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Immunohistochemical differences between hyaluronan- and non-hyaluronan-producing malignant mesothelioma

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Immunohistochemical differences between hyaluronan- and non-hyaluronan-producing malignant mesothelioma. A. Thylén, A-M. Levin-Jacobsen, A. Hjerpe, G. Martensson. ©ERS Journals Ltd 1997.

ABSTRACT: In many but not all cases, malignant mesothelioma is associated with an elevated content of hyaluronan in pleural fluid. The hyaluronan-producing mesothelioma has not yet been immunohistochemically characterized; therefore, the purpose of this study was to compare the immunohistochemical reactivity patterns in relation to the ability of this tumour to produce hyaluronan.

Pleural fluid samples from 33 patients with malignant mesothelioma were analysed for content hyaluronan using a quantitative high performance liquid chromatographic method. Biopsy specimens from the patients were studied immunohistochemically, using monoclonal antibodies against carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), a low molecular weight cytokeratin antigen (CAM 5.2) and vimentin.

An elevated hyaluronan content, *i.e.* >100 mg·L^{-1}, was noted in 23 patients (70%). There was no reactivity to the monoclonal antibody raised against CEA in any case. There was a significantly higher reactivity to EMA (p=0.026), a higher reactivity to CAM 5.2 (p=0.053) and a lower reactivity to vimentin (p=0.057) in the hyaluronan-producing mesotheliomas as compared to those with normal levels of hyaluronan.

Mesotheliomas that produced hyaluronan differed immunohistochemically from those that did not. The connection between the ability to produce different antigens and hyaluronan may relate to the degree of differentiation of the tumour. Both of these characteristics (immunophenotype and ability to produce hyaluronan) may, therefore, be of importance in studies concerning the prognosis and treatment of the malignant mesothelioma.

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Malignant pleural mesothelioma is often caused by occupational exposure to asbestos. There is an increasing incidence of the tumour in the industrial parts of the world. This is not only due to increased use of asbestos [1], but probably also to improved possibilities of establishing the diagnosis. A rising incidence and the legal implications of the disease have focused attention on the diagnostic difficulties caused by the variability in histological expression of the tumour.

The tumour is histologically classified into three major subtypes: epithelial, sarcomatoid, and mixed. Diagnostic difficulties are caused mainly by problems in distinguishing the epithelial subtype of mesothelioma from adenocarcinomas. Introduction of electron microscopy, immunohistochemical techniques and reliable analyses of hyaluronan have greatly reduced this diagnostic dilemma [2].

Immunohistochemical analyses have shown that at least one epitope of carcinoembryonic antigen (CEA) is almost invariably absent in all three subtypes of mesothelioma [3–6]. Antibodies to vimentin strongly react to the sarcomatoid subtype the sarcomatoid part of the mixed subtype of mesotheliomas [3, 4, 6–9]. The epithelial and mixed subtypes in most cases react to antibodies raised against epithelial membrane antigen (EMA) [6–8, 10]. Antibodies to cytokeratin are immunoreactive in all three major subtypes of mesothelioma, and this reaction may be of help in identifying a sarcomatoid component of the tumour [3, 4, 6–8].

Hyaluronan is a high molecular weight glycosaminoglycan. Increased amounts of hyaluronan in the pleural fluid from patients with malignant mesothelioma were first described by MEYER and CHAFFEE [11] in 1939, and the clinical sensitivity of hyaluronan analyses as a means of diagnosing malignant mesothelioma with high specificity (close to 100%) is in the order of 50% [12]. In this way, a number of mesotheliomas will be characterized also according to their hyaluronan production. It is not known, however, whether those tumours with the ability to secrete hyaluronan differ from the non-producing ones in other respects.

The purpose of this study was to compare the immunohistochemical reactivity patterns in relation to the ability of this tumour to produce hyaluronan.

Patients and methods

Pleural fluid samples and biopsy specimens were examined in a prospective study of 33 consecutive patients with biopsy-verified malignant pleural mesothelioma. All patients were males, with a mean age of 63 yrs (range 39–81 yrs), and all except two had known exposure to asbestos.

The histological diagnosis was based on: tissues from thoracoscopy (22 patients); Abrams needle biopsy (1), transthoracic Tru-cut needle (Travenol Laboratory, Deerfield, IL, USA) biopsy with fluoroscopic guidance (5), biopsy of skin metastases (3); and from autopsy (2). The biopsy specimens (2–5 mm) were fixed in 4% buffered formalin and embedded in paraffin. Sections were cut at 4–5 μm, placed on glass slides and coded. The slides were stained with haematoxylin and eosin and with periodic-acid-Schiff-diastase (PAS-D). Re-evaluation of the histological diagnosis was carried out as a joint examination of all sections by two experienced lung pathologists.

Immunohistochemical characterization was performed using a panel of five different antibodies (table 1). After blocking reactions (0.3% H_2O_2 in methanol for 5 min to block endogenous peroxidase and 2% preimmune serum to block nonspecific immunoreactivity), the sections were incubated with the specific antibodies. The antigen-antibody complexes obtained were then visualized using the Multilink Universal HRP kit (Bio Genex Laboratories, San Ramon, CA, USA) with diaminobenzidine (DAB) as the substrate. Appropriate positive and negative controls were performed. For all antibodies, the reactivity was graded as: negative when <2% of the cells were positive; 1+ with 2–25% being reactive; 2+ with 26–50%; 3+ with 51–90%; and 4+ with >90% positive cells. This grading system did not take into consideration the strength of reactivity. Five cases (Nos. 1, 2, 5, 9 and 10) served as controls and were graded as described above every time (seven times) new cases were examined.

The hyaluronan analyses were performed using a quantitative high performance liquid-chromatographic (HPLC) method [13]. The hyaluronan was precipitated from 20 μL of acellular pleural fluid supernatant by adding 80 μL ethanol. The precipitate obtained was centrifuged, and the pellet digested with 100 μL digestion mixture containing chondroitinases and chondroitin sulphatases.

Table 1. – Antibodies used

Antibody	Source	Dilution
Anti-CEA (MoAb)*	Dakopatts, Copenhagen, Denmark	1:25
Anti-CEA (PAb)	Dakopatts, Copenhagen, Denmark	1:100
Anti-vimentin	Dakopatts, Copenhagen, Denmark	1:10
Anti-cytokeratin (CAM 5.2)	Becton & Dickinson, San Jose, CA, USA	Prediluted
Anti-EMA	Dakopatts, Copenhagen, Denmark	1:10

MoAb: monoclonal antibody; PAb: polyclonal antibody; CEA: carcinoembryonic antigen; CAM 5.2: commercial name; EMA: epithelial membrane antigen. *: for case 11 and 26, Dako CEA 11-7 (Dakopatts, Copenhagen, Denmark) was used.

Ten microlitres was then taken for the HPLC analyses, by which the hyaluronan-derived delta disaccharide was separated from others present in the digest. Fisher's exact test and Mann-Whitney U-test were used for the statistical analyses. A p-value of less than 0.05 was considered significant.

Results

All the mesotheliomas showed typical histopathological features. The reactivity to the various antibodies and staining for PAS-D, the histological subtypes and hyaluronan concentrations are presented in table 2. Among the 33 cases, 23 (70%) were of the epithelial, four (12%) of the sarcomatoid, and six (18%) of the mixed histological subtype. The PAS-D stain showed weak positivity in two epithelial mesotheliomas.

CEA

There was no reactivity to the monoclonal antibody raised against CEA in any case.

Vimentin

The reactivity to the vimentin antibody used and the corresponding hyaluronan concentrations are shown in figure 1. The reactivity to vimentin correlates negatively to the capacity to produce hyaluronan ($r=-0.377$; $p<0.05$). The immunohistochemical reaction in the mixed and sarcomatoid mesotheliomas also correlated negatively to their capacity for hyaluronan production ($r=-0.679$; $p<0.05$), whilst for the dominating epithelial group no such correlation was found ($r=0.167$).

CAM 5.2

The reactivity to the CAM 5.2 antibody used and the corresponding hyaluronan concentrations are shown in figure 2. All tumours reacted to the low molecular weight cytokeratin antigen CAM 5.2. No obvious correlation was seen between this reactivity and the capacity to produce hyaluronan.

EMA

The reactivity to the EMA antibody used and the corresponding hyaluronan concentrations are shown in figure 3. The reactivity to EMA was positively correlated to increased levels of hyaluronan in the corresponding pleural fluids ($r=0.458$; $p<0.01$).

Hyaluronan content

The mean (log data), median, range and standard deviation (SD, log data) of the hyaluronan content in the pleural fluids with regard to histological subtype are

Table 2. – Clinical data, hyaluranon content in pleural fluid and immunohistochemical results

Case No.	Age yrs	HYA mg·L^{-1}	Histological subtype	PAS-D	CEA PAb	CEA MoAb	Vimentin	CAM 5.2	EMA
1	79	153	E	1+	1+	-	3+	4+	3+
2	81	264	E	-	1+	-	2+	4+	4+
3	80	231	E	-	3+	-	2+	4+	4+
4	76	42	E	-	-	-	1+	4+	4+
5	74	1380	E	-	-	-	3+	4+	4+
6	75	21	M	-	-	-	4+	4+	4+
7	74	123	S	-	-	-	3+	3+	2+
8	70	117	E	-	-	-	2+	4+	3+
9	70	87	E	1+	-	-	3+	4+	2+
10	70	684	E	-	2+	-	2+	4+	4+
11	68	27	S	-	-	-	3+	1+	-
12	66	84	S	-	-	-	4+	3+	-
13	63	2325	E	-	-	-	3+	4+	4+
14	62	1974	E	-	1+	-	2+	4+	3+
15	64	21	M	-	1+	-	4+	3+	1+
16	61	390	E	-	-	-	1+	4+	4+
17	58	366	M	-	-	-	3+	1+	1+
18	61	546	E	-	1+	-	1+	4+	3+
19	57	93	E	-	-	-	-	2+	4+
20	57	117	E	-	-	-	1+	4+	1+
21	55	1404	E	-	3+	-	1+	3+	3+
22	54	318	E	-	1+	-	3+	4+	3+
23	54	846	E	-	-	-	2+	4+	4+
24	49	750	E	-	-	-	2+	4+	3+
25	47	456	M	-	1+	-	-	3+	3+
26	44	162	M	-	-	-	3+	2+	1+
27	39	969	E	-	-	-	2+	3+	3+
28	77	75	E	-	2+	-	3+	4+	2+
29	64	93	M	-	-	-	2+	2+	2+
30	62	30	S	-	-	-	4+	2+	1+
31	61	228	E	-	1+	-	2+	4+	4+
32	60	258	E	-	-	-	-	3+	2+
33	48	327	E	-	-	-	1+	4+	2+

HYA: hyaluronan; E: epithelial; S: sarcomatoid; M: mixed; PAS-D: periodic-acid-Schiff-diastase. For further definitions see legend to table 1. -: <2% positive cells; 1+: 2–25%; 2+: 26–50%; 3+: 51–90%; 4+ >90% positive cells.

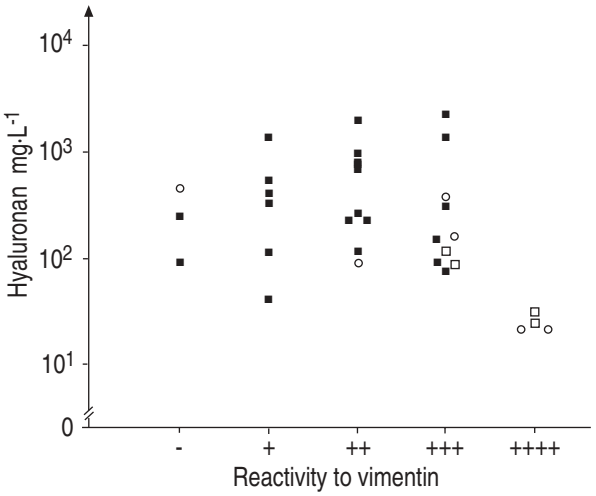

Fig. 1. – Hyaluronan concentration in pleural fluid *versus* reactivity to vimentin. Tumour subtypes: epithelial (■); mixed (○); and sarcomatoid (❑). -: <2% positive cells; =: 2–25%; ++: 26–50%; +++: 51–90%; ++++: >90% positive cells.

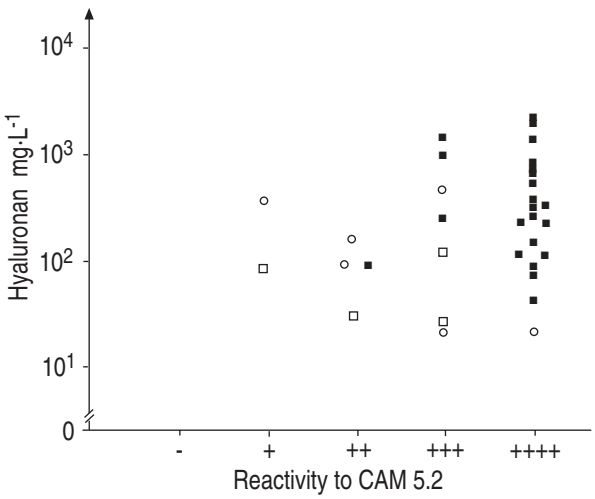

Fig. 2. – Hyaluronan concentration in pleural fluid *versus* reactivity to a low molecular weight cytokeratin antigen, CAM 5.2. Tumour subtypes: epithelial (■); mixed (○); and sarcomatoid (❑). For further definitions see legend to figure 1.

presented in table 3. There was a significantly higher concentration of hyaluronan in the pleural fluid of the epithelial mesotheliomas as compared to the sarcomatoid subtype (p=0.0095), although the latter group comprised only a few cases. A similar difference was also seen when comparing the mixed and sarcomatoid histological subtypes, as one group, to the epithelial group (p=0.0065).

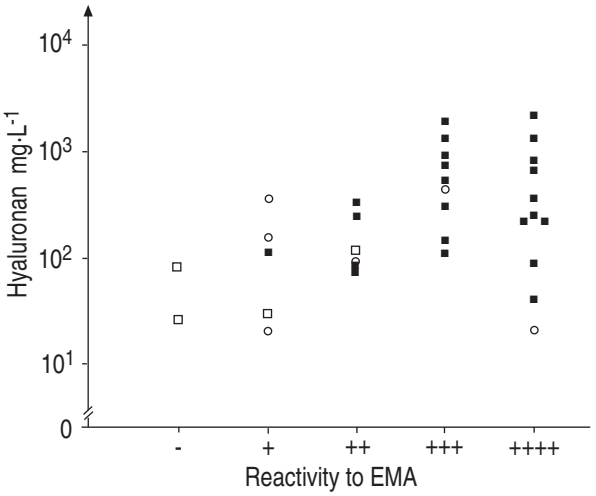

Fig. 3. – Hyaluronan concentration in pleural fluid *versus* reactivity to epithelial membrane antigen (EMA). Tumour subtypes: epithelial (■); mixed (○); and sarcomatoid (❑). For further definitions see legend to figure 1.

Table 3. – Concentration of hyaluronan in pleural fluid samples (mean of log data, median, range, and SD of log data)

	All histological types	Epithelial	Mixed	Sarcomatoid
n	33	23	6	4
Mean (of log data)	2.34	2.53	2.01	1.73
Median	231	318	128	57
Range	21–2325	42–2325	21–456	27–123
SD (of log data)	0.57	0.49	0.59	0.33

The immunohistochemical characterization of the mesotheliomas that produced hyaluronan, using a cut-off level of >100 mg·L^{-1}, as compared to those that did not is presented in table 4. Regardless of the histological subtype, the hyaluronan producing mesotheliomas showed a significantly greater reactivity towards EMA (p=0.026), a higher reactivity to CAM 5.2 (p=0.053), and a lower reactivity to vimentin (p=0.057); the latter two not being statistically significant.

The average hyaluronan content was significantly higher in the group where >50% of the tumour cells were EMA positive, as compared to the group with ≤50% such cells (p=0.0027). There was also a significantly higher hyaluronan content in cases with ≤50% of the cells reacting to vimentin, compared to the group with >50% of the tumour cells being reactive (p=0.0288).

Discussion

In this study of malignant mesothelioma the distribution of the histological subtypes is comparable to that of previous studies [14–16].

There was complete absence of reactivity to the monoclonal antibody used against CEA. This is in accordance with results reported by DEJMEK and HJERPE [5]. Vimentin reactivity, in the present study, was similar to that shown by other authors [3, 4, 6–9]. As described by others [3, 4, 6–8], all cases reacted to CAM 5.2. In the present study, the reactivities to EMA were also in accordance with previous reports [6–8, 10].

The PAS-D staining showed weak positivity in two epithelial mesotheliomas. Autopsy showed no other tumour than mesothelioma in the first of these cases. The second case was a man with more than 30 yrs of heavy exposure to asbestos. Fine PAS-D positive granules, which may be seen in mesotheliomas, could explain the weak PAS-D positivity observed [17].

Among the 33 cases, 23 (70%) had an elevated content of hyaluronan, *i.e.* >100 mg·L^{-1}. Previous studies by our group have also shown a similar clinical sensitivity using this HPLC method [18], and many mesotheliomas remain hyaluranon negative.

The capacity of the tumour cells to produce hyaluronan was correlated to their ability to synthesize EMA (p=0.026) and CAM 5.2 (p=0.053), whilst the opposite was noted for vimentin, *i.e.* increased hyaluronan production was associated with less reactivity to vimentin (p=0.057).

Hyaluronan is synthesized at the cell membrane and deposited in the extracellular matrix of most connective tissues [19]. The EMA positivity in malignant mesothelioma is located at the periphery of cell clusters and individual cells [20]. The labelling by anti-EMA antibodies was shown by VAN DER KWAST *et al.* [21] to occur selectively on the microvilli of the malignant mesothelioma cells. VAN DER KWAST *et al.* [21] also noticed that the microvilli of reactive mesothelial cells were not labelled at all. They, therefore, considered the EMA positivity on malignant mesothelioma cells to be "a differentiation-associated phenomenon", distinguishing malignant from reactive mesothelial cells.

It may well be that the epithelial membrane antigen reactivity and the capacity to synthesize hyaluronan both reflect a more differentiated tissue, which may, therefore, also differ in other biological respects. The occurrence of epithelial membrane antigen reactivity and hyaluronan production are negatively correlated to vimentin reactivity, and this latter epitope may rather indicate a more anaplastic tumour, with similarities to a more primitive fibroblast-like mesenchyma. The hyaluronan producing mesotheliomas, thus, have biological properties

Table 4. – Immunohistochemical reactivity to vimentin, CAM 5.2 and EMA *versus* hyaluronan (HYA) content in pleural fluid above or below 100 mg·L^{-1}

	Vimentin		CAM 5.2		EMA	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
HYA <100 mg·L^{-1}	3	7	4	6	7	3
HYA >100 mg·L^{-1}	16	7	2	21	6	17

Group 1: cases with ≤50% positive cells; Group 2: cases with >50% positive cells. For definitions see legend to table 1.

differing from those of tumours without production of hyaluronan. It may well be that these differences also involve other parameters, such as aggressiveness, prognosis and sensitivity to various therapeutic regimens, although such dependencies remain to be demonstrated.

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References

1. McDonald AD, McDonald CJ. Epidemiology of malignant mesothelioma. *In*: Antman K, Aisner J. Asbestos-related Malignancy. Orlando, Harcourt Brace Jovanovich 1987; pp. 31–55.
2. Martensson G. Diagnosing malignant pleural mesothelioma. *Eur Respir J* 1990; 3: 985–986.
3. Wirth PR, Legier J, Wright GL. Immunohistochemical evaluation of seven monoclonal antibodies for differentiation of pleural mesothelioma from lung adenocarcinoma. *Cancer* 1991; 67: 655–662.
4. Al-Saffar N, Hasleton PS. Vimentin, carcinoembryonic antigen and keratin in the diagnosis of mesothelioma, adenocarcinoma and reactive pleural lesions. *Eur Respir J* 1990; 3: 997–1001.
5. Dejmek A, Hjerpe A. Carcinoembryonic antigen-like reactivity in malignant mesothelioma. *Cancer* 1994; 73: 464-469.
6. Dejmek A, Hjerpe A. Immunohistochemical reactivity in mesothelioma and adenocarcinoma: a stepwise logistic regression analysis. *APMIS* 1994; 102: 255–264.
7. Bolen JW, Hammar SP, McNutt MA. Reactive and neoplastic serosal tissue: a light-microscopic, ultrastructural, and immunocytochemical study. *Am J Surg Pathol* 1986; 10(1): 34–47.
8. Johansson L, Lindén CJ. Review of malignant mesotheliomas in Lund, Sweden, 1980–1990: histopathological and immunohistochemical features - preliminary results. *Eur Respir Rev* 1993; (11): 61–63.
9. Blobel GA, Moll R, Franke WW, Kayser KW, Gould VE. The intermediate filament cytoskeleton of malignant mesotheliomas and its diagnostic significance. *Am J Pathol* 1985; 121: 235–247.
10. Pfaltz M, Odermatt B, Christen B, Rüttner JR. Immunohistochemistry in the diagnosis of malignant mesothelioma. *Virchows Arch A* 1987; 411: 387–393.
11. Meyer K, Chaffee E. Hyaluronic acid in pleural fluid associated with malignant tumour involving pleura and peritoneum. *Proc Soc Exp Biol Med* 1939; 42: 797–800.
12. Nurminen M, Dejmek A, Martensson G, Thylén A, Hjerpe A. Clinical utility of liquid-chromatographic analysis of effusions for hyaluronate content. *Clin Chem* 1994; 40(5): 777–780.
13. Hjerpe A. Liquid-chromatographic determination of hyaluronic acid in pleural and ascitic fluids. *Clin Chem* 1986; 32: 952–956.
14. Chailleux E, Dabouis G, Pioche D, De Lajartre M, Rembeaux A, Germaud P. Prognostic factors in diffuse malignant mesothelioma: a study of 167 patients. *Chest* 1988; 93: 159–162.
15. Antman K, Shemin R, Ryan L, *et al*. Prognostic variables in a registry of 180 patients. *J Clin Oncol* 1988; 6: 147–153.
16. Boutin C, Rey F, Gouvernet J, Viallat JR, Astoul Ph, Ledoray V. Thorascopy in pleural malignant mesothelioma: a prospective study of 188 consecutive patients. Part 2. Prognosis and staging. *Cancer* 1993; 72: 394–404.
17. Corson JM. Pathology of malignant mesothelioma. *In*: Antman K, Aisner J, ed. Asbestos-related malignancy. Orlando, Harcourt Brace Jovanovich 1987; pp. 179–199.
18. Martensson G, Thylén A, Lindqvist U, Hjerpe A. The sensitivity of hyaluronan analysis of pleural fluid from patients with malignant mesothelioma and a comparison of different methods. *Cancer* 1994; 73: 1406–1410.
19. Prehm P. Hyaluronate is synthesized at plasma membranes. *Biochem J* 1984; 220: 597–600.
20. Leong AS-Y, Parkinson R, Milios J. "Thick" cell membranes revealed by immunocytochemical staining: a clue to the diagnosis of mesothelioma. *Diagn Cytopathol* 1990; 6: 9–13.
21. Van der Kwast TH, Versnel MA, Delahaye M, De Jong A, Zondervan PE, Hoogsteden H. Expression of epithelial membrane antigen on malignant mesothelioma cells: an immunocytochemical and immunoelectron microscopic study. *Acta Cytol* 1988; 32(2): 169–174.