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Apoptosis in Normal Rat Embryo Tissues during Early Organogenesis: The Possible Involvement of Bax and Bcl-2

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Summary. **Apoptosis commonly occurs in a variety of developmental processes in mammals. In this study, we investigated the relationship between apoptosis and the expression of both Bax and Bcl-2 during the early organogenesis period (9.5-11.5 days of gestation) of rat embryos. Apoptotic cells detected by the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) method were extremely abundant in the foregut diverticulum at 9.5 days of gestation, while they largely disappeared at 10.5 and 11.5 days of gestation, although they were detected in newly formed mid- and hindgut diverticulum at these times. Real-time RT-PCR analysis of whole embryos revealed that the expression of *bax* mRNA was constant at days 9.5 to 11.5, while the expression of *bcl-2* mRNA gradually increased.**

Immunohistochemical studies of Bax and Bcl-2 expression revealed that these apoptotic cells were exactly positive to Bax in mirror sections, while their expression of Bcl-2 was generally too low to be detected. A disappearance of apoptotic cells was associated with strong Bcl-2 expression in the foregut diverticulum at 10.5 and 11.5 days of gestation. It was similarly observed that apoptotic cells detected in the cardiogenic area at 9.5 days of gestation disappeared with the formation of the primitive heart tube – accompanied by a strong expression of both Bcl-2 and Bax – in the developmental process of the primitive heart. Apoptotic cells were also observed in the primitive brain vesicle, optic vesicle, otic vesicle, and thyroid primordium at 10.5 and 11.5 days of gestation during the developmental process, with a strong expression of Bax. These results indicate that the Bax and Bcl-2 may be important in regulating the induction of embryonic cell apoptosis during early organogenesis.

Cell death is a frequent event in mammalian development (GLÜCKSMANN, 1951; HURLE, 1988; COUCOUVANIS et al., 1995). Programmed cell death (PCD) describes cell death occurring at predictable places and times during development (LOCKSHIN and WILLIAMS, 1964; SAUNDERS, 1966; JACOBSON et al., 1997). Apoptosis is the most common form of PCD in developing embryos, is an active process of cell death that is morphologically characterized by membrane-budding, nuclear and cytoplasmic shrinkage, and chromatin condensation (KERR et al., 1972). Conventional identification of apoptosis has been based primarily on the morphological features of dying cells through the use of electron microscopy, but the number of cells analyzed has been quite limited. TUNEL staining is currently one of the most popular histochemical methods for assessing the cellular characteristics closely associated with apoptosis, permitting the detection of double-stranded DNA breaks occurring in the early stages of apoptosis by terminal deoxynucleotidyl transferase-mediated labeling of a free 3'-OH terminus (GAVRIELI et al., 1992; WANG et al., 1998).

PCD in preimplantation embryos has been extensively studied by many authors. In both mice and humans, 15-50% of embryos die during the preimplantation period, and the developing and arrested embryos contain different propotions of cells with the classic features of apoptosis, including cytoplasmic, nuclear and DNA fragmentation (WARNER et al., 1998; HARDY, 1999). Moreover, embryos also seem to be vulnerable during the postimplantation period, especially when tissues and organs are just being generated – the so-called organogenetic period (MOORE and PERSAUD, 1998). In fact, we have previ-

ously demonstrated that the exposure of rat embryos to hyper- or hypoglycemia during the early postimplantation period, from the headfold stage (day 9) to the period of neural tube closure (day 11), can result in embryonic malformations (FREINKEL et al., 1986; AKAZAWA et al., 1987, 1989; TROCINO et al., 1995). Recently, it has also been suggested that various teratogenic insults associated with embryonic apoptosis occur during this period (CHEN et al., 1994; THAYER and MIRKES, 1995; FINE et al., 1999). Despite their potential importance, studies of the physiological occurrence of apoptosis and its mechanisms in this early organogenesis period have been limited, a fact which prompted us to investigate the occurrence of apoptosis in rat embryo during the early postimplantation period from days 9.5 to 11.5.

The Bcl-2 family of proteins, including pro- and anti-apoptotic molecules, are thought to play a critical role in the regulation of apoptosis, acting as upstream factors of the apoptotic machinery. Bcl-2, a 26-kDa intracellular membrane-associated protein, is homologous to CED-9 which inhibits PCD in *C. elegans* (HENGARTNER and HORVITZ, 1994; GOTOW et al., 2000), and can block apoptosis induced by a variety of treatments in different mammalian cell types (YANG and KORSMEYER, 1996). In contrast, Bax was originally identified as a protein forming a complex with Bcl-2, and later was shown to promote apoptosis (OLTVAI et al., 1993). Analysis of point mutations in the *bcl-2* gene has suggested that the direct binding of Bcl-2 to Bax is essential to the death-repressor activity of Bcl-2 (YIN et al., 1994). Moreover, overexpression of Bax counteracts the death-repressor activity of Bcl-2 (OLTVAI et al., 1993). These accumulated data indicate that the ratio of Bcl-2 to Bax might be essential for determining whether a cell survives or dies following apoptotic signals (KORSMEYER et al., 1993; BABA et al., 1999; DE FELICI et al., 1999; BRAMBRINK et al., 2000).

In the present study we first examined the occurrence of apoptosis in rat embryos by TUNEL-staining during the early organogenetic period. Next, to gain insight into the molecular mechanism underlying the induction of embryonic cell apoptosis, we analyzed the expression of Bax and Bcl-2 at both the mRNA and protein level by quantitative RT-PCR and immunohistochemistry, respectively. Consequently, we found a strict spatial and temporal association between TUNEL-positive cells and Bax expression at each cell basis, especially in the development of the digestive system, primitive heart, and nervous system.

MATERIALS AND METHODS

Animals

Virgin female Wistar rats at 8 weeks of age were obtained from Shizuoka Laboratory (Shizuoka, Japan). All animal procedures were performed according to the Guide for the Animal Care and Use Committee at our institution. Rats were housed in a room where the temperature (23 ± 1℃) and light/dark cycle (12-h light: 12-h dark) were closely controlled. A standard laboratory diet was provided (Oriental Yeast, Tokyo), and water was available *ad libitum*. Female rats were impregnated by exposing them from 18 : 00 to 08 : 00 to males from the same strain. Pregnancy was timed from midnight proceeding the morning on which a vaginal plug was observed, and that midnight was designated as day 0. Pregnant rat were sacrificed by cervical dislocation at days 9.5, 10.5, and 11.5 of gestation, which is the early organogenesis period, and embryos were obtained. For TUNEL and immunohistochemical studies, the embryos were fixed overnight in 4% paraformaldehydrate in PBS at 4℃, and then embedded in paraffin in a routine manner. Three or four embryos of each gestational day were used for the staining. Serial and mirror sections were cut to a thickness of 5 μm. For the RT-PCR analysis, whole embryos at days 9.5, 10.5 and 11.5 of gestation were dissected from decidua under a microscope and immediately frozen and stored at −80℃ until use.

TUNEL staining

TUNEL was performed essentially according to the method described by GAVRIELI et al. (1992) with a slight modification, as described previously (WANG et al., 1998). Briefly, tissue sections were treated with 1 μg/m*l* proteinase K in PBS (Wako Pure Chemicals, Japan) at 37℃ for 15 min. After preincubation with a terminal deoxynucleotidyl transferase (TdT) buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/m*l* BSA, pH 6.6) for 30 min, the sections were reacted with a TdT buffer containing 100 IU/m*l* TdT (Roche Diagnostics, Mannheim, Germany) and 2.5 μM biotinylated 16-dUTP (Roche Diagnostics) at 37℃ for 1 h. The reaction was terminated by three washings with 50 mM Tris-HCl (pH 7.5) for 5 min each. Endogenous peroxidase was inactivated by immersing the sections in 0.3% H_2O_2 in methanol for 15 min, and then washed with 0.075% Brij (Sigma Chem. Co., St. Louis, USA) in PBS. To block the nonspecific reaction, sections were incubated with 500 μg/m*l* normal goat IgG (Sigma Chem. Co.) in 5% BSA/PBS for 1 h, then treated overnight with HRP-

goat anti-biotin (1 : 800, Vector Laboratories Inc. Burlingame, USA) diluted with 1% BSA/PBS. HRP sites were visualized by H_2O_2 and 3, 3'-diaminobenzidine/4HCl (DAB, Dojindo, Japan) for 6 min. In the present study, we added nickel and cobalt ions to enhance sensitivity (ADAMES, 1981). TUNEL staining was expressed as negative (−) or positive (+).

Immunohistochemistry

To localize Bax and Bcl-2 proteins, immunohistochemistry was performed as detailed previously (BABA et al., 1999). For the Bax staining, sections were autoclaved at 121℃ for 10 min in a 0.01 M citrate buffer (pH 6.0) after deparaffinization. The sections were then reacted overnight with rabbit polyclonal anti-Bax (P-19, 1 : 200, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and an anti-Bcl-2 antibody (N-19, 1 : 100, Santa Cruz Biotechnology, Inc.). After washing with 0.075% Brij in PBS, the sections were reacted with HRP-goat anti-rabbit antibody (1 : 200, MBL, Nagoya, Japan) for 1 h at room temperature, and the HRP sites were then visualized as described as above. The sections for Bcl-2 staining were counterstained with methyl green. As a negative control, some slides were reacted with normal rabbit IgG instead of the specific antibodies used above. The protocol was optimized by staining a rat small intestine as a positive control, according to KRAJEWSKI et al. (1994). Intense staining for Bax was observed in the cells within the crypt, and staining for Bcl-2 was more intense in epithelial cells located in the upper portions of the intestinal villi, as described previously. The staining intensities for Bax and Bcl-2 were expressed as negative (−), weak (+), moderate (++), and intense (+++).

Real time quantitative RT-PCR

Total RNA was isolated from the whole embryos at days 9.5 (n=12), 10.5 (n=10), and 11.5 (n=16) of gestation using a commercial reagent kit-Isogen (Nippon Gene, Osaka, Japan), which involves a procedure of acid guanidinium thiocyanate-phenol chloroform extraction. One microgram of total RNA was used to make cDNA using a Thermo script RT-PCR system (GIBcoBRL, Life Technologies, USA). Quantitative PCR analysis was performed as described previously (BRAMBRINK et al., 2000) with slight modifications. By using the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Diagnostics), which is specially adapted for the LightCycler instrument (Roche Diagnostics), the PCR reaction was performed in a final volume of 20μl in the LightCycle glass capillaries according to the manufacturer's recommendations. The primers and probes used in our study for *bax*, *bcl-2*, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) are described in Table 1. The copy numbers in the samples were estimated by interpolation from a plasmid standard curve and an internal control of GAPDH to correct for sample DNA-quality differences. Amplified PCR products were electrophoretically separated in 2% agarose gels and stained with ethidium bromide. The sequence of each PCR product was confirmed to be identical with the published sequence (*bcl-2*, GenBank accession number L14680; *bax*, GenBank accession number AF235993; GAPDH, GenBank accession number AF106860).

Table 1. Primers and probes used for the detection of *bax*, *bcl-2*, and GAPDH mRNA

Gene	Primers and probes			Products size
bax	Primers	Forward	5'-GACAACAACATGGAGCT-3'	205bp
		Reverse	5'-AGCCCATGATGGTTCTGATC-3'	
	Probes	A	5'-GCTGCCACACGGAAGAAGACCT-3' 3'FITC	
		B	5'-TCGGGGGGAGTCTGTATCCACA-3' 5'LC Red640	
bcl-2	Primers	Forward	5'-CATGCGACCTCTGTTTGA-3'	193bp
		Reverse	5'-GTTTCATGGTCCATCCTTG-3'	
	Probes	A	5'-CATACCTGGGCCACAAGTGAGG-3' 3'FITC	
		B	5'-CAGCAGACCTGCCCCAAACAA-3' 5'LC Red640	
GAPDH	Primers	Forward	5'-TGAACGGGAAGCTCACTGG-3'	307bp
		Reverse	5'-TCCACCACCCTGTTGCTGTA-3'	
	Probes	A	5'-CTGAGGACCAGGTTGTCTCCTGTGA-3' 3'FITC	
		B	5'-TTCAACAGCAACTCCCATTCTCCACC-3' 5'LC Red640	

RESULTS

General features of organ development in the early organogenesis of rat embryos

To investigate the role of apoptosis in early organogenesis, we studied rat embryos at 9.5, 10.5, and 11.5 days of gestation, times which correspond to the stages from embryos with 4–5 pairs of somites to 25–30 pairs of somites. During this time, embryos accomplish neural tube formation, the cardiogenic plate differentiates into a primitive heart, and the foregut diverticulum extends to form mid- and hindgut diverticula. The critical developmental stages of rat were defined according to the stages in the mouse as described by KAUFMAN (1992); the development of rat lags just 1 day behind than that of the mouse. The general morphological features of embryos are given in Figures 2A, 3A, 4A, 5A, 6A, and 7A.

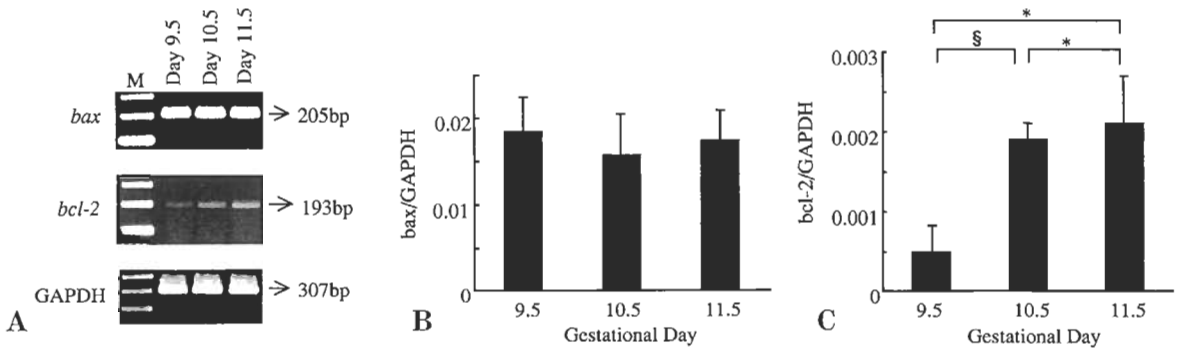

Fig. 1. Quantitative RT-PCR analysis of *bax* and *bcl-2* mRNAs of rat embryos at gestational days 9.5, 10.5, and 11.5. **A.** Amplified PCR products were electrophoretically separated in 2% agarose gels and stained with ethidium bromide. The expected fragments are *bax*, 205bp; *bcl-2*, 193bp; and GAPDH, 307bp. M: marker. **B.** Quantitative results of *bax* mRNA expression. **C.** Quantitative results of *bcl-2* mRNA expression. Values are expressed as means±SD. §$P<0.05$, *$P<0.01$.

Table 2. Presence of TUNEL-positive cells and expression of Bax and Bcl-2 in rat embryos

	Day 9.5			Day 10.5			Day 11.5		
	TUNEL	Bax	Bcl-2	TUNEL	Bax	Bcl-2	TUNEL	Bax	Bcl-2
Primitive heart	+	+*	−	−	+++△	+++	−	+++△	+++
Primitive gut									
Foregut	+	+++*	−	−	−	+++	−	−	+++
Midgut				+	++*	−	+	++*	−
Hindgut				+	++*	−	+	++*	−
Thyroid rudiment				+	+++*	−	+	+++*	−
Nervous system									
Brain vesicle				+	++*	−	+	++*	−
Optic vesicle				+	++*	−	+	++*	−
Otic vesicle							+	++*	−

The results of TUNEL staining are identified as negative (−) and positive (+). The intensities for Bax and Bcl-2 are expressed as negative (−), weak (+), moderate (++), and intense (+++). Two immunostaining patterns for Bax were observed; 1) diffuse cytoplasmic staining (△); 2) staining pattern associated with apoptotic morphologies (*).

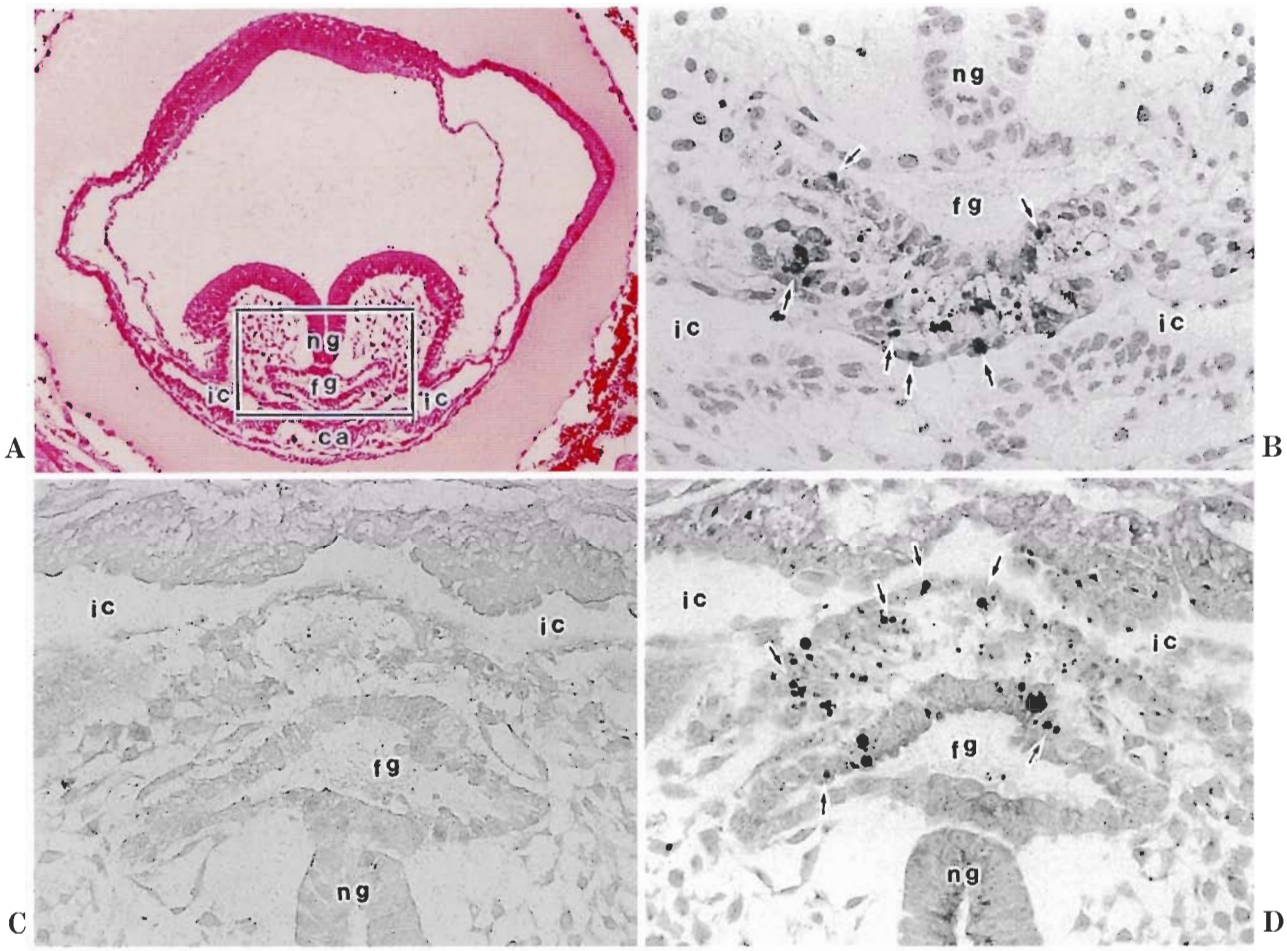

Fig. 2. Staining of rat embryos at day 9.5 of gestation. **A:** Hematoxylin-eosin (H-E) staining (×680), **B:** TUNEL (×2,700), **C:** immunostaining for Bcl-2 (×2,700), **D:** immunostaining for Bax (×2,700). Numerous TUNEL-positive cells are detected in the foregut diverticulum and the endothelial elements that are supposed to form the first pharyngeal arch artery (**B**, *arrows*). Using mirror sections, intense staining for Bax is observed in cells that are also TUNEL-positive (**D**, *arrows*). Note that the staining pattern for Bax is associated with apoptotic morphologies. Immunostaining for Bcl-2 is almost entirely negative (**C**). *ng* Neural groove, *fg* foregut diverticulum, *ic* intra-embryonic cavity, *ca* cardiogenic area

Expression of *bcl-2* and *bax* mRNAs

To confirm the expression of *bcl-2* and *bax* mRNAs during the early organogenesis period, we carried out quantitative RT-PCR using total RNA from whole embryos. The expected size of the products amplified by PCR was 205 bp, 193 bp, and 307 bp for *bax, bcl-2,* and GAPDH mRNAs, respectively (Fig. 1A). Our results revealed that the expression of *bax* mRNA level was relatively high and nearly constant during gestational days 9.5 to 11.5 (Fig. 1B), while *bcl-2* mRNA expression was only marginal (Fig. 1C).

Distribution of TUNEL-, Bcl-2, and Bax-positive cells in rat embryos at day 9.5 of gestation

The primitive gut

At day 9.5 of gestation, the foregut diverticulum was clearly identified, and numerous TUNEL-positive cells were detected in the area (Fig. 2B, Table 2). In the mirror sections, intense Bax staining was found in some cells that were also TUNEL-positive (Fig. 2D, Table 2). Bcl-2 staining was almost entirely negative (Fig. 2C, Table 2).

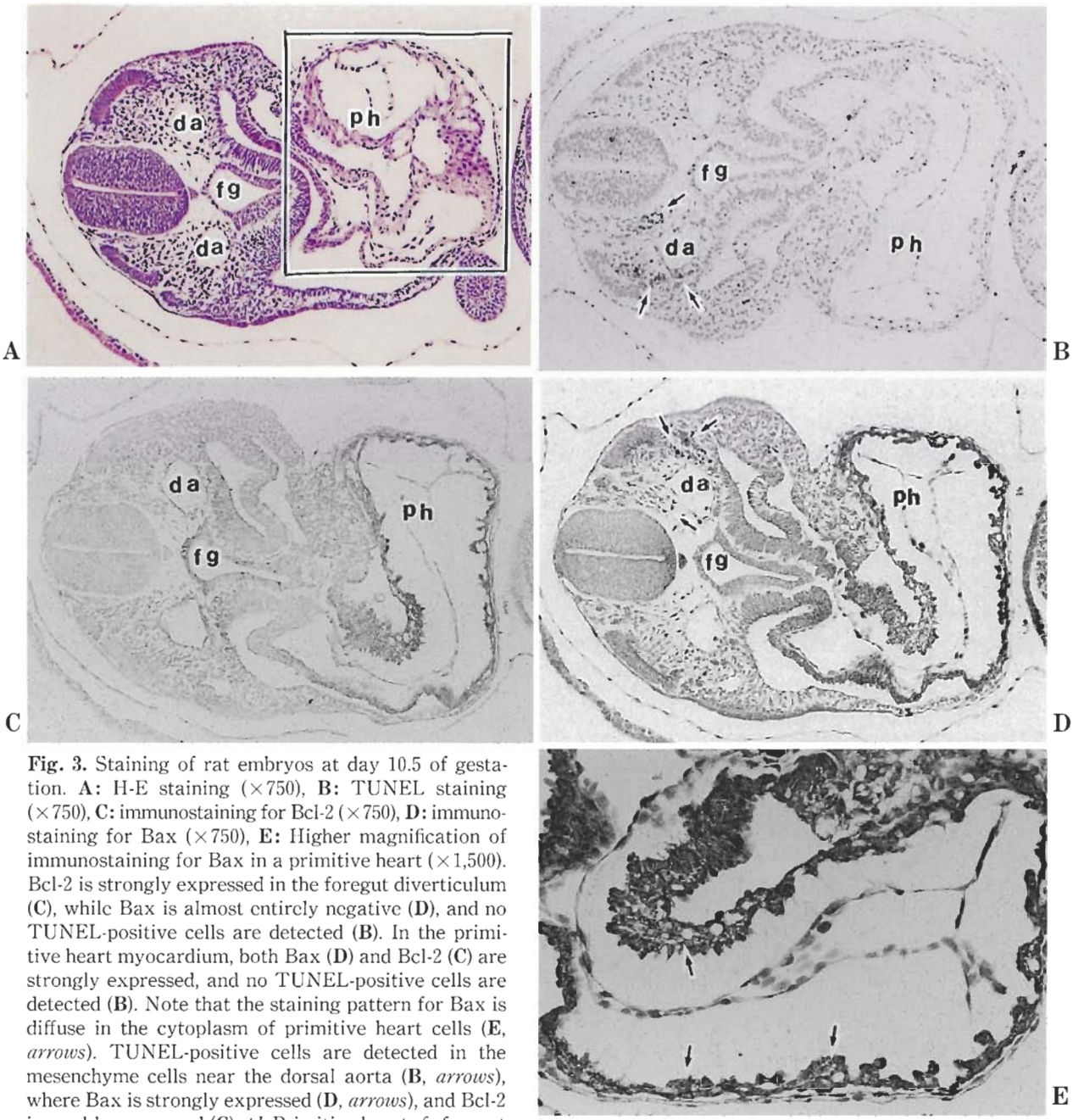

Fig. 3. Staining of rat embryos at day 10.5 of gestation. **A:** H-E staining (×750), **B:** TUNEL staining (×750), **C:** immunostaining for Bcl-2 (×750), **D:** immunostaining for Bax (×750), **E:** Higher magnification of immunostaining for Bax in a primitive heart (×1,500). Bcl-2 is strongly expressed in the foregut diverticulum (**C**), while Bax is almost entirely negative (**D**), and no TUNEL-positive cells are detected (**B**). In the primitive heart myocardium, both Bax (**D**) and Bcl-2 (**C**) are strongly expressed, and no TUNEL-positive cells are detected (**B**). Note that the staining pattern for Bax is diffuse in the cytoplasm of primitive heart cells (**E**, *arrows*). TUNEL-positive cells are detected in the mesenchyme cells near the dorsal aorta (**B**, *arrows*), where Bax is strongly expressed (**D**, *arrows*), and Bcl-2 is weakly expressed (**C**). *ph* Primitive heart, *fg* foregut diverticulum, *da* dorsal aorta

The primitive heart

Numerous TUNEL-positive cells were observed in the areas just under the foregut diverticulum where endothelial elements were amalgamating to form the prospective first pharyngeal arch aorta, (Fig. 2B). In addition, many mesenchymal cells in the cardiogenic area, where the cells routinely cluster to form the ventral pericardial coelum, were also found to be TUNEL-positive (Table 2). Intense Bax staining was observed in TUNEL-positive cells, while Bcl-2 staining was almost entirely negative (Fig. 2C, D, Table 2).

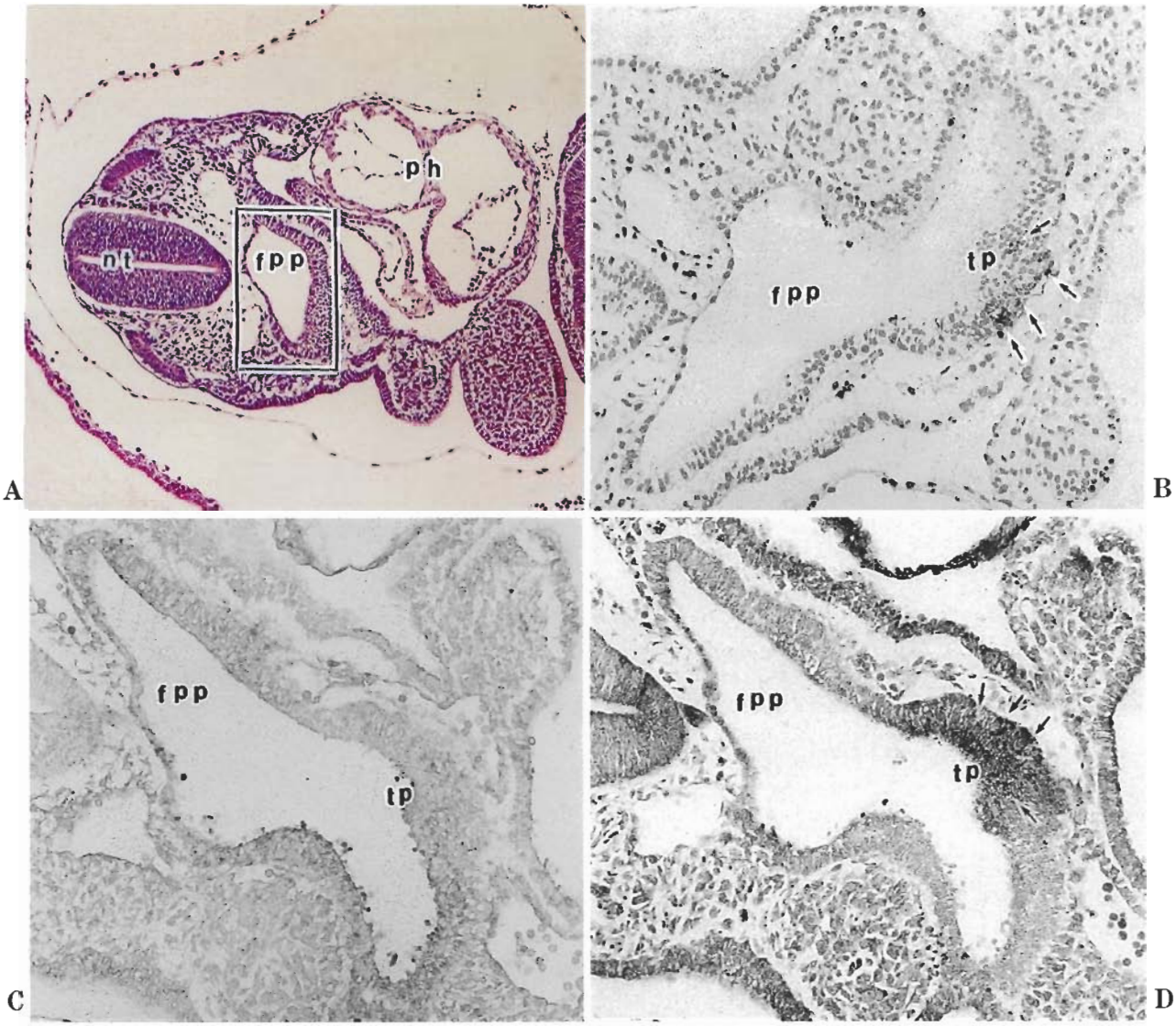

Fig. 4. Staining of rat embryos at day 10.5 of gestation. **A:** H-E staining (×750), **B:** TUNEL staining (×1,500), **C:** immunostaining for Bcl-2 (×1,500), **D:** immunostaining for Bax (×1,500). In thyroid primordium, TUNEL-positive cells are detected (**B**, *arrows*). In mirror sections, a stronger expression of Bax is observed (**B**, *arrows*), and Bcl-2 is only weakly expressed (**C**). *tp* Thyroid primordium, *fpp* first pharyngeal pouch, *ph* primitive heart, *nt* neural tube

The nervous system

Very few TUNEL-positive cells were detected in the neuroepithelium of the neural fold. Intense Bax staining was observed in the TUNEL-positive cell areas, while Bcl-2 staining was almost entirely negative (data not shown).

Distribution of TUNEL-, Bcl-2-, and Bax-positive cells in rat embryos at day 10.5 of gestation

The primitive gut

At day 10.5 of gestation, a rostral extension of the foregut diverticulum began to form the first pharyngeal arch pouch. Along the caudal extension of the gut tube, the mid- and hindgut diverticula were well developed. At this stage, we could not detect TUNEL-positive cells in the foregut diverticulum

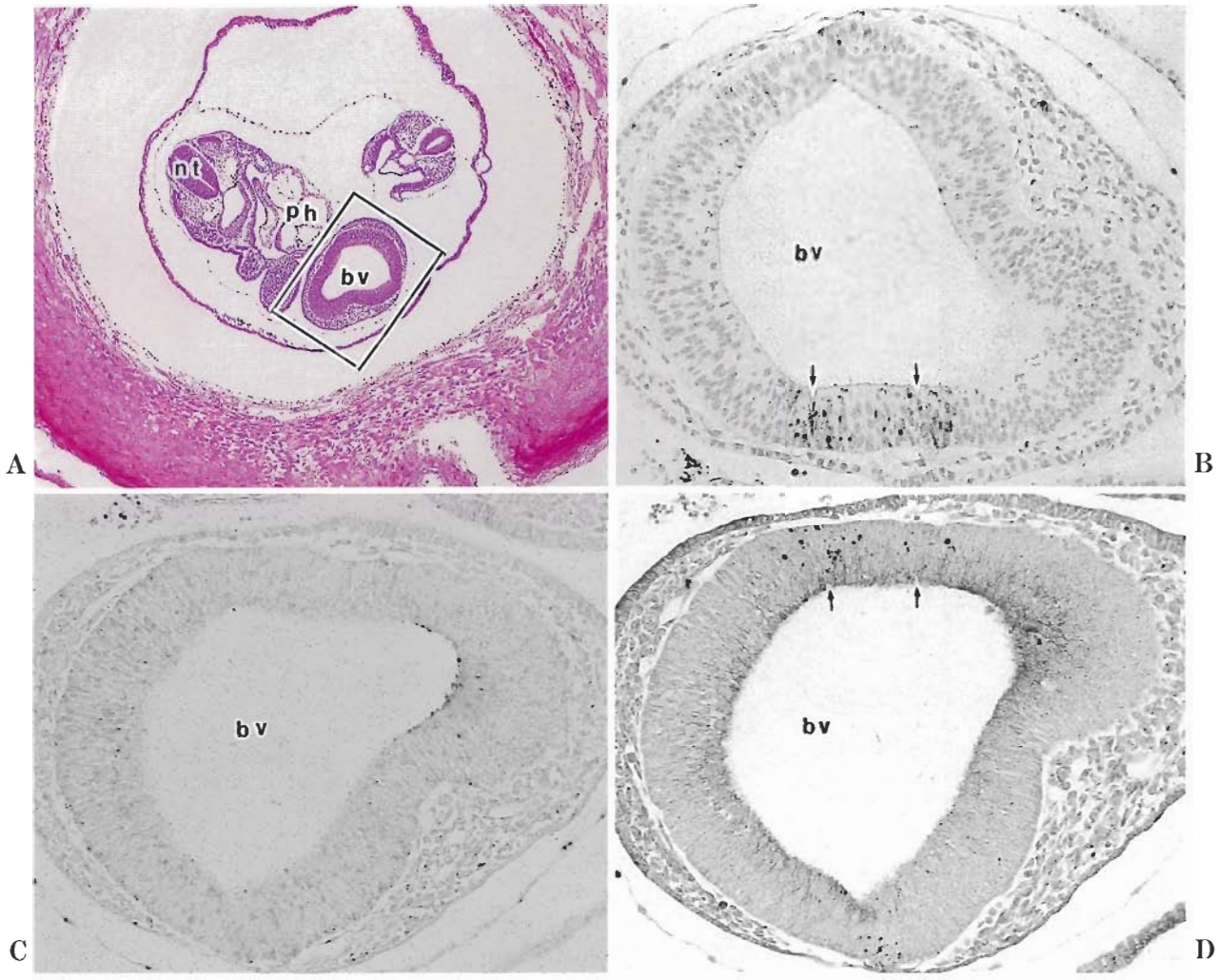

Fig. 5. Staining of rat embryos at day 10.5 of gestation. **A:** H-E staining (×270), **B:** TUNEL staining (×1,350), **C:** immunostaining for Bcl-2 (×1,350), **D:** immunostaining for Bax (×1,350). TUNEL-positive cells are detected in the floor plate of the primitive brain (**B**, *arrows*). Using mirror sections, intense staining for Bax is observed (**D**, *arrows*). However, only weak staining for Bcl-2 is observed (**C**). *bv* Brain vesicle, *nt* neural tube, *ph* primitive heart

(Fig. 3B, Table 2). Intense Bcl-2 staining was observed in the foregut portion (Fig. 3C, Table 2), but Bax staining was almost entirely negative (Fig. 3D, Table 2). However, TUNEL-positive cells appeared in the midgut or hindgut diverticulum, which was undergoing active formation. In addition, TUNEL-positive cells were abundant in the primordial thyroid area (Fig. 4B, Table 2). Again, we found intense Bax staining and weak Bcl-2 staining in this area (Fig. 4C, D, Table 2).

The primitive heart

At this stage, common atrial and ventricular chambers were formed. There were no TUNEL-positive cells observed in the myocardial tissue of the primitive heart (Fig. 3B, Table 2). Here, intensely diffuse cytoplasmic staining for both Bax and Bcl-2 was observed (Fig. 3C, D, Table 2). As shown in Figure 3E, the Bax staining pattern in the primitive heart did not correlated with the occurrence of TUNEL-positive cells.

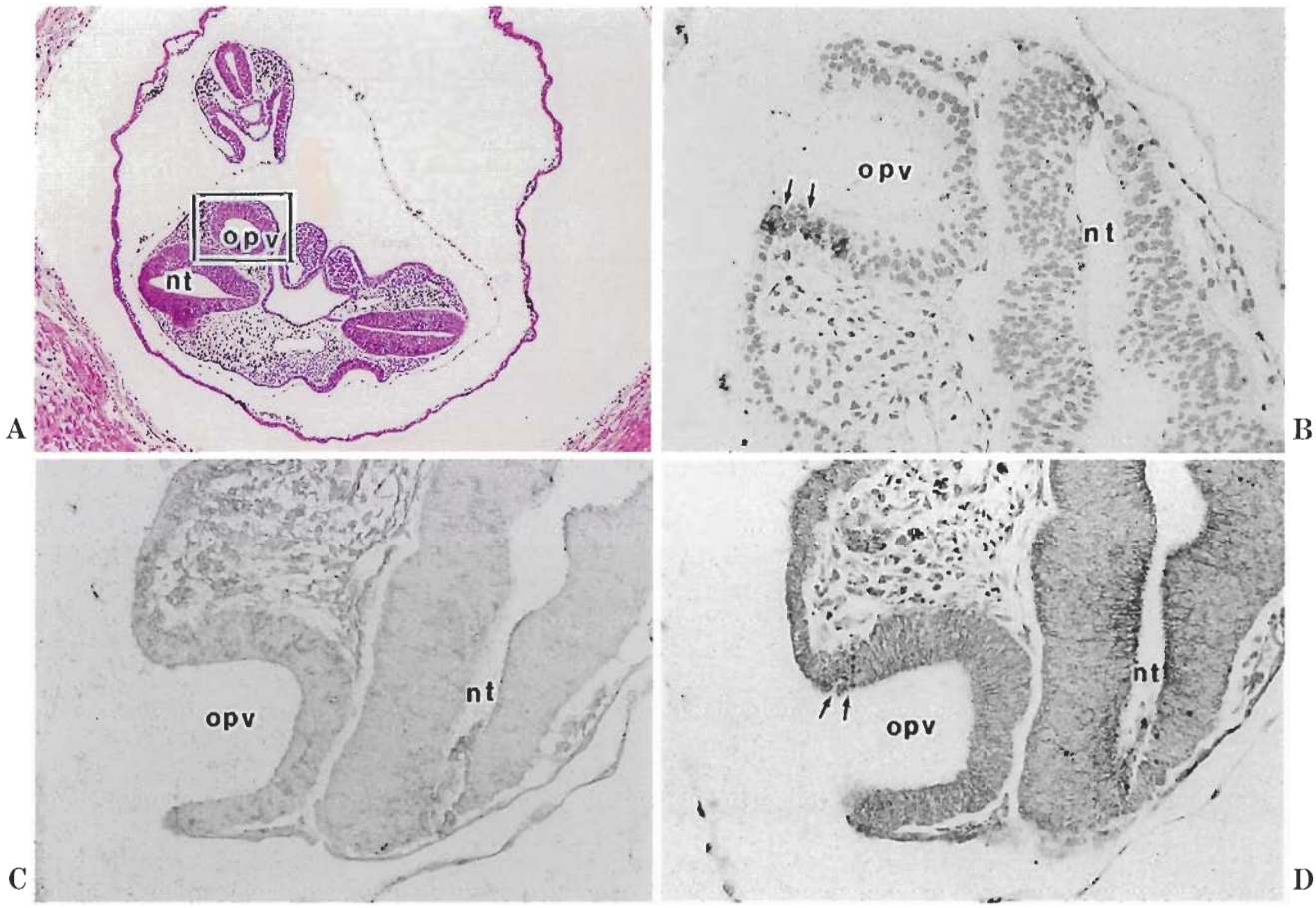

Fig. 6. Staining of rat embryos at day 10.5 of gestation. **A:** H-E staining (×300), **B:** TUNEL staining (×1,500), **C:** immunostaining for Bcl-2 (×1,500), **D:** immunostaining for Bax (×1,500). TUNEL-positive cells are detected in the optic vesicles (**B**, *arrows*). Intense staining for Bax is observed (**D**, *arrows*), while Bcl-2 is almost entirely negative (**C**). *opv* Optic vesicle, *nt* neural tube

The nervous system

As a consequence of closure of the rostral extremity of the neural tube, primitive brain vesicles underwent formation. The caudal extremity of the neural tube was still in the process of closing. Many TUNEL-positive cells were detected in clusters in the roof or floor plate of the primitive brain vesicles (Fig. 5B, Table 2), accompanying both the strong expression of Bax and weak expression of Bcl-2 (Fig. 5C, D, Table 2). During the optic vesicles morphogenesis, TUNEL-positive cells were just detected in those places with actively changing morphology (Fig. 6B, Table 2), intense Bax staining and weak Bcl-2 staining were observed in the area (Fig. 6C, D, Table 2).

Distribution of TUNEL-, Bcl-2-, and Bax-positive cells in rat embryos at day 11.5 of gestation

The primitive gut

Similar to the 10.5-day embryos, TUNEL-positive cells were numerous in the midgut and hindgut diverticulum (Fig. 7B, Table 2) as well as the thyroid primordium, where TUNEL-positive cells were also found to be intensely positive for Bax, but not for Bcl-2 (Fig. 7C, D, Table 2). In addition, the part of foregut that was intensely stained for Bcl-2 was essentially free from TUNEL-positive cells.

The primitive heart

TUNEL-positive cells were not detected in the primitive heart, and intense staining for both Bax and Bcl-2 was observed in this area.

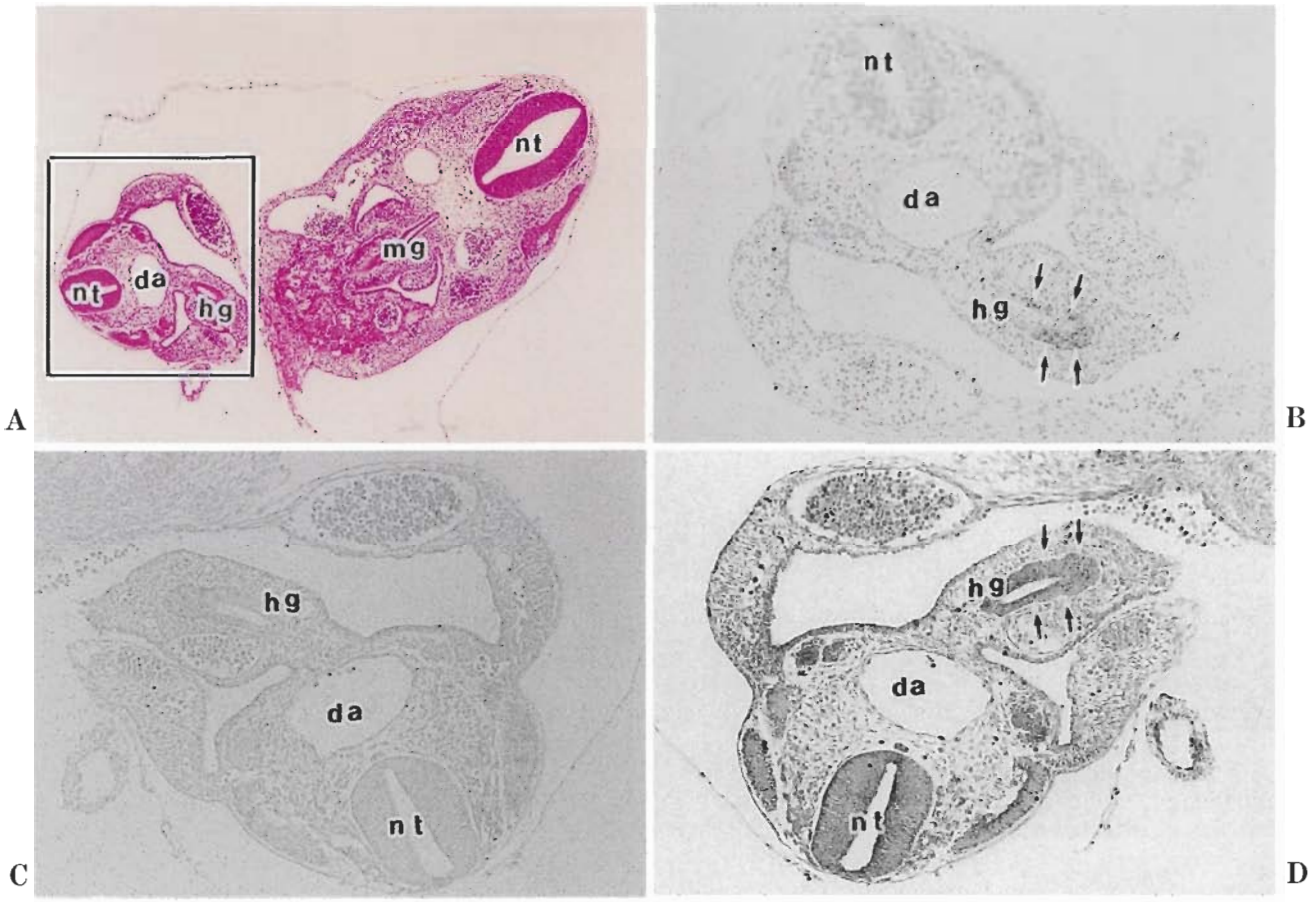

Fig. 7. Staining of rat embryos at day 11.5 of gestation. **A:** H-E staining (×300), **B:** TUNEL staining (×750), **C:** immunostaining for Bcl-2 (×750), **D:** immunostaining for Bax (×750). TUNEL-positive cells are detected in the hindgut diverticulum (**B**, *arrows*). Bax is strongly expressed in the region (**D**, *arrows*), while Bcl-2 is only weakly expressed (**C**). *hg* Hindgut, *nt* neural tube, *mg* midgut, *da* dorsal aorta

The nervous system

Closure of the neural tube was almost complete at day 11.5. TUNEL-positive cells were also frequently detected in the neuroepithelium of primitive brain ventricles and the otic pits, which were in the course of formation, where again intense staining for Bax and weak staining for Bcl-2 were observed.

DISCUSSION

The present study examined the occurrence of apoptotic cell death in various rat embryonic tissues undergoing early organogenesis, confirming the presence of the frequent occurrence of apoptosis strictly in actively differentiating organs. Moreover, we have also found that the balance between Bax and Bcl-2 expression may play an essential role in the regulation of embryonic cell apoptosis.

In the primitive gut, we found numerous apoptotic cells initially in the foregut diverticulum, which then disappeared at days 10.5 and 11.5 but reappeared in the mid- and hindgut diverticulum. Apoptosis in the developing digestive system has been reported at more advanced stages of the human fetus after differentiation of the midgut to the small intestine, where apoptotic enterocytes have rarely been observed between 9 and 17 weeks of gestation, but are regularly detected in villi following this period (VACHON et al., 2000). However, apoptosis during the development of the primitive gut has not been previously reported in any mammalian embryos. Similar to the development of primitive gut, apoptotic cells in

the primitive heart were initially detected in the cardiogenic area at day 9.5 and then disappeared by the time the primitive heart tube was formed at days 10.5 and 11.5. In accord, ZHAO and RIVKEES (2000) have reported no apoptotic cells in the mouse embryonic heart between 9.5 and 10.5 days of gestation. Moreover, they have observed a reappearance of TUNEL-positive cells in the endocardial cushion, myocardium, trabeculae, and papillary muscles of ventricles on later gestational days, when active remodeling of heart tissues occurs. In the nervous system, we detected frequently occurring apoptosis in the roof or floor plate regions of the primitive brain at days 10.5 and 11.5 of gestation. It has been established that neuronal cell death in mice occurs frequently after 14 days of gestation. However, our findings also indicate that a certain population of nerve cells undergoes cell death slightly earlier. In this context, it should be noted that during the early development of the chick embryo, corresponding to day 10 of mouse gestation, massive cell death occurs in a specific population of motoneurons of the ventral cervical spinal cord (SENDTNER et al., 2000).

JACOBSON et al. (1997) have proposed five functions of apoptosis in animal development: 1) structure-sculpting, 2) deletion of unneeded structures, 3) control of cell numbers, 4) elimination of abnormal, misplaced, nonfunctional, or harmful cells, and 5) production of specialized and differentiated cells accompanying a loss of organelles, such as skin keratinocytes and mammalian red blood cells. In fact, the biological significance of the frequent apoptotic cell death occurring in the primordium, such as the foregut diverticulum, the area of the prospective aorta, and thyroid rudiment, is unknown at present. However, most of the apoptotic cells seem to be associated with active morphogenesis of the primordium and may be at least be categorized into function 1) of JACOBSON's classification.

Because the Bcl-2 family plays a crucial role in intracellular apoptotic signal transduction and displays either positive or negative regulatory effects on apoptosis, we investigated the expression of Bax, the death-promoter, and Bcl-2, the death-repressor, in rat embryos. Our immunohistochemical results identified Bax-positive cells in a wide variety of embryonic tissues from days 9.5 to 11.5, while Bcl-2 staining was only marginal throughout the embryo at day 9.5. At days 10.5 and 11.5, some tissues became positive for Bcl-2 immunostaining. During the early organogenesis stage, histochemical analysis further revealed that the TUNEL-positive cells where also positive for Bax in mirror sections, were little or no Bcl-2 staining was detected, strongly indicating that whole-cell staining for Bax is closely associated with apoptosis. However, no TUNEL-positive cells were detected in the primitive heart, where Bcl-2 as well as Bax was strongly expressed. In addition, Bax staining was diffusely detected in the cytoplasm of primitive heart cells, unlike the staining pattern of Bax in TUNEL-positive cells of other primitive organs. It has recently been reported that Bax is mostly localized within the cytoplasm of healthy cells, and after apoptotic signals is translocated to the mitochondria (WOLTER et al., 1997; NOMURA et al., 1999). Bcl-2 is present in the mitochondrial membrane, and exerts its antiapoptotic activity partly by inhibiting the translocation of Bax to the mitochondria by affecting a cytosolic factor (NOMURA et al., 1999; GOTOW et al., 2000). Also, the ratio of Bax to Bcl-2 has been reported to be essential to determining whether a cell survives or dies following apoptotic signals (KORSMEYER et al., 1993; BABA et al., 1999; DE FELICI et al., 1999; BRAMBRINK et al., 2000). Therefore, we can assume that the strong Bcl-2 expression in the primitive heart may inhibit the induction of heart cell apoptosis through the blockage of the translocation of Bax to mitochondria. These results also indicate that the ratio of Bax to Bcl-2 might be important in the regulation of embryonic cell apoptosis.

Despite the expected essential role of Bax and Bcl-2, Bax-deficient mice have been found to be healthy as newborns, though males later exhibit lymphoid hyperplasia and germ cell hypoplasia (KNUDSON et al., 1995). In addition, Bcl-2-deficient mice have survived for 1 week, developing polycystic kidneys, immunodeficiency, and hair hypopigmentation (NAKAYAMA et al., 1993). Therefore, both Bax-deficient and Bcl-2-deficient mice apparently can complete their embryonic development. In this context, it should be noted that other molecules of the Bcl-2 family such as Bak, Bad, and Bcl-x_S, which are known to activate apoptosis, and Bcl-x_L, Bcl-w, and Mcl-1, which inhibit apoptosis, may provide redundancy in death-promoting or death-repressor activity during embryogenesis (YANG and KORSMEYER, 1996).

In conclusion, we detected the frequent occurrence of apoptotic cell death in various embryonic tissues undergoing early organogenesis in the rat embryo, and demonstrated the possible involvement of Bax and Bcl-2 in the induction of normal embryonic apoptosis.

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