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Loss of transcription factor AP-1 DNA binding activity during lymphocyte aging in vivo

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The main feature of cellular senescence is cessation of cell proliferation. Protooncogene *c-fos*, which is required for the cell to enter into DNA synthesis, is repressed in senescent fibroblasts. Diminished expression of *c-fos* and impaired formation of AP-1, which is a complex of c-Fos and c-Jun proteins acting as a transcription factor, was found in lymphocytes derived from old (>18 months) mice and stimulated with Con A. There were no differences in *c-jun* expression and formation of other transcription factors (AP-2 and AP-3) between lymphocytes isolated from old and young mice.

Transcription factor; *c-fos*; *c-jun*; Transcription factor; *c-fos*; *c-jun*; Lymphocyte; Aging

1. INTRODUCTION

The main feature of cellular senescence is cessation of cell proliferation. This phenomenon has been studied most extensively in cultures of human fibroblasts [1]. It is well established that expression of several genes is required for cells to progress through the G_0–S transition and enter DNA synthesis [2]. Senescent human fibroblasts, upon stimulation by serum, express many cell cycle-dependent genes activated in young cells, including regulatory ones like *c-myc* and *c-Ha-ras* [2]. However, serum-stimulated senescent cells fail to express the *c-fos* protooncogene [3]. Additionally, it has been shown that *c-fos* gene and protein expression are necessary for the cells to enter DNA synthesis upon appropriate stimulation [4,5]. All these results strongly suggest the involvement of *c-fos* gene expression in the regulation of cell proliferation. Fos proteins form a stable complex with proteins encoded by members of the *c-jun* family of protooncogenes and this complex, known as transcription factor AP-1, binds to specific regulatory elements termed AP-1 sites [6] and influences the expression of a number of genes. Recently, it was shown that the AP-1 binding activity is necessary for DNA synthesis and that it is impaired during senescence of human fibroblasts in culture [7].

T lymphocytes are physiologically quiescent cells which can be induced to enter the cell cycle in response to specific antigen or mitogenic lectins like concanavalin A (Con A). Cell cycle analyses in T lymphocytes have suggested an age-related decline in the number of cells able to enter the cell cycle [8]. Therefore, we found those cells particularly suitable to test whether aging in vivo may also involve an alteration of AP-1 transcription factor and its components. For our studies we decided to investigate *c-fos* and *c-jun* mRNA levels as well as DNA binding activity of AP-1 in Con A-stimulated T lymphocytes derived from the spleen of aged mice.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Splenocytes were obtained from mechanically disrupted spleens of healthy Swiss albino mice. Lymphocytes were separated by Ficoll gradient centrifugation and then cultivated at a concentration of 2×10^6 cells per 1 ml in RPMI 1640 medium supplemented with 5% fetal calf serum, 20 mM HEPES pH, 24 mM $NaHCO_3$, 5×10^{-5} M mercaptoethanol and antibiotics. Concanavalin A was added at the concentration of 5 μg/ml.

The splenocytes were monitored for the level of stimulation by measuring [^{3}H]thymidine incorporation into DNA (4 μCi/ml for 6 h) 66–72 h following Con A addition.

2.2. *RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)*

Following the desired period of culture, total RNA was isolated by the method of Chomczynski and Sacchi [9]. After the last step of precipitation with 2 vols. of ethanol the RNA was dissolved in diethyl pyrocarbonate-treated (DECP) water and visually checked for integrity and absence of DNA by electrophoresis in a nondenaturing agarose gel containing ethidium bromide. The RNA was then quantified by A_{260} absorbance and used to construct cDNA by the SuperScript Preamplification System (No. 8089SA, Gibco, BRL). 1.5 μg of RNA in 13 μl of DEPC-treated water was mixed with 1 μl of random hexamer, heated to 70°C for 10 min and quick-chilled on ice. The contents of tubes were collected by brief centrifugation and mixed with 10× synthesis buffer, 10 mM dNTP, 0.1 M DTT and 200 units/μl of reverse transcriptase. After 10 min at room temperature, samples were incubated for 50 min at 42°C. Reactions were terminated by immers-

ing the tubes at 90°C for 5 min. Then samples were incubated for 20 min at 37°C with 1 μl of RNase H.

Polymerase chain reaction (PCR) reactions (100 μl of final volume) were conducted in the presence of 400 ng of the 5' and 3' primers. The reagents for PCR were obtained from a PCR kit (Perkin-Elmer Cetus) and each reagent was utilized at the concentration recommended in the manufacturer's instructions. PCR was conducted in a thermal cycler (Perkin-Elmer) for 30 cycles: 1 min denaturation at 94°C, 3 min annealing at 48°C, and 3 min extention at 72°C. The reaction products were visualized by electrophoresing 20 μl of the final reaction in a 2% agarose gel containing 1 μg/ml of ethidium bromide in TBE buffer in the presence of ΦX174 RF DNA/*Hae*III as a size marker.

The primers were synthetized on LKB Gene Assembler Plus according to sequences (*c-fos* 5'ACAGCCTTTCCTACTACCAT, 3'TTGCCCCTTCTGCCGATGCT and *c-jun* 5'GTCTGGAGCGCACGCTCTAA, 3'ACGTGAGAAGGTCCGAGTTC) which were kindly provided by Dr. Rolf Muller.

2.3. *Protein extracts and electrophoretic mobility shift assay (EMSA)*

The nuclear protein extracts were prepared according to Schreiber et al. [10]. After the desired time of culture, typically 2×10^7 cells were washed twice with PBS and suspended in 500 μl cold buffer containing: 10 mM HEPES pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.5 mM PMSF and 10 μg/ml aprotinin. The homogenates were centrifuged for 30 s in a microcentrifuge at 4°C. The nuclear pellets were resuspended in 50 μl of an ice-cold buffer and the tubes were vigorously rocked at 4°C for 15 min. The buffer contained: 20 mM HEPES pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.5 mM PMSF and 10 μg/ml aprotinin. The nuclear extracts were centrifuged for 5 min at 4°C and the supernatants were frozen at −70°C. The protein content was estimated according to Bradford [11].

The AP-1, AP-2 and AP-3 sequences (22 oligomers) from a Stratagene 'gel shift' kit were labeled with terminal transferase and purified on the spun column according to the methods described by Maniatis et al. [12]. Binding reactions and electrophoresis were done as described before [13]. A probe (0.3 ng; 30,000–40,000 cpm) was incubated with 10 μl of incubation buffer containing: 4% Ficoll, 20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.25 mg/ml BSA, 8 μg/ml poly[d(I-C)] and 5 μl of nuclear extract (5 μg of protein). In some experiments 3.0 ng per sample of cold sequences were added for competition. After a 30 min incubation at room temperature, samples were electrophoresed through a 4% polyacrylamide gel (30:1 cross-linked, in 1 mM EDTA, 3.3 mM sodium acetate, 6.7 mM Tris-HCl) for 1.5 h at 120 V. Prior to electrophoresis 0.1% Bromophenol blue was added to the samples. Gels were dried and overnight exposed to X-ray film with intensifying screens.

3. RESULTS AND DISCUSSION

The proliferation responses of splenocytes derived from young (3 months) and old (20 months) mice to Con A stimulation were compared by measuring [^{3}H]thymidine incorporation (Table I). Con A-stimulated lymphocytes from old mice showed diminished proliferation capacity as was reported before [14].

In order to study *c-fos* and *c-jun* gene expression, we decided to use RT-PCR technique. This technique, while not fully quantitative, enabled the investigation of specific gene expression in the small number of cells available in this experiment described herein. In young cells the basal level of *c-fos* mRNA was detectable even in the absence of Con A (Fig. 1A) and then was dramatically elevated 1 h after treatment with mitogen. In contrast, no *c-fos* mRNA was observed in quiescent and cell cycle stimulated T cells from aged animals. On the other hand, *c-jun* mRNA was observed in cells collected from aged animals at even higher levels than in young ones.

Table I

Lymphocyte proliferative response to Con A[a]

Mice	[^{3}H]thymidine incorporation (cpm ± S.E.M.)	
Young (3 months)	56,159 ± 2,309	
Old (18–20 months)	22,179 ± 1,179	$P < 0.001$

[a] The levels of proliferation of T lymphocytes were measured 72 h after Con A treatment by 2 h pulse labeling of [^{3}H]thymidine (4 μCi/ml). The results are given as means ± S.E.M. ($n = 36$) from triplicate culture. Statistical significance was estimated with a one-way analysis of variance.

In the next experiment we investigated whether the AP-1 DNA binding activity was impaired as a result of the reported failure of aged cells to express *c-fos*. As it can be seen in Fig. 2, the AP-1 complex was already detectable in young lymphocytes after 2 h and reached maximum at 6 h following Con A stimulation. There was no detectable AP-1 formation in old lymphocytes at any time measured. The AP-1 specificity of retarded band was confirmed by a control experiment (lane C) in which an excess of cold AP-1 sequence efficiently competed with radioactive probe for protein binding.

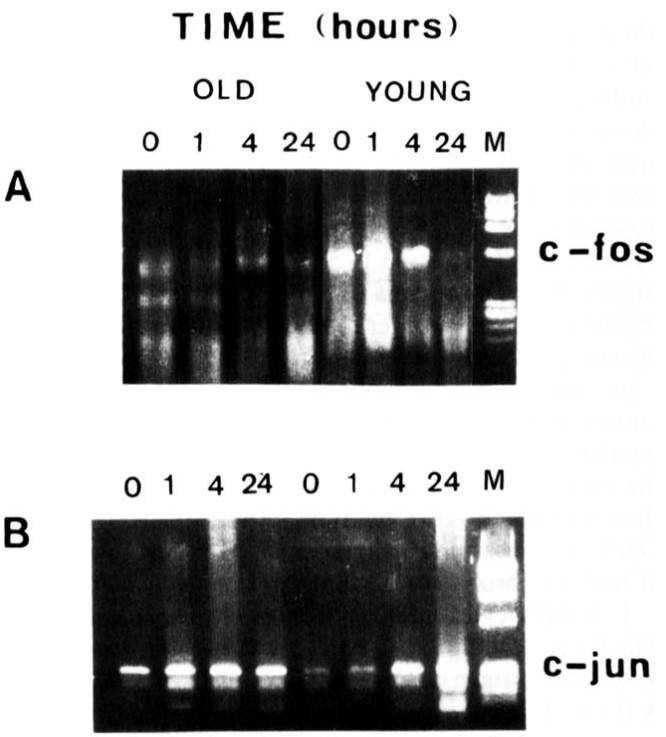

Fig. 1. *c-fos* (A) and *c-jun* mRNA levels (B) in lymphocytes derived from old and young mice. Cells were nonstimulated (0 h) or stimulated to grow with Con A for 1, 4, and 24 h. Total cellular RNA was isolated from 5–6 × 10^6 cells and analyzed by RT-PCR. The last line represents ΦX 174 RF DNA/*Hae*III as a size marker.

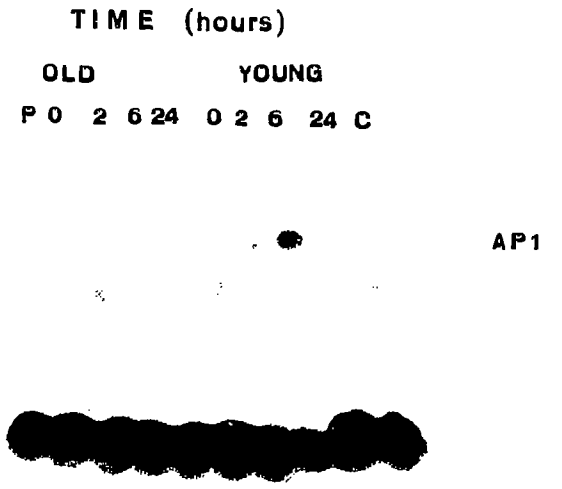

Fig. 2. DNA binding of AP-1 transcription factor in lymphocytes derived from old and young mice nonstimulated (0 h) and stimulated with Con A for 2, 6, and 24 h. EMSA was performed to test AP-1 binding activity in nuclear extracts. The first lane represents a sample with the probe only (P). The last lane shows AP-1 binding activity in extract derived from young lymphocytes stimulated with Con A for 6 h and incubated with a 10× excess of unlabeled oligonucleotide containing AP-1 sequence added for competition (C).

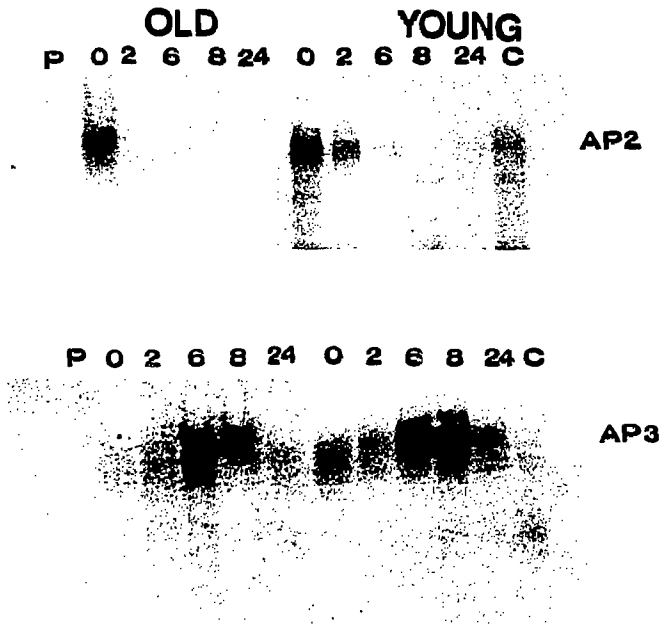

Fig. 3. DNA binding activity of AP-2 and AP-3 transcription factors in lymphocytes derived from old and young mice. Cells were nonstimulated (0 h) or stimulated with Con A for 2, 6, 8, and 24 h. The first lane (P) and the last one (C) show the probe only and test for competition, respectively (see Fig. 1). In the case of AP-2, the test for competition was done with the extract from nonstimulated young lymphocytes and in the case of AP-3, with the extract 6 h after stimulation.

Applying the DNA affinity precipitation assay, Riabowol et al. [7] recently demonstrated that AP-1 binding activity was markedly reduced in old fibroblasts. We therefore extended this study by demonstrating that like in human senescent fibroblasts in vitro, lymphocytes derived from old mice were characterized by a failure of activation of *c-fos* expression and impaired AP-1 formation after cell cycle stimulation.

The observed defect of AP-1 formation could exemplify general alteration of transcription factors in aged cells. To test this suggestion we compared the pattern of expression in young and aged T lymphocytes of two other transcription factors, namely, AP-2 and AP-3. Our results (Fig. 3) indicated that there were no differences between the DNA binding behavior patterns of both transcription factors in young and old lymphocytes. AP-2 DNA binding activity was found only in nonstimulated old and young cells (0 h) and disappeared after Con A stimulation. Contrary to AP-2, AP-3 was found to be inducible and showed the highest binding activity 8 h after lectin stimulation in old and young lymphocytes. It has been previously shown that AP-2 activity increased after the treatment of cells with phorbol ester or cAMP-elevating agents [15]. To the best of our knowledge, there is no data available concerning activation of AP-3 after cell cycle stimulation.

In conclusion, our results showed that the same molecular mechanisms of cessation proliferation could exist in fibroblasts characterized by replicative senescence and in lymphocytes derived from already aged organisms. It is possible that altered prereplicative events in senescent and old cells are clustered in just one or a few pathways that are deficient in these cells. So far, the data concerning senescent human fibroblasts and mice lymphocytes suggest the possibility that *c-fos* may play a key role in a cascade of events that regulates the entry of the cell to the cell cycle.

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