HNK-1 antigens on uveal and cutaneous melanoma cell lines

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The HNK-1 epitope has been associated with the metastatic behaviour of uveal melanomas. We characterized HNK-1 antigens on four human uveal (primary and metastatic) and two primary cutaneous melanoma cell lines by immunocytochemistry and Western blot analysis. We also determined the involvement of the HNK-1 epitope in cell-cell interactions on a matrigel layer. Three uveal melanoma cell lines (one primary and two metastatic) and one cutaneous melanoma cell line showed HNK-1 expression by immunocytochemistry. On matrigel, only the HNK-1-positive cutaneous melanoma cell line Bowes grew in a honeycomb-like structure which disappeared after adding HNK-1 antibodies to the culture medium. Immunoblot analysis of the primary uveal melanoma cell line EOM-3 revealed five HNK-1-positive protein bands with apparent molecular weights of 200, 160, 115, 95 and 75 kDa. The cutaneous melanoma cell line Bowes showed three HNK-1-positive protein bands with apparent molecular weights of 150, 135 and 90 kDa. This study shows that two uveal (primary and metastatic) and one primary cutaneous melanoma cell lines express HNK-1 antigens on immunoblot. Only in the HNK-1-positive cutaneous melanoma cell line Bowes did the HNK-1 epitope have a function in intercellular adhesion. Although the primary uveal melanoma cell line EOM-3 showed a similar HNK-1 immunoreactivity, we could not demonstrate HNK-1-mediated cell adhesion. On immunoblot, the two cell lines displayed different HNK-1 antigens, which may explain the difference in cell adhesion.

Key words: Bowes, cell adhesion, cell line, cutaneous melanoma, HNK-1, matrigel, uveal melanoma

Introduction

Cell-cell interactions, mediated by cell adhesion molecules, play a critical role during embryogenesis and in the homeostasis of eukaryotes. Failures in these cell-cell interactions are suspected to play an essential role in tumour cell detachment, invasion and adhesion in target

tissue.^{1,2} Several cell adhesion molecules, such as N-CAM (neural cell adhesion molecule), MAG (myelin-associated glycoprotein), P₀ (peripheral myelin glycoprotein) and J1 glycoprotein,³⁻⁷ bear a sulphated glucuronyl epitope. This epitope is recognized by the monoclonal antibody HNK-1 (mAb HNK-1), a murine IgM initially raised against the lymphoblastoid cell line HSB-2.⁸ Evidence has been presented that this HNK-1 epitope itself is able to interfere with cell-cell and cell-substrate interactions.⁹ Furthermore, the HNK-1 epitope has been shown to be essential in the migration of neural crest cells.^{10,11}

Uveal and cutaneous melanocytes originate from the neural crest.^{12,13} The differentiation of the neural crest cells to mature melanocytes leads to loss of the HNK-1 antigen expression.¹⁴ However, HNK-1 expression reappears in a small percentage of primary and metastatic cutaneous and uveal melanomas.¹⁵ Furthermore, in a recent study on uveal melanomas it was demonstrated that HNK-1 antigens were significantly less frequently expressed in liver metastases than in other metastatic sites. Therefore, HNK-1 antigens might be involved in the organ-specific metastatic behaviour of uveal melanomas.¹⁵

The aims of the present study were: (i) to determine whether HNK-1 antigens are present on cell lines of uveal and cutaneous melanomas, (ii) to characterize these antigens by Western blot analysis and (iii) to study their influence on cell adhesion.

Materials and methods

Cell lines

The cutaneous melanoma cell line Bowes was obtained from the American Tissue Culture Collection (CRL-9607/US patent 4,766,075; Rockville, MD, USA). The

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cutaneous melanoma cell line BLM is a subline of a highly aggressive and malignant human primary cutaneous melanoma.^{16,17} The uveal melanoma cell lines EOM-3, EOM-29, OMM-1 and OMM-2 were obtained from four different patients.^{18,19} EOM-3 and EOM-29 were derived from primary uveal melanomas and OMM-1 and OMM-2 were obtained from subcutaneous metastatic uveal melanoma lesions.

In brief, cell lines were established as follows: tumour tissue was transported from the operation room in Dulbecco's modified Eagle's medium (DMEM). The tumour was directly minced with a small pair of scissors and strained through a linen cloth by continuous irrigation with DMEM. The resulting single-cell suspension was washed with DMEM depending on the amount of pigment. The cells were cultured in a Falcon flask (T-30) with DMEM supplemented with 10% heat-inactivated fetal calf serum, 0.75 mg/ml penicillin and 1.25 mg/ml streptomycin (full DMEM) at 5% CO₂ and 37°C. After 1 week the medium was renewed and the floating cells were removed. Thereafter, the medium was changed twice a week.

Antibodies

The hybridoma clone producing HNK-1, a mouse IgM antibody, was purchased from the American Tissue Type Culture Collection (TIB 200). The antibody HSP90, an antibody specific for human heat shock protein 90 (HSP90),²⁰ was kindly provided by Dr R. de Crom (Department of Cell Biology and Genetics, Erasmus University Rotterdam, The Netherlands).

Immunocytochemistry

The cells were cultured on glass coverslips for 2 days, washed twice with phosphate-buffered saline (PBS) and fixed in cold acetone (-20°C) for 10 min. Endogenous peroxidases were inhibited by 0.3 % hydrogen peroxide/methanol for 15 min. Subsequently, the coverslips were incubated for 5 min in 2% fetal calf serum/PBS. The antibodies HNK-1 (dilution 1:10) and HSP90 (dilution 1:200) were incubated for 1 h at room temperature. As second step antibodies, rabbit anti-mouse and swine anti-rabbit immunoglobulins (peroxidase-conjugated) were used in a dilution of 1:100 for the HNK-1 and HSP-90 antibody, respectively. The peroxidase was visualized using 3,3'-diaminobenzidine tetrahydrochloride and ureumperoxide (Sigma, St Louis, MO, USA) for 5 min. The cells were counterstained with Mayer's haematoxylin for 1 min, rinsed with water for 10 min, dehydrated and fixed. Between all incubation steps the

coverslips were rinsed with PBS/Tween-20 (0.1%; PBS-T).

Cell culture on matrigel

In a 48-well tissue culture cluster (Costar), 0.1 ml matrigel (Biomedical Products Division, Bedford, MA, USA) per well was polymerized at 37°C for 30 min. Cells were trypsinized and diluted in full DMEM to a concentration of 6×10^6 cells/ml. From the cell suspension, 0.5 ml was brought onto the matrigel layer and was grown at 5% CO₂ (37°C). For each cell line, three wells with matrigel and one without were prepared. HNK-1 mAbs and mouse IgM (Southern Biotechnology Associates Inc., Birmingham, AL, USA) were added in a final concentration of 0.5 µg/ml. Photographs were taken at different times.

Plasma membrane isolation

Plasma membranes were obtained according to a modification²¹ of the protocol of Maeda and co-workers.²²

Sodium dodecylsulphate-polyacrylamide gel electrophoresis and Western blotting

The cells were washed twice with PBS (37°C) and harvested with a rubber policeman. The cell suspension was washed twice with PBS (4°C) and sonicated. Triton X-100 was added to a final concentration of 0.5% and the solution was centrifuged for 15 min at 10,000 g (4°C). The supernatant was directly precipitated with 1:1 cold acetone and resuspended in sample buffer containing 0.1 % dithiothreitol. Subsequently, the sample was boiled for 1 min. Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). Protein analysis was performed on 10% sodium dodecylsulphate polyacrylamide gels with 0.8% cross-linking prepared according to manufacturers' description (Biorad, Veenendaal, The Netherlands).

Subsequently, the proteins were transferred to Immobilon P membrane (Millipore, Etten-Leur, The Netherlands) in a Biorad blot apparatus at a constant current of 1 A for 2 h. Blots were blocked overnight at 4°C in 2% bovine serum albumin (Fraction V, Sigma, Axel, Belgium)/PBS-T and subsequently in 1% normal goat serum (Amersham International, Amersham, UK)/PBS-T for 20 min at room temperature. The blots were incubated with HNK-1 mAb in a dilution of 1:20 for 1 h at room temperature. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark; dilution 1:10,000) was used as a second-step antibody for 1 h at room temperature. Between each incubation step the blots were rinsed five times with PBS-T. The peroxidase

was visualized using the enhanced chemiluminescence detection system (Amersham International).

Analytical two-dimensional electrophoresis

Cells were harvested as described above. The pellet resulting from the acetone precipitation was diluted in sample buffer A containing 0.3% sodium dodecylsulphate, 28 mM Tris/HCl, 22 mM Tris/base and 200 mM dithiothreitol. Two-dimensional electrophoresis was performed with the Millipore Investigator system. Glycer-aldehyde-3-phosphate dehydrogenase carbamalytes (Pharmacia, Woerden, The Netherlands) and prestained high molecular weight markers (BRL Life Technology) were used as iso-electric focusing and molecular weight markers, respectively. For the second dimension 10% duracryl gels were used. Proteins were visualized with the silver staining technique.

Preparative electrophoresis

Preparative one- and two-dimensional electrophoresis was performed to purify proteins for micro-sequence analyses. The samples for the preparative one-dimensional electrophoresis were prepared as described above. For two-dimensional electrophoresis, cell homogenates (protein content 1 mg) were precipitated with cold acetone (-20°C) for 20 min and centrifuged at 12,000 rev/min for 5 min in an Eppendorf microcentrifuge. The pellet was diluted with 300 μl sample buffer A and heated at 55°C for 15 min. First- and second-dimension electrophoresis were performed as described above, but 0.1 mM thioglycolate was added to the upper compartment. The proteins in the gels were transferred to Immobilon P membranes. Blots were stained in parallel with HNK-1 antibodies as described above and with Coomassie Brilliant Blue R-250 (Sigma). The blots were compared and the most intense HNK-1-positive protein spots on the Coomassie blot cut out for micro-sequence analysis.

Using a protein sequencing system (Model 473A, Applied Biosystems, Foster City, CA, USA) the *N*-terminal amino acid sequences of the protein spots of interest were analysed. Additionally, the resulting sequences were compared with known amino acid sequences in the Swiss Protein Databank.

Results

HNK-1 immunoreactivity on melanoma cell lines

By immunocytochemistry, the two metastatic uveal melanoma cell lines (OMM-1, OMM-2) and one of the two

HNK-1 antigens on melanoma cell lines

Table 1. Percentage of the melanoma cells expressing HNK-1 antigens

Uveal		Cutaneous	
Line	Expression(%)	Line	Expression(%)
EOM-3 (primary)	100	Bowes	100
EOM-29 (primary)	0	BLM (primary)	0
OMM-1 (metastatic)	50–0.5 ^a		
OMM-2(metastatic)	8		

^aAfter 20 passages the expression decreased to 0.5%.

primary uveal melanoma cell lines (EOM-3) showed HNK-1 antigen expression, while one of the two primary cutaneous melanoma cell lines tested (the Bowes cell line) expressed HNK-1 epitopes. The HNK-1 expression in the cell lines was quantified by the percentage of HNK-1-positive cells. This expression ranged, depending on the type of cell line, from 0.5 to 100% (Table 1). Positive staining-reactions were characterized by a diffuse cytoplasmic and/or plasma membrane staining. One cutaneous melanoma cell line (Bowes) and one primary uveal melanoma cell line (EOM-3) showed 100% HNK-1-positive immunostaining (Figure 1a and b). In the metastatic uveal melanoma cell line OMM-1, HNK-1 immunoreactivity decreased from 50% (Figure 1c) to 0.5% after 20 passages in culture. The other cell lines kept a stable immunoreactivity.

Cell culture on matrigel and on a plastic culture layer

We investigated whether the HNK-1 epitope was involved in cell-cell interactions in the two 100% HNK-1-positive cell lines Bowes and EOM-3. When both cell lines were cultured on a plastic surface, they grew with a epithelioid-like morphology. In contrast, cultured on a matrigel layer the cells of the cutaneous melanoma cell line Bowes formed branches resembling a honeycomb network (Figure 2). After 24 h the cells aggregated but the aggregates were still connected by branches (Figure 3a). The uveal melanoma cell line EOM-3 did not show this characteristic growth behaviour on matrigel: in this case the cells grew in an epithelioid manner at first but after 24 h aggregated into unconnected colonies.

We added HNK-1 mAbs to both cell lines, cultured on matrigel, in order to investigate the blocking effect of the HNK-1 mAbs on the cells' growth behaviour. Mouse IgM was used as a control antibody to exclude the possibility that non-specific binding of IgM might be causing an effect. In the presence of HNK-1 mAbs the honeycomb network of the cutaneous melanoma cell line Bowes disappeared after 3 h; i.e. the branches of the cells disappeared, leaving the cells unconnected. After 24 h

Figure 1. HNK-1 immunoreactivity of the cell lines: **a** Bowes, **b** EOM-3 and **c** OMM-1 early passage (cytocentrifuge preparation).

the cells aggregated into separate colonies (Figure 3b). HNK-1 mAbs did not alter the growth behaviour of the cells of the cutaneous melanoma cell line Bowes, cultured on the plastic surface. The primary uveal melanoma cell line EOM-3 was not affected by the addition of HNK-1 mAbs on either the plastic surface layer or the matrigel layer. The control mouse IgM did not alter the growth behaviour of the cell lines.

Figure 2. The cutaneous melanoma cell line Bowes displayed a honeycomb-like network, while growing on matrigel for 3 h.

HNK-1 reactivity on immunoblot

The expression of HNK-1-reactive proteins in the cell lines Bowes, BLM, EOM-3, EOM-29 and OMM-1 was determined by Western blot analysis of total cell extracts of the cell lines. In the cutaneous melanoma cell line Bowes, HNK-1 mAb recognized three bands with apparent molecular weights of 150, 135 and 90 kDa (Figure 4, lanes 1 and 2).

BLM and EOM-29 did not react with mAb HNK-1, in accordance with the immunocytochemical results. The total cell extract of the primary uveal melanoma cell line EOM-3 showed five HNK-1-positive protein bands at 200, 160, 115, 95 and 75 kDa (Figure 4, lane 6). A late passage (P24) of the cell line OMM-1 showed a minor band at 45 kDa, but these were of negligible intensity (data not shown).

We isolated plasma membranes of the cell lines EOM-3 and Bowes to compare the HNK-1-immunostaining pattern with the pattern of the corresponding total cell extract. HNK-1 immunostaining of the plasma-membrane extracts of these cell lines showed for the greater part the

Figure 3. The cell line Bowes cultured on matrigel for 24 h **a** without addition of HNK-1 mAbs to the medium and **b** with addition of HNK-1 mAb. Note the disappearance of the honeycomb-like network after addition of HNK-1 mAb.

same HNK-1 antigen pattern (Figure 4, lanes 3, 4 and 5). The 200 and 95 kDa bands in the plasma-membrane extract of the cell line EOM-3 were less prominent and the 160 and 75 kDa bands were more prominent than in the total cell extract. In the cutaneous cell line Bowes the 150 kDa band was less prominent and the 90 kDa band more prominent in the plasma-membrane extract than in the total cell extract. The similar HNK-1 antigen patterns seen with plasma-membrane and total cell extracts indicates that the HNK-1 antigens are mainly localized in or on the plasma membranes.

The total cell extract of the Bowes cell line was analysed by preparative two-dimensional electrophoresis. The HNK-1-positive 90 kDa band was cut out for amino acid sequence analysis. The resulting *N*-terminal amino acid sequence (PEEGQHDDEP) showed 60% homology with 10 amino acids of HSP90. By immunocytochemistry

Figure 4. Western blot analysis of the total cell extract (lanes 1, 2 and 6) and the isolated plasma membranes (lanes 3–5) of the cell lines Bowes (lanes 1–3) and EOM-3 (lanes 4–6), stained with mAb HNK-1. Odd and even lanes were loaded with different amounts of protein.

we observed that 30% of the cultured cells of the Bowes melanoma cell line bound the HSP90 antibody. Also on immunoblot, a 90 kDa protein band was stained by HSP90 antibodies. To investigate whether the HSP90 band showed HNK-1 expression we immunoprecipitated the cells of the Bowes cell line with HSP90 antibodies. We did not observe any HNK-1 reactivity in the immunoprecipitated HSP90, indicating that the 90 kDa HNK-1 antigen is not HSP90.

Analytical two-dimensional electrophoresis

The cell lines EOM-3, OMM-1 and Bowes were analysed by two-dimensional electrophoresis. From each cell line we investigated the total protein pattern by silver-staining and the presence of HNK-1 antigens by immunoblotting. In the total protein pattern of the three cell lines the most intense stained proteins resembled each other in terms of molecular weight and iso-electric points (Figure 5 a-c).

The primary uveal melanoma cell line EOM-3 showed five HNK-1-positive spots on immunoblot (Figure 6a). Three distinguished adjoining spots had molecular weights of approximately 75 kDa and iso-electric points ranging between 5.3 and 5.4 (Figure 6a, proteins 1, 2 and 3). These HNK-1-positive proteins could be matched on the corresponding total protein pattern (Figure 6b, proteins 1, 2 and 3). Two other spots also showed HNK-1 reactivity; this was less intense (Figure 6a, proteins 4 and 5) although the spots corresponded with more material on the total protein pattern (Figure 6b, proteins 4 and 5). The other HNK-1-positive proteins of the primary uveal melanoma cell line EOM-3, identified on the one-dimensional immunoblot (200, 160, 115 and 95 kDa; Figure 4, lane 6), could not be visualized by two-dimensional electrophoresis. The HNK-1-positive proteins of the primary uveal melanoma cell line EOM-3 could be matched with the proteins present in the total protein pattern of the metastatic uveal melanoma cell line OMM-1 (Figure 5b). However, the proteins to which they were matched did not show any HNK-1 antigen activity.

HNK-1 immunostaining of immunoblot of the Bowes cutaneous melanoma cell line resulted in blurry staining, which points to a highly concentrated cell extract. We could not improve the immunoblot by repeating the experiment with less material.

Figure 5. Two-dimensional electrophoresis of the cell lines **a** EOM-3, **b** OMM-1 and **c** Bowes, showing the total protein pattern. Note the resemblance of the primary and metastatic uveal melanoma EOM-3 and OMM-1 and the cutaneous melanoma cell line Bowes. The intensely stained proteins are numbered (1-8).

Figure 6. Two-dimensional electrophoresis of the primary uveal melanoma cell line EOM-3, showing **a** the HKN-1-positive proteins and **b** the total protein pattern. The area shown is an enlargement of the box indicated in Figure 5.

Discussion

We have demonstrated that HKN-1 antigens are expressed heterogeneously in both cutaneous and uveal melanoma cell lines. The HKN-1 antigens were present on the plasma membrane as well as in the cytoplasm. Why some melanomas and not others carry HKN-1 antigens is not clear. A possible consequence of this different expression of the HKN-1 epitope could be that the metastatic behaviour of the melanoma varies in terms of progression or in the organ specificity of the metastatic cells. Recently it has been demonstrated that liver metastases originating from uveal melanomas rarely express HKN-1 epitopes, in contrast to those metastasizing to other sites.¹⁵

In the cutaneous cell line Bowes the honeycomb-like growth behaviour on matrigel was blocked by HKN-1 mAb, indicating that HKN-1 antigens are involved in cell-cell interactions. The primary uveal melanoma cell line EOM-3 did not show the honeycomb-like structure on matrigel, in spite of the high expression of HKN-1 antigens on the plasma membranes.

Further characterization of the HKN-1-epitope-bearing antigens by immunoblotting of the cutaneous melanoma cell line Bowes and the uveal melanoma cell line EOM-3 revealed marked differences in molecular weight. Although the HKN-1 expression of both cell lines was mainly localized on the plasma membranes, the dissimilar HKN-1 antigen pattern on immunoblot may reflect the different role of the HKN-1 epitope in these cell lines and, thus, a different function.

Two-dimensional electrophoresis of the uveal melanoma cell lines EOM-3 (primary) and OMM-1 (metastatic) shows the same protein pattern, but a different pattern of HKN-1 antigen expression. The metastatic uveal melanoma cell line OMM-1 could have lost the HKN-1 epitopes in the process of tumour cell detachment, invasion or adhesion. Or, conversely, it may be that the tumour cells were able to disseminate as a result of the loss of the HKN-1 epitopes.

In cutaneous melanomas a number of cell adhesion molecules have been found to play an important role in tumour progression.²³⁻²⁷ The HKN-1 epitope is present on some of these cell adhesion molecules, for example (chick) integrin (fibronectin and laminin receptor),²⁸ chick NCAM³ and human MUC18/A32.²⁹ For adhesion molecule MUC18/A32 the HKN-1 epitope has been shown to play a role in cell-cell interactions in the cutaneous melanoma cell line WM1366.²⁹ Muc 18/A32 has an apparent molecular weight of 113 kDa, resembling the 115 kDa protein observed in the primary uveal melanoma cell line EOM-3. However, in our study there was no evidence that the HKN-1 epitope of this latter protein was involved in cell-cell interactions in the primary uveal melanoma cell line EOM-3.

Our study shows that HKN-1 antigens are expressed heterogeneously on cutaneous and uveal (primary and metastatic) melanoma cell lines. We found that the HKN-1 epitope has a function in the intercellular adhesion in the cutaneous melanoma cell line Bowes. The fact that many different antigens carry the HKN-1 epitopes may explain the difference in function. Further research will

be needed to characterize the HNK-1-epitope-bearing antigens and to clarify the role of these antigens in cell adhesion and the metastatic process in cutaneous and uveal melanoma cell lines.

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