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Expression of heat shock protein 27 and alpha-crystallins in human retinoblastoma after chemoreduction

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Abstract

Aim—Small heat shock proteins (sHSP) play an important role in the resistance to anticancer drugs. We examined the expression of the sHSP family, HSP27 and α -crystallins, in human retinoblastoma with and without preoperative chemotherapy.

Methods—Eighteen enucleated eyes from patients with retinoblastoma were used. Six patients had undergone chemotherapy before enucleation. Formalin-fixed, paraffin-embedded tissue sections were processed for H&E staining and examined by immunohistochemistry using anti-HSP27 and α -crystallins antibodies.

Results—Eleven of 12 cases with no history of preoperative chemotherapy showed weakly positive or negative staining for HSP27, whereas six and five cases were strongly positive for α A and α B-crystallin, respectively. In the six cases with a history of chemotherapy, several viable retinoblastoma cells remained. Immunoreactivity for HSP27 and α B-crystallin was strongly detected in the cytoplasm of viable retinoblastoma cells, while α A-crystallin immunoreactivity was less marked. Immunoreactivity for HSP27 was significantly higher in retinoblastoma cases with preoperative chemotherapy than in those without chemotherapy ($p < 0.0001$). In contrast, immunoreactivity for α A-crystallin was significantly lower in cases with chemotherapy than in cases without chemotherapy ($p < 0.01$).

Conclusions—HSP27 and α B-crystallin, but not α A-crystallin, were highly expressed in viable tumour cells after chemotherapy, suggesting that HSP27 and α B-crystallin may protect tumour cells from apoptotic signals produced by anticancer drugs in retinoblastoma.

Mammalian heat shock protein (HSP) 27 and α -crystallins belong to the family of small heat shock proteins (sHSP) that display an enhanced synthesis in response to stress. The two α -crystallins, α A and α B, have related amino acid sequences with similar structural properties. The frequent association of HSP27 and α B-crystallin with their structures is linked to the chaperone activity of sHSP. Indeed, molecular chaperones modulate the earliest aberrant protein interactions that trigger pathogenic cascades.¹ Therefore, sHSP may well provide a first line of defence against misfolded, aggregation-prone proteins.¹ Increased cellular resistance to several pro-apoptotic agents or conditions has been observed in cells expressing high loads of HSP27 and α B-crystallin.^{2–6} In contrast, Rao *et al*⁷ recently

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demonstrated that α A-crystallin upregulation was selectively associated with photoreceptor protection against apoptosis caused by oxidative stress.

In malignancy, HSP27 and α B-crystallin increase the ability of cancer cells to resist and evade the apoptotic processes.^{1, 6} The sHSP are highly expressed in human malignant tumours.^{8–12} Moreover, HSP27 and α B-crystallin may be novel prognostic factors in several malignant solid tumours.^{9–11, 13} α B-crystallin expression has been well correlated with poor patient prognosis, but correlation of HSP27 expression and patients' prognosis is controversial.^{13, 14}

Retinoblastoma is an important, life-threatening, malignant retinal tumour. Chemotherapy is a major therapeutic option for retinoblastoma; when used alone, chemotherapy may reduce tumour size, but it does not cure the retinoblastoma.¹⁵ This suggests the existence of pathological mechanisms underlying the survival of retinoblastoma cells after chemoreduction. Nevertheless, sHSP expression in retinoblastoma after chemoreduction has not been elucidated. This study demonstrates the difference in expression of HSP27 and α -crystallins in retinoblastoma with and without a history of preoperative chemotherapy.

MATERIALS AND METHODS

The institutional review board of the University of Southern California approved our use of human specimens. All procedures conformed to the Declaration of Helsinki for research involving human subjects. Using the medical records at Doheny Eye Institute, Pathology Department, from January 2006 through October 2007, we selected 18 cases with retinoblastoma as determined by enucleation. Six of the 18 patients received preoperative systemic chemotherapy, during which the retinoblastomas were treated with 3-monthly cycles of carboplatin (560 mg/m²), etoposide and vincristine sulfate to reduce tumour volume (chemoreduction). One patient underwent local subtenon carboplatin administration.¹⁶ All eyeballs were fixed in 4% paraformaldehyde soon after enucleation.

Immunohistochemistry

The slides were dewaxed, rehydrated and rinsed in phosphate-buffered saline twice for 10 min. As pretreatment, microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). These slides were incubated with 3% hydrogen peroxide for 10 min, then with normal goat serum for 30 min. Sections were incubated with anti-rabbit HSP27, and α A- and α B-crystallin polyclonal antibodies (dilution 1:100; Stressgen, Victoria, Canada) at room temperature for 2 h. Binding of the primary antibody was localised with the Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) for 30 min. Cy3-conjugated mouse IgG without treatment of the primary antibody was incubated to serve as a negative control. Sections were mounted with mounting medium, including 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California). Slides were examined using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, New York).

In the non-necrotic viable tumour tissues, 300 tumour cells were counted from three or four fields of the same slide for each specimen. The number of immunopositive cells was shown as a percentage of viable tumour cells (%). Immunoreactivity for HSP27 and α -crystallins in tumour tissues was evaluated as strongly positive (>30% of tumour cells positive), weakly positive ($\leq$ 30% of tumour cells positive) or negative (background level staining only) according to the previous report.¹⁰

Statistical analysis

Strongly positive immunoreactivity in tumour cells was scored as 2, weakly positive as 1 and negative as 0. Scores were totalled, and the mean and standard deviation were calculated. Statistical evaluations were performed using the Student *t* test. The accepted level of significance for all tests was $p < 0.05$.

RESULTS

Table 1 summarises the clinicopathological profile in patients with retinoblastoma examined in this study. In those patients with no history of chemotherapy (Cases 6–18), the tumour cells represented dense monotonous proliferation with atypical nuclei and scanty cytoplasm (fig 1A). Tissues from 11 of 12 retinoblastoma cases were weakly positive or negative for HSP27 (fig 1E) except one case (Case 8, table 1). On the other hand, tissues from six cases were strongly positive for α A-crystallin, and five for α B-crystallin (table 1). In these cases, the distribution of the immunoreactivity for α A-crystallin (fig 1B–D) and α B-crystallin (fig 1F) in the cytoplasm of tumour cells was heterogeneous.

In six cases with a history of preoperative chemotherapy (Cases 1–6), several histologically intact retinoblastoma cells were present. The tumour cells showed a heterogeneous nuclear staining pattern with obvious nucleoli and irregular cytoplasm. Tumour cells in these eyes formed rough clusters (fig 2A), compared with those cases with no history of chemotherapy. Several melanophages were intermingled in the tumour tissues. Calcified and necrotic tumour cells noted in Cases 1–5 were more prominent than in the no-chemotherapy group (Cases 7–18). Immunoreactivity for HSP27 (fig 2B–D) and for α B-crystallin (fig 2E) was strongly detected in the cytoplasm of histologically intact retinoblastoma cells. In contrast, the tumour cells showed no expression of α A-crystallin in the serial sections (fig 2F). No immunoreactivity for HSP27 and α -crystallins was detected in calcified and necrotic areas of the tumour.

Immunohistochemistry scores revealed that HSP27 immunoreactivity was significantly higher in retinoblastoma cases after chemoreduction than in those cases without chemotherapy (fig 3; $p < 0.0001$). In contrast, immunoreactivity for α A-crystallin was significantly lower in cases after chemoreduction than in cases without chemotherapy (fig 3; $p < 0.01$). There was no significant difference in immunoreactivity for α B-crystallin in retinoblastoma cases with or without chemoreduction.

DISCUSSION

HSP27 and α B-crystallin proteins are known to express similar antiapoptotic properties in cancer cells.¹ We clearly demonstrated that α -crystallins were strongly expressed in retinoblastoma cells without any history of preoperative chemotherapy, whereas HSP27 was not. This suggests that primary retinoblastoma cells may express different sHSP depending on tissue specificity, as shown in thyroid and brain cancers,^{8, 12} for acquisition of immune privilege against apoptotic signal. In contrast, not only α B-crystallin but HSP27 were highly expressed in histologically intact tumour cells in the group treated with chemotherapy, indicating that HSP27 immunoreactivity was particularly enhanced after chemoreduction. Indeed, some of cytostatic anticancer drugs enhance HSP27 and/or α B-crystallin expression, which consequently contributes to cellular resistance to these drugs.^{17, 18} Taken together, these results suggest that HSP27 and α B-crystallin may protect tumour cells from apoptotic signals induced by chemotherapy in retinoblastoma.

α -Crystallins are reported to have different functions; they are active under different conditions, and they modulate different protein interactions.^{19–21} In mammals, α B-crystallin

is a major polypeptide of the eye lens where it is associated with the closely related α A-crystallin to form large hetero-oligomeric structures.²² Although α A-crystallin was strongly expressed in half of primary retinoblastoma cases in the present study, the expression was weak or negative in retinoblastoma tissues after chemoreduction. These results suggest that anticancer drugs selectively involve α A-crystallin-expressing retinoblastoma cells, which might lead to tumour cell apoptosis. On the other hand, α B-crystallin was recently determined to be a novel predictor of resistance to neoadjuvant chemotherapy in breast cancer.⁹ The present study showed strong α B-crystallin expression in retinoblastoma cells, despite preoperative chemotherapy, suggesting that tumour cells use different sHSP to escape from apoptotic signals by anticancer drug administration.

Specific agents including etoposide,^{5, 23} staurosporine,^{20, 24} and cisplatin,²⁵ have mainly been used in chemoreduction related to activation of HSP27 and α -crystallins. Indeed, etoposide and cisplatin can be applied for patients with retinoblastoma. Subsequent studies have proposed that HSP27 and α -crystallins prevent the apoptotic pathway affected by chemoreduction at multiple signal steps, whereas the exact mechanism or regulating targets seem to differ (fig 4). HSP27 prevents apoptosis by interacting with caspase-3 to modulate its activity,²⁴ by interacting with cytochrome *c* to prevent procaspase-9 activation^{5, 23} or by regulating Bid intracellular disruption to block the mitochondrial death pathway.²⁴ In addition, α B-crystallin can suppress the activity of procaspase-3.²⁰ α -Crystallins block upregulation of Bak and translocation of Bax and Bcl-Xs, thereby inhibiting the activation of downstream apoptotic events.²⁰ Recently, Zhang *et al* demonstrated that HSP27 protected cells from cisplatin-induced apoptosis through phosphatidylinositol 3-kinase/Akt activation,²⁵ while the role of α -crystallins in that pathway affected by chemoreduction remains unknown.

Chemotherapy is one of the most important treatments in patients with retinoblastoma; however, chemotherapy alone does not cure retinoblastomas because of their multidrug resistance.¹⁵ As shown in the current study, both HSP27 and α B-crystallin were highly expressed in retinoblastoma cells of cases after chemoreduction. In fact, HSP27 and α B-crystallin expression impairs the efficiency of the clinical treatments including chemotherapeutic agents.^{17, 18} Thus, since HSP27 and α B-crystallin are suggested to have negative activities in retinoblastoma patients treated with chemotherapy, sHSP may be a novel molecular therapeutic target, as it is considered in various systemic cancers.¹

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Figure 1. H&E staining (A), DAPI nuclear staining (B, D, E, F; blue), and immunoreactivity of α A-crystallin (B, D; red), heat shock protein (HSP) 27 (E; red) and α B-crystallin (F; red) in human retinoblastoma with no history of chemotherapy. H&E staining demonstrates that retinoblastoma cells show dense monotonous proliferation with atypical nuclei and scanty cytoplasm (A). Cytoplasmic immunoreactivity for α A-crystallin (Case 15; C, D) and α B-crystallin (Case 15; F) is strongly detected in retinoblastoma cells, while HSP27 immunoreactivity is hardly observed (Case 15; E).

Figure 2.

H&E staining (A), DAPI nuclear staining (B, D–F; blue) and immunoreactivity of heat shock protein (HSP) 27 (C, D; red), α B-crystallin (E, red), and α A-crystallin (F, red) in human retinoblastoma with history of chemotherapy. Several retinoblastoma cells are present with glial reaction. The tumour cells show a heterogeneous nuclear staining pattern with obvious nucleoli and irregular cytoplasm. Tumour cells form rough clusters (A). Immunoreactivity for HSP27 is strongly detected in the cytoplasm of retinoblastoma cells (B–D). Cytoplasmic immunoreactivity for α B-crystallin is also noted in the retinoblastoma cells (E), where no expression of α A-crystallin is observed (F) in the serial sections.

Figure 3.

Scoring of immunoreactivity for heat shock protein (HSP) 27 and α -crystallins in human retinoblastoma cells with or without history of preoperative chemotherapy. The score of HSP27 immunoreactivity is significantly higher in the chemotherapy-treated (chemo+) group ($n = 6$) than in the no chemotherapy (chemo-) group ($n = 12$) ($p < 0.0001$, left side). In contrast, the immunoreactivity score of α A-crystallin reveals significantly higher expression in the chemo- group than that in the chemo+ group ($p < 0.01$, middle side). Immunoreactivity for α B-crystallin shows no significant statistical correlation despite treatment with chemotherapy ($p > 0.05$, right side).

Figure 4.

Proposed mechanism of the apoptotic pathway for chemotherapeutic agent-induced apoptosis, and the presumed protective mechanisms of heat shock protein (HSP) 27 and α B-crystallin affected by chemoreduction in retinoblastoma cells. Anticancer drugs can induce upregulation of Bak and translocation of Bax and Bcl-Xs and the release of cytochrome *c* from the mitochondria. Procaspase-9 is activated by cytochrome *c* release, which causes activation of procaspase-3 and eventually execution of the apoptotic programme. HSP27 inhibits the activation of procaspase-9 and procaspase-3. α -Crystallin blocks upregulation of Bak and translocation of Bax and Bcl-Xs, and suppresses the procaspase-3 directly, thereby inhibiting the activation of downstream apoptotic events.

Table 1

Clinicopathological profiles in retinoblastoma cases examined in this study

No	Age (years)	Gender	Side	Chemo	Diff	Choroidal invasion	ON head invasion	HSP27	aA	aB
1	0.5	F	R	+	Un	-	-	50	10>	50
2	1	F	R	+	Well	-	-	>90	10	70
3	4	M	R	+	Un	-	-	40	10>	30
4	2	F	R	+	Well	-	-	70	10	50
5	1	F	R	+	Un	-	-	80	20	70
6	3	F	R	+	Un	-	-	10	10>	10
7	2	F	L	-	Un	+	-	10>	30	70
8	1	F	L	-	Well	-	+	50	70	60
9	1	M	L	-	Un	+	+	10>	20	20
10	1	F	R	-	Un	-	-	30	80	80
11	3	M	R	-	Un	-	-	10>	10	10
12	2	M	L	-	Un	+	+	10>	20	50
13	0.5	F	L	-	Well	-	-	10>	20	10>
14	2	F	L	-	Un	-	-	10>	90	30
15	1	F	R	-	Un	-	+	10>	70	>90
16	3	M	L	-	Un	-	+	10>	40	<10
17	1	F	L	-	Well	+	-	10>	30	30
18	2	M	R	-	Well	-	-	20	60	30

Diff, differentiation; ON, optic nerve; Un, undifferentiated; Well, well differentiated.