Production and characterisation of an antimelanoma monoclonal antibody KBA.62 using a new melanoma cell line reactive on paraffin wax embedded sections

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Abstract

Aims—To generate new monoclonal antibodies directed against melanoma associated antigens using a new melanoma cell line, KAL.

Methods—The melanoma cell line was established in culture from a lymph node metastasis of malignant melanoma. Normal Balb/c mice were immunised with KAL cells. Splenocytes were used for fusion experiments using standard techniques. Hybridoma supernatants were tested for antibody binding activity using an indirect immunoperoxidase method on frozen sections from KAL tumour cells xenografted onto nude mice and human tonsils. KBA.62 was selected because of its reactivity with melanocytic proliferations on both frozen and paraffin wax sections.

Results—On immunoblotting, KBA.62 reacted with three bands of 140, 135 and 128 kD and two weak bands of 88 and 73 kD. In normal human tissues basal melanocytes in the epidermis did not react with this antibody and only occasional labelling of endothelial cells was noted. Of the human tumours, KBA.62 reacted strongly and uniformly with the majority of benign (21/21) and malignant (75/86) melanocytic proliferations. Staining was localised predominantly to the cell membrane with little or no cytoplasmic reactivity. Negative staining was observed in the majority of human non-melanocytic neoplasms, the exceptions being some carcinomas (11/89), particularly the well differentiated squamous cell type. This, however, was not thought to present a diagnostic problem.

Conclusions—KBA.62 appears to be potentially useful in ascertaining the immunomorphological diagnosis of malignant melanoma in routinely processed paraffin wax sections.

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Keywords: KBA.62, melanoma, paraffin wax sections.

Malignant melanoma may sometimes constitute a diagnostic problem in surgical pathology and may mimic other neoplasms, such as undifferentiated carcinoma, sarcoma or large cell lymphoma. The advent of monoclonal antibodies directed against formalin resistant melanoma associated antigens has provided

a useful tool for the diagnosis of malignant melanomas even in their amelanotic form.¹⁻³ A few antimelanoma antibodies, reactive on paraffin wax sections, are now available. The usefulness of anti-S100 protein and NKI/C-3 in the diagnosis of melanocytic proliferations is limited as these antibodies recognise antigens not restricted to melanoma. Indeed, these antibodies react with a broad range of benign and malignant neoplasms^{2,4-6} and thus must be used in conjunction with other antibodies to allow definitive immunohistochemical diagnosis.^{2,7} As yet, HMB-45 has proved to be the most specific and useful monoclonal antibody in routine histopathology.^{3,8}

Here, we report the production of a monoclonal antibody (KBA.62) directed against a melanoma associated antigen which survives fixation and thus can be used on routinely processed paraffin wax sections. Detailed analysis of the reactivity of KBA.62 on normal and malignant human tissues is presented. These findings in melanocytic proliferations were compared with those obtained with HMB-45, which was used as a reference antibody. Several cases were also immunostained with anti-S100 polyclonal antibody.

Methods

ESTABLISHMENT OF THE KAL CELL LINE AND ITS TRANSPLANTATION INTO NUDE MICE

The melanoma cell line (KAL) was established in culture from a lymph node metastasis of malignant melanoma. A fragment of the fresh tissue was minced with a scalpel blade and incubated in RPMI medium supplemented with 20% fetal calf serum (FCS) and antibiotics at 37°C in a 5% CO₂ humidified environment. After 24 hours, only adherent cells were conserved. After two weeks in culture, cells growing in a monolayer were harvested using trypsin/EDTA (0.05/0.02%) and used for further subculture. The cell line, KAL, has been now maintained in RPMI/10% FCS for more than two years. The inoculation of 10–20 × 10⁶ KAL cells subcutaneously into nude mice caused tumour formation in all inoculated mice, within about three weeks. The KAL tumours from heterotransplanted animals were either snap frozen in liquid nitrogen or fixed in Duboscq-Brasil (ethanol based Bouin's) fluid and prepared for later screening of hybridoma supernatants.⁹

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Table 1 Reactivity of KBA.62, HMB-45 and anti-S100 with melanocytic tumours on paraffin wax embedded sections

Tumour type	KBA.62 (positive/tested)	HMB-45 (positive/tested)	Anti-S100 (positive/tested)
Naevi			
junctional	1/1	ND	ND
intradermal	7/7	3/6*	3/3
compound	8/8	2/5*	3/3
spitz	2/2	2/2	ND
dysplastic	3/3	3/3	ND
total	21/21 (100%)	10/16 (62%)	6/6 (100%)
Melanomas			
primary			
superficial spreading	27/29	12/13	13/13
nodular	6/7	4/4	4/4
acral lentiginous	1/1	1/1	1/1
lentigo	2/2	2/2	2/2
metastatic	39/47	30/39	27/29
total	75/86 (87%)	49/59 (83%)	47/49 (96%)
Total	96/107 (90%)	59/75 (79%)	53/55 (96%)

ND = not done.

* Intradermal naevi and the dermal component of compound naevi were largely non-reactive and only scattered naevic cells in the papillary dermis are labelled with HMB-45.

PRODUCTION OF MONOCLONAL ANTIBODIES AND SCREENING METHODS

Normal Balb/c mice were immunised by four intraperitoneal injections of $10\text{--}20 \times 10^6$ KAL cells at two weekly intervals. Three days after the last injection, mice were sacrificed and the splenocytes were used for fusion experiments using standard techniques.¹⁰ When hybridoma growth could be detected, supernatants were tested for antibody binding activity using an indirect immunoperoxidase method on frozen sections from both KAL tumour cells and human tonsils.⁹ Of six antibody producing

hybridomas reacting with melanoma associated antigens, KBA.62 was selected because of its reactivity with melanocytic proliferations on both frozen and paraffin wax sections (see later). The KBA.62 hybridoma was cloned by limiting dilution and its isotype was determined using immunohistochemistry.¹¹

IMMUNOSTAINING OF VARIOUS HUMAN NORMAL AND NEOPLASTIC TISSUE SECTIONS

Supernatants were produced and used for studying KBA.62 reactivity on tissue sections of different tumours ($n = 289$) and normal tissues ($n = 54$), from our tissue bank, by the Strept-avidin-biotin peroxidase complex (ABC) method using the Dako StreptABCComplex/HRP Duet (Mouse/Rabbit) kit (Dako, Glostrup, Denmark) without prior trypsinisation, as described elsewhere.¹² Some cases were also stained using a Streptavidin-biotin alkaline phosphatase method.¹³ Improved staining (that is, enhanced antigen retrieval), particularly on 10% formalin fixed specimens, was achieved following pretreatment of tissue sections in a microwave oven.¹⁴ Only paraffin wax embedded tissue sections were used. Tissue samples were fixed either in Duboscq-Brasil fluid (Toulouse) or in 10% formalin (Copenhagen). The human tumours investigated in this study included: 107 cases of melanocytic neoplasms (table 1), comprising 21 naevi and 86 cases of malignant melanoma (the latter included 39 primary cutaneous melanomas of different histological types and 47 cases of lymph node metastases); and 182 cases of non-melanocytic tumours of different categories (table 2). The reactivity of KBA.62 with melanocytic proliferations was compared with that of the commercially available HMB-45 monoclonal antibody (Dako A/S, Copenhagen, Denmark), which was used as a reference antibody. Most of the cases were also immunostained with anti-S100 polyclonal antibody (Dako A/S) using the peroxidase-antiperoxidase (PAP) immune complex method with prior trypsinisation.¹⁵ Unexpected reactivities of KBA.62 antibody were also assessed on paraffin wax sections using the multitumour (sausage) tissue block method.¹⁶

Table 2 Reactivity of KBA.62 with non-melanocytic tumours on paraffin wax embedded sections

Tumour type	KBA.62 (positive/tested)
Carcinoma	
skin	
squamous cell	5/6*
basal cell	2/4
lung	
squamous cell	2/7*
adenocarcinoma	0/10
small cell	0/6
gastrointestinal tract	
stomach	1/9
small intestine	0/4
large intestine	0/9
breast	1/8
thyroid	0/11
pancreas	0/1
ovary	0/2
kidney	0/4
prostate	0/2
nasopharynx	0/2
thymus	0/1
unknown origin	0/3
Haemopoietic neoplasms	
non-Hodgkin's lymphomas	0/28
Hodgkin's disease	0/19
Nerve tumours	0/7
Vascular tumours	0/9
Others	
mammary fibroadenoma	0/1
carcinoid	0/6
pheochromocytoma	0/2
adrenocortical carcinoma	0/2
nephroblastoma	0/3
renal oncocytoma	0/1
thymoma	0/2
mesothelioma	0/1
leiomyoma	0/4
histiocytofibroma	0/1
liposarcoma	0/1
synovialosarcoma	0/1
leiomyoblastosarcoma	0/1
rhabdomyosarcoma	0/3
Ewing's sarcoma	0/1
Total	11/182 (6%)

* Particularly well differentiated squamous cell tumour.

IMMUNOBLOTTING ANALYSIS

For immunoblotting analysis, KAL cells were lysed in lysis buffer containing 1% Nonidet P-40 (Sigma, St Louis, Missouri, USA) in 50 mM Tris/HCl (pH 8.0), 0.15 M NaCl, 0.57 mM phenylmethylsulphonyl fluoride, and 0.15 μ M aprotinin at 4°C for 30 minutes. After centrifugation, the samples were separated by SDS-polyacrylamide gel electrophoresis (7.5%) according to Laemmli's method¹⁷ and transferred to nitrocellulose membranes according to the method of Towbin *et al.*¹⁸ Nitrocellulose sheets were incubated with KBA.62 monoclonal antibody and the bound antibody was targeted with rabbit anti-mouse and swine anti-rabbit peroxidase conjugates. The peroxidase activity was revealed using the enhanced chemiluminescence western blotting detection system (Amersham, Little Chalfont, UK).

Results

HYBRIDOMA PRODUCTION AND SELECTION OF KBA.62

Supernatants from six of 924 hybridomas reacted with frozen KAL tumour sections but were negative on human tonsil sections, suggesting that a melanoma associated antigen had been detected. Of these six, the KBA.62 clone (IgG2a subclass) was selected for further detailed analysis because of its reactivity on both frozen and paraffin wax sections. Immunoblotting analysis performed with KBA.62 produced three bands with apparent molecular weights of 140, 135 and 128 kD and two weak bands of 88 and 73 kD (fig 1).

REACTIVITY OF KBA.62 WITH NEOPLASTIC HUMAN TISSUE

As mentioned earlier, paraffin wax sections of different human tumours from our tissue bank (tables 1 and 2) were investigated and the results of KBA.62 staining of melanocytic proliferations compared with those obtained with HMB-45 and anti-S100 antibodies.

Melanocytic tumours

The reactivities of KBA.62 antibody on paraffin wax embedded tissue sections from patients with melanocytic tumours are shown in table 1. KBA.62 was positive with the majority of benign and malignant melanocytic proliferations irrespective of their histological type. In most cases, the staining was strong, predominantly in the cell membrane, and involved all neoplastic cells (fig 2A). Epithelioid melanoma usually showed stronger and more consistent staining than the sarcomatous, spindle cell variant.

Overall, the reactivities of KBA.62 and HMB-45 on melanocytic tumours were comparable, with the notable exception of the dermal component of the intradermal type and compound naevi (figs 2C and 2D). In the latter the intradermal cells were consistently labelled with KBA.62 (fig 2C) and were weakly positive or negative with HMB-45 (fig 2D). In addition,

the staining intensity observed with each antibody varied from case to case (figs 2E and 2F). Furthermore, some cases were positive with KBA.62 and negative with HMB-45, and vice versa. As expected, virtually all melanocytic proliferations were positive on staining with anti-S100.

NON-MELANOCYTIC TUMOURS

Unexpectedly, KBA.62 reacted with paraffin wax sections of non-melanocytic tumours. The results of testing 182 tumour sections are presented in table 2. KBA.62 reacted with some of the carcinomas, of which squamous cell carcinoma of the skin (five of six cases) and of the lung (two of seven cases) predominated. However, staining was mainly present in well differentiated keratinising forms and in contrast to melanoma, positive cells were mainly found at the periphery of malignant lobules.

Figure 1 Western blot analysis of the KAL cell line immunostained with KBA.62 (A) and DBB.42 (B) anti-B monoclonal antibody, which was used as a control. KBA.62 reacts with three bands of 140, 135 and 128 kD and two weak bands of 88 and 73 kD (A).

Figure 2 Lymph node metastasis from malignant melanoma immunostained with KBA.62 (A) and HMB-45 (B). Note the intense membrane labelling of virtually all cells with KBA.62 monoclonal antibody whereas a diffuse cytoplasmic labelling pattern occurs with HMB-45. Compound nevus stained with KBA.62 (C) and HMB-45 (D). KBA.62 reacts with the entire population of intradermal and junctional nevus cells (C) whereas only junctional nevus cells and scattered cells in the superficial intradermal component are stained with HMB-45 (D). Note that the basal cells of the skin also react with KBA.62 (C). Superficial spreading melanoma stained with KBA.62 (E) and HMB-45 (F). Ascending intraepidermal and subepidermal tumour cells are strongly labelled with KBA.62 (E). Staining with HMB-45 (F) is weaker and only a proportion of malignant cells are stained. (Immunoperoxidase technique with nuclear counterstaining.)

REACTIVITY OF KBA.62 WITH NORMAL HUMAN TISSUE

Staining with KBA.62 was negative with virtually all human normal tissues except for the occasional positive endothelial cell. However, clear staining of hair follicles was noted on all skin biopsy specimens after pretreating the sections in a microwave oven. In contrast to normal skin, we noted staining of basal cells of the uninvolved skin localised above intradermal or compound naevi (fig 2C). Basal melanocytes in the normal skin were negative.

Discussion

The in situ immunological identification of human malignant proliferations has been greatly facilitated by the availability of monoclonal antibodies directed against fixative resistant molecules.^{11 19 20} The KBA.62 monoclonal antibody was obtained using the KAL melanoma cell line, underlining the value of new cell lines in generating novel monoclonal antibodies.^{11 21} The potential diagnostic value of this monoclonal antibody stems from the fact that it reacts with a melanocyte associated antigen and can be detected on routinely fixed, paraffin wax embedded tissues. Staining with KBA.62 was positive with all tumours containing naevi irrespective of their histopathological type (table 1). Approximately 87% of malignant melanomas tested in this study were positive. KBA.62 reacted with few non-melanocytic neoplasms (6%). However, it did react with a proportion of cells in well differentiated squamous cell carcinomas of the skin and, to a lesser extent, of the lung. Thus, although KBA.62 is not completely specific for melanocytes, it does distinguish melanoma from poorly differentiated forms of carcinoma, thus resolving a greater practical problem.

Reactivity of KBA.62 was compared with that of HMB-45 because of its restricted reactivity with melanocytic tumours and because the latter can be used on paraffin wax embedded tissue sections. The reactivities of KBA.62 and HMB-45 suggest that the two antibodies are different. This was demonstrated by immunoblotting analysis which showed that KBA.62 reacts with 140, 135, 128, 88, and 73 kD polypeptide chains which are different from those reported for HMB-45: two bands, one 10 kD and the other 100 kD.^{22 23} There are also other differences. In normal tissues KBA.62, in contrast to HMB-45, reacts with endothelial cells. In melanocytic proliferations KBA.62 reacts with two components—that is, junctional and intradermal, of typical naevi. We and others have found that the intradermal naevi and the dermal component of compound naevi reacted to varying degrees with HMB-45, but were mostly negative.^{3 24 25} In malignant melanomas, despite the overall comparable reactivities of KBA.62 and HMB-45, some cases were positive with KBA.62 and negative with HMB-45, and vice versa. In addition, KBA.62 usually stains membranes which is strikingly different from the cytoplasmic staining observed with HMB-45 (figs 2A and 2B). However, the precise immunolocalisation of the antigen re-

cognised by KBA.62 on KAL cells, with and without membrane permeabilisation, revealed that the antigen is probably on the inner part of the cell membrane (data not shown). KBA.62 reacts with some epithelial tumours, which is also the case with HMB-45.^{8 26} The reactivity of melanocytic neoplasms with anti-S100 is well documented.^{1 27} However, anti-S100 antibodies are of limited diagnostic value because of their broad spectrum of reactivity with non-melanocytic tumours.^{4 5} Thus, the latter marker has to be used in conjunction with other tissue specific immunomarkers.

In summary, KBA.62 is a new monoclonal antibody suitable for use on routinely processed paraffin wax sections. This antibody, in association with other antibodies, may prove to be valuable for a definitive immunomorphological diagnosis of malignant melanoma in surgical pathology practice.

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