J Gashegu
N Vanmuylder
C Philippson
M Choa-Duterre
M Rooze
S Louryan

Correlation of Hsp110 expression with caspase-3 and -9 during apoptosis induced by *in vivo* embryonic exposition to retinoic acid or irradiation in early mouse craniofacial development

Authors' affiliations:
J. Gashegu, N. Vanmuylder, M. Choa-Duterre, M. Rooze, S. Louryan, Laboratory of Human Anatomy and Embryology, Université Libre de Bruxelles, Faculté de Médecine, Brussels, Belgium
C. Philippson, Service of Radiation Therapy, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Brussels, Belgium

Correspondence to:
S. Louryan, MD PhD
Laboratory of Human Anatomy and Embryology
Faculté de Médecine
Université Libre de Bruxelles
C.P. 619, Route de Lennik 808
B-1070 Brussels
Belgium
Tel.: 32 2 555 6376
Fax: 32 2 555 6378
E-mail: slouryan@ulb.ac.be

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Structured Abstract

Authors – Gashegu J, Vanmuylder N, Philippson C, Choa-Duterre M, Rooze M, Louryan S

Objective – To analyze the expression and role of three proteins (HSP110, caspase-3 and caspase-9) during craniofacial development.

Design – Seven pregnant C57Bl/6J mice received, by force-feeding at gestation day 9 (E9), 80 mg/kg of all-trans retinoic acid mixed to sesame oil. Seven pregnant NMRI mice received two grays irradiation at the same gestation day. Control mice of both strains (seven mice for each strain) were not submitted to any treatment. Embryos were obtained at various stages after exposition (3, 6, 12 and 24 h), fixed, dehydrated and embedded. Coronal sections (5 μm) were made. Slide staining occurred alternatively using anti-Hsp110, anti-caspase-3 and anti-caspase-9 immunohistochemistry.

Results – Expression of HSP110, caspase-3 and caspase-9 was found in cells of well-known locations of programmed cell death. After retinoic acid exposure, expressions were increased especially in neural crest cells of mandibular and hyoid arches. Quantification of positive cells shows that caspase-9 and Hsp110 were expressed before caspase-3. After irradiation, the expression of the three proteins quickly increased with a maximum 3 h after irradiation. For all three models of apoptosis (physiological, retinoic-induced and irradiation-induced) HSP110 positive cells were more numerous than caspase-3 positive cells. Caspase-3 positive cells were more numerous than caspase-9 positive cells especially in mesectodermal irradiation-induced apoptotic cells.

Conclusion – The findings show a potential function of HSP110 in apoptosis during embryo development. Caspase-3-expressing cells are more numerous than cells expressing caspase-9, especially irradiation-induced apoptotic neural crest cells. This suggests that other caspases, still to be identified, may activate caspase-3 in this model.

Key words: apoptosis; caspases; embryology; heat shock proteins; teratology

Introduction

Apoptosis has been recognized as an essential event in normal and teratogenic embryonic development, tissue homeostasis and genesis of different pathology. Programmed cell death is controlled by the cell, and has been preserved through evolution (1). Commitment to apoptosis is the result of interconnected cascades of biochemical events. The core component of this 'suicide machinery' is a proteolytic system involving a family of proteases known as caspases (2).

At present, at least 14 mammalian caspases have been identified. They are classified in three functional groups. The first group contains caspases involved in the inflammatory process including caspase-1, caspase-4, caspase-5 and caspase-11. These enzymes are also known as 'ICE-like' caspases. The second group or initiator caspases contains caspase-2, caspase-8, caspase-9 and caspase-10 and are considered as 'signaling' caspases. The third group or 'effector' caspases includes caspase-3, caspase-6 and caspase-7. The most recently identified caspase-12, caspase-13 and caspase-14 are not sufficiently described to be placed in one of these groups (2,3). In healthy cells, caspases are present as proenzymes containing prodomains that must be cleaved off to activate the caspase.

Two pathways of caspase activation have been described: an extrinsic pathway mediated by death receptor (the Fas death pathway), and an intrinsic pathway (mitochondrial pathway). The Fas receptor pathway is triggered by binding to Fas ligand that induces receptor clustering. Activated Fas receptor then recruits a cytosolic adapter protein Fas associated death domain protein (FADD) at death domain (DD) site. FADD, in turn, recruits and binds to procaspase-8 via common death effector domains (DED) to form a complex known as death-inducing signaling complex (DISC). Within the DISC, procaspase-8 is autocatalytically processed to yield active caspase-8. Once activated, caspase-8 in turn processes effector caspase-3 (2–4). During the intrinsic pathway, diverse proapoptotic signals converge at mitochondrial level, provoking a mitochondrial permeability transition (MPT), which has several consequences including rupture of the outer mitochondrial membrane. This rupture leads to the release in cytoplasm of proteins, which normally reside in the intermembrane space of the mitochondria including cytochrome C. The cytosolic cytochrome C binds to Apaf-1 'apoptosis activator factor 1', a cytosolic protein containing a caspase recruitment domain (CARD). Binding of Apaf-1 to cytochrome C exposes the CARD domain of Apaf-1, which subsequently recruits procaspase-9 and facilitates its autoactivation. Once activated, caspase-9 activates caspase-3 (2,3,5,6).

Caspase-9 and caspase-3 have been shown to play an important role in the central nervous system development. The major reported phenotypes in either caspase-3 knockout or caspase-9 knockout mice manifest within the central nervous system and are related to reduction of developmental apoptosis (7,8). A recent work suggests that caspase-3 may play a crucial role in bone development and metabolism (9). The role of the caspase-9–caspase-3 cascade has not yet been demonstrated in craniofacial development.

On the other hand, heat shock proteins (HSPs) are an essential component of the cell defense mechanism against a range of harmful conditions (temperature, alcohols, heavy metal exposition, oxidative stress…) Under normal conditions, some HSPs function as molecular chaperone: they are involved in protein folding, assembly and transport. In cellular stress conditions, HSP bind to damaged proteins. Thus, they prevent irreversible aggregation or misfolding of damaged proteins (10–13). Some stress proteins have been shown in cells undergoing apoptosis. Particularly, HSP110 have been shown to be expressed in chondrocytes of the growth

plate undergoing apoptosis (14), in apoptotic neural crest cells (15,16) and mouse embryonal F9 cells (17). It has been demonstrated that HSP110 was expressed in TUNEL positive apoptotic cells but the mechanism has yet to be understood (16). HSP110 belongs to HSP100 proteins family that has various functions as ATPase activity and oligomerization. Additionally, HSP100 proteins play a crucial role in tolerance to severe stresses and multiple mild stresses. HSP100 proteins have an indirect proteolysis function thought their capacity to affect the structure of their substrates, thereby providing better substrates to associated protease (14).

The objective of this study was to demonstrate the expression of three proteins (HSP110, caspase-3 and caspase-9) within physiological cell death during early craniofacial development. Both retinoic acid and irradiation were used to develop teratogenic models. Retinoic acid and irradiation have been shown to enhance apoptosis during early mammalian craniofacial development, and give rise to craniofacial defects (18–25). The relationship between HSP110 and caspase-3 and -9 is also described.

Materials and methods

Preparation of tissue sections

Mature and nullipare female mice were used. Mating occurred overnight. The next day was considered day 0 of gestation (E0) when vaginal plug was found. On day 9 of gestation, a group of seven pregnant NMRI mice was irradiated (two grays) using linear accelerator (Clinac 2100C; Varian Medical Systems, Palo Alto, CA, USA) with an energy of 6 MV. Seven other pregnant NMRI mice were kept as controls and did not receive any irradiation. The irradiation protocol was previously validated (18).

On the same day, another group of seven pregnant C57Bl/6J mice received 80 mg/kg of all-trans-retinoic acid mixed to sesame oil by forcible feeding. A group of seven C57Bl/6J pregnant mice was kept as control and did not receive any treatment. Previous experiments have permitted to validate this protocol (16,22). C57Bl/6J mouse strain was chosen because it showed sensitivity to retinoic acid teragenocity (20,22,25). NMRI mice showed less sensibility to retinoic acid teratogen. However, cell death pattern appears to be very similar in both strains (19,20).

Pregnant female mice were killed by cervical dislocation under ether anesthesia on E9 and E9 plus 3, 6, 12 or 24 h. Uteri were collected in physiological Locke buffer before the embryos were harvested. Embryos were fixed for 2–3 h in Serra's fixative medium. After dehydratation in graded alcohols, embryos were embedded in paraffin according to standard procedures. Coronal sections (5 μm) were obtained. Alternative staining of the slices was performed to obtain HSP100, caspase-3 and caspase-9 immunohistochemical expression at a same region of each embryo.

Caspase-3 immunohistochemistry

Tissue sections mounted on slides were deparaffinized and rehydrated through graded alcohol and water. To permeabilize the cellular membrane, slides were placed in citrate buffer and irradiated in microwave at 650 w for 2 min. After progressive cooling in citrate and washing in phosphate-buffered saline (PBS), endogenous peroxidase was blocked by incubation in methanol containing 0.3% of hydrogen peroxide. After washing in PBS containing 0.1% triton X-100® to further permeabilize the cytoplasmic membrane, the slides were incubated in normal goat serum (NGS) (IHC Select Chemicon, Temecula, CA, USA) for 120 min to block non-specific binding sites. NGS was removed and slides were incubated overnight in a humidified chamber with polyclonal rabbit anti-caspase-3 (BD Biosciences Pharmagen, Southampton, NY, USA), diluted 1/500 in PBS. This antibody is recognized as specific to active caspase-3 form. The slides were washed in PBS and incubated with goat anti-rabbit IgG (IHC Select Chemicon) for 30 min. After washing with PBS, slides were incubated avidin–biotin–peroxidase complex (ABC) (IHC Select Chemicon) for 30 min. After PBS washing, the slides were incubated with peroxidase substrate solution of diaminobenzidine (DAB) (Vector Industries, Edmonton, Canada) for 4 min. Finally, the slides were rinsed in tapwater, deshydrated in graded alcohol, mounted in DPX mounting medium (BDH Lab Supplies, Poole, UK) and examined under optic microscopy.

Caspase-9 and HSP110 immunohistochemistry

We used the same method to identify HSP110 or caspase-9 positive cells. For caspase-9, we used the

polyclonal rabbit anti-caspase, specific to caspase-9 active form (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), diluted 1/50 in PBS. For HSP110, we used the polyclonal (rabbit) anti-HSP104 (Affinity Bioreagens Inc., Golden, CA, USA), diluted 1/250 in PBS. This antibody is directed against yeast *saccharomyces cerevisiae* HSP104. It is specific to mammalian (human and mouse) HSP100 and HSP105. These two mammalian proteins belong to HSP110 family (26).

Quantification HSP110-positive, caspase 3-positive and caspase 9-positive cells

Quantification of HSP110-positive, caspase-3-positive and caspase-9-positive cells was performed at right side in the first visceral arch of (E9 + 3, E9 + 6, E9 + 12, and E9 + 24) mouse embryos, both in control and after irradiation and RA administration according to previous study (16).

Additional embryos of control NMRI or C57Bl/6J harvested at gestational E9 served for comparison. For each stage, three embryos were randomly chosen, a total of 50 embryos were used. The same level sections were used for the three proteins expressing cells to insure that the quantification is performed at similar region of embryos. Data were collected from three consecutive sections from each embryo. The data acquisition system was as follows: each slide was digitalized by a CCD camera (Higekami, Japan) and aframe-grabber (Data Translation 2871, Bissingen, The Netherlands).

The digitalized image was sent to a TV monitor, for visual control. One square of labeled cells per section was chosen and remains the same for all the process. We determined for each protein the cell labeling to be considered as positive. Positives spots only were then automatically counted. For each case, mean and standard error were calculated.

Results

During normal embryonic development, caspase-3, caspase-9 (caspase-3 and caspase-9 are understood in this paper as active form) and HSP110 were expressed in cells in the neural tube, optic vesicle, neural crest of the first and second visceral arches, otocyst as well as cells of various ganglia of visceral arches (V, VII, and VIII). These zones are already known to be the site of programmed cell death in early craniofacial development. The expression of these proteins was similar in both strains (NMRI or C57Bl/6J) (Figs 1A, C, 2A, C and 3A, C). The number of cells expressing these proteins was appreciably increased during retinoic-induced apoptosis or irradiation-induced apoptosis (Figs 4 and 5).

After retinoic acid exposure, the expression of these proteins was increased by cells of ectomesenchyme, ganglia of visceral arches, optic vesicle and otocyst. We did not observe any increase of caspase-3 and -9 by neural cells. Although strains sensibility differences cannot be formally exclude, neural crest cells of mandibular and hyoid arches mesectoderm showed a susceptibility to teratogenic retinoic acid exposure (Figs 1B, 2B and 3B). During retinoic-induced apoptosis, the number of cells that expressed these proteins gradually increased with the maximal expression 12 h after exposure for HSP110 and caspase-9. Caspase-3 expression continued to rise even 24 h after exposition (Fig. 6). Therefore, HSP110 and caspase-9 appeared precociously compared with caspase-3 expression.

In irradiation-induced apoptosis, HSP110, caspase-3 and caspase-9 expressions were very increased, showing the severity of irradiation teratogenic exposure (Figs 1D, 2D and 3D).

The maximal expression appeared 3 h after irradiation (Fig. 7). We found differences in the regression of numbers of cells expressing these three proteins. Whereas caspase-3 and caspase-9 positive cells regressed similarly, the expression of HSP110 regressed more quickly (Fig. 7). We observed also a 'rebound expression' of these three proteins after 24 h of irradiation (Fig. 7). This is a delayed effect of DNA damaging action of irradiation. It is known that irradiation induces apoptosis in two temporally distinctive waves: a group of post-mitotic and non-S-phase proliferating cells die rapidly, followed by a later death of proliferating cells including those that were irradiated during S-phase (27). The number of positive cells was increased and disseminated in different regions but neural tube cells showed particular responsiveness to this teratogenic aggression (Fig. 3B, D). Cells expressing these three proteins were increased in the neural tube whatever the level of the neural anlage. Caspase-3 and HSP110 positive cells were also increased in ectomesenchyme. However, caspase-9 positive cells were not particularly increased in ectomesenchyme of the mandibular and hyoid arches (Fig. 7).

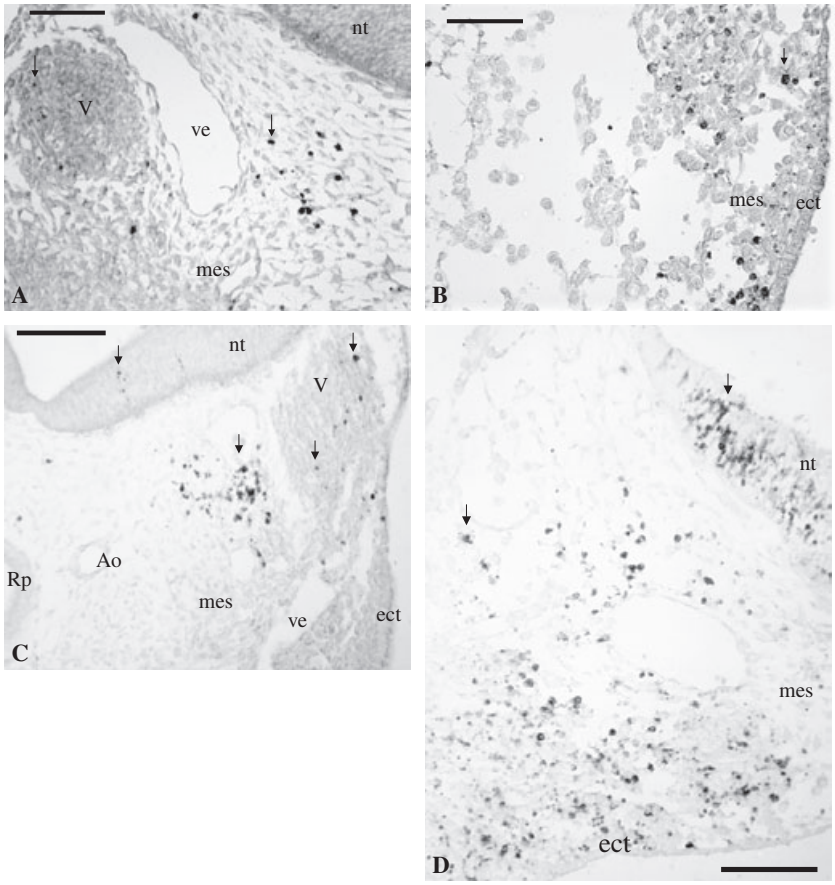

Fig. 1. Sections through the first visceral arch of control, retinoic treated or irradiated mouse embryo. Caspase-3-positive cells are showed. (A) E9 + 12 C57Bl/6J control embryo. Caspase-3-positive cells (arrow) are seen in trigeminal ganglia and mesectoderm. (B) E9 + 12, C57Bl/6J retinoic acid treated embryo. We observe an increase of intracellular space me mesectoderm and increasing expression of caspase-3 (arrow). (C) E9 + 3 NMRI control embryo. Caspase-3 positive cells (arrow) are seen in neural tube (mesencephalon), trigeminal ganglia, mesectoderm and ectoderm. (D) E9 + 3 NMRI irradiated embryo. Numerous cells in neural tube are caspase-3 positive (arrow). There is also an increase of mesectodermal caspase-3-positive cells (arrow). Same ectodermal cells are caspase-3 positive.

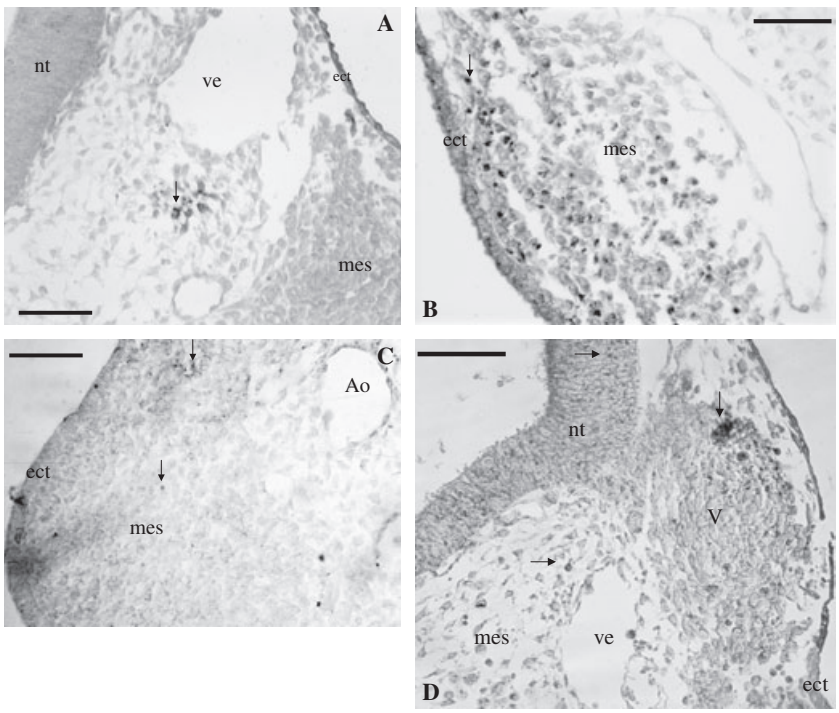

Fig. 2. Sections trough the first visceral arch of control, retinoic treated or irradiated mouse embryo. Caspase-9-positive cells are showed. (A) E9 + 12 C57Bl/6J control embryo. Cells expressing caspase-9 (arrow) are seen in mesectoderm. (B) E9 + 12, C57Bl/6J embryo exposed to the acid retinoic. Cells expressing caspase-9 (arrow) are increased in mesectoderm. (C) E9 + 3, NMRI control embryo. Cells expressing caspase-9 (arrow) are seen in mesectoderm. (D) E9 + 3, NMRI irradiated embryo. Cells expressing caspase-9 (arrow) are particularly increased in neural tube. Increase of caspase-9 positive cells is also seen in the trigeminal ganglia and lesser in mesectoderm.

In addition, we noted that in these three models of apoptosis (physiological, retinoic-induced and irradiation-induced), HSP110-positive cells were more numerous than caspase-3-positive cells, whereas caspase 3-positive cells were more numerous than caspase 9-positive cells (Figs 4 and 5). Thus, HSP110

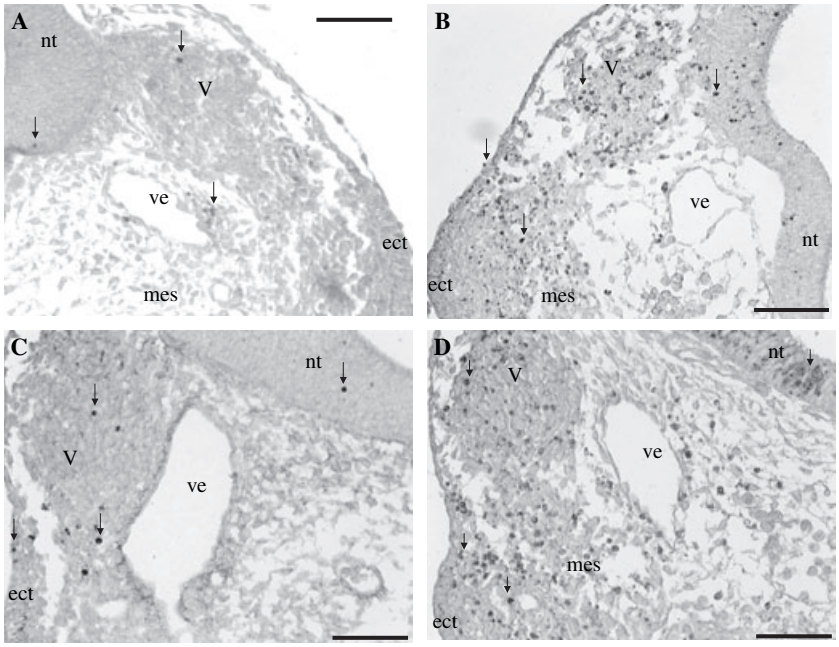

Fig. 3. Sections trough the first visceral arch of control, retinoic treated or irradiated mice embryo. HSP110-positive cells are showed. (A) E9 + 12, C57Bl/6J control embryo. Cells expressing HSP110 (arrow) are seen in neural tube, trigeminal ganglia and mesectoderm. (B) E9 + 12, C57Bl/6J embryo exposed to the acid retinoic. Cells expressing HSP110 (arrow) are increased in neural tube, trigeminal ganglia and mesectoderm. (C) E9 + 3, NMRI control embryo. Cells expressing HSP110 (arrow) are seen in neural tube, trigeminal ganglia, mesectoderm and ectoderm. (D) E9 + 3, NMRI irradiated embryo. Cells expressing HSP110 (arrow) are increased in neural tube, trigeminal ganglia, mesectoderm but not in ectoderm. Ao, aorta; ect, ectoderm; mes, mesectoderm; Rp, Rathke's pouch; nt, neural tube; V, trigeminal ganglia; ve, anterior cardinal vein; Bar: 100 μm.

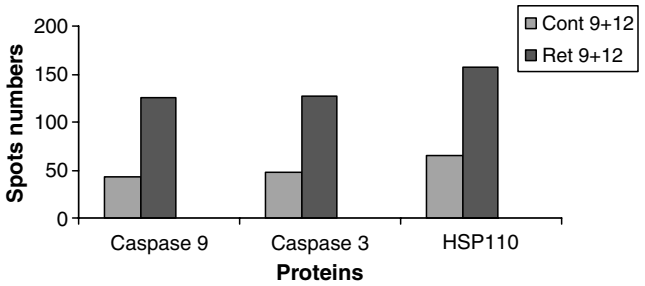

Fig. 4. Graph showing the evolution of HSP110, caspase-3 and caspase-9 expression in control B57Bl/6J embryo and after embryonic exposure to retinoic acid.

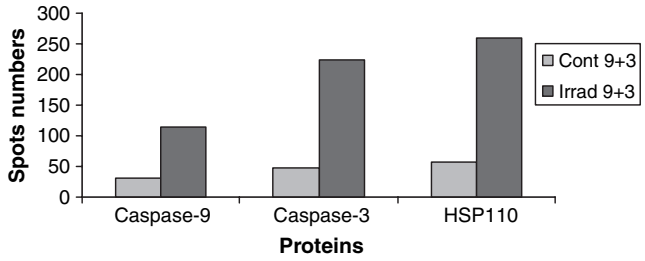

Fig. 5. Graph showing the evolution of HSP110, caspase-3, caspase-9 expression in control NMRI embryo and after irradiation of the embryo.

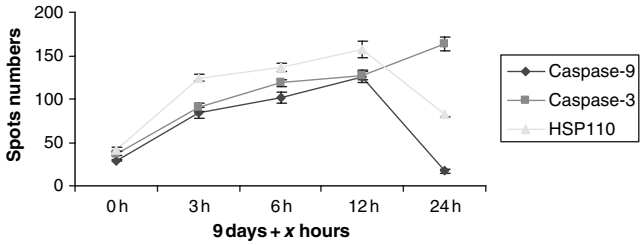

Fig. 6. Graph showing the evolution of HSP110, caspase-3 and caspase-9 expression after embryonic exposure to retinoic acid.

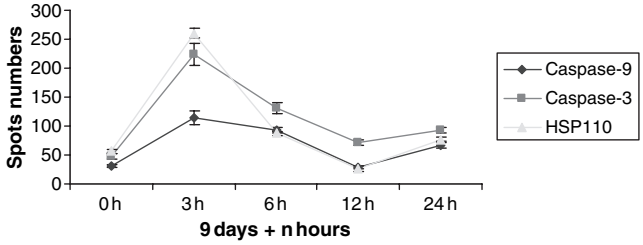

Fig. 7. Graph showing the evolution of HSP110, caspase-3, caspase-9 expression after irradiation of the embryo.

may be expressed in both caspase-3 dependent apoptosis and caspase-3-independent apoptosis. It is also possible that HSP110 is expressing by same 'stressed-cells' which are not undergoing apoptosis. In addition, especially in irradiation-induced apoptosis, caspases other than caspase-9 may activate caspase-3.

Discussion

Evidence of waves of programmed cell death in early craniofacial development has been established for decades. Programmed cell death (PCD) plays an essential role during mammalian development (28–33). Studies from various authors showed evidence of waves of PCD in mesectoderm populations during cephalogenesis. These waves of PCD could contribute to

regulate the number of cells, and foreshadow the migratory route of growing neuritis to the corresponding first two visceral arches (19). In addition, former studies showed that the exposure of embryos to teratogenic agents (retinoic acid and irradiation) increases cell death in the developing face and subsequently gives rise to craniofacial defects (18,20,33,34).

It is now clear that caspase activation is a hallmark of almost all apoptotic systems. Caspase-3 is a central effector caspase in many cell death models. In the developing central nervous system, caspase-3 and -9 have been shown to play an essential role in physiological apoptosis (6,8,35). We observed that in control embryos caspase-3, caspase-9 and HSP110 positive cells have a spatial pattern that corresponds closely with the areas in which programmed cell death occurs in early craniofacial development. It is known that caspase-9 is activated by the apoptosome of the mitochondrial cell death pathway. We show that caspase-9 active form is expressed during normal craniofacial development. This demonstrates that the mitochondrial pathway may play a role in developmental apoptosis during early craniofacial development. After teratogenic exposure to all-trans retinoic acid, the number of caspase-3 and caspase-9 positive cells increased. This shows that exposure to exogenous retinoic acid triggers an episode of apoptosis.

Our data confirm previous studies that showed neural crest sensibility to retinoic acid. Those studies have demonstrated that embryonic exposure to retinoic acid results in cranial neural crest-derived structures defects (22,25). Retinoic exposure to the embryos disturbs the physiological RA gradient especially on the rombomeres level from which neural crest cells of the two first arches arise. This disturbance of RA gradient initiates a rapid alteration of Hox gene expression, thus changing the neural cell specification. Some studies suggest a relationship between retinoic-induced cell specification and reactivity to growth factors, which control cell proliferation, as mechanism of apoptosis induced by retinoic acid in neural crest cells (31,33,36–41). Growth factors related apoptosis is mediated by mitochondrial pathway. We show here that caspase-9 expression precedes that of caspase-3 in retinoic-induced cell death. Therefore, it is likely that caspase-3 is activated by caspase-9 in retinoic-induced apoptosis. Thus, the mitochondrial cell death pathway is implicated in this model.

After irradiation, caspase-3-positive and caspase-9-positive cells were remarkably increased showing the severity of irradiation teratogenic effects. We showed also a 'rebound expression' of caspases after 24 h of irradiation. These two caspases are strongly expressed by neural tube cells whatever the level of neural tube. This is a supplementary evidence of neural tube susceptibility to irradiation and the implication of mitochondrial pathway in irradiation-induced apoptosis (18,21,23,24). Embryo irradiation causes damage to genomic DNA. DNA damaged embryonic cells are eliminated by p53-dependent apoptosis that converge on mitochondrial pathway (27,42,43).

We observed that, unlike apoptotic cells of the neural tube which expressed similarly the two caspases investigated, caspase-9-positive neural crest cells of the first two arches were less numerous than those expressing caspase-3. Thus, if caspase-9 can be considered as the principal activator of caspase-3 in irradiation-induced apoptotic neural cells, other caspases, still to be identified, may activate caspase 3 in neural crest derived cells. A previous study suggests the coexistence in the same cells of several caspase activation pathways (44).

We suggest that endoplasmic reticulum apoptosis pathway may be investigated in these cell death models. Indeed, endoplasmic reticulum stresses (alteration in calcium homeostasis, accumulation of unfolded proteins, exposure to free radicals, glucose starvage) lead to apoptosis. In cytoplasmic reticulum apoptosis pathways, caspase-3 is activated by caspase-4 or caspase-12 (45–48).

Our results also show that HSP110 is related to physiological, retinoic-induced or irradiation-induced apoptosis. Although our data do not formally exclude a possible protective function of the HSP110 expression, the HSP110-positive cells spatial pattern that is closely similar to that of caspase-3 in the three models. A previous study showed the expression of HSP110 within TUNEL-positive cells. This is evidence that HSP110 is expressed in apoptotic cells in craniofacial development (15,16). Furthermore, it have been demonstrated that HSP105α of HSP110 family proteins is involved in increasing reactive oxygen, release of cytochrome C and finally activation of caspases (17). This apoptotic action of HSP105α seems to be specific

to embryonic cells as it has an anti-apoptotic action in mature neural cells (17,49).

It is possible that HSP110 acts in the same way as HSP60 (50) and plays a role in activating caspase-9 or other component of apoptosis machinery. Thus, it is challenging to find out the mechanisms of the dual functions of HSP110 as member of HSP100 proteins (51). In one hand, HSP100 proteins appeared to mediate the repair of substrates already damaged, rather than to protect substrate from damage, by resolubilization of proteins aggregates, subsequently assuring survivor of cells. But HSP100 proteins also have a proteolytic function. Same proteins in their folded state are protease-resistant, but when held in an unfolded conformation by HSP100 oligomer, the surface of the substrate would be exposed to protease hydrolysis. The outcome of this action can be cell survival or cell death depending on the substrates (12,14). It is exciting to know which components of 'apoptosis machinery' are substrates of HSP110 protease activity.

Conclusion

This study shows that HSP110, caspase-3 and caspase-9 are expressed in cell undergoing physiological, retinoic-induced or irradiation-induced apoptosis in early craniofacial development. The HSP110 is at least expressed spontaneously with caspase-9. The pattern of HSP110 expression is similar with the caspase-3. Thus, our data and previous findings show a potential function of HSP110 in apoptosis during embryo development. Caspase-3-expressing cells are more numerous than cells expressing caspase-9, especially irradiation-induced apoptotic neural crest cells. This suggests that other caspases, still to be identified, may activate caspase-3 in this model.

The present results must be correlated to some pathways observed in human malformative syndromes. Recent observations have demonstrated that mandibulofacial dysostosis is associated to a high rate of apoptosis in the neural folds (32). It could be interesting to see if our results can be extrapolated to this model, which appears to be very similar to the effect of retinoic administration in the mouse.

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References

1. Jacobson DM, Weil M, Raff CM. Programmed cell death in animal development. *Cell* 1997;**88**:347–54.
2. Howard YC, Xiaolu Y. Proteases for cell suicide: functions and regulation of caspase. *Microbiol Molec Biol Rev* 2000;**64**:821–46.
3. Blatt BN, Glick DG. Signaling pathways and effectors mechanisms pre-programmed cell death. *Bioorg Medic Chem* 2001;**9**:1371–84.
4. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Feng LJ, Tomaselli K et al. Two CD95 (APO1/Fas) signaling pathways. *EMBO J* 1998;**17**:1675–87.
5. Xiaodong W. The expanding role of michondria in apoptosis. *Genes Develop* 2001;**15**:2922–33.
6. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H et al. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 1998;**94**:325–37.
7. Kuida K, Hydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H et al. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 1998;**94**:325–37.
8. Kuida K, Zheng ST, Na S, Kuan CY, Yang D, Karasuyama H et al. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* 1996;**384**:368–75.
9. Miura M, Chen XD, Allen MR, Bi Y, Gronthos S, Seo BM et al. A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. *J Clin Invest* 2004;**114**:1704–13.
10. Burdan H. Heat shock and the heat shock proteins. *Biochem J* 1986;**240**:313–24.
11. Alexandrov V. Functional aspects of cell response to heat shock. *Int Rev Cytol* 1994;**148**:171–227.
12. Sreedhar SA, Csermely P. Heat shock proteins in regulation of apoptosis: new strategies in tumor therapy, a comprehensive review. *Pharmacol Therapeut* 2004;**101**:227–57.
13. Schirmer EC, Glover JR, Singer MA, Lindquist S. HSP100/Clp proteins: a common mechanism explains diverse functions. *TIBS* 1996;**21**:289–96.
14. Vanmuylder N and Dourov N. Strong expression of heat shock proteins in growth plate cartilage, an immunohistochemical study of HSP28, HSP70 and HSP110. *Anat Embryol* 1995;**195**:359–62.
15. Evrard L, Vanmuylder N, Glineur R, Louryan S. Cytochemical identification of HSP110 during early mouse facial development. *J Craniofac Genet Dev Biol* 1999;**19**:24–32.
16. Evrard L, Vanmuylder N, Dourov N, Hermann C, Biermans J, Werry-Huet A et al. Correlation of HSP110 expression with all-trans retinoic acid-induced apoptosis. *J Craniofac Genet Dev Biol* 2000;**20**:183–92.
17. Yamagishi N, Saito Y, Ishahara K, Hatayama T. Enhancement of oxidative stress-induced apoptosis by hsp105α in mouse embryonal F9 cells. *Eur J Biochem* 2002;**269**:4143–51.
18. Glineur R, Louryan S, Philippson C, Evrard L, de Vos L. Effects of irradiation on facial development in mouse embryos. *Eur J Morphol* 1998;**36**:245–52.
19. Louryan S, Glineur R. Les foyers de degenerescence physiologique dans les arcs brachiaux de l'embryon de souris: étude morphologique, histochimique et applications tératologiques. *Bull Assoc Anat* 1993;**237**:19–22.
20. Louryan S, Glineur R, Tainmont S, Vandam P. l'acide 13-cis rétinoïque sur les ébauches mandibulo-otiques de l'embryon de souris: appoche histologique et histochimique. *Bull GIRSO* 1990;**33**:147–53.
21. Miller RW, Mulvihill JJ. Small head size after atomic irradiation. *Teratology* 1976;**14**:355–7.

22. Mulder B.G, Maneley N, Grant J, Schmidt K, Zeng W, Eckhof F.C et al. Effects of excess vitamin A on development of cranial neural crest-derived structures: a neonatal and embryologic study. *Teratology* 2000;**61**:297–304.
23. Murakami U, Kameyana Y, Majima A, Sakurai T. Radiation malformations belonging to cyclopia-arrhinencephalia-otocephalia group in mouse foetus. *J Embryol Exp Morphol* 1962;**10**:64–72.
24. Pampfer S, Streffer C. Prenatal death and malformation after irradiation of mice zygotes with neutrons or X-rays. *Teratology* 1988;**37**:599–607.
25. Sulik KK, Johnston MC., Smiley JS, Speight SH, Jarvis EB. Mandibulofacial dysostosis (Treacher–Collins syndrom): a new proposal for its pathogenesis. *Am J Medic Genet* 1987;**27**:359–72.
26. Parsell A, Sanchez Y, Stitzel JD, Linquist S: HSP104 is highly conserved protein whith two binding sites. *Nature* 1991;**353**:270–3.
27. Borges HL, Chao C, Xu Y, Linden R, Wang JYJ. Radiation-induced apoptosis in developing mouse retina exhibits dose-dependent requirement for ATM phosphorylation of p53. *Cell Death Differ* 2004;**11**:494–502.
28. Evrard L, Louryan S, Vanmulder N, Glineur R., Rooze M, Dourov N. Le rôle de l'apoptose durant le développement cranio-facial: concepts et importance en pathologie. *Rév méd Brux* 1998;**1**:20–7.
29. Ilies A. La topographie et dynamique des zones nécrotiques chez l'embryon humain. *Rev Roum Embryol Cytol* 1967;**4**:51–84.
30. Liu-Bin xx, Milaire J. Spatial and temporal patterns of physiological cell death in the somite of mouse embryos. *Arch Biol Bru* 1982;**93**:99–109.
31. Louryan S, Biermans J, Flemal F. Nerve growth factor in the developing craniofacial region of the mouse embryo. *Eur J Morphol* 1995;**33**:415–9.
32. Milaire J, Rooze M. Hereditary and induced modifications of normal necrositic patterns in developing limb buds of rat and mouse: facts and hypothesis. *Arch Biol Bru* 1983;**94**:459–90.
33. Sulik KK, Cook CS, Webster WS. Teratogens and craniofacial malformations: relationship to cell death. *Development* 1988;**103**(Suppl.):213–32.
34. Dixon J, Brakebusch C, Fässler R, Dixon MJ. Increased levels of apoptosis in the prefusion neural folds underlie the craniofacial disorder, Traecher–Collins disorder. *Human Mol Genet* 2000;**9**:1473–80.
35. Woo M, Hakem R, Soengas S.M, Duncan S.G, Shahinian A, Kägi D et al. Essential contribution of caspase 3/CPP33 to apoptosis and its associate nuclear changes. *Genes Develop* 1998;**12**:806–19.
36. Barrow JR, Stadler H-S, Capecchi MR. Roles of Hoxa1 and Hoxa2 in patterning the early hindbrain of the mouse. *Development* 2000;**127**:933–44.
37. Kochhar MD. Teratogenicity of retinoic acid. *Teratology* 2000;**62**:178–80.
38. Maden M, Graham A, Gale E, Rollinson C, Zile M. Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. *Development* 1997;**124**:2799–805.
39. Scheineider AR, Hu D, Rubenstein LJ, Maden M, Helms AJ. Local retinoid signal coordinates forebrain and facial morphogenesis by maintaining FGF 8 and SHH. *Development* 2001;**128**:2755–67.
40. Dupé V, Lamslen A. Hindbrain patterning involves graded responses to retinoic acid signaling. *Development* 2001;**128**:2199–2208.
41. Frenz D, Liu W. Treatment with all-trans-retinoic acid decreases levels of endogenous Fgf-ß1 in the mesenchyme of the developing mouse inner ear. *Teratology* 2000;**61**:297–3045.
42. Gahegu J, Vanmuylder N, Kassengera Z, Evrard L, Glineur R, Philippson C et al. L'expression de la caspase 3 et 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* 2005;**89**:82–9.
43. Heyer BS, Auley AM, Behrendtsen O, Werb Z. Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. *Genes Develop* 2000;**14**:2072–84.
44. Haken R, Haken A, Duncan S.G, Henderson T.H, Woo M, Soengas S.M et al. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* 1998;**95**:339–52.
45. Hitomi J, Katayama T, Egushi Y, Kudo T, Tanigushi M, Koyama Y et al. Involvement of caspase 4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death. *J Cell Biol* 2004;**164**:347–56.
46. Hitomi J, Katayama T, Tanigushi M, Honda A, Imaizumu K, Tsujimoto Y et al. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci Lett* 2003;**357**:127–130.
47. Rao VR, Hermel E, Castro-Obregon S, del Rio G, Ellenby ML, Ellenby HM et al. Coupling endoplasmic reticulum stress to cell death program. *J Biol Chem* 2001;**276**:33869–74.
48. Wootz H, Hansson I, Korhonen L, Näpänkangas U, Lindholm D. Caspase 12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS. *Biochem Biophy Res Commun* 2004;**322**:281–6.
49. Hatayama T, Yamagishi N, Minobe E, Sakai K. Role of hsp105α in protection against stress-induced apoptosis in neural PC12 cells. *Biochem Biophys Res Commun* 2001;**288**:528–34.
50. Xanthoudakis S, Roy S, Rasper D, Hennessey T, Aubin Y, Cassady R et al. Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *EMBO J* 1999;**18**:2049–56.
51. Abdelwahid E, Eriksson ??, Pelliniema LJ, Jokinem E. Heat shock proteins, HSP25 and HSP70, and apoptosis in developing endocardial cushion of mouse heat. *Histochem Cell Biol* 2001;**115**:95–104.