Acta Biochimica Polonica

Vol. 40 No. 2/1993

QUARTERLY

The impaired transcription factor AP-1 DNA binding activity in lymphocytes derived from subjects with some symptoms of premature aging

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Received 5 May, 1993

The study of human disorders known as premature aging syndromes may provide insight into the mechanisms of cellular senescence. The main feature of cellular senescence *in vitro* is cessation of cell proliferation. Down syndrome (DS) and neuronal ceroid-lypofuscinosis (NCL) are clinically characterized by the premature onset of numerous features normally associated with human aging. Phytohemagglutinin stimulated lymphocytes derived from DS subjects showed a statistically significant diminished proliferation capacity in comparison with lymphocytes derived from NCL and healthy individuals. We demonstrated, by applying the electrophoretic mobility shift assay, slightly impaired AP-1 DNA binding activity in NCL lymphocytes and strong in DS ones. Our results showed that the same molecular mechanisms of proliferation cessation could exist in fibroblasts characterized by replicative senescence and in lymphocytes derived from individuals with premature aging syndromes (Down).

The main feature of cellular senescence *in vitro* 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-depended 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]. Fos proteins form a stable complex with 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 and influences the expression of a number of genes [4]. 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 [5] and splenocytes derived from old mice [6].

T lymphocytes are physiologically quiescent cells which can be induced to enter the cell cycle in response to a specific antigen or mitogenic lectins like phytohemagglutinin (PHA)[1]. Cell cycle analyses in T lymphocytes

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[1]Abbreviations: DS, Down syndrome; PHA, phytohemagglutinin; NCL, neuronal ceroid-lipofuscinosis; PMSF, phenylmethyl sulphonyl fluoride

have suggested an age related decline in the number of cells able to enter the cell cycle [7].

People with Down syndrome (DS) and neuronal ceroid-lypofuscinosis (NCL) show some signs of premature aging [8, 9]. Major manifestations of DS include growth retardation, heart defects, hypotonia, alteration of the immune system similar to those of aged people, increased susceptibility to infections and leukemia, and the ultimate development of Alzheimer disease-type pathology [8]. The NCL is a progressive encephalopathy and is characterized by neural and extraneural (also in lymphocytes) accumulation of ceroid- and lipofuscin-like storage cytosomes [10].

For our studies we decided to investigate DNA binding activity of AP-1 in phytohemagglutinin stimulated peripheral blood lymphocytes derived from patients with DS and with late infantile type NCL. We applied the electrophoretic mobility gel shift assay for separating complexes of specific nuclear proteins with ^{32}P-labeled AP-1 oligonucleotide sequence.

MATERIAL AND METHODS

Cell culture. Lymphocytes were obtained from peripheral blood of persons with DS (range of age 13 - 31), NCL (range of age 5 1/2 - 9) and healthy controls (range of age 7 - 39) (Fig. 1). 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 (GIBCO BRL) supplemented with 5% fetal calf serum, 20 mM Hepes, 24 mM $NaHCO_3$, and antibiotics. The cells were stimulated to proliferate with phytohemagglutinin (10 µg/ml) and monitored for the level of stimulation by measuring [^{3}H]thymidine incorporation into DNA (4 µCi/ml for 6 h) 66 - 72 h following PHA addition. At the end of incubation period, cells were harvested and washed by a multiple cell culture harvester (Skatron, Norway) and radioactivity was measured by liquid scintillation counting.

Statistical significance of proliferation ability was estimated by a one-way analysis of variance.

Protein extracts and electrophoretic mobility shift assay (EMSA). The nuclear protein extracts were prepared according to Schreiber *et al.* [11]. After the desired time of culture, typically 2×10^7 cells were washed twice with phosphate-buffered saline and suspended in 500 µl cold buffer containing: 10 mM Hepes, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethyl sulphonyl fluoride (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 dithiothreitol, 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 [12].

The AP-1 sequence (22 oligomer) from a Stratagene "gel shift" kit was labeled with terminal transferase and purified on the spun column. Binding reactions and electrophoresis were done as it was described before [6]. A probe (0.3 ng; 30000 - 40000 c.p.m.) was incubated with 10 µl of incubation buffer containing: 4% Ficoll, 20 mM Hepes, pH 7.8, 50 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.25 mg/ml BSA, 10 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 sequence was 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, pH 7.5) for 1.5 h at 120 V. Prior to electrophoresis 0.1% bromophenol blue was added to the samples. Gels were dried and exposed overnight to X-ray film with intensifying screens.

RESULTS AND DISCUSSION

The proliferation response of peripheral blood lymphocytes derived from DS, NCL and healthy individuals to PHA stimulation were compared by measuring [^{3}H]thymidine incorporation (Fig.1). PHA-stimulated lymphocytes from DS subjects showed statistically significant diminished proliferation capacity in comparison with NCL and healthy individuals ($p < 0.001$). It has been shown by others [8] that there was no difference in proliferation capacity of lymphocytes derived from healthy and DS

	Down Syndrome					Neuronal Ceroid Lipofuscinosis				Control				
Individuals	D1	D2	D3	D4	D5	N1	N2	N3	N4	C1	C2	C3	C4	C5
Age (years)	29	18	31	31	13	5 1/2	9	5 1/2	8	11	7	39	26	27

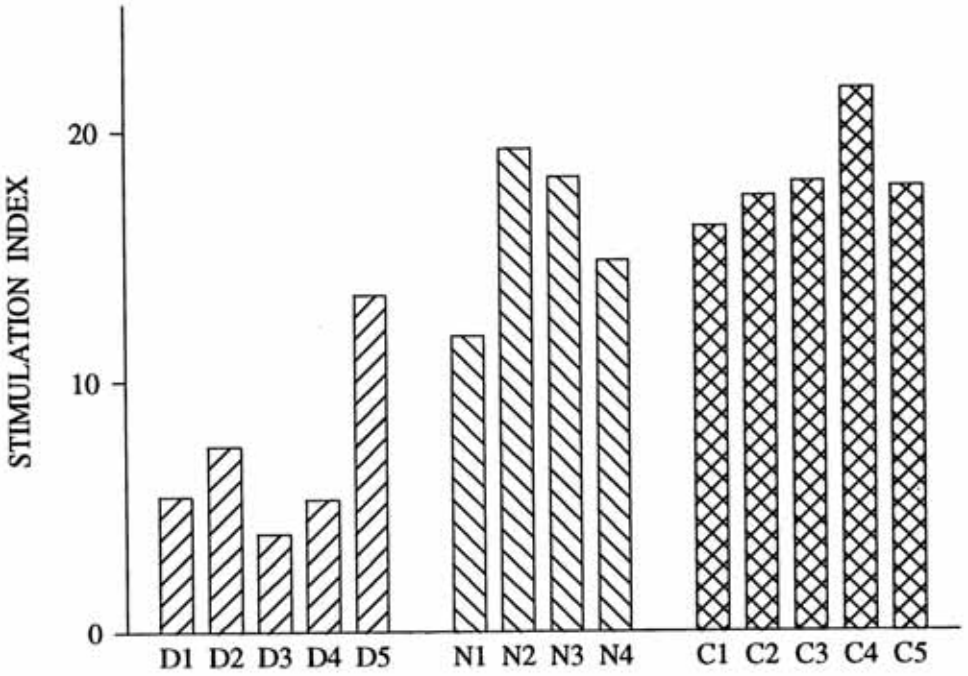

Fig. 1. Proliferation ability of T cells stimulated with PHA (10 μg/ml) of DS (D1-D4), NCL (N1-N4) and healthy controls (C1-C5).
The proliferation ability is expressed as stimulation index ([^{3}H]thymidine incorporated by PHA-stimulated cells divided by the value of incorporation by nonstimulated cells)

children. Our patients with one exception (D5) were above 18 years old. Four NCL subjects were children and did not show statistically significant impairment of proliferative capacity in comparison with normal controls. To our knowledge there is no data in literature concerning the proliferation ability of any cells derived from individuals with NCL syndrome.

The AP-1 DNA binding activity in lymphocytes derived from all DS, NCL and healthy individuals was measured 5 h after PHA stimulation (Fig. 2) as at that time the maximum AP-1 formation in lymphocytes derived from healthy subjects was observed (data not shown). Very strong AP-1 DNA binding activity was observed in lymphocytes isolated from all tested control subjects, and slightly weaker activity in three from four NCL ones. There was no visible complex in extracts derived from all DS and one NCL (N4) lymphocytes in the position of AP-1 migration. The AP-1 specificity of the retarded band was confirmed by a control experiment (lane L) in which an excess of cold AP-1 sequence efficiently competed with radioactive probe for protein binding. The additional bands visible especially in lymphocytes derived from one DS (lower) and one NCL (upper) subjects could suggest other specific binding to the AP-1 DNA sequence in these cells. The formation of Jun-Jun homodimer in DS lymphocytes .cannot be excluded and the further experiments will be performed to explore this possibility. So far our results show a good correlation between the proliferation ability of human lymphocytes derived from healthy and DS individuals and AP-1 DNA binding activity. A similar correlation between the AP-1 DNA binding activity and proliferation ability of splenocytes derived from old and young mice has been shown [6]. The AP-1 DNA binding activity in NCL lymphocytes, although lower than in healthy ones,

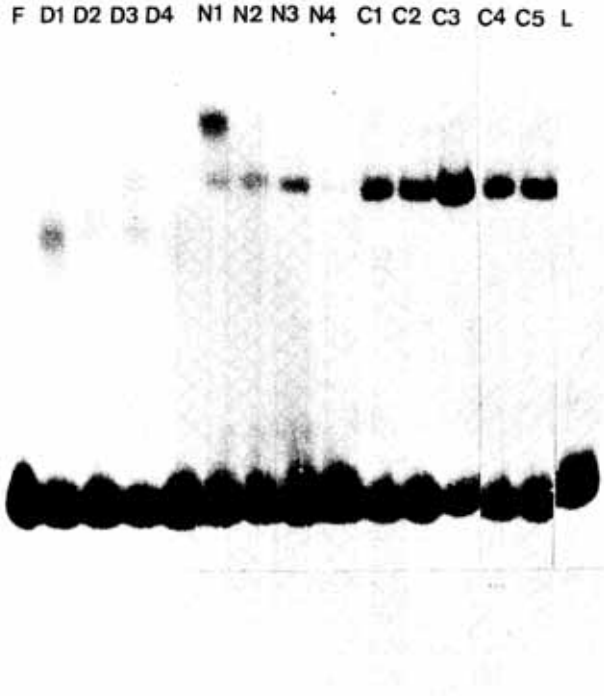

Fig. 2. Formation of AP-1 transcription complex in lymphocytes derived from DS (D1-D4), NCL (N1-N4) and control subjects (C1-C5) and stimulated for 5 h with PHA.

F lane represents a probe only and L lane shows AP-1 binding activity in extract derived from C3 subject incubated with 10-fold excess of cold oligonucleotides containing AP-1 sequence added for competition.

probably was high enough to ensure almost normal cell proliferation.

Our results suggest that the same molecular mechanisms of proliferation cessation could exist in fibroblasts characterized by replicative senescence [3, 5] and in lymphocytes derived from already aged organisms [6], as well as in lymphocytes derived from individuals with premature aging syndrome, e.g., DS (this paper). It is possible that *c-fos* and AP-1 transcription factor may play a key role in the cascade of events that regulates the entry of the cell to the cell cycle.

We wish to thank Dr Leszek Kaczmarek for reading the paper and for valuable suggestions. We are grateful to Prof. Barbara Grzelakowska-Sztabert for the critical review of the manuscript.

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