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C-Fos Is Not Essential for Apoptosis

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The transcription factor AP-1, made up of dimers of Fos and Jun proto-oncogene products, is involved in distinct cellular processes, including cell proliferation, differentiation and apoptosis. In this study, we have used mice in which both copies of the c-*fos* gene were disrupted by targeted mutagenesis in order to analyze how the apoptotic response was affected in these mice. We prepared primary cultures from the lymphoid organs, spleen and thymus, obtained from both wild-type and c-*fos* -/- mice and analyzed the induction of apoptosis in these cultures in the absence and presence of etoposide, an inducer of apoptosis in distinct cell types. Primary cultures from both organs, spleen and thymus, isolated from wild-type mice underwent apoptosis after 3 and 6 h of culture, respectively. Addition of etoposide enhanced the apoptotic response and c-*fos* mRNA levels in both spleen and thymic cells. Nevertheless, we found that induction of apoptosis in primary cultures of cells obtained from spleen and thymus of c-Fos-deficient mice was practically identical to that observed in wild-type mice. These results demonstrate that c-Fos is not essential for apoptosis and that cells lacking c-Fos may undergo normal apoptosis.

Apoptosis is a genetically encoded cell death program defined by characteristic morphologic and biochemical changes, such as condensation of chromatin, blebbing of the cell surface and transient increase in buoyant density (1,2). An early and crucial event in the development of apoptosis is the internucleosomal DNA digestion by an endogenous endonuclease (3), which represents a point of irreversible commitment towards death. As apoptosis can be prevented in several systems by the presence of inhibitors of RNA or protein synthesis (3–6), it has been postulated that apoptosis is an active process requiring gene transcription and translation.

The c-Fos protein is a major component of the AP-1 transcription factor and is a member of a multigene family, including the *fos*-related genes *fos* B, *fra*-1 and *fra*-2 (7). The function of c-Fos as a transcription factor is dependent on formation of heterodimers with members of the Jun family (c-Jun, Jun B, Jun D) or with other leucine zipper-containing molecules (7).

A great number of reports have implicated c-*fos* in important physiological processes, including cell proliferation, cell differentiation and apoptosis. It has been shown that continuous c-*fos* expression precedes induction of apoptosis *in vivo* (8). Furthermore, a number of reports have demonstrated c-*fos* expression before promotion of apoptosis in distinct cell types through growth factor deprivation or upon addition of several agents (8–12). It has been reported that etoposide, an inhibitor of topoisomerase II induces c-*fos* expression in various cell types (13–15), and a correlation between c-*fos* expression and induction of apoptosis has been found in cells exposed to a variety of DNA-damaging agents, including etoposide (13,15–18). Also, addition of antisense oligonucleotides directed against c-*fos* have been shown to increase survival of growth factor-deprived lymphoid cells (10), and antibodies against the Fos family proteins protected nerve growth factor-deprived neurons from apoptosis (12). However, a conclusive statement on the actual role of c-*fos* for programmed cell death remains to be established.

To understand better the putative role of c-*fos* in the apoptotic process, we have used mice lacking c-*fos* and studied the induction of apoptosis *in vitro* in thymic and spleen cells. c-Fos-

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deficient mice have been shown to develop osteopetrosis as a primary pathology (19), as a result of a defect in the differentiation of the osteoclast-macrophage lineage (20).

MATERIALS AND METHODS

Materials. RPMI-1640 culture medium, fetal calf serum and L-glutamine were purchased from GIBCO/BRL (Gaithersburg, MD). Antibiotics were from Laboratorios Llorente (Madrid, Spain). Etoposide was purchased from Sigma (St. Louis, MO). Restriction enzymes were from Pharmacia (Uppsala, Sweden). The 123-bp DNA ladder was from GIBCO/BRL. All other chemicals were from Sigma or Merck (Darmstadt, Germany).

Mice. c-Fos deficient mice were bred and their genotype ascertained as previously described (19). Mice between 4 and 7 weeks were used for this study. Genotypes were confirmed by Southern blot analysis of tail DNA (19). Heterozygous c-*fos* +/− and wild-type mice were used as controls.

Southern blot analysis. Genomic DNA was digested with *Sac*I, separated on an agarose gel, transferred to Hybond-N nylon membranes (Amersham, IL) and hybridized with a c-*fos*-specific DNA probe as previously described (19).

Primary cultures of thymic and spleen cells. Spleen and thymus were obtained from wild-type and c-Fos-deficient mice under sterile conditions. Tissues were carefully dispersed in RPMI using forceps and transferred to a 15-ml tube. Large pieces of tissue were allowed to settle down and the cell suspension was decanted into another tube and pelleted by centrifugation. Cells were then resuspended and cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 24 μg/ml gentamicin. Cells were incubated at 37°C in a humidified CO_2/air (1:19) atmosphere. The viability of the cells was consistently above 95%, as assessed by the trypan blue exclusion method. Etoposide was added to the cell cultures at 3 μg/ml for the times indicated in the respective Figures.

Northern blot analysis. Total RNA was isolated from 70×10^6 cells by the guanidine thiocyanate/CsCl method (21). Samples of 10 μg of RNA were electrophoresed on 0.9% (w/v) agarose-formaldehyde gels, and then transferred to Hybond-N nylon membranes as previously described (22). A ^{32}P-labeled c-*fos* cDNA probe was prepared using the random hexanucleotide priming method (22,23) (Oligo-Labeling Kit, Pharmacia) to a specific radioactivity $>7 \times 10^8$ c.p.m./μg of cDNA. Conditions for blot hybridization and washing have been described elsewhere (22).

DNA fragmentation assay. To assess DNA fragmentation, we isolated fragmented DNA as previously described (24). 3×10^6 cells were used for each experimental condition and aliquots of the fragmented DNA were analyzed by electrophoresis on 1% agarose gels in 0.5 × TBE buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA), with 0.5 × TBE buffer as running buffer. A 123-bp DNA ladder was used as standard. DNA was visualized after electrophoresis by ethidium bromide staining.

RESULTS AND DISCUSSION

We have studied the role of c-*fos* in the induction of programmed cell death by analyzing and comparing the internucleosomal DNA digestion in spleen and thymic cells isolated from wild-type and c-*fos*-deficient mice. As shown in Fig. 1, spleen and thymic cells cultured in RPMI containing 10% fetal calf serum underwent apoptosis after 3 hours (spleen cells; Fig. 1, *lane 6* and 6 hours) (thymic cells; Fig. 1, *lane 12*) of incubation, as evidenced by degradation of DNA into oligonucleosome-length fragments. The induction of apoptosis in thymic cells was highly increased by addition of etoposide (3 μg/ml) to the culture medium (Fig. 1, cf. *lanes 8,9* and *12,13*). Etoposide has been shown to induce apoptosis in a number of cell types (13,25,26), being this etoposide-induced apoptosis inhibited by protein and RNA synthesis inhibitors (26,27). Addition of etoposide to primary cultures of spleen cells also increased DNA fragmentation, although to a lower degree than in the culture of thymic cells (Fig. 1, cf. *lanes 6,7* and *lanes 10,11*). This could be due to the fact that thymic cells are more resistant to etoposide-induced apoptosis than spleen cells. Thus, after 3 hours of culture no apoptosis was observed in cultured thymic cells, except etoposide is present in the medium, whereas cultured spleen cells displayed already a DNA ladder (Fig. 1, cf. *lanes 6,8* and *9*).

Treatment of thymic cells (Fig. 2) or spleen cells (data not shown) with etoposide induced a transient increase in c-*fos* mRNA levels. Enhanced levels of c-*fos* mRNA were also detected during the culture of thymic and spleen cells undergoing apoptosis in the absence of any drug (data not shown). Thus, an apparent correlation was found between c-*fos* induction and promotion of apoptosis.

However, the accumulating evidence so far cannot distinguish among three possible scenarios concerning the role of c-*fos* in apoptosis (Fig. 3): a) c-*fos* expression may play a crucial role and

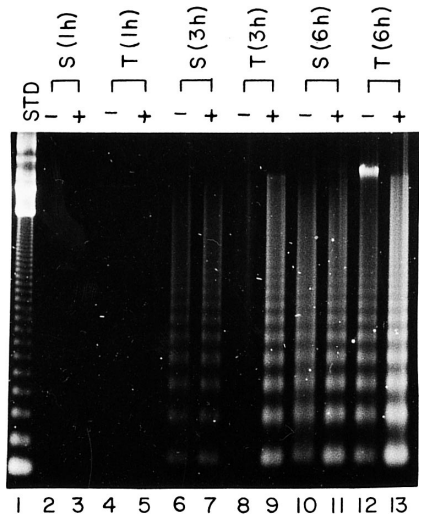

FIG. 1. Time-course of apoptosis in untreated and etoposide-treated primary cultures of spleen and thymic cells isolated from wild-type mice. Cell suspensions prepared from spleen (S) and thymus (T) of wild-type mice were assayed immediately for DNA fragmentation, as described under 'Experimental Procedures,' after incubation in RPMI culture medium containing 10% fetal calf serum in the absence (−, *even lanes*) or in the presence (+, *odd lanes*) of etoposide for the times indicated. (*Lane 1*) 123-bp DNA ladder. Fragmented DNA from 7×10^5 cells was loaded in each lane.

constitutes a prerequisite for the initiation of apoptosis; b) apoptosis is triggered by alteration (activation or inhibition) of certain cell death specific signal transduction mechanisms, and some of these signaling modifications may induce c-*fos* expression as a side effect in the signaling process leading to programmed cell death (in this case apoptosis would be accompanied by c-*fos* expression); c) c-*fos* expression may constitute a side effect of the initiation of apoptosis due to the fact that the intracellular signal transduction mechanism involved in apoptosis may result, through cross-talk of signaling processes, in a coincidental induction of c-*fos*, without a direct connection between both events (in this case apoptosis may be accompanied or not by c-*fos* expression).

To distinguish among the above three putative mechanisms in which c-*fos* expression can occur during the triggering of apoptosis, and to verify the actual role of c-*fos* in the induction of programmed cell death, we studied the induction of apoptosis in thymic and spleen cells from homozygous c-*fos* -/- mice, and compared it with that observed in wild-type mice. We used 4–7 week-old c-*fos* -/- mice, and found a significant reduction in the size of the thymus with no apparent change in the spleen size, in agreement with a previous report (19). It has been previously shown

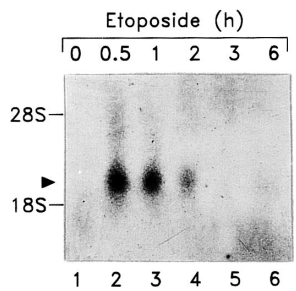

FIG. 2. Expression of c-*fos* proto-oncogene in etoposide-treated thymic cells. Northern blot analysis of mRNA levels after treatment with 3 μg/ml etoposide for 0, 0.5, 1, 2, 3 and 6 h of cell suspensions prepared from thymus of wild-type mice. Autoradiogram was developed after 24 h of exposure. The positions of ribosomal RNA as molecular size markers are indicated.

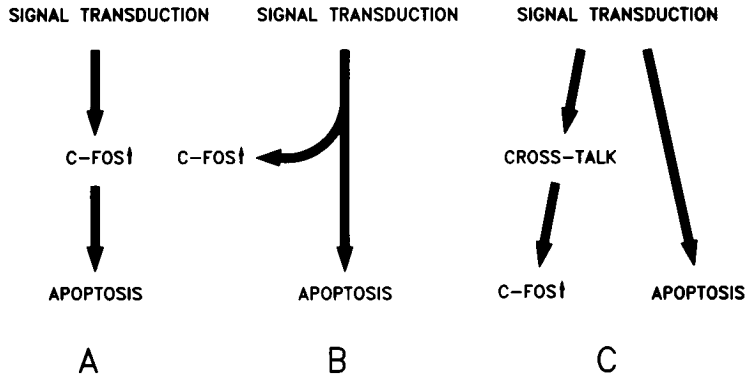

FIG. 3. Schematic views showing c-Fos induction during apoptosis. This is a schematic representation designed to outline three putative scenarios that can explain c-*fos* induction during apoptosis. See text for further details.

that c-Fos-deficient mice have normal hematopoietic stem cells (28,29), and that c-Fos is not absolutely required for the development and function of T cells (29) and myeloid cells (28). A defect in the B-cell development was observed in c-*fos* -/- mice, due to an impaired bone marrow environment (28). However, no studies on the induction of apoptosis have been performed in c-Fos-deficient mice. We prepared primary cultures from spleens and thymuses of c-*fos* -/- mice in RPMI containing 10% FCS, and found internucleosomal DNA breakdown in both spleen and thymic cells after 3 h and 6 h of culture, respectively (Fig. 4, *lanes 6* and *12*). This observation was practically identical to that found in primary cultures from thymus and spleen obtained from wild type mice (cf. Figs. 1 and 4). Also, we found that addition of etoposide reduced the time required for these cells to undergo apoptosis and increased the apoptotic response in both spleen and thymic cells from c-*fos* -/- mice (Fig. 4); the same result as that observed in wild type mice (cf. Figs. 1 and 4). Thus, these data demonstrate conclusively that the presence of c-*fos* is not required for the induction of apoptosis in primary cultures obtained from thymus and spleen. We did not even find a delay in the induction of apoptosis in c-*fos* -/- mice when compared to that observed in wild-type

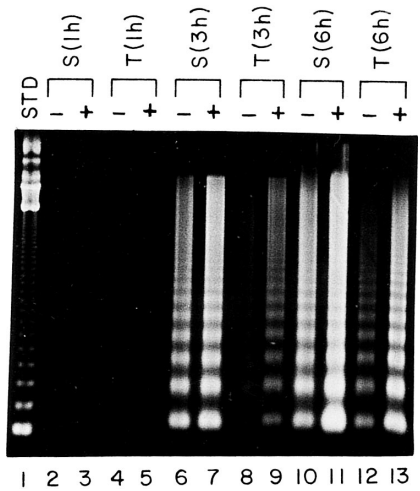

FIG. 4. Time-course of apoptosis in untreated and etoposide-treated primary cultures of spleen and thymic cells isolated from c-Fos-deficient mice. Cell suspensions prepared from spleen (S) and thymus (T) of homozygous c-*fos* (−/−) mice were assayed immediately for DNA fragmentation after incubation in RPMI culture medium containing 10% fetal calf serum in the absence (−, *even lanes*) or in the presence of (+, *odd lanes*) etoposide for the times indicated. DNA extraction and analysis were as in Fig. 1. (*Lane 1*) 123-bp DNA ladder. Fragmented DNA from 7×10^5 cells was loaded in each lane.

mice (cf. Figs. 1 and 4), indicating that c-*fos* expression is not a prerequisite for the induction of apoptosis, and it is not essential for the events leading to apoptosis in our system. Previous reports have found that etoposide induces both c-*fos* mRNA expression and apoptosis in HL-60 cells (13,15), suggesting a putative role of c-*fos* in the initiation of apoptosis. However, we found that etoposide induced an apoptotic response in both thymic and spleen cells in c-*fos*-deficient mice (Fig. 4). Thus, these results clearly indicate that induction of apoptosis by etoposide does not require c-*fos* expression. The results herein reported also indicate that the pathway a) represented in Fig. 3 can be ruled out. The proto-oncogene c-*fos* is not essential for apoptosis in c-*fos* -/- mice, but may play a role during induction of cell death in wild-type mice. In c-*fos* -/- mice, the lack of c-*fos* gene expression may be compensated by the presence of other members of the AP-1 complex family. Alternatively, the pathways b) and c) depicted in Fig. 3 may take place. As there are a large number of reports showing c-*fos* expression prior to the appearance of apoptosis under many different experimental conditions (8–12), we can envisage that c-*fos* expression can occur, in many cases, before induction of internucleosomal DNA breakdown as a side effect during the signaling mechanism directly involved in cell death induction, but it does not participate in the cascade of events leading to cell death. Thus, c-*fos* expression could be indicative of an ongoing apoptotic process, despite not having a direct role in this process. However, as c-*fos* expression results from the activation of certain signaling processes not necessarily or specifically involved in the triggering of apoptosis, c-*fos* could also be induced in other non-apoptotic physiological processes, such as cell proliferation and differentiation, as has been widely documented. Thus, the present results give an explanation for the expression of c-*fos* under very different physiological responses.

Recent evidences with a c-Jun dominant negative mutant (30) and with intracellular microinjections of neutralizing antibodies (12) indicate that the AP-1 transcription factor is involved in neuronal apoptosis and provide support for a main involvement of c-*jun* in this process. Nevertheless, it has not been reported whether apoptosis is abnormal in c-Jun knockout mice which show a complex phenotype and die during embryonic development at about 12.5 days (31,32). In this regard, the present study constitutes the first report which directly addresses the putative involvement of a major component of the AP-1 transcription factor in apoptosis by the use of c-Fos knockout mice.

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