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Evaluation of Apoptosis and Immunohistochemical Expression of the Apoptosis-related Proteins in Mesothelioma

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ABSTRACT

We evaluated apoptosis and the expression of apoptosis-related proteins in 3 mesothelioma cell lines and 34 paraffin-embedded tissue specimens. Apoptosis was evaluated by the TUNEL method, while expression of the apoptosis-related proteins, bax, bcl-2, survivin, caspase-3 and cleaved caspase-3 was evaluated by immunohistochemical staining. The mean apoptotic index of mesothelioma tissue was 17.6 (ranging from 0 to 41.9), which was significantly lower than that of other carcinomas. Thirty-one of 34 cases showed caspase-3 expression. However, the cleaved caspase-3 index in mesothelioma was only 14.7 (ranging from 0 to 36.5). There was a direct correlation between apoptotic index and cleaved caspase-3 index (p value = 0.03). All cases of mesothelioma tissue showed bax expression, while only 2 cases showed bcl-2 expression. Thirty of 31 mesothelioma cases showed cytoplasmic expression of survivin, and 16 cases out of 30 cases showed diffuse staining while 11 cases showed strong staining. Three mesothelioma cell lines also showed high cytoplasmic expression of bax, caspase-3 and survivin, while there was no expression of bcl-2, and apoptosis and cytoplasmic expression of cleaved caspase-3 were limited. mRNA expression of survivin was confirmed by RT-PCR and its protein was confirmed by western blotting. In conclusion, apoptosis is an uncommon event in mesothelioma and low mean cleaved caspase-3 index, suggesting the role of low activation of caspase-3 for inhibition of apoptosis. High expression of survivin in mesothelioma may play a role in inhibition of apoptosis.

Key words: *Mesothelioma, Apoptosis, Survivin, Caspase-3*

Mesothelioma, arising from the mesothelial cell linings of the pleural, peritoneal and pericardial cavities and tunica vaginalis, is mainly induced by exposure to asbestos[1]. Although the latent period between the initial exposure to asbestos and the development of mesothelioma ranges from 15 to 40 years of exposure to asbestos[14], mesothelioma is characterized by rapid growth and a grim prognosis.

Cell homeostasis is maintained by the balance between proliferation, growth-arrest, and apoptosis. It has been proposed that neoplastic cells acquire resistance to apoptosis by overexpression of the inhibitor of apoptosis proteins (IAPs)[5].

Apoptosis is primarily implemented by a family of cysteine proteases called caspases. Caspases are normally present in the cell in an inactive proenzyme form and require limited proteolysis for enzymatic activity[13] Activated caspase-3 cleaves DNA Fragmentation Factor-45 (DFF-45), leading to DNA fragmentation and apoptosis[10]. Two important groups of proteins that regulate apoptosis are Bcl-2 family and the inhibitor of apoptosis proteins (IAPs). The Bcl-2 family consists of pro-apoptotic protein, bax, which down-regulates anti-apoptotic protein, bcl-2. Bcl-2 inhibits the release of cytochrome c, leading to caspase-9 activation, and Smac, inhibiting function of IAPs[20]. To date, eight human IAPs have been identified: c-IAP1, c-IAP2, neuronal apoptosis inhibitory protein, survivin, XIAP, apollon, testis-specific IAP, and livin[2]. In general, IAPs inhibit the apoptotic action of caspases by preventing proteolytic cleavage of caspase proforms and/or directly inhibiting activated caspases[4]. Among these IAPs, the strongest evidence for IAP involvement of survivin in cancer has been report-

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ed[8]. Overexpression of survivin has been reported in human cancers, including mesothelioma[7, 21].

The aim of this study was to examine the level of apoptosis in mesothelioma and the expression of apoptosis-related proteins: survivin, bcl-2, bax, caspase-3 and cleaved caspase-3.

MATERIALS AND METHODS

Cell lines and tissue samples

Three mesothelioma cell lines were obtained from the following sources: ACC-MESO-1[19] and ACC-MESO-4[19] from the RIKEN BioResource Center (Tokyo, Japan) and NCI-H2452 from the American Type Culture Collection (Manassas, VA, USA). All of the mesothelioma cells were cultured in RPMI-1640 Glutamax supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2.

Thirty-four cases of mesothelioma, including 23 of the epithelioid type, 9 of the sarcomatoid type and 2 of the biphasic type, were obtained from the surgical and autopsy archives of the Department of Pathology, Hiroshima University. The male to female ratio was 26:8, and the average age of the mesothelioma patients was 59.2 years (range, 23-81 years). The microscopic slides were reviewed and reclassified by three pathologists (V.J.A., Y.T. and K.I.) using the current histological classification of lung and pleural tumors (WHO), 2004[1].

Apoprosis assays by TUNEL immunostaining

Apoptosis was determined by the TUNEL method (terminal deoxynucleotidyl transferase end labeling) using the ApopTag Peroxides *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA), following the manufacturer's instructions with a slight modification. In brief, after deparaffinization and rehydration, the sections in aminopropyltriethoxysilane-coated slides were incubated with Proteinase K (20 μg/ml, TaKaRa, Shiga, Japan) at room temperature for 15 min. Endogenous peroxidase activity was quenched in 3.0% hydrogen peroxide in PBS (pH 7.2). The free 3'-OH end of DNA termini *in situ* were labeled with digoxigenin-labeled nucleotides by terminal deoxynucleotidyl transferase for 75 min, followed by incubation with antidigoxigenin conjugate. The color was developed by peroxidase substrate containing diaminobenzidine. The sections were lightly counterstained with 2% methyl green. For the control, tissue sections from tonsils showing abundant apoptotic B cells within germinal centers were used. The nuclei of apoptotic cells were indicated by brown coloration.

Determination of the apoptotic index

Apoptotic bodies were defined as small, positively labeled globular bodies in the cytoplasm that could be found either singly or in groups. Apoptotic cells and bodies were counted from several areas of each case. The apoptotic index (AI) was estimated as the number of apoptotic cells and/or bodies per 1000 tumor cells.

Immunohistochemical staining

Immunohistochemical staining was performed on sections from formalin-fixed, paraffin-embedded tissue using Histofine Simple Stain MAX PO (MULTI) kit (Nichirei, Tokyo, Japan). The primary antibodies used in the present study were as follows; survivin (prediluted, Spring BioScience, Pleasanton, CA, USA), bax (1:50, Dako, Glostrup, Denmark), bcl-2 (prediluted, Nichirei BioScience, Tokyo, Japan), caspase-3 (1:50, Cell Signaling Tech., Beverly, MA, USA) and cleaved caspase-3 (1:100, Cell Signaling Tech., Beverly, MA, USA). Antigen retrieval was done by autoclaving the tissue section at 121°C for 20 min, except for survivin which was carried out by microwave for 10 min. The expression of survivin, bax, bcl-2 and caspase-3 was evaluated as follows: -, no immunoreactivity; +, <25% of tumor cells showing cytoplasmic positivity; ++, ≧25% of tumor cells showing cytoplasmic positivity. In addition, the expression of survivin was also evaluated as weak or strong reactivity. The cleaved caspase-3 index was estimated as the number of tumor cells with cleaved caspase-3 expression per 1000 tumor cells.

Survivin expression in mesothelioma cell lines

RNA isolation from 1×10^5 cultured mesothelioma cells followed by reverse transcription to cDNA was performed using the Power SYBR Green Cells-to-CT Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. PCR amplification for survivin (BIRC5bF, TCCGGTTGCGCTTTCCT and BIRC5bR, TCTTC TTATTGTTGGTTTCCTTTGC, 121 bp) and a housekeeping gene (beta-actin sense GCCAACCG CGAGAAGATGA and anti-sense CATCACGATGC CAGTGGTA, 120 bp) was performed by using the KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA, USA) according to the manufacturer's instructions in an Mx3000P real-time PCR system (Agilent Technologies, CA, USA). The cyclic conditions for both the products were as follows: initial denaturation at 95°C for 10 min, 30 cycles of 95°C for 15 sec, and 62°C for 1 min, followed by dissociation analysis from 55°C to 95°C.

Protein was extracted from 1×10^6 mesothelioma cells using the Cell-LyEX1 protein extraction kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer's protocol, and its concentration was determined by a NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Tokyo, Japan). The extracted protein was separated on 10% SDS-PAGE and transferred onto PVDF membrane (HybondTM-LFP, GE Healthcare Bio-Sciences,

Tokyo, Japan). Immuno-labeling with anti-survivin antibody (NB500-237, clone 32.1, 1:500, Novus Biologicals, Littleton, CO, USA) and anti-actin antibody (SC-1616-R, 1:1000, Santa Cruz, CA, USA) using Western Dot 625 Kits (Invitrogen, Eugene, OR, USA) were performed in Western Q (SciTrove Inc, Tokyo, Japan) according to the manufacturer's protocols.

Statistical analysis

Descriptive statistics, Pearson's correlation and Unpaired Student's t-tests were used to determine statistical significance. Statistical significance was attributed to p-values lower than 0.05.

RESULTS

Apoptosis and cleaved caspase-3 expression in mesothelioma tissue and cell lines

The AI in mesothelioma tissue ranged from 0 to 41.9 (17.6 ± 11.3) (Table 1, Fig. 1. C). We also examined mean AI in other cancers including lung, stomach and colon cancers (Table 2). The mean AI of epithelioid mesothelioma (15.2) was lower than that of sarcomatoid mesothelioma (23.5), but it was not statistically significant (p=0.063). The mean AI in mesothelioma cell lines was 63.9 (Table 1, Fig. 1. D).

The cleaved caspase-3 index in mesothelioma ranged from 0 to 36.5 (14.7 ± 10.6) (Table 1, Fig. 1. K). Statistical analysis showed positive correlation between AI and cleaved caspase-3 index (p=0.036), but Pearson's correlation coefficient was not very high (r=0.373) (Table 3). The mean cleaved caspase-3 index in mesothelioma cell lines was 92.2 (Table 1, Fig. L).

Bax, caspase-3, bcl-2 expression in mesothelioma tissue and cell lines

Cytoplasmic expression of bax was found in all (100%) and caspase-3 was found in 31 (91.2%) of 34 of the mesothelioma cases (Table 1, Fig. 1. E, I). Expression of bcl-2 was detected in the cytoplasm of only 2 (5.9%) cases of mesothelioma (Table 1, Fig. 1. G). All three mesothelioma cell lines showed cytoplasmic expression of bax and caspase-3 (Table 1, Fig. 1. F, J), and non-expression of bcl-2 (Table 1, Fig. 1. H).

Survivin expression in mesothelioma tissue and cell lines

The cytoplasmic expression of survivin was found in 30 (96.8%) of 31 mesothelioma cases (Table 1, Fig. 1. M). Among them, 16 (51.6%) cases showed survivin expression in more than 25% of tumor cells and 11 (35.5%) cases showed strong reactivity. All three mesothelioma cell lines also showed cytoplasmic expression of survivin (Fig. 1. N). Survivin expression was also confirmed by expression of mRNA by real time RT-PCR and expression of protein by western blot (Fig. 2).

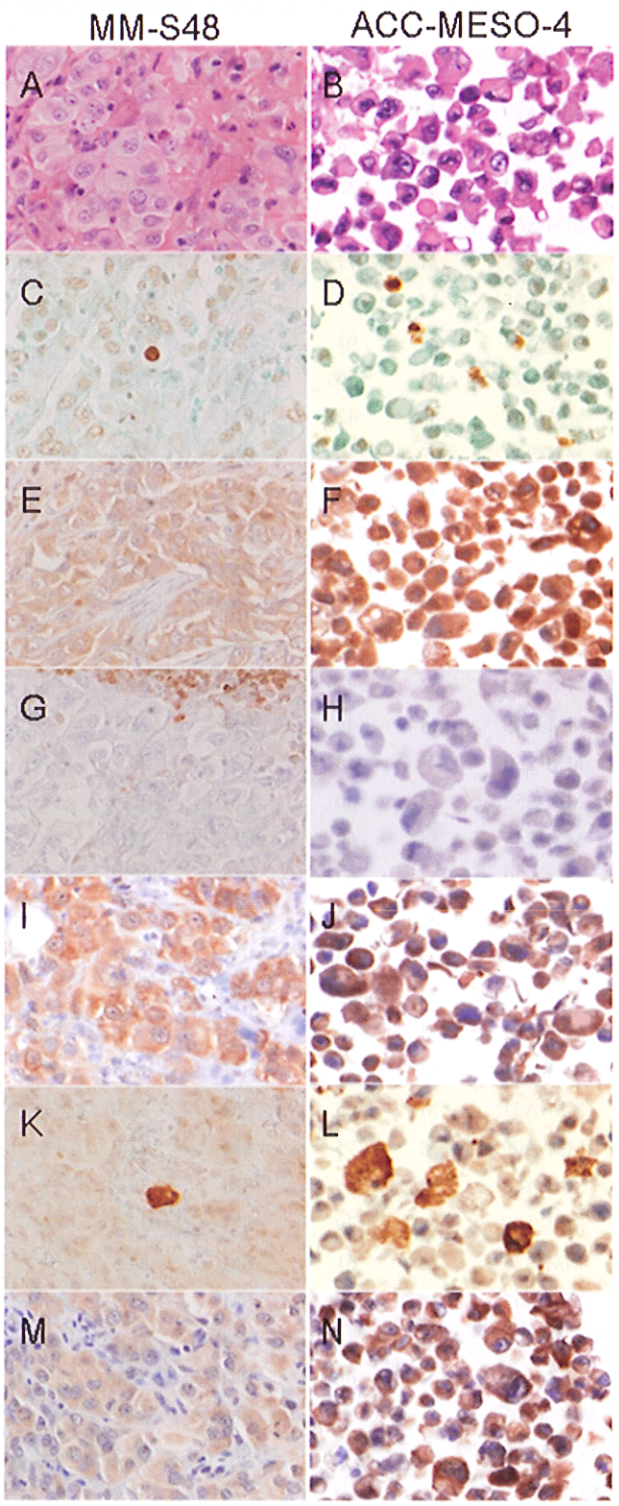

Fig. 1. MM-S48 is epithelioid mesothelioma showing solid growth (A) and ACC-MESO-4 is mesothelioma cell line obtained from epithelioid mesothelioma (B). Very few apoptotic bodies were detected by the TUNEL method (C and D). Immunohistochemically, both of them showed diffuse cytoplasmic expression of bax (E and F), no expression of bcl-2 (G and H), and diffuse cytoplasmic expression of caspase-3 (I and J). Very few tumor cells showed cytoplasmic expression of cleaved caspase-3 (K and L). Survivin expression was detected in the cytoplasm (M and N).

Table 1. Expression of apoptosis related proteins and apoptotic index in mesothelioma tissues and cell lines

Cases	Sex	Age	Histology	AI	Bax[a]	Bcl-2[a]	Survivin[a]	Caspase- 3[a]	Cleaved caspase-3 index[a]
MM-A26	F	52	EM	0	++	-	+/W	+	10.6
MM-S01	M	26	EM	2.0	++	-	++/S	++	15.7
MM-A04	M	23	EM	5.0	++	+	+/S	-	35.6
MM-S46	F	48	EM	6.9	++	-	++/W	++	4.0
MM-S17	M	77	EM	7.0	++	-	++/S	++	14.8
MM-A35	M	57	EM	8.0	++	-	++/S	++	17.4
MM-S16	M	61	EM	9.2	++	-	+/W	++	21.0
MM-S42	M	56	EM	9.8	++	-	++/S	++	2.0
MM-A41	F	42	EM	12.5	++	-	+/S	-	12.3
MM-S39	F	39	EM	12.7	++	-	++/W	++	8.8
MM-S31	M	72	EM	13.9	++	-	++/S	++	19.4
MM-A06	F	74	EM	15.0	++	+	NI	++	2.0
MM-S35	M	46	EM	17.4	++	-	++/S	++	1.0
MM-S41	M	81	EM	17.7	++	-	+/W	++	1.9
MM-S36	M	46	EM	19.8	++	-	+/W	++	14.4
MM-A40	M	75	EM	21.7	++	-	++/W	-	25.8
MM-A19	M	49	EM	22.1	++	-	++/W	+	18.8
MM-S50	F	48	EM	23.1	++	-	++/W	++	0
MM-S52	M	60	EM	32.5	++	-	+/S	++	8.0
MM-S48	M	69	EM	32.9	++	-	+/S	++	18.4
MM-S20	M	67	EM	34.9	++	-	++/W	++	24.4
MM-A10	F	76	EM	NI	++	-	++/W	+	35.1
MM-A30	M	73	EM	NI	++	-	+/W	++	2.0
MM-S15	M	54	SM	5.0	++	-	NI	++	16.0
MM-A02	M	64	SM	7.0	++	-	-	+	1.0
MM-A21	M	49	SM	15.0	++	-	++/W	+	2.0
MM-A31	M	65	SM	22.0	++	-	+/W	+	25.0
MM-A09	M	75	SM	23.0	++	-	+/W	+	3.0
MM-A18	M	65	SM	32.7	++	-	+/W	+	12.3
MM-A38	M	79	SM	32.7	++	-	+/W	+	21.7
MM-S26	M	61	SM	34.5	++	-	NI	++	22.9
MM-S02	M	68	SM	41.9	++	-	++/W	++	36.5
MM-S33	M	51	BM	11.1	++	-	++/S	++	12.8
MM-A39	F	66	BM	23.0	++	-	+/W	++	24.5
ACC MESO 4			EM	58.3	++	-	++/S	++	92.8
ACC MESO 1			SM	57.2	++	-	++/S	+	74.6
NCI-H2452			SM	76.3	++	-	++/S	++	109.2

AI, apoptotic index; BM, biphasic mesothelioma; EM, epithelioid mesothelioma; F, female; M, male; NI, not informative; S, strong; SM, sarcomatoid mesothelioma; W, weak

[a]Details of immunohitochemical scoring in mesothelioma described in methods

Table 2. Apoptotic index in mesothelioma, lung carcinoma, gastric adenocarcinoma and colon adenocarcinoma

		Apoptotic index			
	No. of cases	Range	Mean	SD	p-value[a]
Mesothelioma	32	0 - 41.9	17.6	11.1	
Epitheliod mesothelioma	21	0 - 34.9	15.2	9.9	0.063[b]
Sarcomatoid mesotehlioma	9	5.0 - 41.9	23.5	12.9	
Biphasic mesothelioma	2	11.1 - 23.0	17.1	8.4	
Lung carcinoma	10	10.0 - 57.8	28.6	15.8	0.018[c]
Gastric adenocarcinoma	10	6.5 - 57.0	30.1	16.4	0.009[c]
Colon adenocarcinoma	10	12.8 - 71.3	31.4	17.3	0.005[c]

[a] Student t- test

[b] Epithelioid mesothelioma versus sarcomatoid mesothelioma

[c] Versus mesothelioma

Table 3. Apoptotic index and cleaved caspase-3 index in mesothelioma

	No. of cases	Range	Mean	SD	r	p-value*
Apoptotic index	32	0 - 41.9	17.6	11.3	0.373	0.036
Cleaved caspase-3 index	34	0 - 36.5	14.7	10.6		

* Pearson's correlation used 32 cases, except for 2 cases as not informative of apoptosis.

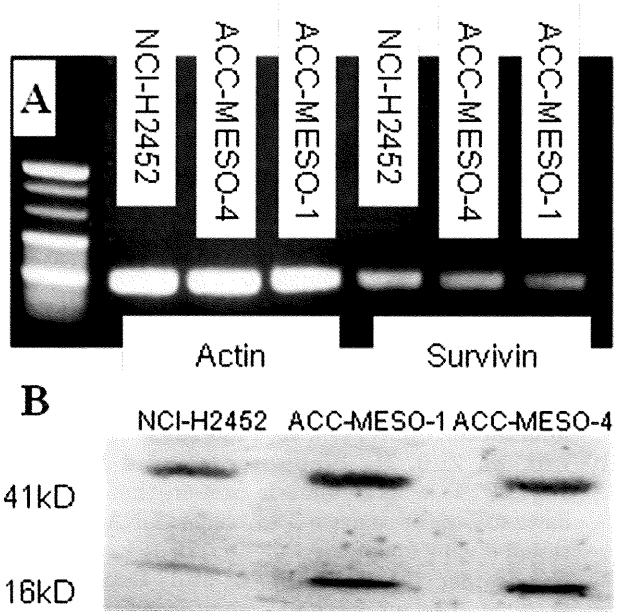

Fig. 2. (A) Electrophoresis of RT-PCR product of survivin mRNA and actin mRNA amplification shows 121 bp and 120 bp fragments. (B) Western blot analysis shows expression of survivin protein (16 kD) and actin (41 kD) in all three of the mesothelioma cell lines.

DISCUSSION

In the present study, the mean apoptotic index (AI) in mesothelioma tissue was 17.6, which is similar to those reported previously in mesothelioma[6, 16]. With similar techniques, mean AI in various other human malignancies of lung, stomach and colon were 28.6, 30.1 and 31.4, respectively (Table 2). The mean AI in non-small cell lung carcinoma has been previously reported to be as low as 13.9[9] or 20.7[12]. This discrepancy of AI in our result may be due to the difference in technique or the kit used for the TUNEL method. Therefore, the mean AI in mesothelioma is significantly lower than that in other malignancies, suggesting the more aggressive nature of mesothelioma.

Caspase-3 is an inactive proenzyme and its cleavage by other upstream proteases such as active caspase-8 and caspase-9 leads to its active form, called cleaved caspase-3[13, 18]. IAPs inhibit the proteolytic cleavage of caspase proforms caspase-3 and caspase-9[8, 11, 17] and/or directly inhibits activated caspases[4]. In the present study, we used two different anti-caspase-3 and anti-cleaved caspase-3 antibodies. The former can detect full length caspase-3 (35 kD) and the large fragment of caspase-3 resulting from cleavage (17 kD), and the latter can specifically detect the large fragment (17/19 kD). Mesothelioma

tissue and cell lines showed high cytoplasmic expression of caspase-3 and very low expression of cleaved caspase-3. We only found a positive correlation between AI and cleaved caspase-3 expression in mesothelioma tissue. Soini et al reported high expression of caspase-3 but no association was found between apoptotic index and caspase-3 immunoreactivity in mesothelioma[15]. They used an anti-caspase-3 antibody that detects both inactive 32 kD pro-enzyme and the active 17 kD fragment[15]. Our result using cleaved caspase-3 is more specific and, therefore, we found a positive correlation of AI to cleaved caspase-3 expression.

Mesothelioma tissue and cell lines showed high cytoplasmic expression of bax and low cytoplasmic expression of bcl-2. This result is similar to a previously published study of mesothelioma[16]. The previous report found no significant difference in bcl-2 mRNA expression between mesothelioma and normal pleural tissue[3], and suggested that apoptosis in mesothelioma has no direct relation to bax or bcl-2 expression. Survivin is the smallest protein among the IAPs[8], and it directly inhibits activation of caspase-3[8, 11]. In the present study, mesothelioma tissue and cell lines showed high cytoplasmic expression of survivin. The immunohistochemical expression of survivin is reinforced by mRNA expression by real time RT-PCR and protein expression by western blot. Falleni et al. have reported high expression of survivin mRNA in mesothelioma tissue compared to corresponding normal tissue[3].

In conclusion, apoptosis is an uncommon event in mesotheliomas. Although apoptosis-inducing proteins such as bax and caspase-3 were highly expressed in mesothelioma tissue and cell line, the expression of cleaved caspase-3 indicated that low activation of caspase-3 was responsible for the inhibition of apoptosis. Furthermore, high expression of survivin, a known inhibitor of caspases, may also play a role in the inhibition of apoptosis in mesothelioma.

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