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Small stress protein Hsp27 accumulation during dopamine-mediated differentiation of rat olfactory neurons counteracts apoptosis

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Abstract

The small stress protein Hsp27 is expressed during mammalian neural development. We have analyzed the role of this protein in immortalized rat olfactory neuroblasts. In the presence of dopamine a fraction of these cells differentiate into neurons while the remaining cells undergo apoptosis. We report here that the dopamine induced differentiation and apoptosis are associated with a transient and specific accumulation of Hsp27. Moreover, transfection experiments have shown that Hsp27 overexpression drastically decreases the fraction of cells undergoing apoptosis. In contrast, reduction of the endogenous level of Hsp27 led to abortion of differentiation and, therefore, drastically increased the number of apoptotic cells. Furthermore, in the normal cell population we show that Hsp27 accumulation takes place only in differentiating cells that were not undergoing apoptosis. We therefore conclude that Hsp27 may represent a key protein that controls the decision of olfactory precursor cells to undergo either differentiation or cell death.

Keywords: apoptosis; differentiation; dopamine; Hsp27; olfactory neurons; stress protein

Abbreviations: sHsp, small heat shock protein; DA, dopamine

Introduction

Small stress proteins (sHsp) belong to the family of heat shock (or stress) proteins.[1] Between species, sHsp show greater variation than other stress proteins (i.e. Hsp70), in sequence, in molecular mass and in number.[2] For example, four sHsp exist in *Drosophila* and mammals (Hsp27, αB-crystallin, Hsp20 and HspB2)[2–4] while plants contain more than 20 different sHsp.[5] sHsp are oligomeric proteins which share homology to the major eye lens proteins, αA- and αB-crystallin.[6] Mammalian Hsp27 and αB-crystallin are phosphoproteins[2] whose expression confers cellular thermotolerance,[7,8] resistance to cytotoxic drugs[9–12] and protection against oxidative stress- and TNFα-induced cytotoxicity.[9,13–17] Recently, we and others have reported that sHsp are also negative regulators of apoptosis.[18–20] For example, constitutive expression of mammalian Hsp27 can interfere with the death mediated by numerous apoptotic stimuli, such as Fas/APO-1 activation, staurosporine and etoposide. Moreover, we have recently observed that the *in vitro* anti-apoptotic effect of Hsp27 leads *in vivo* to an increased tumorigenesis of rat colon cancer cells.[21]

The molecular basis of the sHsp protective function is still unknown but could be related to the *in vitro* ATP-independent chaperone activity associated with these proteins that prevents the heat-induced aggregation of substrate proteins.[22,23] In heat-treated cells, large oligomers of sHsp are thought to serve as reservoirs of non native protein intermediates maintained in a folding competent state[24,25] hence preventing further aggregation of non-native proteins and therefore enhancing their refolding by ATP-dependent chaperones (i.e. Hsp70 and co-chaperones). We have recently proposed that the large oligomers of mammalian Hsp27 represent the active form of this protein during early differentiation[26,27] or in response to oxidative stress.[18,28] A specific protective activity against the stress-induced disruption of the actin microfilament network was also proposed,[17,29] which probably results from the *in vitro* actin capping-decapping activity associated with Hsp27 small oligomers.[30] Finally, we have suggested that sHsp also have the ability to modulate reactive oxygen species and glutathione levels, a phenomenon leading to the inhibition of cell death.[16]

sHsp share the intriguing property of tissue specific expression during development (reviewed in[2,31]), particularly during maturation of the nervous system (reviewed in[32]). This phenomenon has been extensively studied in *Drosophila* where three out of the four sHsp display a pattern of developmental expression in gonads, imaginal discs and the nervous system.[33–35] In mammals, while Hsp27 (denoted Hsp25 in mouse) expression is restricted to specific motoneurons and primary sensory neurons of adult rat brain,[36] a massive expression of this protein is detected, through days 13 to 20 of mouse development, in neurons of the spinal cord and Purkinje cells[37] or at the area of neural tube closure.[38] The role of sHsp expression during nervous system development is unknown but could be related to a specific role during cell differentiation. Indeed, *in vitro* sHsp share the property of transient

expression during cellular differentiation of all cell types so far tested.[2] In mouse embryonic stem cells, inhibition of this expression leads to abortion of differentiation and massive commitment to apoptosis.[27]

The role of Hsp27 during neural differentiation was studied in immortalized rat olfactory neuronal progenitor 13.S.1.24 cells. In the presence of dopamine, a fraction of these cells undergo neuronal differentiation while the remaining population die by apoptosis.[39] We show here that dopamine provokes a transient and specific accumulation of Hsp27. Modulation of Hsp27 level led to the conclusion that this protein is a key element which controls the balance between differentiation and cell death. Furthermore, we present evidence which suggest that differentiating neural cells escape from apoptosis by transiently accumulating high levels of Hsp27.

Results

We reported recently that specific subpopulations within cultured 13.S.1.24 cells were committed to either death or neuronal differentiation when exposed to DA or DA agonists.[39] Several DA concentrations (20–80 μM) have been assessed and have been shown to lead to either more differentiation (low DA concentration) or to more cell death (high DA concentration). For these experimental conditions, we have determined the level of Hsp27 by immunoblot analysis. Figure 1 shows the kinetic analysis of the level of Hsp27 in 13.S.1.24 cells treated with the two extreme DA concentrations, 20 (A) or 80 (B) μM DA. A low level of Hsp27 (see also Figure 2A where ten times more total protein has been loaded) is expressed in untreated neuronal progenitors while a high level of this protein can be detected after DA treatments. This accumulation is transient and peaks 12–24 h after DA treatment. A similar effect was observed with intermediate doses of DA such as 40 and 60 μM (not shown). A control experiment was performed by analyzing the level of the main stress protein, Hsc70/Hsp70. An immunoblot analysis using an antiserum raised against Hsc and Hsp70 has shown that the highest DA treatment (80 μM) leads to a slight decrease of Hsc70 while Hsp70 remains non detectable (Figure 1C). A densitometric analysis of Hsp27/Hsc70 behavior in response to DA is presented in Figure 1D. Hence, DA treatment does not induce a stress response (i.e. monitoring by Hsp70 accumulation) but leads to a drastic and specific accumulation of Hsp27 in 13.S.1.24 cells.

We next investigated whether Hsp27 accumulation in response to DA was related to the process of differentiation. 13.LA.tet2 cells, derived from 13.S.1.24 cells by stable integration of tTA transactivator gene, were transiently transfected with either a control vector (cont.) or vectors containing human Hsp27 coding sequence placed in normal (hsp27+) or reverse orientation (hsp27−) under the control of tTA dependent promoter (see Materials and Methods). Transfection efficiency was more than 50% (not shown). Immunoblot analysis of the sHsp cellular contain was performed 24 h after transfection (Figure 2A). The accumulation of Hsp27 represented about 220% of that of the endogenous protein. The reverse phenomenon was observed when the anti-sense vector was used. In this case, the level of endogenous Hsp27 was decreased by about 70% (Figure 2A).

The transiently transfected cells described above were treated with 40 μM DA and expression of the olfactory marker protein (OMP), a specific marker for differentiated olfactory neurons, was determined. An intermediate dose of DA (40 μM) was chosen since this concentration allows a relatively equal amount of cell death and differentiation (Figures 2B and 3B). Figure 2B describes a quantitative

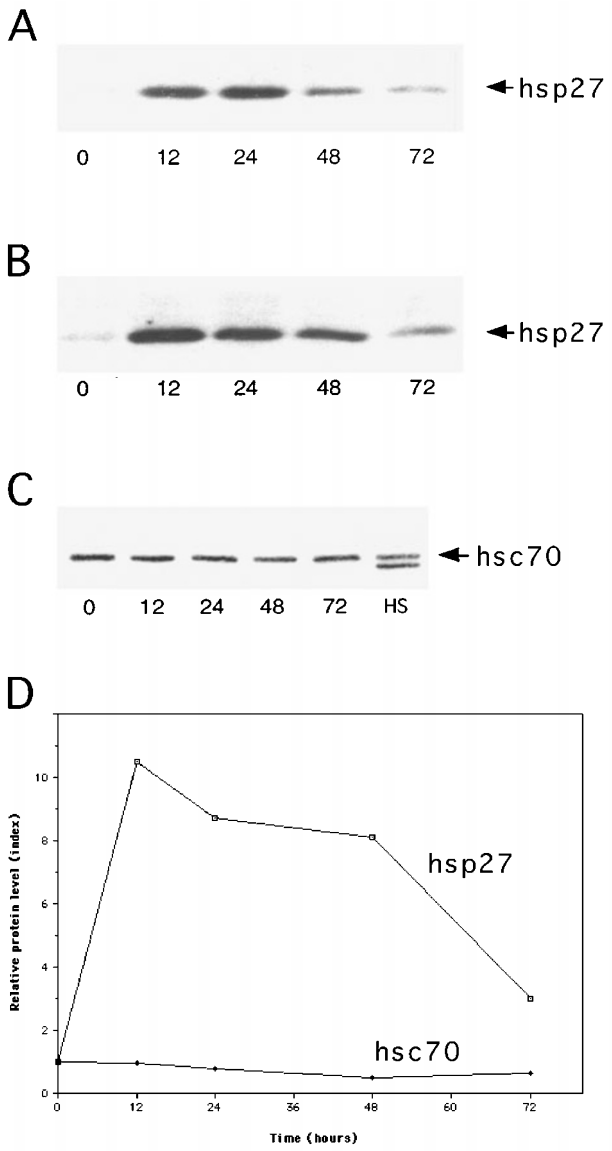

Figure 1 Dopamine induces a specific and transient Hsp27 accumulation in 13.S.1.24 cells. 13.S.1.24 cells were either kept untreated (0) or treated for the time indicated with 20 μM (**A**) or 80 μM (**B** and **C**) DA in the presence of 100 μM ascorbic acid. Immunoblot analysis of 10 μg of total protein was then performed as described in Materials and Methods using antibodies raised against either Hsp27 (**A** and **B**) or Hsc/Hsp70 (**C**). In **C**, a heat shock of 30 min at 43°C followed by 20 h of recovery has been performed (HS). The detection was performed using ECL chemiluminescence system. (**D**) Densitometric analysis of **B** and **C**. Note the strong but transient accumulation of Hsp27 12 h after dopamine treatment

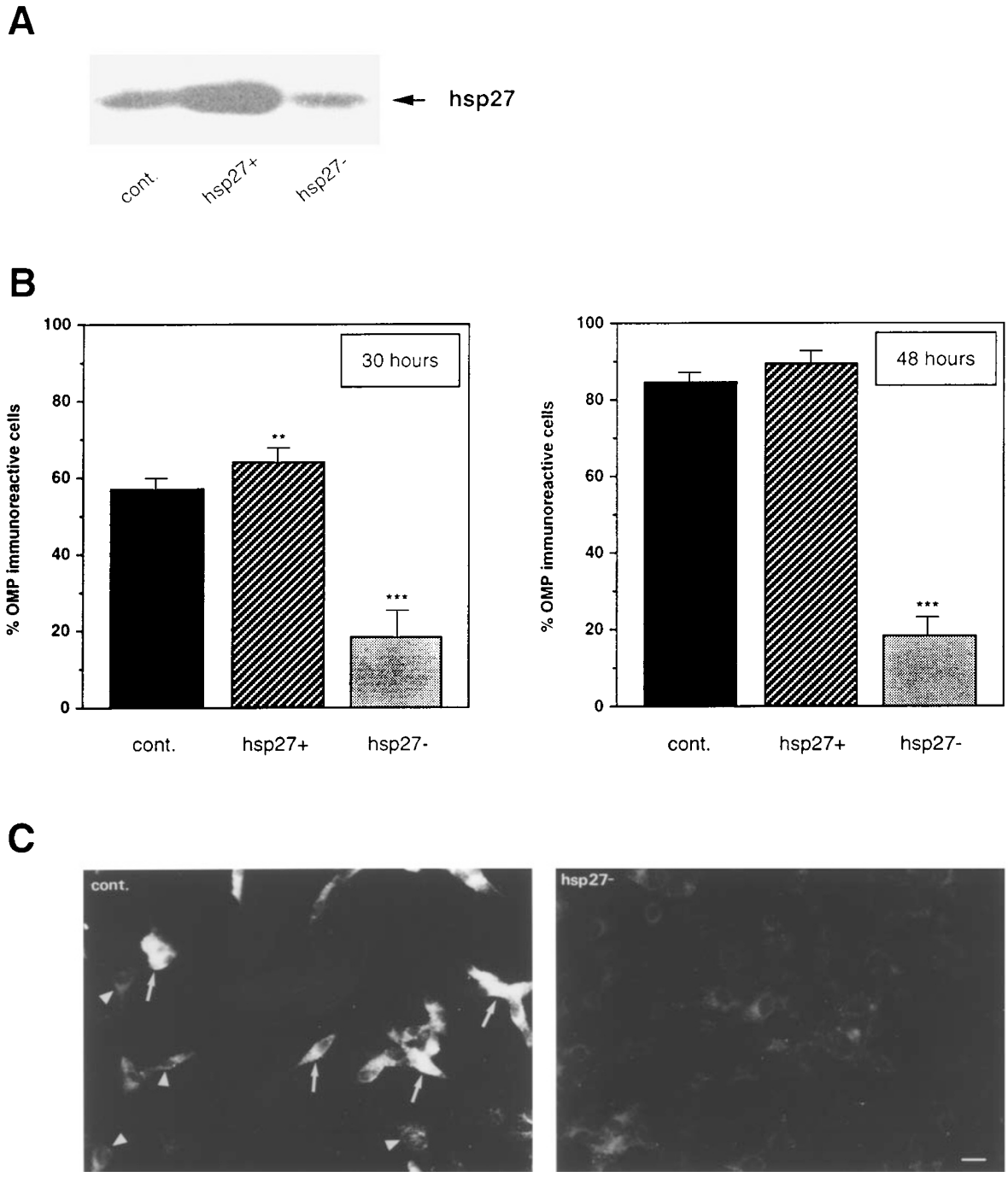

Figure 2 Change in Hsp27 level drastically modulates DA-mediated differentiation of olfactory neuroblasts. 13.LA.tet2 cells were transfected with control pUH10-3 (cont.), Hsp27 sense pUH10-3Hhsp27 (hsp27+) or anti-sense pUH10-3Hanthsp27 (hsp27−) vectors. Twenty-four hours after transfection, cells were either immediately harvested and loaded (100 μg of total protein) on a gel for subsequent Hsp27 immunoblot (**A**) or treated with 40 μM DA in the presence of ascorbic acid for 30 or 48 h (**B** and **C**) before being fixed and prepared for immunofluorescence analysis performed with OMP antiserum (see Materials and Methods). (**A**) Immunoblot analysis of Hsp27 cellular content. The increased signal in hsp27+ cells reflects the accumulation of human Hsp27. The decreased level in hsp27− cells is due to the lack of production of endogenous Hsp27. (**B**) Differentiation of olfactory progenitors was monitored as the percentage of OMP immunoreactive cells calculated by dividing the number of OMP positive cells by the total number of cells. This experiment is one of three distinct experiments leading to similar results. An average number of 975 cells was counted in 20 fields. Significantly different from control: ***$P<0.001$, **$P<0.01$ (non linear binomial test). (**C**) Immunofluorescence detection of OMP in 13.LA.tet2 cells submitted for 30 h to 40 μM of DA. (cont.) 13.LA.tet2 cells transfected with control vector: note that numerous cells display a strong cytoplasmic immunoreactivity (arrows) whereas other cells showed only a faint granular autofluorescence (arrowheads). Both types of cells are either scattered in the plate or gather into small clusters. (hsp27−) 13.LA.tet2 cells transfected with Hsp27 anti-sense vector: note that most cells display the faint granular autofluorescence typical of non-immunoreactive cells. Scale bar: 20 μm

analysis of the number of transfected 13.LA.tet2 cells expressing OMP in response to DA. After 30 h of treatment, a slight but significant increase in the number of OMP positive cells was detected in the human Hsp27 expressing (hsp27+) cellular population (control: 56%$\pm$2.6, hsp27+: 64.8$\pm$3.52). After 48 h of DA treatment, this slight increase in Hsp27 overexpressing cells is no longer significant, hence suggesting that expression of human Hsp27 only slightly and transiently increased the rate of OMP immunoreactivity. However, after 30 or 48 h of DA treatment, only a small number of OMP positive cells was observed among cells underexpressing Hsp27 (hsp27$-$) compared to the control population (30 h: 18.6%$\pm$6.44 *versus* 56%$\pm$2.6) (Figure 2B and C). Morphological analysis based on the bipolar appearance of differentiated cells led to the similar conclusion (Figure 2C and not shown) that underexpression of the endogenous Hsp27 drastically reduced the frequency of the differentiated phenotype. Hence, the level of Hsp27 probably represents a key element in the differentiation of progenitors into mature neurons.

As DA treatment not only provokes differentiation but also cell death, we have analyzed the effect mediated by different levels of Hsp27 on DA induced cell death. 13.LA.tet2 cells were transfected as described above and cell death was monitored 60 h after addition of 40 μM DA (see Materials and Methods). At this particular time point, the switch between differentiation and death has been achieved. Cell death, which begins to be detectable less than 24 h after adding DA to cells,[39] was monitored by flow cytometry analysis of propidium iodine (PI) incorporation (see Materials and Methods). Cells not treated with DA demonstrated a low rate of death (about 7%) (Figure 3A and not shown). In contrast, a 40 μM DA treatment induced the death of about 59% of the control cells (Figure 3B). Overexpression of Hsp27 led to an important decrease of DA-mediated cell death (30%), while a massive increase in the percentage of dying cells was observed in cells underexpressing Hsp27 (82%) (Figure 3C and D). Hence, the level of Hsp27 appears to play a major role in the control of DA-mediated cell death of olfactory progenitor cells.

To more precisely understand the role of Hsp27 in DA treated olfactory progenitor cells, we have compared the level of this protein in differentiating or pre-apoptotic cells. 13.S.1.24 cells were treated for 24 h with DA, then cell sorting of either living (gate R1) or early apoptotic cell (gate R2) sub-populations was performed as described in Materials and Methods. The Western blot analysis presented in Figure 4 shows that Hsp27, which strongly accumulates 24 h after DA addition (Figure 1A and B), is concentrated in differentiating cells and almost absent in cells undergoing apoptosis. Hence, these results strongly suggest that Hsp27 accumulates in differentiating cells to allow their escape from apoptosis.

Discussion

The present study has led to the determination of a role for Hsp27 during the differentiation of olfactory neuronal progenitors. It is, to our knowledge, the first report describing a sHsp function in a neuronal cell line. We show here that Hsp27 transiently and specifically accumulates in differentiating neuronal progenitors. As already observed in several other differentiating systems, Hsp27 accumulation peaks during early differentiation, and the level of this protein drastically recedes soon after the appearance of differentiation markers.[26,27,40–42] The results presented here therefore confirm that Hsp27 plays a role during early differentiation and is probably of no more use during late differentiation.

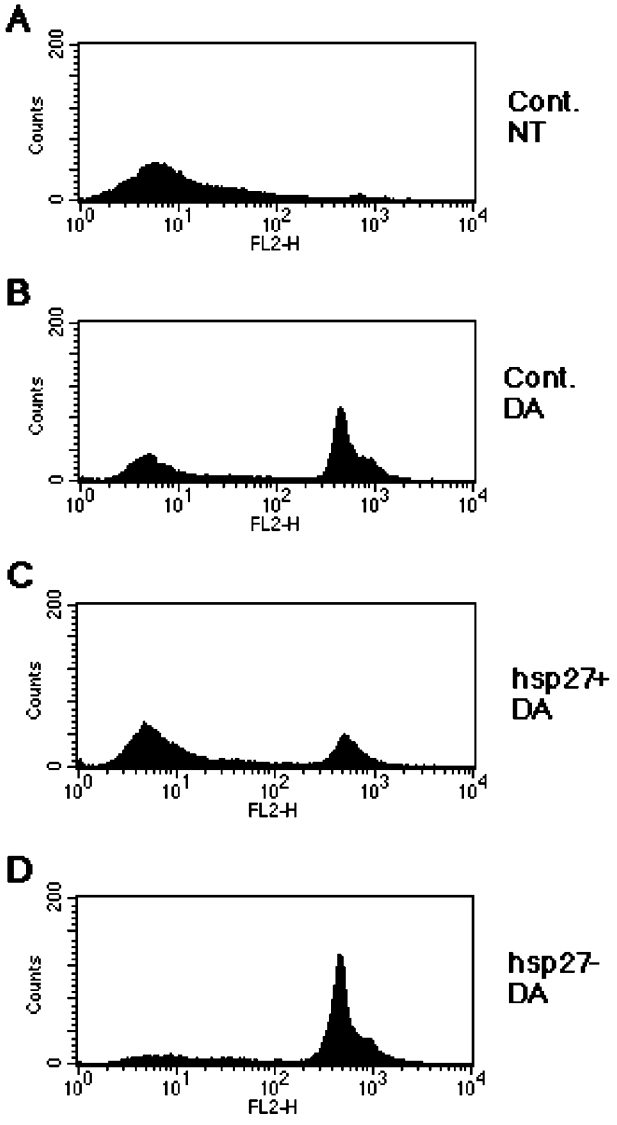

Figure 3 Change in Hsp27 levels modulates dopamine induced cell death. 13.LA.tet2 cells were transfected with control pUH10-3 (cont.) (**A** and **B**), Hsp27 expressing pUH10-3Hhsp27 (hsp27+) (**C**) or Hsp27 anti-sense pUH10-3Hanthsp27 (hsp27$-$) (**D**) vectors. Twenty-four hours after transfection, cells were treated (DA) or not (NT) with 40 μM dopamine in the presence of ascorbic acid for 48 h. Cell death was monitored by PI incorporation as described in Materials and Methods. Results are presented as histogram plots showing the number of cells (counts) *versus* PI fluorescence (FL2-H). Note the direct relation between Hsp27 expression and cell survival

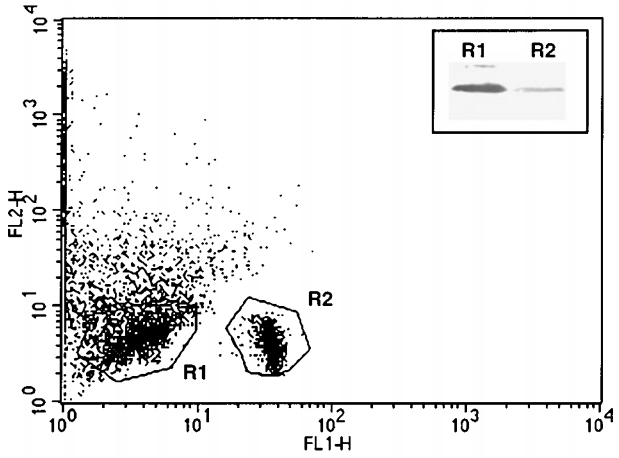

Figure 4 Hsp27 specifically accumulates in cells escaping from apoptosis. 13.S.1.24 cells were treated for 24 h with 40 μM DA in the presence of ascorbic acid. FACS analysis was performed using PI and annexin V labelling as described in Materials and Methods. Cell sorting was performed on either 'PI and annexin V negative cells' (alive cells, gate R1) or 'PI negative and annexin V positive' (cells undergoing early apoptosis, gate R2) subpopulation. 5.10^5 cells of each sub-population were then loaded on a 15% acrylamide gel and Hsp27 level was determined by immunoblot analysis using ECL+ chemiluminescence system. Note that Hsp27 is present in live cells and almost absent in cells undergoing apoptosis

Remarkably this *in vitro* system allows the analysis of both differentiation and apoptosis, since large subpopulations of cells were committed to either death or to neuronal differentiation. We show here that variations in Hsp27 levels lead to a modulation of both differentiation and apoptosis processes. While high levels of Hsp27 provoke a slight increase in the rate of differentiation and an important decrease in cell death, the underexpression of endogenous Hsp27 is associated with a complete failure of differentiation and a massive commitment to cell death. These results confirm our previous observation that Hsp27 underexpression aborts the differentiation of totipotent murine embryonic stem cells[27] and that sHsp can interfere with Fas/ APO-1, staurosporine and etoposide induced apoptosis.[18,19]

The fact that Hsp27 overexpression only slightly and transiently modulates the differentiation efficiency but strongly blocks the process of neural cell death suggests that Hsp27 does not really act as a mediator of differentiation but rather as an inhibitor of cell death. Besides, our finding that Hsp27 accumulates only in cells that have escaped from apoptosis leads to the same conclusion and strongly suggests that Hsp27 is a key element in the control of cell death during neuronal differentiation. Interestingly, Hsp27 definitely differs from the other anti-apoptotic proteins by the fact that it naturally accumulates during differentiation and specifies, in the same population, differentiating cells from cells which will undergo apoptosis. How and why, in the same population submitted to DA, some neural progenitors accumulate Hsp27 and consequently undergo differentiation and others do not accumulate this protein and die remains to be shown. A preliminary study has suggested that the switch allowing either accumulation of Hsp27 or cell death is downstream of the DA D2 receptor since cAMP, its second messenger, allows a similar accumulation of Hsp27 (Ducasse and Arrigo, unpublished observations). Further studies need now to address the signal transduction pathway(s) leading to this differentiation-dependent accumulation of Hsp27.

The molecular mechanism allowing Hsp27 to protect differentiating neural cells from apoptosis is unknown. We have reported previously that Hsp27 protects against Tumor Necrosis Factor induced cell death via the control of reactive oxygen species (ROS). However, the protection observed here is probably related to other mechanisms since the dopamine induced apoptosis in 13.S.1.24 cells occurs even in the presence of anti-oxidant.[39] However ROS are not the only mediator of cell death related to redox modulation. Indeed, several pieces of evidence have converged toward the importance of glutathione in the control of apoptosis.[43] In this respect, we have shown that Hsp27 expression in cells, that are normally devoid of this protein, upregulates glutathione levels leading to decreased ROS level and a global pro-reduced state.[16] Remarkably, during differentiation of embryonic stem cells Hsp27 transient accumulation is accompanied by an increase in glutathione.[44] On the other hand, several data have strengthened the hypothesis that Hsp27 induced protection against apoptosis can be independent of glutathione (Mehlen and Arrigo, unpublished observations) but rather dependent upon its ability to bind specific proteins such as actin[30] or proteasome subunit (Boelens, personal communication). Hence, a protein controlling actin polymerization could be of great interest, particularly during differentiation of cells with specific morphology such as neural cells. Likewise, proteasome activity seems to be involved both in neural cell death[45] and in neural differentiation.[46] A protein controlling proteasome activity could then represent a check point between apoptosis and differentiation. Future studies need to address the molecular function of Hsp27 in this system.

It is now tempting to speculate that this *in vitro* transient Hsp27 accumulation observed in differentiating olfactory progenitors could then be related to the *in vivo* cell-specific and developmental stage-specific pattern of sHsp expression in the developing nervous system.[34,35] Hence, in these neuronal tissues, one *in vivo* function of Hsp27 could be of selecting differentiating neurons either to die or to carry on their differentiation process. To clarify this point, we are currently investigating the *in vivo* relationship between Hsp27, apoptosis and differentiation in the rat olfactory system.

Materials and Methods

Cells and dopamine treatment

The immortalized neuroblast 13.S.1.24 cells have been described previously.[39] They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco, BRL) and 0.3 mg/ml of gentamycine (Gibco, BRL). For dopamine treatments, cells were plated at a density of 50 000 cells/

cm^2 and fed with DMEM 10% FCS (day 0) then DMEM 5% (day 1). On day 2 of culture, 20–80 μM of dopamine and 100 μM of ascorbic acid were added to the media and cell death or differentiation was monitored 24–48 h later.

Immunoblotting

One dimensional immunoblots using antibodies raised against Hsp27[47] or Hsp70 (StressGen, Tebu, France) were performed as already described.[15,47] They were revealed with the ECL or ECL+ kit from Amersham Corp. (UK) and autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co, Rochester). A Bioprofil system (Vilber Lourmat, France) was used for quantification. The analysis was performed within the range of proportionality of the film.

Transfections

13.LA.tet2 cells were derived from 13.S.1.24 cells by the stable transfection of modified pUHD15-1 plasmid coding for Tetracycline repressor-herpes simplex virus transactivator protein VP16 (tTA).[48] Briefly, 13.S.1.24 cells (10^6) were plated in 100 mm dishes and transfected using lipofectamine procedure according to manufacturer instruction with 2 μg of pUHD15-1-puro plasmid and 9 μl of lipofectamine. Forty-eight hours after transfection, puromycin was added and clones were isolated about 3 weeks later. Characterization of positive clones was performed as described previously.[48] 13.LA.tet2 cells have been shown to behave as 13.S.1.24 cells in their ability to differentiate or undergo apoptosis in response to dopamine treatment. To modulate Hsp27 level, 13.LA.tet2 cells, grown in absence of tetracycline, were transiently transfected with control pUH10-3,[48] pUH10-3Hhsp27, and pUH10-3Hanthsp27 vectors using lipofectamine procedure.[16,18] pUH10-3Hhsp27 and pUH10-3Hanthsp27 plasmids were derived from pUH10-3 plasmid by cloning the *Eco*RI-*Eco*RI fragment containing the coding sequence of human Hsp27[14,27] respectively in normal or reverse orientation in *Eco*RI site of pUH10-3 polylinker. Cells were plated at a density of 50 000 cells/cm^2 24 h before transfection and were dopamine treated 24 h later.

Analysis of dopamine induced apoptosis

Apoptosis of 13.S.1.24 or 13.LA.tet2 cells was analyzed 24 h after dopamine treatment using the annexin V procedure following the manufacturer's instructions (Boehringer Mannheim, France). Briefly, collected cells were washed in Ca^{2+} containing buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) and incubated for 10 min in the presence of annexin V. Cells were then washed prior to propidium iodine (PI) addition. Flow cytometry analysis was performed with a FACS-Calibur (Becton Dickinson, Le Pont de Claix, France) using 488 nm excitation. Emission filter was 530 nm for annexin and 610 nm for PI.

Analysis of dopamine induced cell death

Sixty hours after dopamine treatment, cell death (necrosis or late apoptosis) was analyzed in 13.LA.tet2 using PI staining. Briefly, cells were washed in PBS buffer and then incubated with Propidium Iodine. As above, flow cytometry analysis was performed with as above using 488 nm excitation and 610 nm emission filters.

Dopamine induced differentiation analysis

Differentiation in 13.S.1.24 or 13.LA.tet2 cells was monitored by immunochemistry using an antibody raised against OMP (Olfactory Marker Protein) as described previously.[39] Prior to transfection, cells were plated in 10 mm wells containing coverslips. After transfection and dopamine treatment, cells were rinsed in PBS and fixed for 30 min at 4°C in Bouin's fixative. Immunocytochemistry was then performed using a goat polyclonal antiserum raised against OMP (1:1000 dilution).

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