Programmed cell death in the absence of c-Fos and c-Jun

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SUMMARY

Programmed cell death, or apoptosis, is a normal process in the development of a variety of embryonic and adult tissues, and is also observed in several pathological conditions. Several recent studies, using both expression and functional assays, have implicated the transcription factor, AP-1, in the regulation of programmed cell death, and specifically implicate the genes c-fos and c-jun, as well as some other family members. If the products of the c-fos and/or c-jun genes are essential components in the cascade of events that leads to programmed cell death in mammalian cells, it follows that cell death would not occur in mice lacking functional copies of these genes. We have made use of null mutations in the c-fos and c-jun genes that were produced by gene targeting (Johnson, R. S., Spiegelman, B. M. and Papaioannou, V. E. (1992). Cell 71, 577-586; Johnson, R. S., Van Lingen, B., Papaioannou, V. E. and Spiegelman, B. M. (1993). Genes Dev. 7, 1309-1317) to investigate this possibility. Cell death was assayed using an in situ apoptosis assay in c-fos null embryos and adults, c-jun null embryos, and c-fos/c-jun double null embryos compared with control mice. The occurrence of cell death in c-fos null mice was also assessed in two experimental conditions that normally lead to neuronal cell death. The first was unilateral section of the sciatic nerve in neonates, which leads to the death of anterior horn cells of the spinal cord on the operated side. The second was a genetic cross combining the weaver mutation, which causes death of cerebellar granule cells, with the cfos mutation. Our results show that programmed cell death occurs normally in developing embryonic tissues and adult thymus and ovary, regardless of the absence of a functional c-fos gene. Furthermore, absence of c-fos had no effect on neuronal cell death in the spinal cord following sciatic nerve section, or in heterozygous weavers’ cerebellae. Finally, the results show that programmed cell death can take place in embryos lacking both Fos and Jun.

Key words: AP-1, c-fos, c-jun, apoptosis, programmed cell death, mouse

INTRODUCTION

Programmed cell death is a normal and essential process in the development of a variety of embryonic tissues, and in the physiological turnover of numerous adult tissues, as well as a mechanism for the removal of excess or superfluous cells and a means of shaping structures during morphogenesis (Saunders and Fallon, 1967; Hinchcliffe, 1981; Ellis et al., 1991; Oppenheim, 1991). Developmentally regulated cell death commonly displays the characteristics of apoptosis, a type of cell death that features clumping of chromatin, the condensation of nucleus and cytoplasm, and the formation of membrane blebs that contain fragments of the nucleus and cell organelles. A hallmark of apoptosis is the activity of Ca2+-dependent endonucleases that induce chromatin cleavage, resulting in the formation of nucleosomal DNA fragments (Kerr et al., 1987). Apoptosis is an active, regulated and spatially restricted cell phenomenon, whereby particular cells are destined to die as part of their normal developmental fate (Biggers, 1964; Glucksman, 1951). Programmed cell death is a decision that requires the expression of specific genes in the cells fated to die, and implementation of the cell death program in different cell types is likely to involve different controlling mechanisms. Several well-characterized examples of programmed cell death, such as hormonally controlled prostate regression (Buttyan et al., 1989), cytokine-sensitive, glucocorticoid-induced lymphocyte apoptosis (Walker et al., 1993), and NGF-controlled nerve cell death (Estus et al., 1994), provide the opportunity to explore the pathways between external stimuli, intracellular signalling and alterations in gene expression controlling the process. Factors such as changes in hormone and growth factor levels have been shown to effect cell death (Oppenheim, 1989; Williams et al., 1990; Raff, 1992; Luciano et al., 1994) and various genes such as tumor suppressors and transcription factors, including AP-1, have been implicated in the pathway(s) leading to apoptosis (Estus et al., 1994; Hengartner et al., 1992; Marti et al., 1994; Sikora et al., 1993; Gagliardini, 1994).

The AP-1 transcription factor is a dimeric protein made up
of heterodimers between Fos and Jun family gene products or homodimers of Jun family gene products. Several studies have implicated AP-1 in the regulation of programmed cell death of different cell types. AP-1 (c-Fos/JunD) is induced during programmed cell death in the involuting mouse mammary gland and rat ventral prostate in vivo (Buttyan et al., 1988; Marti et al., 1994). Colotta et al. (1992) found that both c-fos and c-jun are rapidly induced after growth factor deprivation in cytokine-dependent mouse lymphoid cell lines in vitro, a condition that leads to apoptosis. In this model, antisense oligonucleotides directed against c-fos and c-jun protected cells from death induced by growth factor deprivation, indicating a role for these genes in activating the genetic program responsible for cell death in lymphoid cells. An association has also been found between expression of c-fos and c-jun in rat thymocytes undergoing cell death following heat shock or dexamethasone treatment in vitro, but, paradoxically, expression is also seen upon stimulation of proliferation with Concanavalin A (Grassilli et al., 1992; Sikora et al., 1993). Estus et al. (1994) documented changing levels of expression of different Fos and Jun family members in cultured neuronal cells undergoing apoptosis in response to nerve growth factor (NGF) deprivation in vitro, and subsequently used antibody injection to block the function of either Fos or Jun family members, and also specific Jun family members (c-jun and JunB). They showed that antibodies to either family, or to c-jun alone, can protect NGF-deprived cells from apoptosis, and concluded that c-jun and at least one member of the Fos family are necessary for neuronal apoptosis. In a similar system, Ham et al. (1995) found that overexpression of c-jun alone can trigger cell death in neurons and that expression of a c-jun dominant negative mutation protects sympathetic neurons from cell death upon NGF deprivation.

Several reports point to involvement of c-fos expression in apoptosis during embryonic development. Expression of c-fos has been shown to correlate with the naturally occurring cell death in interhemispheric cortex neurons of the rat (Gonzalez-Martin et al., 1992). Recently, a more extensive correlation has been documented between the expression of c-fos and impending cell death in a variety of developing neural and nonneural tissues, and in several pathological conditions in which neurons die in the brain and spinal cord, leading to the hypothesis that c-fos is involved in changes in gene expression in the pathway to apoptosis (Smytne et al., 1993). In this study, the expression of c-fos was demonstrated in mice carrying a fusion transgene with the c-fos promoter driving the marker bacterial gene encoding β-galactosidase (Smytne et al., 1992). Expression of the transgene was seen in atretic follicles in the adult ovary, and in embryonic heart valve cushions, secondary palate, nasal septum and developing tooth germ, all areas where programmed cell death normally occurs.

In the same study, neurons were seen to express the fusion transgene in three pathological conditions that normally lead to neuronal cell death: (1) in neurons susceptible to excitotoxic cell death following treatment with kainic acid, (2) in motor neurons of the anterior horn of the spinal cord after section of the sciatic nerve, an operation that leads to cell death when performed on neonatal mice, and (3) in neurons vulnerable to the actions of a naturally occurring murine mutation known as weaver (wv). In mice carrying this spontaneous mutation, cell death is found in the brain in both cerebellum and midbrain (see Roffler-Tarlov, 1992 for review). In homozygous mutants, large numbers of granule cells die during the first two postnatal weeks, and Purkinje cell numbers are low, resulting in a small and disordered cerebellum. A less severe loss of granule cells also occurs in the heterozygote; in this case the cerebellum is somewhat smaller than in the wild type, and the normal trilaminar structure of the cerebellar cortex is disturbed, caused

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Fig. 1. In situ detection of apoptosis during embryogenesis. Positively stained (red/brown) apoptotic nuclei were observed in both the c-fos heterozygous (A,C,E) and the c-fos null (B,D,F) embryos in the dorsal root ganglia (A,B), myotome (C,D) and heart valve (F) at 13.5 days p.c.. No nuclear staining was detected in control sections (myotome shown), in which TdT enzyme was omitted from the end-labelling cocktail (E). Bar, 50μm.
both by the lack of cells and by incomplete migration of surviving cells (Rezai and Yoon, 1972; Rakic and Siman 1973). In their experiments, Smeyne et al. (1993) bred mice carrying both wv and the c-fos-lacZ fusion gene and showed lacZ expression in heterozygous weavers on post-natal day 4 in cerebellar zones occupied by the developing, and vulnerable, granule cells.

If the products of the c-fos and/or c-jun genes are essential for the cell death program in mammalian cells, it follows that cell death would not occur in mice lacking functional copies of these genes. We have made use of null mutations in the c-fos and c-jun loci that were produced by gene targeting (Johnson et al., 1992, 1993) to investigate the possibility that c-fos and/or c-jun induction are required components in the cascade of events that leads to programmed cell death. Mice lacking c-jun survive only to midgestation and die from as yet undetermined causes during organogenesis (Hilberg et al., 1993; Johnson et al., 1993). They progress far enough in development, however, that disturbances in normal programmed cell death should be apparent. Animals lacking a functional c-fos gene are viable but suffer from toothlessness, impaired fertility and severe osteopetrosis that can be attributed to a cell autonomous block in osteoclast differentiation (Johnson et al., 1992; Wang et al., 1992; Grigoriadis et al., 1994). There is no abnormality apparent in the palate, a tissue where remodelling is accompanied by extensive apoptosis (Mori et al., 1994), and no syndactyly that might indicate an effect on apoptosis in mesodermal web of the interdigital zone of the developing limb buds, as occurs in the mutants polysyndactyly (Johnson, 1969) and fused toes (van der Hoeven et al., 1994). We previously reported that c-fos null females had an abnormally high proportion of atretic follicles in their ovaries, as judged by the presence of pycnotic nuclei (Johnson et al., 1992), but did not show that this cell death was apoptotic in nature.

In this study, we have used c-fos and c-jun null mutant mice to further examine the involvement of Fos and Jun in programmed cell death pathways of a variety of cell types in vivo. We assayed cell death in c-fos and c-jun null mutants, double c-fos/c-jun null mutants and control mice, using an in situ assay for apoptosis. We examined developing embryonic tissue, adult thymus and ovary, and also carried out two types of experiments to test the hypothesis that the c-fos product is involved in neuronal cell death. The first was to determine whether c-fos is required for the death of anterior horn cells after sciatic nerve section; the second was to determine the requirement for c-fos in the death of cerebellar granule cells caused by the wv mutation.

**MATERIALS AND METHODS**

**Mice**

The c-fos mutation, which was produced by gene targeting (Johnson et al., 1992, 1993)...

![Fig. 2. In situ detection of apoptosis in adult organs. Apoptotic thymocytes were detected in both wild-type (A) and homozygous c-fos null (B) mice; negative controls, in which TdT enzyme was omitted, are shown in C and D, respectively. Apoptotic granulosa cells were detected in atretic follicles in the ovaries of both wild-type (E) and c-fos homozygous null (F) mice. Whereas all nuclei stained positively in DNase-pretreated positive control sections (G), no staining was observed in negative controls, in which TdT enzyme was omitted (H). Bar, 50 μm.](image-url)
et al., 1992), was maintained in mice of mixed genetic background, derived from (C57BL/6J×129SvEvF1)F1 mice. Homozygous c-fos null mice can be recognized by their lack of teeth on postnatal day 12, nonetheless the genotype of all animals was confirmed by PCR screening of tail, liver or embryonic limb DNA using primers specific for both the introduced neomycin sequence and the endogenous c-fos sequence (Johnson et al., 1992). The c-jun mutation, also produced by gene targeting, was maintained on a 129 background derived from two 129 substrains. Mice and embryos from the c-jun mutant stock were genotyped by PCR using primers specific for the targeted and endogenous c-jun alleles (Johnson et al., 1993). c-fos/c-jun double heterozygotes were produced by breeding heterozygotes from the two mutant stocks together. Embryos for experiments were collected at 13.5 days post coitum (p.c.) from matings between c-fos heterozygotes, where 1/4 of the embryos are expected to be c-fos homozygous null. From matings between double heterozygotes for c-fos and c-jun, where 1/16 of the embryos are expected to be null for c-fos and c-jun, embryos were collected at 11.5 days p.c., prior to the time of c-jun null mutant death.

Homozygous wv/+ females were produced by mating pairs of heterozygous wv/+ mice on the B6CBAc-A/J background, obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were recognized as homozygous wv by a behavioral phenotype that includes ataxia, hypotonia, and fine tremor. Homozygous mutant females were mated with c-fos heterozygous males, offspring were genotyped for c-fos, and double heterozygotes were bred together to produce experimental litters. These would be expected to contain 1/16 double null embryos, 3/16 of each single null, as well as heterozygous and wild-type embryos.

**In situ apoptosis assay**

Embryos were fixed overnight at 4°C in 4% paraformaldehyde, paraffin embedded, and sectioned. A portion of one limb from each embryo was taken for genotyping by PCR and embryos of specific genotypes were selected for processing; these included one c-fos null and one c-fos heterozygous embryo from the mixed background, as well as the embryos indicated in Table 1. Ovaries and thymuses were collected from 6-week-old wild-type and c-fos null adult female mice, and were similarly fixed, embedded and sectioned. The apoptosis assay relies on the detection of broken DNA strands resulting from the nucleosomal DNA fragmentation characteristic of apoptotic nuclei (Gavrieli et al., 1992). Terminal deoxynucleotidyl transferase (TdT), a template-independent polymerase, is used to incorporate biotinylated nucleotides using the 3' -OH ends as primers. The incorporated biotin is then detected by streptavidin-peroxidase staining using conventional in situ histochemical methods. The method used was essentially as described by Gavrieli et al. (1992), except that biotinylated dUTP was substituted with Bio-11-dCTP (US Biochemical Corp., Cleveland, OH), and that the Extra-avidin Peroxidase detection system was replaced by a streptavidin-peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Peroxidase activity was visualized using 3-amino-9-ethylcarbazole (AEC) or a Vector VIP substrate kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with Mayer’s hematoxylin and mounted in an aqueous mounting medium (Crystal Mount; Biomedica, Foster City, CA) to preserve the red/brown AEC or purple VIP staining.

**Sciatric nerve sections**

Seventeen neonates from three litters of c-fos heterozygous pairs were operated on 3 or 4 days postnatally. The pups were deeply anaesthetized by hypothermia and, under an operating microscope, a skin incision was made in the upper posterior right thigh. The sciatic nerve was often visible through and between the bellies of the muscles after the skin was opened. After the gluteral muscles were separated with microforceps, the sciatic nerve was identified between the bellies of the muscles and sectioned near the pelvis with sharp micro-vanna scissors. The completeness of sciatic nerve section was verified by observing both cut ends of the nerve. The skin was closed with sutures. The operation was completed in about 5 minutes. After the operation, the pups were warmed until they were awake and moving normally, and were then returned to their mother’s cage. Successful operations resulted in weakness of the right leg shortly after surgery.

The pups were examined and killed 18 or 19 days after sciatic nerve section. The leg weakness was usually improved by this time but effects of the operation were still visible; for example, the grasp of a pencil by the hindfeet was abnormal in all the operated mice. After the designated survival period, the mice were anaesthetized with Nembutal (40 μg/g body weight) and perfused through the heart with a solution of 4% paraformaldehyde and 5% sucrose; a sample of liver and/or tail was taken for DNA extraction and genotyping by PCR. The mice were postfixed for several hours. The lumbar-sacral spinal cords were removed, placed in 20% sucrose, and later frozen. On the basis of genotype and litter, animals were selected for further analysis. A series of 40 μm-thick transverse sections were made through the lumbar enlargement and sacral regions containing the motor neurons that supply the sciatic nerve. The sections were mounted on glass slides and Nissl stained with cresyl violet. Sections from a total of 11 operated mice were examined by an investigator who did not know the genotype of the animals. The sections were from 3 wild-type mice, 3 heterozygotes, and 5 c-fos null mice. Cell counts were made of the motor neurons on the left and right sides of lumbar and sacral sections of spinal cord to determine the effect of sciatic nerve section in c-fos null and normal littermates.

**c-fos/weaver interaction**

All offspring of c-fos/wv double heterozygous matings were genotyped for c-fos by PCR screening of tail DNA, and the behavioral weaver phenotype of severe ataxia was recorded, if present. Twenty animals between 7 and 17 weeks of age were selected for histological analysis; these included 9 c-fos nulls, and 11 sex-matched, littermate controls that included 5 heterozygotes and 6 wild-type mice (Table 2). All the mice were anaesthetized with tribromoethanol (Papaoianou and Fox, 1993) and perfused through the heart with a solution of 4% paraformaldehyde and 5% sucrose. Liver samples were taken to reconfirm the c-fos genotype by PCR and the brains were removed, postfixed overnight, placed overnight in 10% sucrose, and

### Table 1. Number of 11.5 day p.c. embryos of each genotype that were examined for apoptosis from c-fos/c-jun double heterozygous crosses

<table>
<thead>
<tr>
<th>c-jun genotype</th>
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+ and – indicate the wild-type and mutant alleles respectively.

### Table 2. Number of mice of particular c-fos genotypes classified for weaver genotype on the basis of cerebellar morphology

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<th>c-fos genotype</th>
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<td>3</td>
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<td>+/-wv</td>
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<tr>
<td>wv/wv</td>
<td>+/-wv</td>
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stored in 20% sucrose at 4°C until sectioning. A series of 30 μm-thick sagittal sections through the midline vermis of the cerebellum was mounted on slides, Nissl stained with cresyl violet and examined for morphological characteristics of wild type, heterozygous weaver, or homozygous weaver by an investigator who knew neither the c-fos genotype nor the behavioral phenotype of the animals.

RESULTS

Apoptosis in c-fos null, c-jun null and c-fos/c-jun double null embryos and c-fos null adults

In several embryonic tissues where apoptosis is known to be involved in the normal developmental program, in situ labelled apoptotic nuclei were detected in both a homozygous c-fos null embryo and a heterozygous control embryo at 13.5 days p.c. These tissues included the dorsal root ganglia, myotomes, and endocardial cushions of the atrioventricular heart valves (Fig. 1). Apoptosis also occurs normally in some adult tissues and, similar to the embryonic results, apoptotic nuclei were observed in both homozygous c-fos null adult animals and wild-type controls. Tissues examined were the thymus, where thymocytes are undergoing normal, selective cell death, and the ovaries, where apoptotic granulosa cells were detected in the atretic follicles of both control and mutant adult females (Fig. 2). These observations indicate that normal, physiological cell death can occur in the absence of a functional c-fos gene in both embryonic and adult tissues.

Embryos lacking a functional c-jun gene die during midgestation, but not before organogenesis is well underway. In order to assess the effect of a lack of Jun on apoptosis, and at the same time to assess the effect of the lack of both Fos and Jun, we examined embryos from double heterozygous crosses at a time when most c-jun null embryos would be expected to be alive. From the double heterozygous, c-fos/c-jun cross, 77 embryos were dissected at 11.5 days p.c. and genotyped for both genes. Among these, 6 double null mutants were recovered, which is close to the expected 1/16, or 4.8, double null embryos (χ² = 0.32, P > 0.05). Only two of these double null embryos were dead at the time of dissection; the other 4 were morphologically normal with beating hearts and no obvious abnormalities. In addition, there were 17 c-jun null embryos that were wild type or heterozygous for c-fos, only one of which was dead at the time of dissection. This indicates that the time of death of the c-jun null embryos is not markedly affected by the lack of c-fos.

One living double null embryo, along with 4 c-jun null/c-fos heterozygous, 1 c-jun null/c-fos wild-type, and 5 double or single heterozygous embryos were selected for the apoptosis assay (Table 1). In embryos of all genotypes, similar numbers of apoptotic cells were observed in a variety of tissues known to be undergoing programmed cell death as a normal part of development. These tissues included dorsal root ganglia (data not shown), myotome, sclerotome, heart valve and mesonephros (Fig. 3). The results indicate that the absence of a functional c-jun gene does not prevent apoptosis from occurring during the development of embryonic tissues, and furthermore, that the lack of both c-fos and c-jun gene products does not prevent apoptosis from occurring.

The c-jun null and c-fos/c-jun double null embryos selected for the apoptosis assay were all alive and appeared normal immediately prior to the time of dissection, and showed no obvious signs of their impending demise. However, in addition to the appropriate, developmental apoptosis observed in tissues where it also appeared in control embryos, apoptosis was observed in the liver and neural ectoderm of the double null embryo and 4 of the 5 c-jun null embryos (data not shown). Only very low levels were observed in these tissues in the control embryos.

Cell death following sciatic nerve section

The large motor neurons in the anterior horns of the spinal cord were readily identifiable in the wild-type mice. The spinal cords from the c-fos null mice were flattened compared to wild type, probably due to changes in bone structure caused by the mutation. The anterior horn cells in several of the c-fos null mice were very lightly stained and difficult to count. Nevertheless, comparison of numbers of identifiable motor neurons in the anterolateral quadrant on the operated and the unoperated side showed reduction of motor neurons on the operated side regardless of the genotype of the mouse. Approximately 50% of the large motor neurons on the operated side of wild-type animals and the c-fos null mutants were killed by sciatic nerve transection. In wild-type mice (3 mice), the operated side contained 47±4% of the number of motor neurons on the unoperated side. In the c-fos null group (5 mice), the operated side contained 51±3% of the number of motor neurons on the unoperated side. In addition, gliosis was evident in all mice, including the c-fos null mice, as shown by increased numbers of small darkly staining glial cells in the anterolateral quadrant of the operated side. The numbers of anterior motor neurons on the unoperated side of c-fos null mice were not increased compared to wild type, indicating that lack of c-fos product did not interfere with naturally occurring cell death which reduces the number of cells in the anterior horn by more than half during early post-natal development. In short, lack of c-fos did not spare anterior horn cells from cell death after sciatic nerve section (Fig. 4), nor from cell death that occurs normally during innervation of the limbs.

Cell death in the cerebellum of c-fos/wv double mutants

Among 185 offspring from c-fos/wv double heterozygous matings that survived to weaning, no double homozygous null mice were recovered. Taking into account that weaver homozygotes survive normally, but that only 60% of c-fos null mice survive to weaning (Johnson et al., 1992), we would have expected to find about 7 double null mice in this group. This is a significant deficiency (χ² = 7.8, 1 df, P < 0.01), possibly indicating that the double homozygous individuals are at a more severe disadvantage for early survival than mice lacking either c-fos or wv alone.

Midsagittal sections of Nissl-stained cerebella from 20 selected mice were examined histologically to determine the cerebellar phenotype, with respect to characteristics of wild type, heterozygous weaver or homozygous weaver. Among the 17 mice analyzed that had shown no behavioral phenotype, there were two clear histological phenotypic classes (Table 2). One was normal, in which neurons were arranged in three distinct laminae: the cell-poor molecular layer, the Purkinje cell layer and the granule cell layer. The other corresponded to the heterozygous weaver phenotype, with indistinct laminae
due to the presence of ectopic granule cells in the molecular layer, fewer than normal granule cells in the internal granule cell layer, and out-of-order Purkinje cells. The three animals that had displayed a behavioral weaver phenotype of severe ataxia were classified as homozygous weaver upon histological examination. Their cerebella were very small, and on the midline contained very few granule cells and a jumble of Purkinje cells, the trilaminar organization being destroyed. The weaver homozygous phenotype was seen only in c-fos wild-type and heterozygous mice. The weaver heterozygous phenotype was seen in mice of all three c-fos genotypes, indicating that programmed cell death takes place in the cerebellum in spite of the lack of c-fos. Evidence that cell death took place in the heterozygous weaver/c-fos null mice was supported by the finding that the size of the cerebellum was reduced compared to that of wild-type weaver, regardless of c-fos genotype. The estimated area of midline sagittal sections of heterozygous weavers, whether c-fos null or normal, was 80-85% that of wild type.

DISCUSSION

The identification of genes involved in apoptosis is a key goal for the understanding of programs regulating cell death in development and tissue regression. Although the best understood system is undoubtedly C. elegans (Driscoll and Chalfie, 1992, for review), a number of genes have been identified in mammals that play some role in programmed cell death pathways (e.g. bcl-2, Piacentini and Autuori, 1994; Veis et al., 1993; p53; Yonish-Rouach et al., 1991; c-myb, Evan et al., 1992). Prominent among these genes are c-fos and c-jun, components of the AP-1 transcriptional regulatory factor. An association of programmed cell death with increased AP-1 activity or increased gene expression of c-fos and/or c-jun has been found in a number of studies involving a variety of adult and embryonic tissues (Buttyan et al., 1988; Colotta et al., 1992; Gillardon et al., 1994; Gonzalez-Martin et al., 1992; Ham et al., 1995; Marti et al., 1994; Smeyne et al., 1992, 1993). Furthermore, functional inhibition of c-fos or c-jun inhibits cell death in lymphoid cells (Colotta et al., 1992) and neuronal cells (Estus et al., 1994; Ham et al., 1995) in vitro. We have made use of an in situ assay for apoptosis in mice carrying c-fos and c-jun null mutations (Johnson et al., 1992, 1993) to examine the requirement for these genes in programmed cell death in vivo, both during normal development and in pathological situations leading to apoptosis. Our results indicate that neither gene is essential for programmed cell death to occur in a wide range of tissues in the embryo and the adult, including embryonic dorsal root ganglia neurons (Oppenheim, 1991), myotome (Glucksmann, 1951), developing heart valve (Pexieder, 1975) and adult thymus (Surh and Sprent, 1994). In animals lacking c-fos, apoptosis was observed in appropriate embryonic and adult tissues, and although cell death was not quantified, no differences between genotypes were observed in any of the tissues examined, with the exception that there were many more atretic follicles in the c-fos null females than in controls, as reported earlier (Johnson et al., 1992). From the c-fos/c-jun double heterozygous cross, several c-jun null embryos and one c-fos/c-jun double null embryo were recovered and examined for apoptosis. At the time of dis-
section, all of these embryos appeared normal and vital with beating hearts, and were thus recovered prior to the embryonic death brought about by the c-jun mutation, which usually occurs around 12.5 days p.c. (Johnson et al., 1993). Apoptosis was detected in the same tissues in the c-jun null and the c-fos/c-jun double null embryos as in the controls, providing no evidence for a decrease in apoptosis even in the absence of both genes. However, the death of c-jun null mutants at midgestation limits the range of tissues that can be assessed. Our observation of elevated levels of apoptosis in liver and neural ectoderm in the double null embryo and in most of the c-jun null embryos may be an early indication of their impending death.

In the development of the nervous system, the periphery plays an important role in controlling the number of cells in spinal ganglia and the ventral horn of the spinal cord (Hamburger and Levi-Montalcini, 1949; reviewed by Hinchcliffe, 1981). Cell death normally occurs during the differentiation of all the spinal ganglia, with differential levels of death accounting for differences in the final size of these ganglia; lower levels of cell death are seen in the brachial and lumbar-sacral ganglia as the result of limb bud innervation. Similarly, amputation of limbs or nerve resection causes degeneration of motor cells at corresponding levels in the ventral horn as the feedback from the periphery is removed. Our results give no indication of alterations in the extent of cell death in c-fos null mice, either in the dorsal root ganglia during development or in the ventral horn following section of the sciatic nerve shortly after birth. Furthermore, the motor pool looked about the same size as normal in the c-fos null group, indicating that naturally occurring cell death must have taken place normally. Because two studies have specifically implicated c-jun in the cell death pathway in developmental neuronal cell death (Estus et al., 1994; Ham et al., 1995) it is unfortunate that the early death of c-jun null mutants precludes their use in the assessment of nerve cell death postnatally.

The results of experiments using the wv mutation were limited by the lack of recovery of live-born animals that were both c-fos null and homozygous wv. Cell death in the cerebellum is most evident in homozygotes where the granule cell

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**Fig. 4.** Transverse Nissl-stained sections of lumbar spinal cords from (A) a c-fos null mouse and (B) a wild-type mouse. Sections are from 21-day-old mice in which the right sciatic nerve had been sectioned on postnatal day 3. The non-lesioned (left) side is marked by the partial circles placed in the dorsal horns of the tissue section. The open arrows on the left side of each section point to motor neurons. The solid arrows on the right indicate the regions of greatest loss of motor neurons on the lesioned side. Bar, 400 μm.

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**Fig. 5.** Photomicrographs of Nissl-stained sections of cerebella from (A) wild-type weaver/c-fos null, (B) heterozygous weaver/c-fos null, and (C) heterozygous weaver/c-fos wild-type mice. The cerebellum from a mouse wild-type at the wv locus and null at the c-fos locus (A) has three clearly defined tiers in cerebellar cortex showing distinct molecular (M), Purkinje cell (P) and granule cell (G) layers. The arrow points to a Purkinje cell. Heterozygous weaver mice, whether (B) c-fos null or (C) wild type, could be identified by the disordered structure of the cerebellar cortex. In the heterozygous weaver’s cerebellum, Purkinje cells (arrows in B and C) are embedded among the granule cells. Numerous ectopic granule cells are found in the molecular layer (arrowheads in B and C). Bar, 100 μm.
population is nearly decimated. However, the heterozygous weaver animal also suffers death of granule cells, albeit more moderately, which has the characteristics of apoptosis as assessed by in situ end labelling (Harrison and Roffler-Tarlov, 1995). In fact, it was a heterozygous wv/+ animal in the study of Smeyne et al. (1993) in which the lac-Z reporter gene indicated that c-fos expression precedes granule cell death.

The trilaminar organization of the cerebellum is distorted in the heterozygous weaver mouse because of reduction of the number of granule cells and Purkinje cells, and because of incomplete migration of some of the surviving granule cells, which are stranded in the molecular layer and never reach the internal granular layer. The Purkinje cells, which normally form an orderly line of single cells between molecular and granular layers, are scattered among the cells of the internal granular layer. Because of the death of neurons, the overall size of the heterozygous weaver’s cerebellum is reduced. The severity of the phenotype depends upon the genetic background (Roffler-Tarlov et al., 1994). In our study, cerebella were examined from c-fos null animals that were either wild-type or heterozygous for the weaver gene. The absence of a functional c-fos gene neither affected the normal wild-type pattern, nor did it correct the heterozygous phenotype. In each of the weaver heterozygotes examined, whether wild-type, heterozygous or null for the c-fos allele, the trilaminar organization of the cerebellum was largely destroyed. The heterozygous weaver phenotype expressed was uniformly severe in all mice examined. Granule cells were ectopically placed in the molecular layer and Purkinje cells were buried among the granule cells in the internal granular layer. The overall size of the heterozygous weaver cerebellum was reduced compared to that of the wild type, indicating that cell death had indeed taken place in the disordered cerebellar cortex in the c-fos null as well as in the wild-type mice.

There are two possibilities to account for the lack of effect of the absence of c-fos and/or c-jun on apoptosis in the various situations tested in the current study. A straightforward explanation, one that is supported by our results and other in vivo studies, is that there is no causal relationship between either of these genes and the control or execution of the cell death pathway, even though the expression of c-fos and c-jun coincides with apoptosis in many cell types. In this scenario, the expression of c-fos that accompanies cell death could result from disruption of homeostasis in apoptotic cells rather than being a causal factor in the cell death pathway. The in vitro functional inhibition studies, however, argue against this explanation, and a second possibility is that AP-1 is indeed a required component of the cell death pathway, but that different Fos or Jun family members are normally involved or can substitute opportunistically in the absence of c-fos and/or c-jun. In fact, the members of the AP-1 complex activated during apoptosis vary considerably among different systems, and in our studies only a single Fos and/or Jun family member is disrupted, leaving other family members intact. Marti et al. (1994) reported increased c-fos, junB, junD and to a lesser extent c-jun mRNA levels in involuting mouse mammary glands; similarly, in the involuting rat ventral prostate, the AP-1 complex consisted mainly of c-fos/junD. In a study by Gillardon et al. (1994), increases in c-jun, junB, and c-fos mRNA were seen in rat skin following UV irradiation. In this study, inhibition of c-fos did not significantly affect the formation of apoptotic cells, indicating that c-fos does not play a major role in UV-induced apoptosis. Other studies, however, implicate specific family members. Antisense oligonucleotide inhibition studies specifically implicate c-fos and c-jun in lymphoid cell lines undergoing cell death following growth factor deprivation (Colotta et al., 1992). Antibody inhibition studies point specifically to c-jun and at least one Fos family member as essential for neuronal cell death in response to NGF deprivation in vitro (Estus et al., 1994; Ham et al., 1995).

The occurrence of programmed cell death in animals lacking functional genes for c-fos and/or c-jun clearly demonstrates that neither gene is essential for apoptosis to take place normally in certain tissues in vivo, nor is the c-fos/c-jun heterodimer essential for programmed cell death to occur in certain embryonic tissues. We cannot rule out, however, the possibility that AP-1 might normally play a role in the control of apoptosis, but that different heterodimers can substitute in different cell types, or that alternative pathways are brought into play in the absence of specific gene products.

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REFERENCES


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