ORIGINAL ARTICLE

Eur. J. Anat. 19 (2): 189-195(2015)

Caspase-2 immunohistochemistry in differentiating cells during mouse cephalic development

Nathalie Vanmuylder, Marie Lejong, Myriam Choa-Duterre and Stéphane Louryan

Laboratoire d'Anatomie, Biomécanique et Organogenèse, Université Libre de Bruxelles, Faculté de Médecine, Bruxelles, Belgium

SUMMARY

Caspases are proteases primarily involved in the process of apoptosis; however, caspases can exert non-apoptotic functions. The purpose of this work was to use immunohistochemistry to analyse the expression sites of caspase-2 during normal mouse cephalic development and in embryos exposed to irradiation. Control embryos from embryonic day 9 (E9) to E17 were analysed, and E9 and 10 irradiated embryos were removed and observed after administration of 2 Gy irradiation at embryonic day 9. Surprisingly, not only apoptotic cells expressed caspase-2. In addition, numerous cell populations in normal and experimental embryos displayed transient but intense caspase-2 immunoreactivity, with nuclear and cytoplasmic localisation. This immunoreactivity was not observed with caspase-3 and -9 antibodies.

Cranial neural crest cells, premuscular blastemata, cartilage, teeth, the heart, the eye and some other structures displayed caspase-2 expression, with progressive changes during embryonic development. These changing patterns evoke progressive waves of cell differentiation in specific cell populations. Little is known regarding the non-apoptotic functions of caspase-2. Despite the difficulty in understanding the role of this protease during cell differentiation, the fact that caspase-2 is known to prevent DNA damage and to protect the cell cycle could be closely associated with our observations, which point to the need for further research, particularly in caspase-2 knockout mice.

Key words: Caspase-2 – Embryo – Development – Head – Cell differentiation

INTRODUCTION

Caspases (Cysteine-dependent aspartate-specific proteases) are proteases involved in several biological processes, including programmed cell death. At least 14 caspases have been identified; some of these caspases function in the initiation and execution of apoptosis (Chang and Yang, 2000). Caspase-2 is considered an initiator of apoptosis (Dostyn et al., 2012). Caspase- 2 plays a role in cell cycle regulation. Caspase-2-deficient mice exhibit a higher rate of cell proliferation. Caspase-2 is also involved in DNA damage repair, in apoptosis in p53-deficient cells, and in cell death induced by heat shock, oxidative stress and aging (Dorstyn and Kumar, 2009)

During vertebrate development, Caspase-3 and -9 are expressed by apoptotic cells under normal conditions and when cell death is provoked by a teratogen (Gashegu et al., 2005, 2006, 2007); thus, immunodetection of caspase expression constitutes an interesting method to detect cell death during embryogenesis, in parallel with TUNEL and HSP110 staining (Gashegu et al., 2006). Caspase expression participates in the "caspase cascade", which controls the process of cell death (Ren et

Corresponding author: Stéphane Louryan. Laboratoire d'Anatomie, Biomécanique et Organogenèse, Université Libre de Bruxelles, Faculté de Médecine, route de Lennik, 808, B 1070 Bruxelles (Belgium). E-mail: slouryan@ulb.ac.be

Submitted: 8 September, 2014. *Accepted:* 6 March, 2015.

al., 2005).

The initial purpose of this work was to build on previous observations by analysing caspase-2 expression during normal mouse cephalic development and during abnormal embryogenesis induced by a single dose of irradiation. This model, which generates severe craniofacial malformations, has previously been validated (Glineur et al., 1998) and is characterised by a spectacular increase in cell death (Gashegu et al., 2006). However, based on the initial results, the objectives were modified to explore normal caspase-2 expression stage by stage in various mouse embryonic tissues independent of the apoptotic process.

MATERIAL AND METHODS

Mice and teratogenic models

Mature and nulliparous NMRI female mice were mated overnight with a male, and the day on which vaginal plugs were found was considered day 0 of gestation (E0).

On day 9 of gestation, a group of pregnant NMRI mice was irradiated (2 Gy) using a linear accelerator (Clinac 2100C, Varian Medical Systems, Palo Alto, Ca) with an energy of 6 MV. Other pregnant NMRI mice were kept as controls and did not receive any irradiation. This irradiation protocol had been previously validated (Glineur *et al*. 1998).

Pregnant females were killed by CO_2 exposition on embryonic day 9 (E9), or on day 9 plus 3, 6, 12 or 24 h. Embryo staging was performed by determining the crown–rump length and by counting the number of somites (Theiler, 1987) to ensure that embryos were at the same stage of development.

Preparation of sections and caspase-2 immunohistochemistry

Embryos were fixed for 2-3 h in Serra's fixative medium. After dehydration in alcohol and paraffin embedding, 5 µm coronal sections were placed on slides and stored until further processing. For each case, serial alternating sections were prepared. The tissue sections, which were mounted on slides, were deparaffinised and rehydrated through a graded series of alcohol and water.

To permeabilise the cellular membranes, the slides were placed in citrate buffer and irradiated in a microwave at 650 W for 2 min. After progressive cooling in citrate and washing in phosphate-buffered saline (PBS) containing 0.1% Triton X-100® to further permeabilise the cytoplasmic membrane, the slides were incubated in normal blocking reagent (Millipore, Temecula, Ca, USA) for 120 min to block non-specific binding sites. Then, this reagent was removed, and the slides were incubated overnight in a humidified chamber with rabbit polyclonal anti-caspase-2 (Abcam, Cambridge, UK), which was diluted 1:250 in PBS. This antibody is specific for the active form of caspase-2. The slides were washed in PBS and Triton X-100®, and endogenous peroxidase was blocked by incubation in methanol containing 0.3% hydrogen peroxide.

Then, the slides were incubated with goat anti-rabbit IgG (Millipore, Temecula) for 30 min. After washing with PBS, the slides were incubated with streptavidin HRP (Millipore, Temecula) for 30 min. After PBS washing, the slides were incubated with a peroxidase substrate solution containing diaminobenzidine (Dako, Carpinteria, CA, USA) or Sigmafast DAB tablets with metal enhancer (Sigma, St Louis, MO, USA) for 4 min. In some cases, both DAB and DAB-metal were used on the same specimen, in order to reinforce the contrast between isolated positive cells (brown) and their environment (gray-blue), because of frequent background in embryo sections. Finally, the slides were rinsed in tap water, dehydrated through a graded alcohol series, mounted in DPX and examined under light microscopy. In order to correlate the caspase-2 immunoreactivity with the process of cell proliferation, a double labelling with PCNA and caspase-2 was performed. The slices were incubated overnight with anti-caspase antibody (1/50), goat anti- PCNA (Santa Cruz, Dallas, Texas, USA) (1/50), normal goat serum (1/100) and normal rabbit serum 1/100). After washing, the slices were incubated during 30 min with mouse-anti-rabbit (1/200) and donkey anti-goat (1/200) antibodies (Jackson, West Grove, PA, USA).

Negative controls were performed by missing of primary or secondary antibodies, and by exposition to rabbit and/or goat serum. Corresponding sections displayed no significant staining.

Serial sections of irradiated embryos were stained using TUNEL method (Evrard et al., 2000) in order to identify apoptotic cells. To correlate expression of caspase-2 with the apoptotic process, a double labelling in fluorescence was performed with TUNEL and caspase-2 identification, using the in situ cell death detection kit, POD (Roche, Mannheim, Germany), incubated at 37°C in the dark during 60 min. The same mouse-anti rabbit secondary antibody was used (30 min).

RESULTS

The essential data are summarised in Table 1.

At day 9, two populations exhibited strong immunoreactivity for caspase-2: the neural crest (Figs. 1A and 2B) cells and the paraxial mesoderm-derived cells (Fig. 1B). Close correlations with previous classical work (Le Lièvre, 1978, Louryan, 1990) enabled both populations on the histologic sections to be identified precisely. Caspase-2-positive cells were also present in the heart primordium (Fig. 3A and B).

At day 10, the premuscular blastemata originating from the paraxial mesoderm, as well as the cranial nerve ganglions, remained positive for caspase-2 expression (Fig. 1C and D).

Table 1. Chronology of appearance of Caspase 2 immunoreactivity.

Early stages (E9-11)	Middle stages (E12-14)	Late stages (E15-17)
Neural crest cells	Cartilage and perichondrium	Cartilage
Ganglia	Nerve fibres neuroepithelium	Premigratory neuroblasts
Paraxial mesoderm and premuscular blastemata	Myoblasts	
Heart	Heart	Heart: conduction tissue, valvular areas, and arterial outflow
Digestive endoderm	Digestive endoderm	Digestive and respiratory endoderm
Notochord		
Retinal pigment epithelium	Retinal pigment epithelium Lens	Lens
Meningeal primordia	Meninges	
	Odontoblasts and dental epithelium	
	Stellate reticulum	
	Nasal and otic epithelium (including the vomeronasal organ)	
	Rathke's pouch	

The notochord, the retinal pigment epithelium, the cranial meningeal membranes and the digestive endoderm strongly expressed caspase-2.

Several apoptotic cells exhibited immunoreactivity in both control specimens and irradiated embryos (Fig. 2to E). If physiologic apoptotic areas were found in classical regions of embryonic cell death (pharyngeal arches, mesenchyme ventral to the otic capsule, etc.), irradiation-induced apoptotic cells were observed diffusely in the embryo, with predominance in the neural epithelium (Fig. 2B to E). Close correlation between caspase-2 immunoreactivity with TUNEL staining is clearly demonstrated (Fig. 2 C to E). In control embryos, no immunoreactive apoptotic cells are observed in the neuroepithelium. Caspase-2 staining in non- apoptotic populations were identical as in control embryos.

During the "middle stages", immunoreactivity was observed in differentiating tissues, including the muscles, heart, eye, cartilages, neural tissue, meninges, teeth and several epithelial structures (Figs. 4 to 6). In later stages, caspase-2 remained visible on the cartilage, neuroblasts, valvular and outflow parts of the heart, conduction tissue, and lens (Figs. 6 and 7).

Comparison with PCNA showed that, if PCNA and caspase-2 were co-expressed in numerous cells in the same areas, there was no absolute correlation between both staining patterns; caspase 2 was clearly less expressed than PCNA, which appeared ubiquitously (Figs. 8 and 9).

DISCUSSION

Our results demonstrate that caspase-2 is expressed in numerous cell populations of different origins, in parallel with the cytodifferentiation process of these populations, but not restricted to proliferating cells. A close correlation exists between several transient patterns of caspase 2 expression and some other histochemical properties. For example, the pattern of caspase-2 expression in premuscular primordia is similar to the pattern of alkaline phosphatase activity (Louryan, 1990). Specific immunostaining of the roof of the nasal fossa evokes HNK1/NCAM fixation in the same area (Louryan et al., 1996)

In Fig. 8, we compare a schematic drawing of the location of mesencephalic neural crest cells in a stage 19 chick embryo (after Le Lièvre, 1978) with an immunoreactive cell population in similar neural crest cells in an E9 mouse embryo, which strongly suggests specific transient caspase-2 expression in neural crest cells. Similar comparisons could be performed with the paraxial mesoderm and with the premuscular blastemata.

The location of the caspase-2 protein is both nuclear and cytoplasmic, following the stage and the cell type. This finding is consistent with the fact that caspase-2 is known as a unique caspase with nuclear expression.

Caspase-2 is expressed on apoptotic cells; its pattern is consistent with TUNEL labelling. The localisation of positive cells are similar to anterior results relative to caspase-3 and 9 immunoreactivity (Gashegu et al., 2005, 2006) and the cell death areas are consistent with previous cytological studies (Vanmuylder et al., 2004).

Little is known regarding the non-apoptotic functions of caspase-2. Several studies have demonstrated that caspase-2 could play a role in osteoblastic (Mogi et al., 2005) and neuronal differentiation (Shimohama, 2001; Pistritto et al., 2012), as well in erythroblast and macrophage formation (Launay, 2005). Other caspases are involved in normal lens and monocyte differentiation (Abrahams and Shaham, 2004) and in muscle development (Schwerk et al., 2003)

Caspase-2 is involved in DNA damage repair (Dostyn and Kumar, 2009) and in tumour suppression (Ho et al., 2008). Caspase- 2-/- cells display resistance to drug-induced apoptosis, suggesting

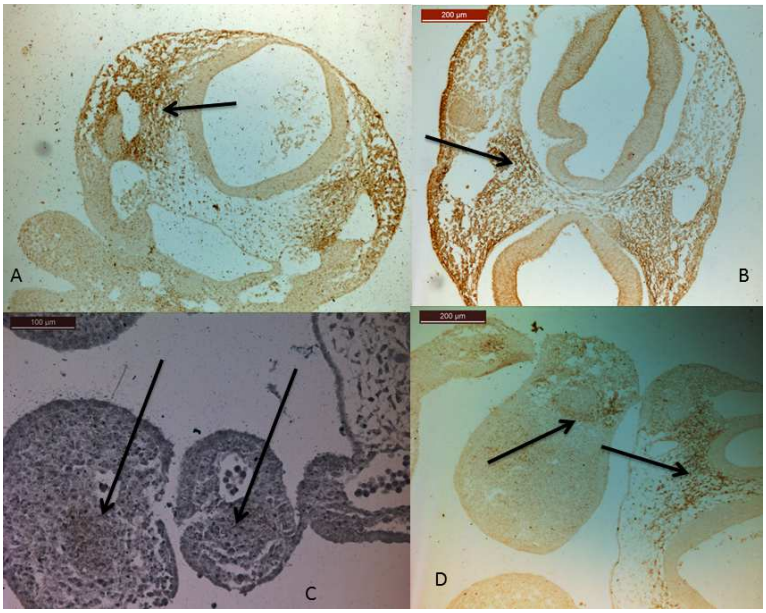

Fig. 1. (A) E9 embryo. Caspase-2 is strongly expressed in mesectodermal cells arising from the neural crest (arrow). NT: neural tube; PA1: first pharyngeal arch. **(B)** E9 embryo. Mesectodermal population between the midbrain and hindbrain exhibits intense caspase-2 immunoreactivity. Di: diencephalon; Rh: rhombencephalon. **(C)** Irradiated E9 embryo. Caspase-2 is present in the premuscular branchial primordium (arrows). PA: pharyngeal arches. **(D)** E10 embryo. Caspase-2 is present in the premuscular mandibular primordium (left arrow) and in the mesenchyme surrounding otic vesicle (right arrow). PA1, first pharyngeal arch; NT, neural tube.

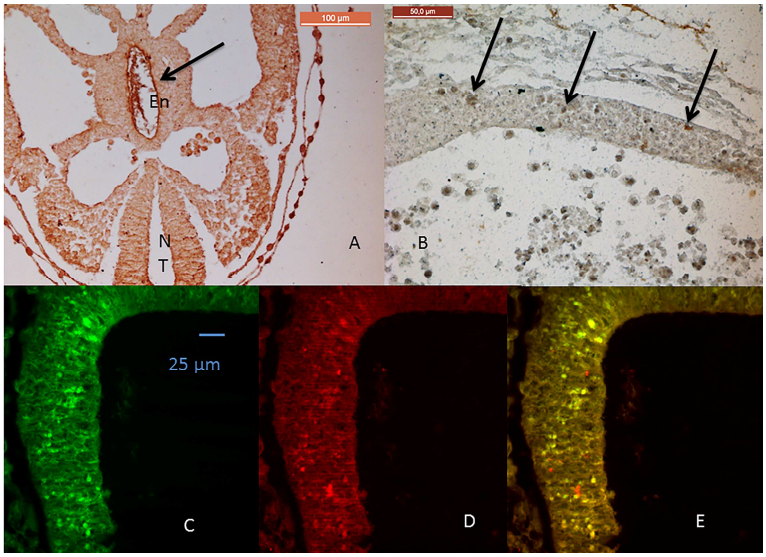

Fig. 2. (A) E9 embryo. The digestive endoblast exhibits intense immunostaining (arrow). NT: neural tube. **(B)** E9 irradiated embryo. TUNEL in visible light. Apoptotic cells (arrows) in the neural epithelium are positive for caspase-2 (arrows). NT neural tube. **(C,D,E)** Double labelling in fluorescence (C: TUNEL, green, D: caspase-2, red, E: fusion of both stainings) in the neuroepithelium of a E9 irradiated mouse embryo. Tunnel-positive apoptotic cells express simultaneously the caspase-2 antibody.

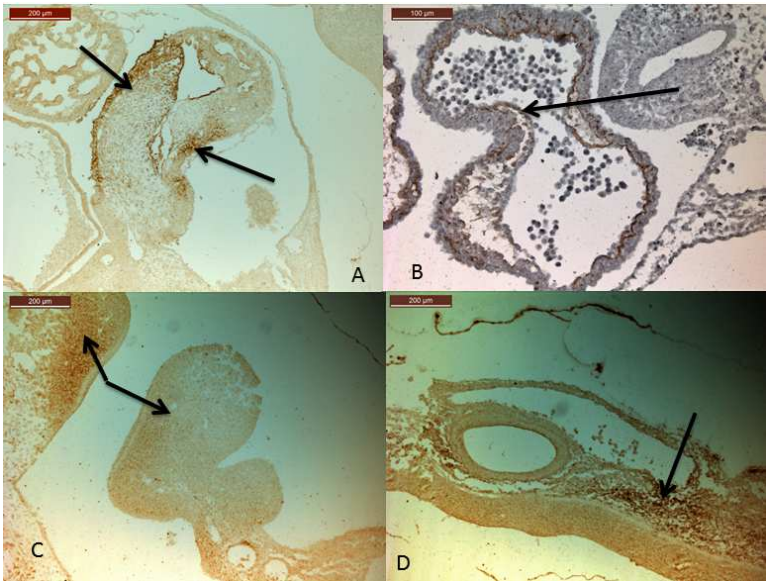

Fig. 3. (A) E9 embryo. Intense immunoreactivity is present in the external layer of the heart primordium (arrows). H: heart. **(B)** E9 irradiated embryo. Immunoreactivity is well visible in the endocardiac layer (arrow) of the cardiac tube. H: heart. **(C)** E9 embryo. Diffuse immunoreactivity in the juxta-oral area of the maxillary (left arrow) and mandibulary (right arrow) buds. PA1: first pharyngeal arch. **(D)** E10 embryo. The mesenchyme surrounding the otic vesicle is diffusely stained (arrow). ACV, anterior cardinal vein; OV, otic vesicle; Rh, rhombencephalon.

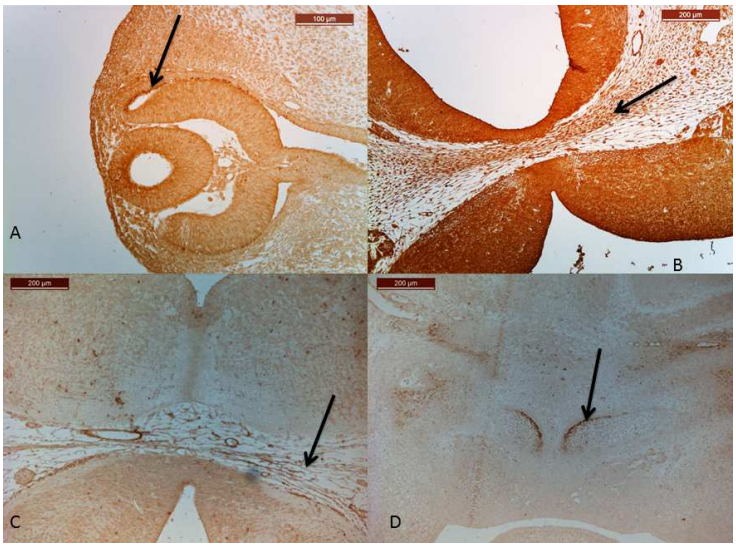

Fig. 4. (A) E11 embryo. Immunopositive cells are visible in the external epithelial layer of the optic vesicle. **(B)** E11 embryo. Caspase-2 in expressed in the Meckel's cartilage (arrow) and surrounding mesectoderm . PA: pharyngeal arches. **(C)** E12 embryo. Persistence of Caspase-2 expression (arrow) in the meningeal fibres. **(D)** E12 embryo. The perichondrium tissues of both Meckel's cartilages are positive (arrow). Several chondroblasts also express caspase 2.

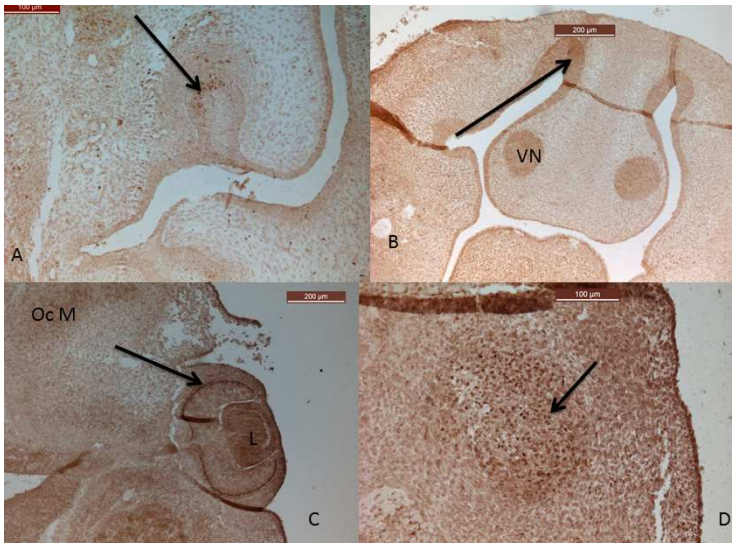

Fig. 5. (A) E12 embryo. Immunoreactivity is visible in the enamel epithelium (arrow) and in several dental mesenchyme cells. **(B)** E12 embryo. The epithelium of the roof of the nasal fossa and the vomero-nasal organ exhibit caspase-2 immunoreactivity. T: tongue. **(C)** E13 embryo. Persistence of immunoreactivity in the outer epithelium of the optic vesicle. **(D)** E13 embryo. The mandibulary premuscular primordium express caspase-2 and is morphologically well delineable.

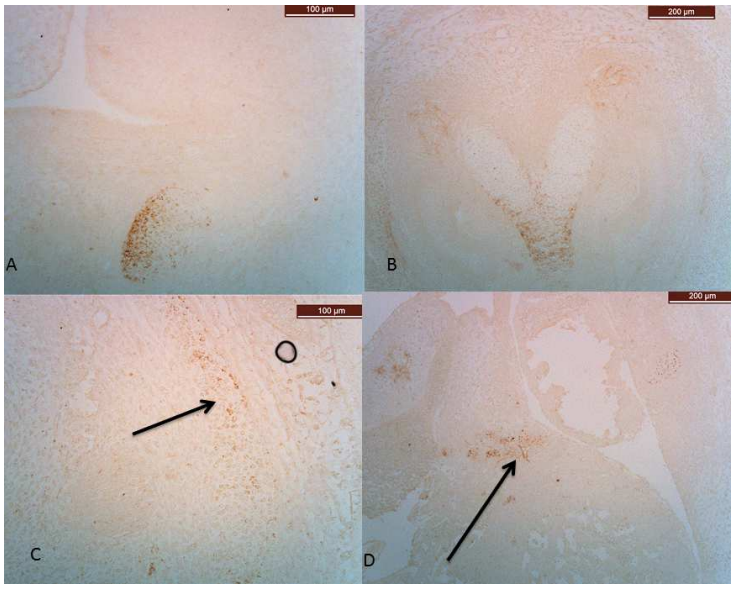

Fig. 6. (A) E14 embryo. Caspase-2 immunoreactivity in chondroblasts of Meckel's cartilage. MC: Meckel's cartilage. **(B)** E14 embryo. Caspase-2 immunoreactivity in the area of fusion of both Meckel's cartilages at the midline. MC's: Meckel's cartilages. **(C)** E15 embryo. Positive myoblastic cells in the temporal area. **(D)** E15 embryo. Positive cells in the outflow tract of the heart. H, heart.

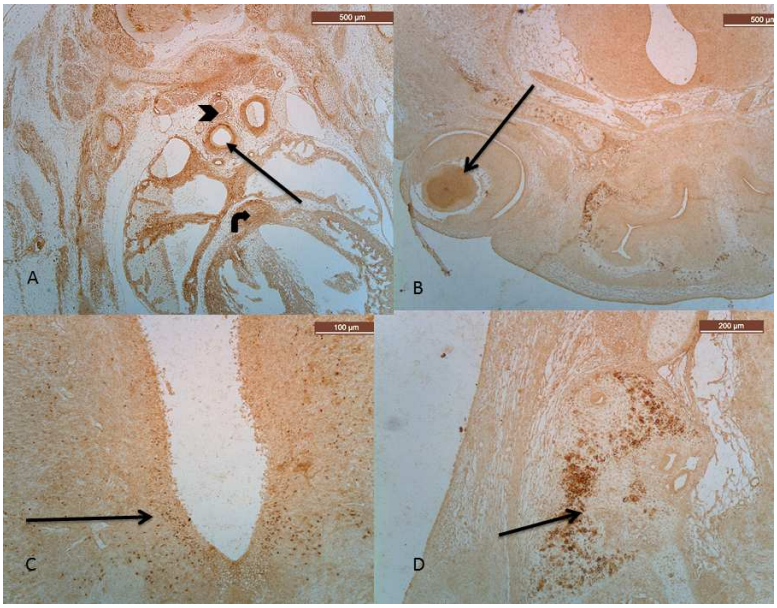

Fig. 7. (A) E15 embryo. Positive cells in the external layers of the trachea (arrow) and the oesophagus (arrowhead). H: heart. **(B)** E15 embryo. Caspase-2-positive cells are visible in the lens (arrow). Numerous positive cells are also present in cartilaginous structures. **(C)** E16 embryo. Numerous neuroblasts of the ventricular layer are positive (arrow). NT: neural tube. **(D)** E16 embryo. Some chondroblasts are stained in the otic cartilaginous capsule; however, numerous deep cells remain immunonegative. OC, otic capsule.

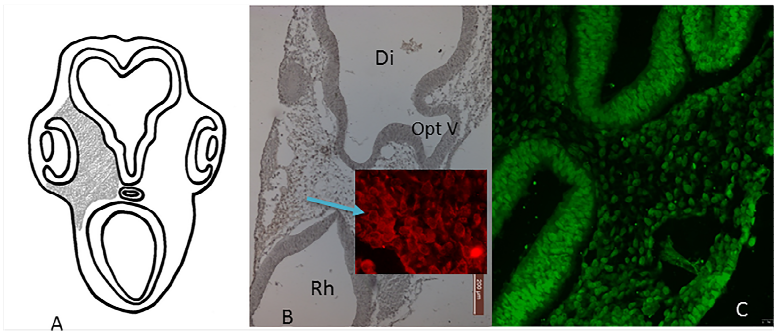

Fig. 8. Comparison of a schematic drawing of the mesencephalic neural crest area in the chick (after Le Lièvre, 1978) **(A)** with caspase-2 **(B)** and PCNA **(C)** stainings. In B, the corresponding cell population exhibits diffuse caspase-2 immunoreactivity, on DAB section as well in fluorescence (high magnification). **(C)** PCNA staining demonstrates a high rate of proliferative cells. Di, diencephalon; OpV, optic vesicle; Rh, rhombencephalon.

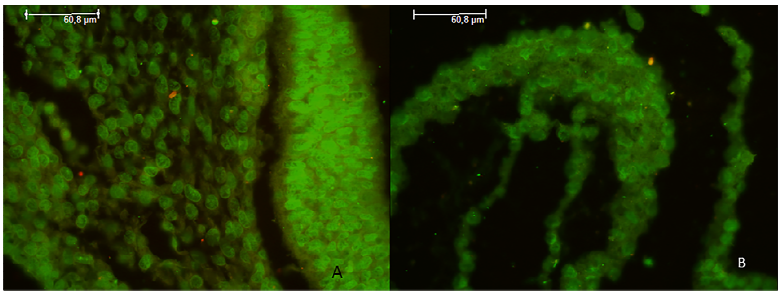

Fig. 9. Double labelling PCNA (green) and caspase-2 (red) in mandibular arch **(A)** and heart **(B)** of a E9 embryo, with images fusion, showing that, if both stainings occur in the same areas, caspase-2 is also expressed by non-proliferative cells.

that caspase-2 exerts a tumour suppressive function (Ho et al., 2008). More recently, Dorstyn et al. (2012) demonstrated that a loss of caspase-2 leads to a defective apoptotic response to DNA damage. The use of inhibitors seems to demonstrate that the loss of caspase-2 and -9 does not disturb the preimplantation stages of mouse development (Busso et al., 2010).

The ability of caspase-2 to stabilise DNA to guarantee cell cycle maintenance (Dorstyn et al., 2012) could be correlated with the putative role of caspase-2 during cellular differentiation in several embryonic populations.

The nuclear localisation of caspase-2 is closely associated with its functions in cell cycle regulation; thus caspase-2 expression during this process could be considered a “protection mechanism” to avoid DNA damage during differentiation. Another hypothesis could be related to the role of caspase-2 in cell proliferation, and could suggest that caspase-2 expression in differentiating cells is associated with a reduced proliferation rate during differentiation.

The fact that the role of caspase-2 during apoptosis is considered redundant (Dorstyn and Kumar, 2009) could explain its poor expression in apoptotic embryonic cells compared with that of caspase-3 (Gashegu et al., 2006). However, the fact that caspase- 2-/- mouse embryos are viable (but display premature ageing features (Zhang et al., 2007)) suggests that its presence in differentiating cells may not be mandatory.

Further immunohistochemical investigations with

caspase- 2-/- mouse embryos would constitute an interesting way to elucidate this question.

ACKNOWLEDGEMENTS

The authors thank Mrs Tara Chapman for linguistic assistance.

REFERENCES

ABRAHAMS MC, SHAHAM S (2004) Death without caspases, caspases without death. *Trends Cell Biol*, 14: 184-193.

BUSSO D, DOMINGUEZ C, PEREZ-ACLE T, MORENO RD (2010) Life-giving caspases: revealing new roles during mouse embryo preimplantation development. *Int J Dev Biol*, 54: 857-865.

CHANG HY, YANG X (2000) Proteases for suicide: functions and regulation of caspases. *Microbiol Molec Biol Rev*, 64: 821-846.

DORSTYN L, PUCCINI J, WILSON CH, SHALINI S, NICOLA M, MOORE S, KUMAR S (2012) Caspase-2 deficiency promotes aberrant DNA-damage response and genetic instability. *Cell Death Differentiation*, 19: 1288-1298.

DORSTYN L, KUMAR S (2009) Putative functions of caspase-2. *Biol Rep,* 1: 96. doi: 10.3410/B1-96.

EVRARD L, VANMUYLDER N, DOUROV N, HERMANS C, BIERMANS J, WERRY-HUET A, ROOZE M, LOURYAN S (2000) Correlation of HSP110 expression with all-trans retinoic acid-induced apoptosis. *J Craniofac Genet Dev Biol*, 20: 183-192.

GASHEGU J, VANMUYLDER N, KASSENGERA Z, EVRARD L, GLINEUR R, PHILIPPSON C, ROOZE M, LOURYAN S (2005) L'expression de la caspase 3 et de la protéine p53 au cours de l'apoptose physiologique et induite par 3 agents tératogènes au cours du développement crâniofacial précoce de l'embryon de souris. *Morphologie,* 89: 82-89.

GASHEGU J, VANMUYLDER N, PHILIPPSON C, CHOA-DUTERRE M, ROOZE M, LOURYAN S (2006) Correlation of Hsp110 expression with caspase-3 and-9 during apoptosis induced by in vivo embryonic exposition to retinoic acid or irradiation in early craniofacial development. *Orthod Craniofacial Res*, 9: 84-92.

GASHEGU J, LADHA R, VANMUYLDER N, PHILIPPSON C, BREMER F, ROOZE M, LOURYAN S (2007) HSP110, caspase-3 and -9 expression in physiological apoptosis and apoptosis induced by in vivo embryonic exposition to all-trans retinoic acid or irradiation during early eye development. *J Anat,* 210: 532-541.

GLINEUR R, LOURYAN S, PHILIPPSON C, EVRARD L, DE VOS L (1998) Effects of irradiation on facial development in mouse embryos. *Eur J Morphol*, 36: 245-252.

HO LH, TAYLOR R, DORSTYN L (2009) A tumor suppressor function for caspase-2. *PNAS*, 106: 5336-5341.

LAUNAY S, HERMINE O, FONTENAY M, KROEMER G, SOLARY E, GARRIDO C (2005) Vital functions for lethal caspases. *Oncogene*, 24: 5137-5148.

LE LIÈVRE CS (1978) Participation of neural crest-derived cells in the genesis of the skull in birds. *J Embryol exp Morphol*, 47: 17-37.

LOURYAN SM (1990) Morphogenèse des ébauches musculaires branchiales chez l'embryon de souris: correlation avec les données recueillies chez le poulet, chez *Mabuia Megalura* (Lacertilia: Scincidae) et chez *Scyllium Canicula* (Chondrichtyes: Selachii). *Eur Arch Biol*, 101: 65-75.

LOURYAN S, WERRY-HUET A, VAN SINT JAN S (1996) Comparison between two HNK-1-related antibodies immunoreactivity (HNK-1-anti-leu 7 and anti-HNK-1/N-CAM) during rat cephalogenesis. *Eur J Morphol*, 35: 295-300.

MOGI M, TOGARI A (2003) Activation of caspases is required for osteoblastic differentiation*. J Biol Chem*, 278: 47477-47482.

PISTRITTO G, PAPALEO V, SANCHEZ P, CECI C, BARBACCIA ML (2012) Divergent modulation of neuronal differentiation by Caspase-2 and-9*. PloS ONE* 7, e36002. doi: 10.1371/journal.pone.0036002. Epub 2012 May 18.

REN J, SHI M, LIU R, YANG QH, JOHNSON T, SKARNES WC, DU C (2005) The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. *PNAS*, 102: 565-570.

SCHWERK C, SCHULTZE-OSTHOFF K (2003) Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem Pharmacol*, 66: 1453-1458.

SHIMOHAMA S, TANINO H, FUJIMOTO S (2001) Differential expression of rat brain caspase family proteins during development and ageing. *Biochem Biophys res commun*, 289: 1063-1066.

THEILER K (1989) The house mouse. Atlas of embryonic development. Springer, Berlin.

VANMUYLDER N, EVRARD L, GLINEUR R, SALMON I, GASHEGU J, ROOZE M, LOURYAN S (2004) Aspects histologiques et ultrastructuraux de la mort cellulaire programmée et induite par deux agents tératogènes dans les arc viscéraux de l'embryon de souris. *Rev Méd Brux*, 25: 14-20.

ZANG Y, PADALECKI SS, CHAUDHURI AR (2007) Caspase-2 deficiency enhances aging-related traits in mice. *Mech Ageing Dev*, 128: 213-221.