Histol Histopathol (2001) 16: 809-820

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

The immunohistochemical expression of stress-response protein (srp) 60 in human brain tumours: Relationship of srp 60 to the other five srps, proliferating cell nuclear antigen and p53 protein

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Summary. This study analyzed the expression of stress-response (heat-shock) protein 60 (srp 60) in a series of 158 human brain tumours. Immunohistochemical procedures were employed; cells of the human cervical cancer line HeLa S3 exposed to hyperosmolar stress served as positive controls. Deposits of reaction products were found in the cytoplasm. Approximately half of the glioblastomas multiforme (17/31), breast carcinoma metastases (6/10), and lung carcinoma metastases (5/11) as well as about one-third of the astrocytomas (5/13) and meningiomas (8/23) had tumour cells that expressed srp 60. A positive reaction for srp 60 was also seen in some medulloblastomas (2/16), primitive neuroectodermal tumours (PNETs) (2/11), schwannomas (2/21), and pituitary adenomas (2/7), but no positive reactions were observed with oligodendrogliomas and ependymomas. Compared with srp 60-negative tumours, srp 60-positive tumours coexpressed one or more stress-related proteins, among which srp 90, srp 72, srp 27, alphaB-crystallin and ubiquitin occurred with higher frequencies; a high correlation between srp 60 and the other five srps (0.88 - 0.97, $p<0.01$, Pearson correlation coefficient) was observed in srp 60-positive tumours. In contrast, the correlation coefficient in srp 60-negative tumours was not significant (-0.26 - 0.71). There was a tendency for the proliferating cell nuclear antigen (PCNA)-labeling index to be higher in glioblastomas, astrocytomas, medulloblastomas, PNETs, and breast and lung carcinoma metastases that expressed srp 60 than in those that did not. No significant immunohistochemical reactions of srp 60, PCNA and p53 protein were seen with sections of normal brain tissues. We conclude that primary and metastatic tumours of the brain produce srp 60 and that srp 60 in certain brain tumour cells may coexpress the other five srps. In addition, srp 60 expression might depend, in part, on proliferating potential.

Key words: Brain tumours, Stress-response protein 60, Stress-response proteins (heat-shock proteins), Proliferating cell nuclear antigen, p53 protein

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Introduction

Under various conditions of harmful stresses, stress-response proteins (srps) are induced in prokaryotic and eukaryotic species, as a means for protection (Lindquist and Craig, 1988; Morimoto et al., 1990; Ellis and van der Vies, 1991). There have been extensive studies to evaluate the physiological significance of these srps in the maintenance of cell integrity at the cellular level (Lindquist and Craig, 1988; Morimoto et al., 1990; Ellis and van der Vies, 1991). These srps are classified into different families according to their apparent molecular mass. The srp 60 kDa family (srp 60), which retains an uniquely high level of sequence conservation during evolution, is expressed in response to severe circumstances at the cellular level (Jindal et al., 1989; Itoh et al., 1995).

Recently, srp 60 has been considered to be involved in the pathogenetic mechanism of autoimmune diseases (Lanks, 1986; Haregewoin et al., 1989, 1991; Selmaj et al., 1991, 1992; Boog et al., 1992; Lohse et al., 1993; Brosnan et al., 1996). Srp 60 is reported to develop in the target tissues of autoimmune diseases such as synovial membranes in juvenile chronic arthritis (Boog et al., 1992), oligodendrocytes in multiple sclerosis (Selmaj et al., 1991, 1992; Brosnan et al., 1996) and hepatocytes in autoimmune hepatitis (Lohse et al., 1993). Furthermore, srp 60 is thought to be one of the epitopes of gamma delta T lymphocytes that play an important role in immune responses (Lanks, 1986;

Haregewoin et al., 1989, 1991). When srp 60 is induced to protect the self structure from harmful stresses, the gamma delta T lymphocytes recognize the srp 60 as an antigen (Haregewoin et al., 1989, 1991). These gamma delta T cells attack the target cells expressing srp 60 as well as the activated alpha beta T cells, resulting in persistent inflammation; i.e., an autoimmune inflammation (Collins and Hightower, 1982; Haregewoin et al., 1989, 1991; Selmaj et al., 1991, 1992; Boog et al., 1992; Ferm et al., 1992; Lohse et al., 1993; Xu et al., 1994; Kato et al., 1995; Brosnan et al., 1996; Martins et al., 1996).

Among the primary brain tumours, glioblastoma multiforme (GBM) is one of the most malignant tumours (Kleihues et al., 1993; Lantos et al., 1997). This type is histologically characterized by pseudopalisading around areas of tumour necrosis, mitotic activity, poorly differentiated fusiform cells, pleomorphic cells including multinucleated giant cells, vascular proliferation including endothelial proliferation, and often mononuclear cell infiltration including perivascular lymphocytic cuffing. The lymphocytic cuffing, which predominantly consists of T cells, may represent the host immune system's immune response to GBM cells (Lantos et al., 1997). Lymphocytic cuffing is thought to be a disadvantageous response to the GBM cells themselves: there are some reports that the GBM patients with lymphocytic cuffing have a better prognosis compared to the GBM patients without the lymphocytic cuffing (Brooks et al., 1978; Palma et al., 1978). However, other researchers failed to note any correlation with patient survival (Burger and Vollmer, 1980; Ito et al., 1994). It has even been reported that the presence of mononuclear cell infiltration in malignant glioma is associated with a poor prognosis (Safdari et al., 1985). To date, little is known about the exact mechanism of the mononuclear cell infiltration in GBM.

Although GBMs cannot readily be compared with autoimmune diseases such as juvenile arthritis, multiple sclerosis and autoimmune hepatitis, the perivascular lymphocytic cuffing observed in GBMs leads us to the notion that GBM cells in the vicinity of this cuffing would express srp 60. Based on these considerations, we have carried out retrospective immunohistochemical studies on srp 60 expression in GBMs, comparing the results with those obtained from other tumours of the central nervous system (CNS) including metastatic brain tumours. Moreover, to gain insight into the biological significance of srp 60 expression in brain tumour cells, comparative immunohistochemical studies were carried out to characterize the expression of the other srp families (srp 90, srp 72, srp 27, alphaB-crystallin, and ubiquitin), proliferating cell nuclear antigen (PCNA) and p53 protein in these tumours.

Materials and methods

Tissue collection

This retrospective study was carried out on biopsy samples of 137 primary brain tumours, biopsy specimens of 10 CNS metastatic breast carcinomas, and autopsy samples of 11 CNS metastatic lung carcinomas. The tumour tissues were fixed in 10% phosphate-buffered formalin (pH 7.4) and then embedded in paraffin. The brain tumours were classified according to the WHO Histological Classification of Tumours (Kleihues et al., 1993). Eight of the 14 astrocytomas were fibrillary, 3 were protoplasmic, and 3 were pilocytic. Of the 11 primitive neuroectodermal tumours (PNETs) examined, 9 were located in the cerebrum and 2 in the spinal cord. Of the 20 meningiomas studied, 10 were classified as meningotheliomatous, 4 as fibroblastic, 5 as transitional, and 1 as psammomatous. We examined autopsy specimens of brain tissues (cerebral cortex, basal ganglia, thalamus, white matter, brain stem, cerebellum, and spinal cord), as well as pituitary glands from 20 neurologically and neuropathologically normal individuals (aged 15-75 years [yr]).

Histopathology and immunohistochemistry

As a control, we included cells of the continuous human cervical tumour line HeLa S3 (Herz et al., 1977; Hatayama et al., 1986; Rattner, 1991). Two types of cultures were examined: cells grown in regular medium

Table 1. Primary antibodies used in this study.

ANTIBODY AGAINST	CLONALITY	DILUTION	SOURCE (REFERENCE)
Stress-response protein 60	M	1:1,000	Clone LK-1; StressGen, Victoria, Canada
Stress-response protein 90	M	1:5,000	Clone 3B6; Affinity BioReagents, Neshanic Station, NJ, USA
Stress-response protein 72	M	1:500	Code RPN1197; Amersham, Little Chalfont, UK
Stress-response protein 27	M	Ready-to-use	Clone G3.1; BioGenex, San Roman, CA, USA
AlphaB-crystallin	P	1:250	Dr. J.E. Goldman (Iwaki et al., 1989, 1990)
Ubiquitin	P	1:800	Dr. S.-H. Yen (Lee et al., 1989)
p53 Protein	M	1:200	Clone DO-1; Oncogene Science, Uniondale, NY, USA
Proliferating cell nuclear antigen	M	1:200	Clone PC10; Oncogene Science, Uniondale, NY, USA
CD45RO	M	1:100	Clone UCHL1; Nichirei, Tokyo, Japan
CD20	M	Ready-to-use	Clone L26; Nichirei, Tokyo, Japan
CD68	M	1:100	Clone PG-M1; Dako, Glostrup, Denmark

M: monoclonal; P: polyclonal.

and cells grown under conditions of hyperosmolar stress (Kato et al., 1992a). Eagle's minimum essential medium containing 10% fetal bovine serum was used. Cells were grown in chamber slides (Herz et al., 1985; Kato et al., 1992a) and the osmolality of one set of cultures was increased from 290 mOsm/kg to 490 mOsm/kg by the addition of 100 mM NaCl (from an autoclaved 3 M stock solution) 24 hours (h) after cell transfer. The cell monolayers were fixed 72 h later and stored before use at 4 °C in phosphate-buffered saline, pH 7.4 (PBS).

Multiple sequential sections (6-micrometers-thick) were prepared from each specimen. One section was stained with haematoxylin and eosin (H&E) and the others were employed in the immunohistochemical assays. The sources of the primary antibodies and the dilutions used are listed in Table 1. Sections were deparaffinized, endogenous peroxidase activity was quenched by incubation for 30 minutes (min) with 0.3% H_2O_2 and then they were washed in PBS. Normal sera homologous with the secondary antibody were used as blocking reagents. Tissue sections were incubated with the antibodies listed in Table 1 for 18 h at 4 °C. Only the HeLa S3 cell monolayers were incubated with the antibody against srp 60. Sections exposed to PBS served as controls. With respect to the preabsorption test, some sections and HeLa S3 cell monolayers were incubated with anti-srp 60 antibody that had been preabsorbed with an excess of the recombinant human srp 60 antigen (StressGen, Victoria, Canada). Bound antibodies were visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using the appropriate Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen. In some sections and some HeLa S3 cell monolayers, methyl green was used as counterstain.

The labeling of two antigens in the same tissue section was also performed. After the visualization of the first immunoreaction products by the ABC method using DAB as a brown chromogen, the DAB sections were rinsed in PBS and twice incubated for 60 min at room temperature with glycine-hydrochloric acid buffer solution (pH 2.2) to elute the bound antibody. The completeness of the elution process was verified by obtaining a negative reaction after repeating the immunohistochemical assay with the appropriate ABC kit employing a secondary antibody and 3-amino-9-ethylcarbazole (AEC) (red, Vector) as chromogen on the eluted sections. Subsequently, the sections were rinsed with PBS and incubated with normal sera homologous with the secondary antibody as blocking reagents. These sections were treated with an avidin-biotin blocking kit (Vector), incubated with the second primary antibody for 18 h at 4 °C, and then the immunoreactivity was visualized by the ABC method using Vector SG (grey/blue, Vector).

For the assessment of PCNA immunostaining, only neoplastic areas delineated by the histological features seen on contiguous, H&E-stained serial sections were evaluated; 1,000 tumour cells from the randomly selected high power fields (x400) were counted, excluding the areas with excessive or scant immunostaining. All neoplastic cell nuclei stained by the respective primary antibody were considered to be PCNA-positive. The PCNA-labeling index (LI) was expressed as the percentage of PCNA-positive tumour cell nuclei. The p53 immunoreaction was considered to be positive when the cell nuclei were stained, irrespective of the percentage of positive cells. All procedures were performed blind by two observers. Each was analyzed by the Fisher exact test, Wilcoxon rank-sum test, and Pearson correlation coefficient.

Results

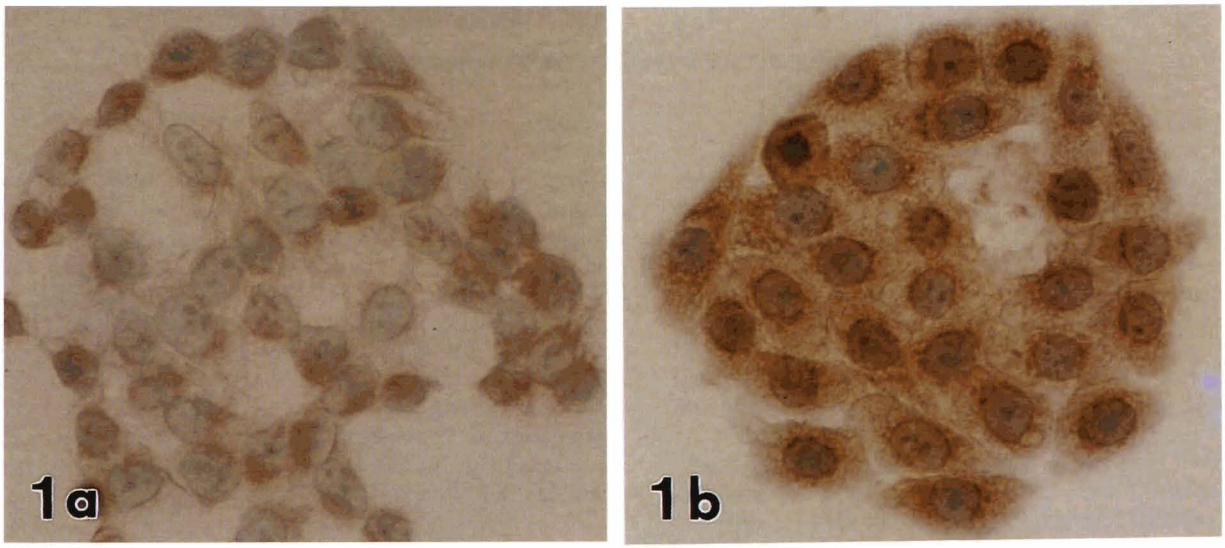
When representative paraffin sections and HeLa S3 cell monolayers were incubated with PBS, no staining was detected. In addition, the anti-srp 60 antibody pretreated with an excess of the recombinant human srp 60 antigen did not stain sections or HeLa S3 cells with any immunoreaction products. No significant reaction was seen with normal human brains and the other normal nervous tissues examined.

Although srp 60-positive HeLa S3 cells were detected in cultures grown in regular medium, the staining intensity of stained cells was not very pronounced (Fig. 1a). As was to be expected (Stege et al., 1995), the staining intensity of srp 60-positive cells in cultures grown under hyperosmolar conditions was greater than that in cells grown in regular medium: the deposits of reaction products were found in the cytoplasm (Fig. 1b).

The staining intensity and proportion of positively-stained versus unstained tumour cells varied from sample to sample. Cytoplasmic staining with anti-srp 60 antibody was observed in 17 of the 31 GBMs (Figs. 2, 3). Table 2 listed the frequencies of srp 60 immunoreactivity and perivascular cuffing among the 31 specimens with GBMs. In 13 (41.9%) of the 31 GBMs examined, perivascular cuffing was evident. Whether the occurrence of perivascular cuffing coincided with srp 60 immunoreactivity was determined by the Fisher exact test, yielding a probability of 0.1582. Statistically, the presence of perivascular cuffing was not more common in GBM specimens with srp 60-positive tumour cells as compared to those with srp 60-negative cells: there was no specific linkage between the srp 60 expression and the presence of perivascular cuffing. Even in srp 60-

Table 2. Relationship between stress-response protein (srp) 60 immunoreactivity and perivascular cuffing in 31 specimens in patients with glioblastomas multiforme.

	SRP 60 IMMUNOREACTIVITY		TOTAL
	Positive	Negative	
Perivascular cuffing	9	4	13
No perivascular cuffing	8	10	18
Total	17	14	31



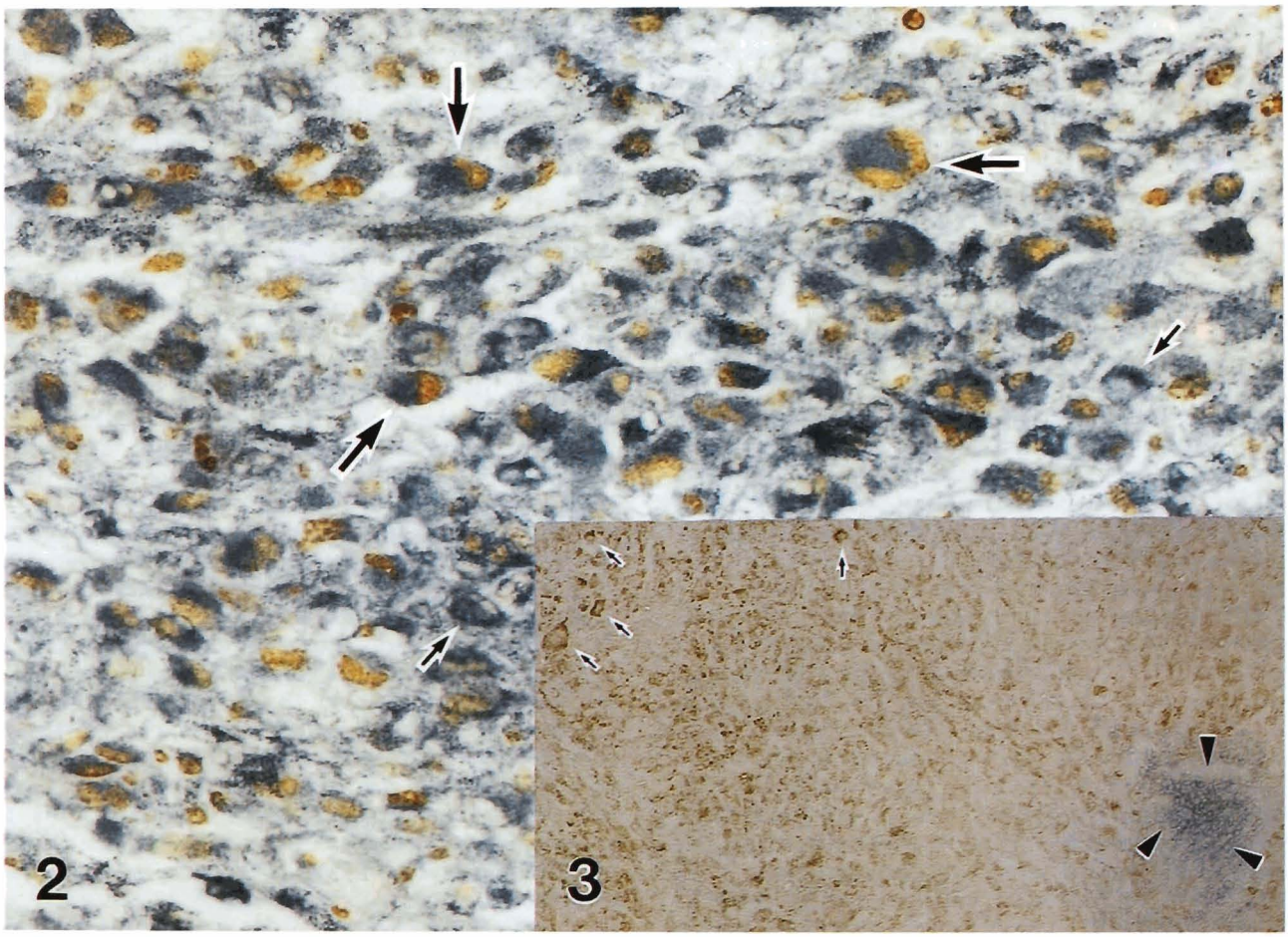
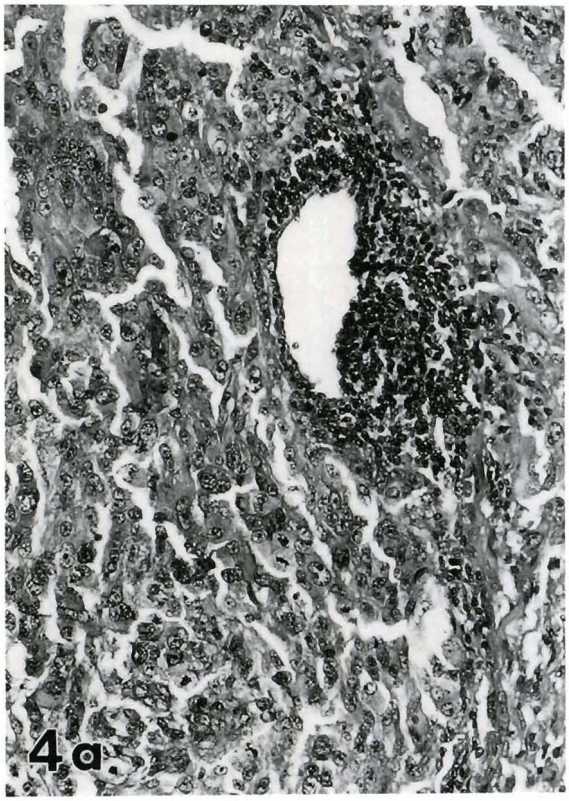
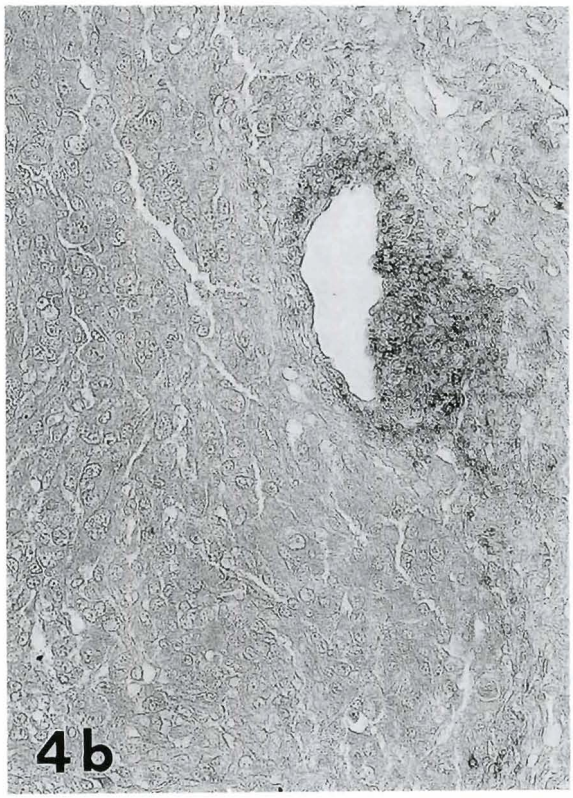


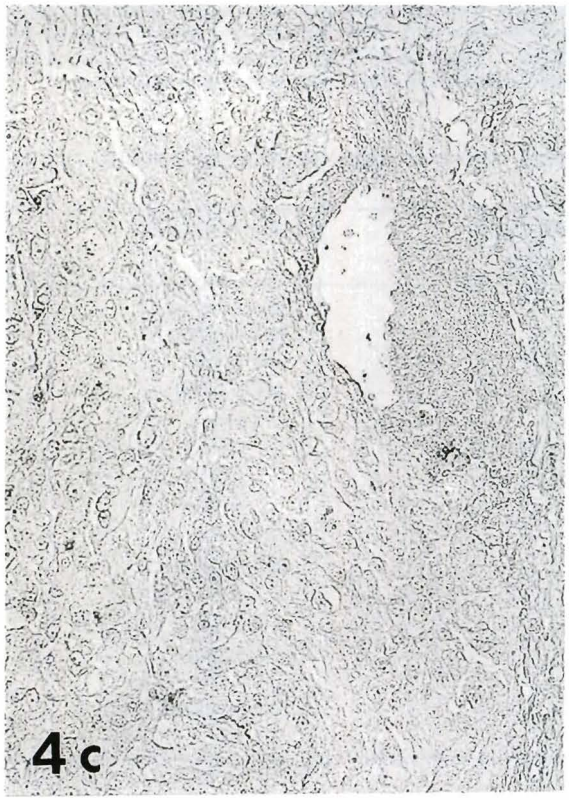
Fig. 1. Immunocytochemical demonstration of stress-response protein (srp) 60 in the human cervical tumour cell line HeLa S3. **a.** Srp 60 of cells grown in regular medium; some cells are positively stained. **b.** Srp 60 of cells grown for 72 h in hyperosmolar medium. The number of positive cells is increased and staining intensity is greater than in cells in regular medium (**a**). Counterstained with methyl green. x 725

Fig. 2. Light micrograph of a glioblastoma multiforme. Section stained by double-labeling with antibody against srp 60 (LK-1) using Vector SG (grey/blue) and antibody to proliferating cell nuclear antigen (PCNA) (PC10) using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (brown). Cytoplasmic staining with anti-srp 60 antibody (grey/blue) is observed in many tumour cells, and nuclear staining with anti-PCNA antibody (brown) is also seen. Over 30% of the tumour cells are intensely stained by anti-srp 60 antibody, and this tumour shows a high PCNA-labeling index, with an average of 49.0%. Many srp 60-positive tumour cells have PCNA-positive nuclei (large arrows), but some srp 60-positive cells have PCNA-negative nuclei (small arrows). No counterstaining. x 545

Fig. 3. Light micrograph of a glioblastoma multiforme. Double labeling technique using anti-srp 60 antibody (brown) and anti-CD45RO antibody (UCHL1) (grey/brown). Srp 60-positive tumour cells (arrows) are observed not only at the marginal region of the CD45RO-positive cell (T cell) infiltration (arrowheads) but also at the distant region from the CD45RO-positive cell infiltration. No counterstaining. x 150







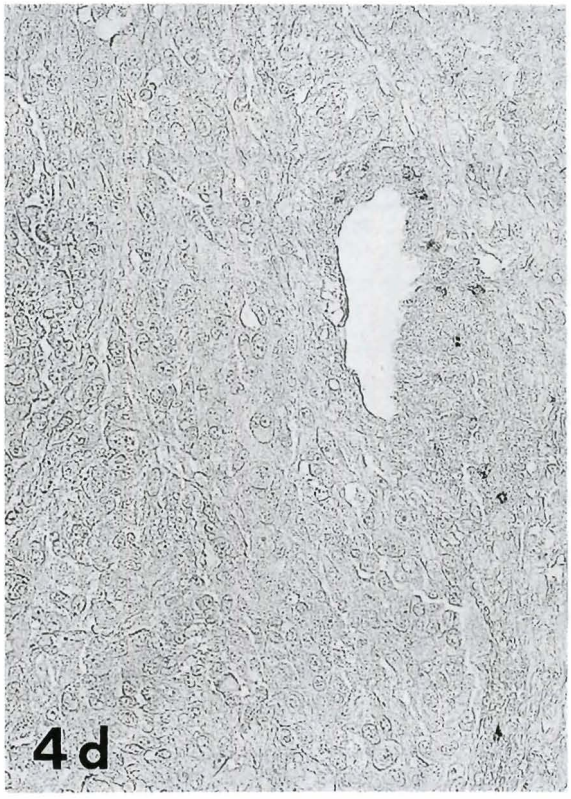


Fig. 4. Serial sections **(a-d)** of perivascular cuffing in a glioblastoma multiforme. Perivascular cuffing mainly consists of mononuclear cells **(a)**. A predominance of CD45RO (UCHL1)-positive cells (T cells) can be observed **(b)**. CD68 (PG-M1)-positive cells (macrophages) cannot be seen in this cuffing **(c)**, but CD20 (L26)-positive cells (B cells) are present in smaller numbers **(d)**.
a, Haematoxylin and eosin, x 235;
b, CD45RO, No counterstaining, x 235;
c, CD68, No counterstaining, x 235;
d, CD20, No counterstaining, x 235

Table 3. Relationship of stress-response protein (srp) 60 to the other five srps (srp 90, srp 72, srp 27, alphaB-crystallin and ubiquitin), p53 protein, and proliferating cell nuclear antigen labeling index (PCNA-LI) in 137 primary brain tumours and 21 brain metastases.

DIAGNOSIS	Srp 60		Srp 90	Srp 72	Srp 27	αBC	Ubi	p53	PCNA-LI
	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(%)*
GBM	17(54.8%)		10	10	11	13	7	13	24.8±3.3
[31]		14	3	2	6	7	0	10	18.1±1.8
Ast	5(38.5%)		3	1	1	4	1	2	1.5±0.6
[13]		8	3	2	0	2	0	3	1.3±0.6
Olig	0(0%)		0	0	0	0	0	0	0
[10]		10	0	0	0	0	0	3	1.7±0.5
Epen	0(0%)		0	0	0	0	0	0	0
[5]		5	1	1	1	3	0	3	2.5±0.9
Med	2(12.5%)		2	2	0	1	0	1	27.2, 40.1
[16]		14	1	0	1	2	0	4	17.4±2.5
PNET	2(18.2%)		1	1	1	1	0	0	23.4, 39.0
[11]		9	1	1	1	0	0	1	16.6±3.0
Schw	2(9.5%)		1	1	2	2	0	1	1.3, 1.6
[21]		19	1	1	16	14	1	8	1.3±0.2
Men	8(34.8%)		6	7	5	1	1	2	1.5±0.3
[23]		15	5	6	2	0	1	6	1.4±0.6
Pt-Ad	2(28.6%)		0	1	2	0	1	0	0.3, 1.8
[7]		5	0	1	0	1	0	1	1.2±0.4
Br-Mt	6(60.0%)		4	6	6	2	2	2	26.5±5.2
[10]		4	1	2	2	0	1	2	21.6±7.9
L-Mt	5(45.5%)		2	2	3	0	1	2	11.6±3.9
[11]		6	0	0	0	0	2	2	9.0±2.3

Srp 90: Stress-response protein 90; Srp 72: stress-response protein 72; Srp 27: stress-response protein 27; αBC: alphaB-crystallin; Ubi: ubiquitin; p53: p53 protein; PCNA-LI: proliferating cell nuclear antigen labeling index. (+): positive; (-): negative. GBM: glioblastoma multiforme; Ast: astrocytoma; Olig: oligodendroglioma; Epen: ependymoma; Med: medulloblastoma; PNET: primitive neuroectodermal tumour; Schw: schwannoma; Men: meningioma; Pt-Ad: pituitary adenoma; Br-Mt: breast carcinoma metastasis; L-Mt: lung carcinoma metastasis. *: values each represent a mean ± standard error of the mean. The total number of each tumour type is in brackets. For each type of tumour, the percentage expressing a given protein is in parentheses.

Table 4. Comparison of the expression of stress-response protein (srp) 60 and proliferating cell nuclear antigen labeling index (PCNA-LI) in srp 60-positive primary and metastatic brain tumours.

DIAGNOSIS	PERCENTAGE OF SRP 60- POSITIVE TUMOR CELLS			PCNA-LI RANGE (%)
	<15%	15-30%	>30%	
Glioblastoma	12			6.8-41 (19.2)a
multiforme		3		13.4-49 (34.1)
			2	41.0, 49.0
Astrocytoma	5			0.1-5.2 (1.5)
Medulloblastoma	1			27.2
			1	40.1
Primitive	1			23.4
neuroectodermal tumour		1		39.0
Schwannoma	2			1.3, 1.6
Meningioma	7			0.5-2.5 (1.3)
		1		2.9
Pituitary adenoma	2			0.3, 1.8
Breast cancer	4			3-32 (22)
metastasis		2		30, 41
Lung cancer	4			2.1-17.7 (9.0)
metastasis		1		22

a: the mean value of the PCNA-LI of given tumours is in parentheses.

positive specimens with CD45RO-positive cell (T cell) infiltration, srp 60-positive tumour cells were observed not only at the marginal regions of the CD45RO-positive cell infiltration but also at the distant regions from CD45RO-positive cell infiltration (Fig. 3). Namely, srp 60 expression and the perivascular cuffing were independent of each other. Immunohistochemical results of the perivascular mononuclear cell phenotypes revealed that a predominance of CD45RO-positive cells (T cells) was generally observed and that CD68-positive cells (macrophages) and CD20-positive cells (B cells) appeared to be present in smaller numbers (Fig. 4a-d).

The results on 137 primary brain tumours and 21 carcinoma metastases to the CNS are summarized in Table 3. Approximately half of the GBMs (17/31), breast carcinoma metastases (6/10), and lung carcinoma metastases (5/11) as well as about one-third of the astrocytomas (5/13) (Fig. 5) and meningiomas (8/23) had tumour cells that expressed srp 60. An srp 60-positive reaction was also seen in some medulloblastomas (2/16) (Fig. 6), PNETs (2/11), schwannomas (2/21), and pituitary adenomas (2/7), but no positive reactions were observed with the oligodendrogliomas and ependymomas.

Table 3 also revealed a striking variability with respect to expression of the proteins examined. Although the staining intensity and proportion of positively-stained tumour cells varied, the reaction products with the antibodies to srp 60, srp 90, srp 72, srp 27 and alphaB-crystallin were seen only in the cytoplasm; patchy staining patterns were observed in the specimens, including those with cytoplasmic staining for ubiquitin. No significant immunoreactivity with respect to p53 was seen in normal brain tissues. In contrast, the brain tumour specimens were stained by the antibody to p53 protein; granular or diffuse reaction product deposits were seen. However, the staining intensity varied from one tumour cell to another. Normal brain tissues were consistently negative for PCNA. By comparison, diffuse or granular nuclear staining was observed in the tumour tissues, with the reaction intensity varying among the PCNA-positive cells.

Only the oligodendrogliomas failed to be immunostained by the anti-srp 60 antibody as well as the other antibodies against srp 90, srp 72, srp 27, alphaB-

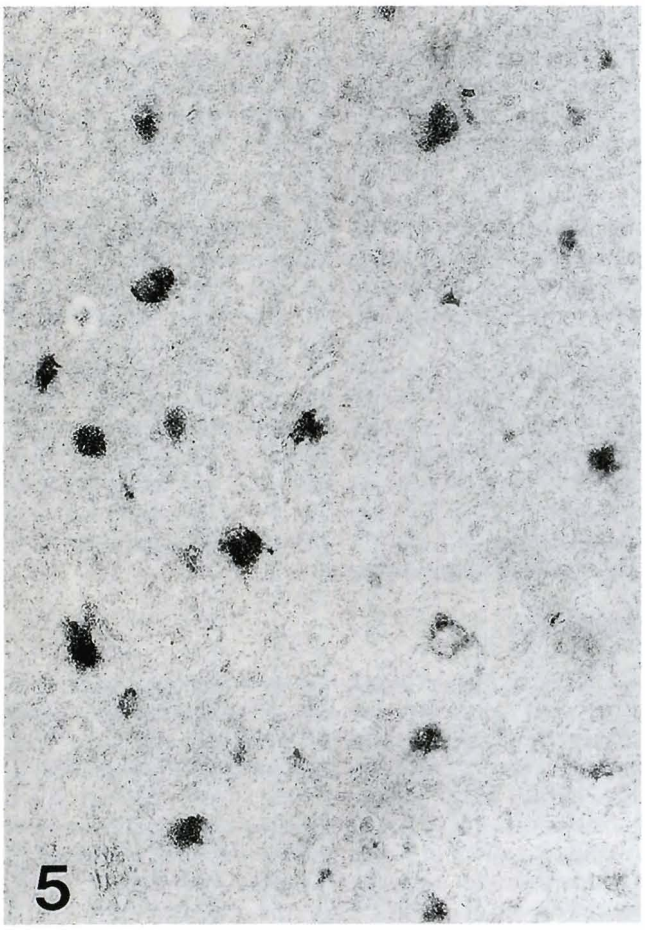


Fig. 5. Immunostaining for srp 60 of a protoplasmic astrocytoma. Several tumour cells with a positively-stained cytoplasm can be seen. No counterstaining, x 230

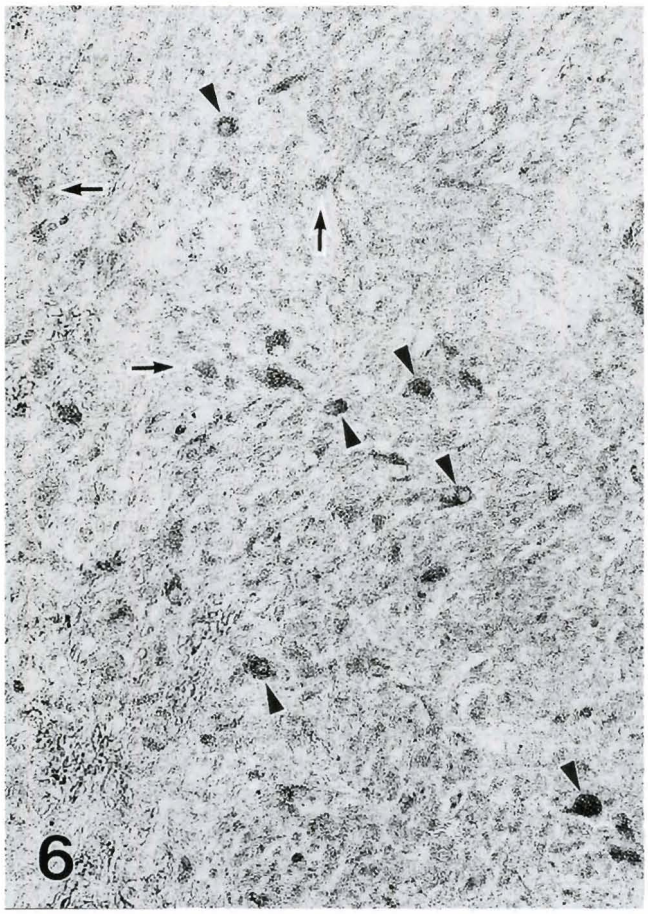


Fig. 6. Immunostaining for srp 60 of a medulloblastoma. The cytoplasm of the tumour cells is positively stained. The staining intensity varies from one tumour cell to another. Some tumour cells are intensely stained (arrowheads), but others are weakly stained (arrows). Weakly counterstained with methyl green, x 230

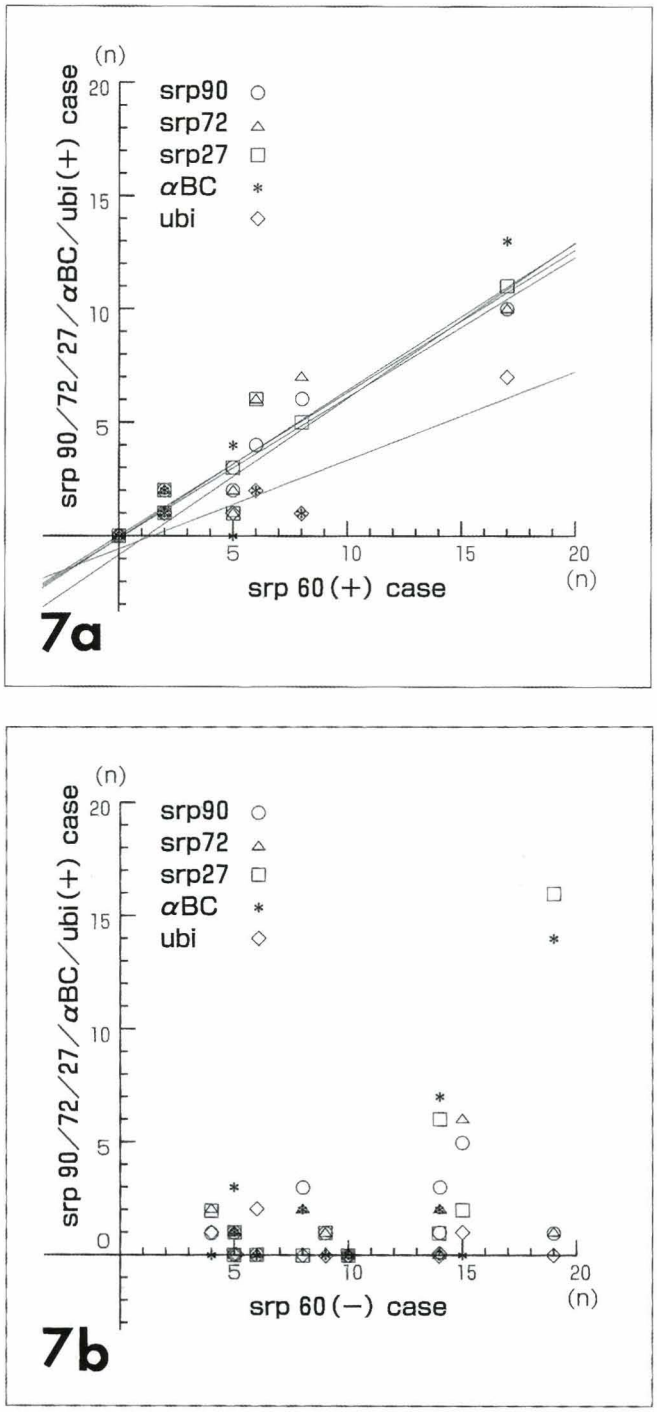


Fig. 7. Correlation between srp 60 and the other five srps (srp 90, srp 72, srp 27, alphaB-crystallin or ubiquitin) in srp 60-positive samples **(a)** and in srp 60-negative samples **(b)**. **a.** In the srp 60-positive group, a high correlation between srp 60 and the other five srps is observed. Srp 60 and srp 90 data (open circles): Pearson correlation coefficient (r) = 0.97, y = 0.62x - 0.11, $p < 0.01$. Srp 60 and srp 72 data (open triangles): r = 0.92, y = 0.63x + 0.02, $p < 0.01$. Srp 60 and srp 27 data (open squares): r = 0.94, y = 0.65x - 0.10, $p<0.01$. Srp 60 and alphaB-cristallin data (αBC, asterisks): r = 0.88, y = 0.69x - 0.87, $p<0.01$. Srp 60 and ubiquitin data (ubi, open diamonds): r = 0.94, y = 0.39x - 0.57, $p<0.01$. **b.** In the srp 60-negative group, the correlation coefficient (-0.26 - 0.71) is not significant. (n) = number, (+) = positive, (-) = negative.

crystallin and ubiquitin. All other tumour types had cells that were positively stained for one or more srps. The proportion of tumour types positively immunostained for a given srp varied. When we focused on the srp 60-positive tumours in comparison to the srp 60-negative tumours, it is evident that the srp 60- positive tumours expressed one or more stress-related proteins (i.e., srp 90, srp 72, srp 27, alphaB-crystallin and ubiquitin) with higher frequencies. Most notably, the srp 60-positive GBMs showed higher expressions of srp 90 (10/13), srp 72 (10/12), srp 27 (11/17), alphaB-crystallin (13/20) and ubiquitin (7/7). Quantitatively, similar observations were made in breast cancer metastases and lung cancer metastases: in the srp 60-positive breast cancer metastases, higher frequencies of positive reactions for srp 90 (4/5), srp 72 (6/8), srp 27 (6/8), alphaB-crystallin (2/2) and ubiquitin (2/3) were observed. Likewise, srp 60-positive lung cancer metastases showed higher frequencies of srp 90 (2/2), srp 72 (2/2), srp 27 (3/3) and ubiquitin (1/3). Namely, a high correlation between srp 60 and the other five srps (0.88 - 0.97, $p<0.01$, Pearson correlation coefficient) was noted in srp 60-positive tumours (Fig. 7a). In contrast, the correlation coefficient in srp 60-nagative tumours was not significent (-0.26 - 0.71) (Fig. 7b).

Although we could recognize no statistically significant relationship between srp 60 expression and PCNA-LI in primary and metastatic brain tumours, there was a tendency for the PCNA-LI to be higher in GBMs, astrocytomas, medulloblastomas, PNETs, breast carcinoma metastases and lung carcinoma metastases that expressed srp 60 than in those that did not (Table 3). Especially, GBMs, medulloblastomas, PNETs, breast carcinoma metastases and lung carcinoma metastases with a high proportion of cells expressing srp 60 tended to have a higher PCNA-LI than those with a low proportion of srp 60-positive cells (Table 4). Thus, the highest PCNA-LI (41.0% and 49.0%) was seen in the two GBMs that had more than 30% srp 60-positive cells. Compared with those two GBMs, the 12 GBMs with less than 15% srp 60-positive cells had a lower PCNA-LI (average 19.2%). No evidence of a relationship between srp 60 expression and p53 protein was seen in primary and metastatic brain tumours.

Discussion

Adverse physiological and environmental conditions cause cells to produce a variety of ubiquitous and highly conserved proteins, collectively called "stress-response proteins (srps)" (Lindquist and Craig, 1988; Morimoto et al., 1990; Ellis and van der Vies, 1991). As a consequence, these proteins also present under normal conditions, albeit in much smaller amounts, increase and accumulate in cells (Lindquist and Craig, 1988; Morimoto et al., 1990; Ellis and van der Vies, 1991). Much information has accumulated regarding the role of srps in cells and tissues subjected to stress (Lindquist and Craig, 1988; Morimoto et al., 1990; Ellis and van

der Vies, 1991), and a variety of functions has been ascribed to them under physiological conditions. They include synthesis, assembly and translocation of proteins (Chirico et al., 1988; Kang et al., 1990); binding of ATP (Milarski and Morimoto et al., 1989); signal transduction (Michishita et al., 1991); regulation of cell proliferation (Pechan, 1991); and complexing with the nuclear phosphoprotein encoded by the tumour suppresser gene p53 (Pinhasi-Kimhi et al., 1986; Finlay et al., 1988). The role of srps in apoptosis (Schett et al., 2000) and tumorigenesis (Soldes et al., 1999) has also reported. Among srp families, srp 60 has been shown to be one of the critical antigens in a number of autoimmune diseases (Collins and Hightower, 1982; Lanks, 1986; Haregewoin et al., 1989, 1991; Selmaj et al., 1991, 1992; Boog et al., 1992; Ferm et al., 1992; Lohse et al., 1993; Xu et al., 1994; Brosnan et al., 1996; Martines et al., 1996). The first member of this srp 60 family to be recognized was the GroEL protein that was identified as a protein essential for the morphogenesis of bacteriophage lambda, functioning as a scaffold for the assembly of the head and tail whilst not becoming incorporated into the final assembled phage (Itoh et al., 1995; Brosnan et al., 1996). Homologous forms of this protein have been identified in most living organisms, but in eukaryotes, constitutive expression of srp 60 is principally associated with mitochondria where it participates in the folding and assembly of transported proteins into the mitochondrion in an ATP-dependent manner (Itoh et al., 1995; Brosnan et al., 1996).

Monoclonal antibodies that are unique for the bacterial or mammalian forms of srp 60 have been developed, allowing identification of possible sources of altered expression of this protein in various disease states (Itoh et al., 1995; Brosnan et al., 1996). Especially, the monoclonal antibody LK-1 used in this study recognizes an epitope located between amino acids 383-447 of the human srp 60 sequence (Boog et al., 1992). This amino acid sequence does not share homology with any subunit of chaperonin containing TCP-1 (CCT) in Protein Bank. Together with the fact that srp 60 has been reported to be expressed in response to conditions of cell stress at the cellular level (Jindal et al., 1989; Itoh et al., 1995), HeLa S3 cells exposed to hyperosmolar stress reacted strongly with the monoclonal antibody LK-1. In the CNS tumours found to express srp 60, the stained cells were accompanied by tumour cells that failed to react with the monoclonal antibody against srp 60. This lack of uniformity, also encountered with CNS tumours examined for the expression of srp 90 (Kato et al., 1995), srp 72 (Kato et al., 1992c, 1993), srp 27 (Kato et al., 1992b, 1993), alphaB-crystallin (Kato et al., 1993) and ubiquitin (Kato et al., 1993), could reflect tumour heterogeneity, as well as the possibility that stress is being exerted on some, but not all the cells of the same tumour cell population. Our data that the srp 60-positive brain tumours have coexpressed the other five srps with higher frequencies are a novel finding suggesting that srp 60 in brain tumour cells may function in concert with the other srps (90 kDa, 72 kDa, 27 kDa, alphaB-crystallin and ubiquitin) in response to stress conditions; a high correlation between srp 60 and the other five srps (0.88 - 0.97, $p<0.01$, Pearson correlation coefficient) was observed in srp 60-positive tumours. In contrast, the correlation coefficient in srp 60-negative tumours was not significant (-0.26 - 0.71). This is compatible with the Hartl's hypothesis that srp 60 functions in combination with the other srps in highly coupled sequential chaperone pathways (Hartl and Martin, 1995).

As for the relationship between srp 60 expression in tumour cells and mononuclear cell infiltration including T cell lymphocytes, we could not identify a specific linkage between the two phenomena. Therefore, the possibility has to be considered that increased srp 60 expression by certain CNS tumour cells may be an intrinsic characteristic of some neoplasmic cells. This may reflect our finding that HeLa S3 cells constitutively expressed srp 60 in regular medium and the staining intensity of HeLa S3 cells exposed to hyperosmolar shock was amplified.

PCNA, a 36-kDa non-histone nuclear protein, is highly conserved not only in mammals, but also in plants (Suzuka et al., 1989). In eukaryotic cells, this protein is directly involved in the synthesis of leading strand DNA and in DNA repair, acting as a cofactor of DNA polymerase-delta (Bravo et al., 1987; Nishida et al., 1988; So and Downey, 1988). Nuclear accumulation of PCNA begins in the late G_1 phase and reaches a maximum in the S phase of the cell cycle (Celis and Celis, 1985). When the expression of PCNA was used as an indicator of cell proliferation (Morris and Mathew, 1989), we did not find that PCNA-LI of srp 60-positive primary and metastatic brain tumour cells was statistically higher than that of srp 60-negative cells. However, there was a tendency for the PCNA-LI to be higher in glioblastomas, medulloblastomas, PNETs, and metastatic brain tumours that expressed srp 60 in a high proportion of cells. In particular, two GBMs that had more than 30% srp 60-positive cells also had high growth potential. This suggests that srp 60 might play a role in the proliferation of diverse brain tumour cells. However, each individual tumour cell observation failed to demonstrate that all srp 60-expressing cells were indeed PCNA-positive nuclei-bearing cells, i.e., proliferating cells. To conclusively elucidate the relationship between srp 60 expression and PCNA expression for individual tumour cells, a more complete understanding of the molecular mechanism of srp 60 expression in human brain tumour cells would be necessary.

Wild-type (normal) p53 protein is a 53 kDa, 393 amino acid nuclear phosphoprotein that functions as a check point controller in the G_1 phase of the cell cycle in response to DNA damage (Lane, 1992; Louis, 1994). The human p53 gene is located on chromosome 17p13.1 (Benchimol et al., 1985). Deletion of one allele (loss of heterozygosity) is associated with human brain tumours (el-Azouzi et al., 1989; Fultz et al., 1989; James et al.,

1989; Nigro et al., 1989; Frankel et al., 1992). Furthermore, the normal function of the other allele is impaired by a point mutation that results in the loss of the normal function of the gene product (Lane, 1992). Although the half-life of wild-type p53 protein of most cells is only 20 to 30 min, the prolonged half-life of mutant p53 protein allows its immunohistochemical detection (Levine et al., 1991). This reflects our finding that normal brain tissues display no significant reactivity with respect to p53 protein. There is evidence that mutant p53 protein preferentially binds to srps, especially the srp 70 family, and forms a p53-srp complex that extends the protein's half-life (Pinhashi-Kimhi et al., 1986; Finlay et al., 1988). Although no direct correlation between srp 60 and p53 protein was statistically confirmed in primary and metastatic brain tumours, the observation that certain intracranial tumours express srp 60 showed that the p53 gene as a normal tumour suppressor gene was impaired.

Although srp 60 has been detected in primary brain tumours and CNS metastases, the ultimate biological significance of these observations and their possible relationship to tumour behavior have not been established. Nevertheless, it would appear that in certain primary and metastatic tumours of the brain, srp 60-positive tumours have expressed the other five srps (90 kDa, 72 kDa, 27 kDa, alphaB-crystallin and ubiquitin) with higher frequencies. In addition, there seems to be a tendency for the PCNA-LI to be higher in these primary and metastatic brain tumours that have expressed srp 60 in a higher proportion of cells: srp 60 expression might depend, in part, on the proliferating potential. Whether these considerations apply to brain tumours and metastases to the CNS, whose cells do not have normal proliferation control mechanisms, and how the present findings on srps expression and proliferating capacity of a given tumours are correlated, remain to be determined.

Acknowledgements. We express our appreciation to Dr. James E. Goldman for kindly providing the antibody against alphaB-crystallin and Dr. Shu-Hui Yen for the antibody to ubiquitin.

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Accepted April 16, 2001