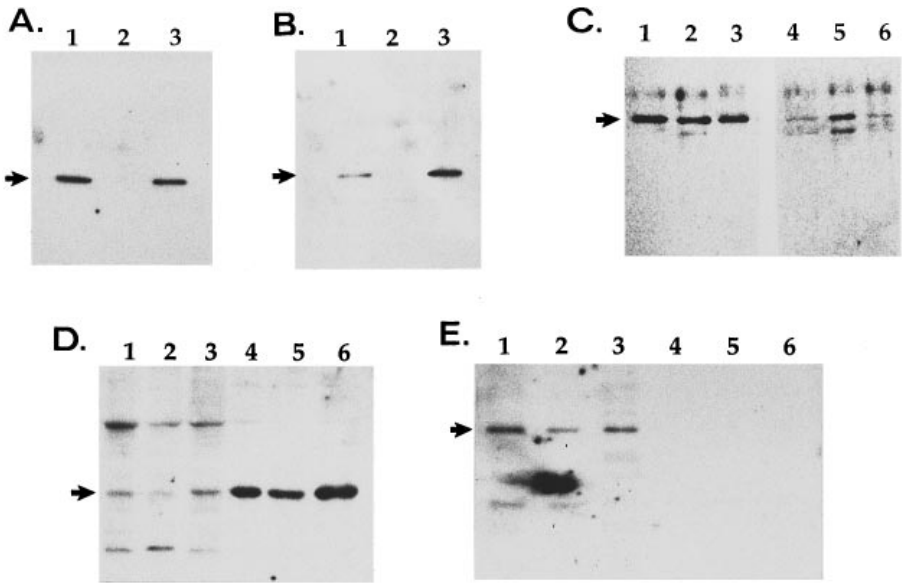

Figure 5 Protein levels of NF-κB components in proliferating, senescent and SV-40-immortalized fibroblasts

Western blot assays of protein levels of p50 (**A**), p52 (**B**), p65 (**C**), IκB-α (**D**) and Bcl-3 (**E**) components in nuclear and cytoplasmic fractions of human WI-38 fibroblasts. Lanes 1, 2 and 3, nuclear fractions of young, senescent and SV-40-immortalized fibroblasts respectively; lanes 4, 5 and 6, cytoplasmic fractions of young, senescent and SV-40-immortalized fibroblasts respectively. Arrows show the positions of the specific protein.

(Figure 4). This phenomenon appeared only with NF-κB-binding activity but not with Sp-1-binding activity (Figure 4). The nuclear binding activity of Sp-1 was strongly up-regulated by SV-40 immortalization and down-regulated by aging (Figure 4).

Western blot assays of the NF-κB components showed that nuclear levels of p50 (Figure 5A) and p52 (Figure 5B) proteins were strongly decreased in senescent WI-38 fibroblasts, but that of p65 (Figure 5C) decreased only slightly. Surprisingly, the level of cytoplasmic p65 protein was up-regulated in senescent WI-38 fibroblasts (Figure 5C). Cytoplasmic levels of p50 and p52 proteins were low and not affected by replicative senescence (results not shown).

The inhibitory IκB-α was located mostly in the cytoplasm (Figure 5D) but Bcl-3 was present only in nuclei (Figure 5E). Senescence of WI-38 fibroblasts decreased the levels of inhibitory IκB-α and Bcl-3 proteins to some extent.

Transfection assays with the NF-κB enhancer-driven CAT reporter gene plasmid were performed to show whether the decreased binding activity of NF-κB and the decreased protein levels of p50 and p52 affect the NF-κB-driven promoter activity. Young (passage 20) and senescent (passage 33) WI-38 fibroblasts were co-transfected with NFκB-TK10-CAT and pCH110 plasmids (see the Materials and methods section), in four separate experiments with each category of fibroblast. In young cells the promoter activity was 165 ± 38 pg of CAT per unit of β-galactosidase; in senescent cells the activity was 106 ± 14 (means $\pm$ S.E.M.; $P < 0.01$). These results show a significant down-regulation of the NF-κB-mediated promoter activity in senescent WI-38 fibroblasts.

DISCUSSION

Aging of tissues *in vivo* as well as the replicative senescence of cultured cells affect the expression patterns of a number of genes [1,2,6]. Several lines of evidence suggest that aging could be due to programmed changes in gene expression [1,6]. Programming could occur via the modulation of the profile and the function of transcription factors. However, there have been few studies on the expression level and the binding activities of transcription factors during aging and in replicative senescence [7–10,21].

Ammendola et al. [7] observed a strong and consistent decrease in the binding activity of Sp-1 in rat tissues such as brain and liver. In agreement with the observations of Ammendola et al. [7] we observed a strong down-regulation of Sp-1 in mouse brain and heart and in rat liver and heart. We could not find in rat brain any age-related differences in Sp-1 binding activity. Ammendola et al. [7] reported that aging does not affect the protein level of Sp-1 although the binding activity is markedly decreased. The binding activity of Sp-1 could be decreased by several regulatory mechanisms. We have observed that Sp-1 as well as AP-1 and CREB binding factors are sensitive to oxidative stress induced by the treatment of nuclear brain extracts with hydrogen peroxide *in vitro* [17]. The inhibition of DNA-binding activity of these factors is due to the oxidation of the thiol groups of cysteine residues in the DNA-binding domain [23,24]. The reduction of oxidized proteins with DTT and mercaptoethanol could totally restore the binding activities of AP-1 and CREB factors, but not Sp-1 binding [17]. Hence the oxidation of Sp-1 factor could permanently inhibit the DNA-binding activity of this factor and down-regulate the expression of several housekeeping genes that contain GC-boxes during aging.

The nuclear DNA-binding activity of the NF-κB factor strongly increased during aging in all tissues of rats and mice studied. This is in contrast with the down-regulation of the binding activity of Sp-1 and also that of AP-1 in some tissues. The increase in nuclear NF-κB-binding activity could be observed in, for example, liver tissue even in middle-aged rats, which shows that the process coincides with aging. There are several possible reasons why the nuclear binding activity of NF-κB could increase during aging. The most frequent cause of nuclear up-regulation is the activation of an inactive NF-κB complex in the cytoplasm and the translocation of NF-κB dimers to the

nuclei [25]. This seems unlikely during aging, however, because the protein levels of cytoplasmic p50, p52 and p65 components, as well as that of inhibitory IκB-α, were unaffected by aging. Instead we observed a marked increase in the nuclear concentration of the p52 component of the NF-κB complex, which could enhance the formation of the NF-κB complex in the nuclear fraction. A high level of the p52 component could also be observed in NF-κB-binding complexes in supershift assays. In addition to the changes in p52 protein level, changes in the binding affinities of complexes due to the phosphorylation of the p50 [26] and p65 [27] protein components could also play a role. The phosphorylation of the NF-κB components could strongly enhance the DNA-binding affinity of proteins and improve the stability of DNA–protein complexes during PAGE.

Protein p52 can form dimers with p50 and p65 components and bind to the κB sites [25]. Protein p52 does not contain the transactivation domain and hence the dimers of p50/p52 and p52/p52 are repressive to transactivation. Supakar et al. [28] have also observed a marked increase in NF-κB-binding activity in rat liver during aging. Supakar et al. [28] observed that NF-κB acts as a negative regulator for the rat androgen receptor gene in liver. They did not analyse the protein components of the NF-κB complexes. However, it seems that the increased NF-κB-binding activity during aging reflects an inhibitory activity in gene expression. The response of NF-κB to aging seems to be analogous to the down-regulation of the heat-shock response during aging [29] although this down-regulation is induced by decreasing the binding activity of heat shock factor.

Replicative senescence of human WI-38 fibroblasts is frequently used as a model of aging *in vitro* [2]. Contrary to the aging-induced up-regulation of nuclear NF-κB binding activities in tissues, we observed a marked down-regulation of NF-κB and Sp-1 transcription factors in cultured senescent human WI-38 fibroblasts. Western blot assays showed that the protein levels of p50 and p52 were down-regulated in the nuclear fraction. The cytoplasmic and nuclear levels of IκB-α were also slightly decreased in senescent fibroblasts. We also observed a significant down-regulation of NF-κB enhancer-driven CAT reporter gene in senescent WI-38 fibroblasts. It seems that replicative senescence down-regulates the NF-κB transcription factor signalling pathway in human WI-38 fibroblasts, as we have observed in UVB-induced activation of the NF-κB system (M. Helenius, unpublished work). NF-κB responses to aging are divergent in tissues *in vivo* and cultured fibroblasts *in vitro*. However, the aging responses could be repressive in the function of the NF-κB signalling system both in tissues and cultured fibroblasts. The repressive gene regulation of NF-κB could contribute to the progressive loss of physiological capacities observed during aging [1,2].

Several lines of evidence show that oxidative stress is one of the most important contributors to the aging process in animal tissues [30,31]. The redox status of cells, and especially several oxidants, can regulate the activation of the NF-κB signalling pathway by activating stress-responsive protein kinases that activate the cytoplasmic NF-κB complex [15,24]. However, the sustained activation of the stress-responsive NF-κB signalling pathway during aging seems unlikely because, for instance, the protein levels of cytoplasmic p50, p52 and IκB-α were not affected by aging in tissues. It seems that the expression of the p52 component increases during aging in tissues, because we could not see any pathological changes, such as inflammation, in liver and heart tissues of rat, which excludes the possibility of invading cells as an origin of NF-κB changes (M. Helenius, unpublished work). The expression of the p50 component of the NF-κB complex has been reported to be greatly increased in some cancers, e.g. in lung carcinoma, leaving the expression of p52 unaffected [32]. It seems that the p50 and p52 components of the NF-κB complex might have divergent functions in the regulation of the NF-κB signalling pathway.

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