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Binding of ovarian cancer cells to immobilized hyaluronic acid

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Ovarian cancer has the highest mortality rate of any gynaecological malignancy. This is caused by metastatic deposits obstructing the intestinal tract. Very little is known about the molecules involved in the initial attachment of the metastatic tumour cells to the peritoneal mesothelial lining. Previously, we showed that many ovarian tumour lines express the adhesion molecule, CD44, on their cell surface. The major ligand for CD44 is the extracellular matrix glycosaminoglycan, hyaluronic acid (HA). Because mesothelial cells have a pericellular cost that contains large amounts of HA, it was postulated that the CD44/HA interaction is an important stage in ovarian cancer spread. However, it was difficult to demonstrate this interaction in an *in vitro* adhesion assay with mesothelial cells as most of the HA, and presumably the bound tumour cells, were lost from the mesothelial cells during the washing steps of the assay. In order to try and clarify the situation, the adhesion of six ovarian tumour lines to immobilized HA was measured. Four lines expressed high levels of CD44 and two lines expressed negligible amounts. Preliminary experiments were carried out with one of the CD44-expressing lines. After coating a plate overnight with 3 mg ml^{-1} HA, the 5 min adhesion of this line varied between 2% and 73% according to the type of plate that was used. Falcon Micro Test III flexible plates gave the highest adhesion and was used for further experiments. Plates were coated with concentrations of HA between 0.001 mg ml^{-1} and 3 mg ml^{-1}. All CD44 expressing lines adhered to HA, but the maximum adhesion and the adhesion strength varied with the line studied and was not closely related to the total CD44 expression. These results suggest that CD44 on ovarian tumour cells binds to HA on mesothelial cells. As much of the HA can be very easily lost from the mesothelial cell surface, additional factors such as the strength of the CD44/HA interaction, and the formation of bonds by the tumour cells with other membrane adhesion molecules, such as integrins, are also important in promoting tumour spread.

***Keywords*: cell adhesion, CD44, hyaluronic acid, metastasis, ovarian cancer, pericellular coat**

Introduction

Ovarian cancer is a lethal disease and is the fourth leading cause of cancer-related death in women [1]. The most common mechanism for the metastasis of ovarian cancer is by shedding of cells from the primary growth into the peritoneum and the implantation of these cells on the omental and bowel surfaces. The outer lining of these sites is comprised of a single layer of flattened epithelial cells called mesothelium. Very little is known about the molecules involved in the initial attachment of the ovarian tumour cells to the mesothelium lining. This would seem to be a critical step in establishing metastatic growth and an ideal point at which to prevent tumour spread.

A previous study [2] detected high expression of the highly-glycosylated glycoprotein CD44 on 11/13 ovarian tumour lines. A major ligand for CD44 is the glycosaminoglycan, hyaluronan (HA) (see reference [3] for review). This molecule is a high molecular weight polymer consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetylglucosamine, and it is found in association with extracellular membranes and sometimes in association with the cell membrane as a pericellular coat (PC) [3]. Recently, we showed that peritoneal mesothelial cells produced a PC that contained large amounts of HA [4], but about 90% of this HA was very easily removed from the cells by sucking off all the medium with a micropipette [4].

These studies suggested that CD44 on the ovarian tumour cells could interact *in vivo* with HA on the mesoathelial cells. However, when we tried to measure the adhesion of tumour cells to the mesothelial cells *in vitro*, we found that the results were difficult to interpret. Although tumour lines that expressed CD44 gave higher adhesion to mesothelial cells the overall values were low (11.2–35.6%), and much higher values were obtained when the HA coat was removed (see Table 1). We explained these findings by the fact that in the presence of the coat, the cells that were bound to the HA were lost in the washing steps of the adhesion assay; whereas in the absence of the coat, some

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Table 1. Adhesion of ovarian tumour lines to mesothelial cells in the presence and absence of the HA/coat.

Ovarian tumour line	*With HA coat (% adhesion)*	*Without HA coat (% adhesion)*	*CD44 (RMF)*
'2'	11.2 [7.7, 14.6]	21.7 [14.9, 28.4]	1.2
'6'	17.3 ± 7.2 (10)	57.0 ± 11.3 (10)	95
'10'	17.3 ± 5.3 (5)	73.6 ± 9.3 (5)	67
'42'	35.6 ± 6.9 (4)	70.7 ± 2.5 (4)	32
'59'	24.6 ± 10.4 (7)	78.5 ± 10.0 (7)	100
'180'	11.8 ± 3.2 (5)	15.3 ± 1.8 (5)	1.9

Cell adhesion (mean ± SD) was measured by adding fluorescently-labelled tumour cells to mesothelial monolayers in multiwell plates and removing the tumour cells after 5 min contact. In some wells the HA/coat was left undisturbed by leaving half the medium on the mesothelial cells, and in other wells 90% of the HA/coat was removed by sucking off the medium with a Pasteur pipette and replacing with fresh medium. Number of experiments are given in round brackets. CD44 expression was determined by flow cytometry using mouse anti-CD44 antibody. RMF is the relative median fluorescence: the medium fluorescence of the test cells divided by the median fluorescence of the control cells; RMF values of 1–2 indicate negligible CD44 expression. All data taken from reference [3].

cells probably bound initially to residual HA (not removed by sucking off the medium), but stronger bonds were quickly established by other adhesion molecules, such as integrins. It was assumed that when the full HA coat was present it would be more difficult for integrins to rapidly form these bonds [2].

The aim of this study was to measure the adhesion of different tumour cells lines to HA immobilized on to plastic surface of microtitre plates and determine if their adhesion was related to the expression of CD44.

Materials and methods

Six well characterized ovarian tumour lines were used in this study (OAW '42', '59', and '180' from Dr A. Wilson, Derby, UK; OVMZ '2', '6', and '10' from Dr V. Mobus, Ulm, Germany). Details of the culture methods, FACS analysis and the measurement of the cell adhesion to mesothelial monolayers have been previously described [2, 4, 5].

The adhesion of tumour cells to microtitre plates coated with HA (Sigma, UK) was carried out as follows. A plate was incubated with different concentrations of HA in PBS (3–0.001 mg ml^{-1}) overnight. After this treatment the plate was extensively washed with a 50:50 mix of Earles/Ham F-12 medium containing 0.2% (w/v) bovine serum albumin (EHB), before being blocked with EHB. Fluorescently-labelled [5] tumour cells were added to each well, the plate was centrifuged and the total fluorescence was measured using a Cytofluor 2000 (Millipore, UK). Five minutes later, the medium was removed, the unattached cells were washed away and the fluorescence remeasured. Tumour cell adhesion was expressed as a percentage and for each plate the average from six to eight wells was calculated. Microtitre plates from four manufacturers (Maxisorp, NUNC; Immunolon 4, Dynatech; Microtest III-tissue culture plates; and Microtest III-flexible, Becton Dickinson) were used in preliminary experiments, but the Microtest III-flexible plate was used routinely.

Results

Table 2 shows the binding of tumour line '42' to different microtitre plates coated with HA at 1 mg ml^{-1} and 3 mg ml^{-1}. Results from uncoated, but bovine serum albumin (BSA) blocked, wells are given for comparison. The adhesion to the different plates varied considerably. The highest adhesion was obtained with Microtest III flexible plate, which bound ~70% of the cells after 5 min when coated with 1 mg ml^{-1}. Table 3 shows the adhesion of ovarian tumour lines to flexible Microtest III plates precoated with different concentrations of HA. Table 4 summaries these results. The highest adhesion (~90%) was obtained for tumour lines '10' and '59'. Only about 50% of cells for lines '6' and '42' attached after 5 min. No attached-cells could be detected for the CD44 negative cell lines '2' and '180', which after correction for background gave adhesion values of approximately 0%. The strength of the attachment could be estimated from the coating concentration at which adhesion was half its maximum value. Using this measurement, the strength adhesion to HA for the different tumour lines was found to be in the order '59' > '10' > '42' = '6'.

Discussion

This study showed that all ovarian tumour lines that expressed high amounts of CD44 adhered to HA immobilized

Table 2. Adhesion of ovarian tumour line '42' to different plastic microtitre plates coated with HA

Microtitre plate	*5 min cell adhesion (%)*		
	0 mg ml^{-1} HA	*1 mg ml^{-1} HA*	*3 mg ml^{-1} HA*
Immulon 4	1.0 ± 0.3	1.9 ± 0.3	1.8 ± 0.5
Maxisorp	1.2 ± 0.2	29.9 ± 16.7	34.1 ± 15.7
Micro Test III Tissue Culture	14.8 ± 6.4	24.1 ± 11.8	73.1 ± 6.0
Micro Test III Flexible	10.2 ± 2.5	69.3 ± 9.5	65.0 ± 11.2

Values shown are mean ± SD from four experiments. The binding assay and HA precoating were carried out as described in the Materials and Methods.

Table 3. Adhesion of ovarian tumour lines to Flexible Micro Test III plates coated with different concentrations of HA.

HA Coating conc. (mg ml^{-1})	*Ovarian Tumour Line (% Adhesion)*			
	'6'	*'10'*	*'42'*	*'59'*
0.001	–	1.6, 0.7	–	1.5, 2.4
0.01	0.3, 0.5	21.9, 34.7	1.1, 0.6	56.8, 58.4
0.05	6.9, 3.5	–	–	86.0 ± 3.1
0.1	7.3, 4.0	66.3, 73.9	8.4, 4.0	90.1 ± 2.5
0.25	31.8 ± 8.0	85.8 ± 4.0	43.8 ± 7.6	84.7 ± 8.2[a]
0.5	33.1 ± 12.5	87.0 ± 4.7	53.1 ± 8.1	82.2 ± 3.6
1.0	38.7 ± 12.7	86.4 ± 5.0	59.1 ± 8.1	88.2 ± 5.5[b]
3.0	45.4 ± 9.1	85.6 ± 7.5	54.8 ± 9.7	83.4 ± 5.6

The binding assay and the HA precoating were carried out as described in the experimental section. All values are the mean ± SD from four experiments except where otherwise indicated, [a] eight experiments, [b] 10 experiments. All adhesion values were corrected for adhesion to plates in the absence of HA precoating. The control values for lines '2', '6', '10', '42', '59', and '180' were [mean ± SD%(*n*)]; 1.9 ± 0.7 (4), 4.0 ± 1.6 (6), 3.6 ± 1.3 (6), 7.5 ± 4.6 (6), 3.8 ± 1.7 (10), 2.2 ± 1.5 (4) respectively.

Table 4. Maximum adhesion and adhesion strength of ovarian tumour lines immobilized to HA.

Cell Line	*Maximum adhesion (%)*	*Adhesion strength (mg ml^{-1} HA)*
'6'	45.4	0.25–0.1
'10'	85.6	0.1–0.01
'42'	54.8	0.25–0.1
'59'	83.4	0.01–0.001

Adhesion strength was calculated from the data given in Table 3, and was defined as the range of HA concentrations over which adhesion was at least 50% of the maximum adhesion.

to plastic. These findings provide support for the idea that CD44-expressing tumour cells could bind strongly to the HA coat on mesothelial cells. It seems likely that the previously observed low adhesion of the tumour cells to mesothelial cells was caused by the type of the assay procedure used, which washed away the bound cells.

It was also shown that the ability of a line to bind to immobilized HA was not just related to total CD44 expression. For example, lines '6' and '59' had similar CD44 expressions but the maximum adhesion and adhesion strength of '59' was much greater than that of line '6' (Table 4). On the other hand, the adhesion properties of line '6' were similar to those observed for line '42', although the latter expressed 1/3 of the CD44 of the former (Table 4).

There are two possible explanations for the lack of close correlation between total CD44 expression and adhesive properties with immobilized HA. Tumour cells may express CD44 splice variants, some of which are known to bind HA weakly [6]. This appears unlikely, however, as RT/PCR studies have shown that the major CD44 species on these tumour lines is CD44H, the form that binds HA strongly (unpublished observations). Alternatively, CD44 may have different post-translational modifications, such as altered glycosylation or the addition of chondroitin sulphate, which could effect the binding to CD44. Treatment with neuraminidase and tunicamycin has been shown to increase the HA-binding of some cells [7, 8].

In conclusion, the *in vivo* interaction between tumour cell CD44 and HA may be important in ovarian cancer spread, but as the HA coat on mesothelial cells will be continually lost into the peritoneal cavity by the circulation of fluid, high affinity of CD44 for HA and rapid formation of bonds with other adhesion molecules are also necessary components for successful invasion. These aspects will be the subject of future investigations.

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