Expression of Ligands to NKp46 in Benign and Malignant Melanocytes

Emanuela Cagnano[1,6], Oren Hershkovitz[2,3,6], Alon Zilka[2,3], Ahuva Bar-Ilan[2,3], Alexandra Golder[2,3], Netta Sion-Vardy[1], Alexander Bogdanov-Berezovsky[4], Ofer Mandelboim[5], Daniel Benharroch[1] and Angel Porgador[2,3]

Human melanoma cell lines were shown to express ligands for the natural cytotoxicity receptor, NKp46, expressed by natural killer (NK) cells. We aimed to examine the expression of ligands for NKp46 by various primary human melanocytic cells and melanocytic lesions. Sections from primary nevi and melanomas were tested for expression of NKp46 ligands employing chimeric NKp46-Fc for staining. The melanocytes present in the reticular dermis were negative for NKp46 ligands in common nevi; in malignant melanocytic lesions, the deeper melanocytes were focally positive. In dermoepidermal junction of all melanocytic lesions, the melanocytes showed enhanced expression of NKp46 ligands. Melanophages in all lesions were consistently positive for NKp46 ligands. These observations establish the expression of NKp46 ligands by primary-transformed melanocytes. Normal melanocytes did not express ligands to NKp46. Therefore, the results show (i) a correlation between the malignant potential of the lesion and the expression of NKp46 ligands in the reticular dermis, and (ii) enhanced expression of NKp46 ligands in the active proliferation zone (dermoepidermal junction) of nevi and melanomas. Ligands to NKp46 were expressed on the membrane and within the cells. The physiological role of NKp46 ligands in the progression of malignancy within melanocytic lesions should be explored further.

Journal of Investigative Dermatology (2008) **128,** 972–979; doi:10.1038/sj.jid.5701111; published online 1 November 2007

INTRODUCTION

Natural killer (NK) cells are the principal effector cells of the innate immune system and have a well-established role in anti-tumor responses and cancer immunosurveillance (Dunn *et al.*, 2002). The NK–tumor direct interaction is essentially mediated through the repertoire of ligands to NK receptors manifested by the cancer cells (Biassoni *et al.*, 2001). NK cells response is the outcome of a delicately regulated balance between activating and inhibitory signals delivered by a multitude of NK receptors (Biassoni *et al.*, 2001; Moretta *et al.*, 2001). Binding of major histocompatibility complex class I molecules to NK inhibitory receptors results in an inhibition signal and preclude lysis by NK cells. In contrast, cells that express insufficient levels of major histocompatibility complex class I molecules, a phenomenon that frequently accompanies tumor transformation, become susceptible to NK killing (Karre, 2002); the ground is set for tumor cell lysis by NK cells, mediated by signaling through the NK-activating receptors (Bakker *et al.*, 2000; Cerwenka *et al.*, 2000; Biassoni *et al.*, 2001; Moretta *et al.*, 2001; McQueen and Parham, 2002).

A prime player within these NK-activating receptors is the NKp46 receptor, considered as a major member of the natural cytotoxicity receptors (NCRs) group that also includes the NKp30 and NKp44 receptors (Biassoni *et al.*, 2001; Moretta *et al.*, 2001). NKp46 is highly NK-specific and can trigger NK-mediated lysis of various tumor cells through direct engagement of membranal ligands expressed by the cancerous cell (Moretta *et al.*, 2001). We and others published that NKp46 recognizes cellular ligands expressed on a wide variety of tumor cell lines, including melanoma cell lines (Moretta *et al.*, 2000, 2001; Arnon *et al.*, 2001, 2004; Biassoni *et al.*, 2001; Mandelboim and Porgador, 2001; Mandelboim *et al.*, 2001). Furthermore, it has been established that NKp46 is a primary NK-activating receptor for the recognition and lysis of human melanoma cell lines by fresh NK (Moretta *et al.*, 2000; Pende *et al.*, 2002).

[1]*Department of Pathology, The Soroka University Medical Center, Beer-Sheva, Israel;* [2]*The Shraga Segal, Department of Microbiology and Immunology, Ben-Gurion University of the Negev, Beer-Sheva, Israel;* [3]*The Cancer Research Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel;* [4]*Department of Plastic Surgery, The Soroka University Medical Center, Beer-Sheva, Israel and* [5]*The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel*

[6]*These authors have contributed equally to this work.*

Correspondence: Dr Angel Porgador, Department of Microbiology and Immunology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.
E-mail: angel@bgu.ac.il

Abbreviations: CN, compound nevi; DEJ, dermoepidermal junction; DysN, dysplastic nevi; IN, intradermal nevi; NCR, natural cytotoxicity receptor; NK, natural killer; NKp46D2, membrane-proximal domain of NKp46; SSMM, superficial spreading malignant melanoma

Received 23 January 2007; revised 18 June 2007; accepted 15 August 2007; published online 1 November 2007

In this study, we further explored the expression of ligands to NKp46 on human primary melanocytic nevi and melanomas. True melanocytic tumors are defined by a sustained proliferation of transformed melanocytes. They include three main categories: nevi, dysplastic nevi (DysN), and melanomas (Wick and Patterson, 2005). Benign nevi (predominantly junctional, compound, and intradermal nevi (IN)) show increased nevus cells numbers that retain more melanin and lie next to each other or in nests. The cells of melanoma behave similarly, but they proliferate without control to eventually metastasize. The DysN were first described as large, clinically atypical nevi presenting in several members of the same family, of which two members at least developed melanoma. The DysN have specific histological features; these include a lentiginous architecture, with melanocytes proliferating from the basal epidermis into the papillary dermis and show cellular atypia (Wick and Patterson, 2005).

The role of NK cells in response to melanocytic nevi and melanoma has been studied with the immunoperoxidase method, using the B73.1 antibody, raised against an Fc receptor present on NK cells and neutrophils. Rare NK cells were identified in about 10% of DysN and in 1 of 8 primary melanomas. In contrast, NK cells were much more frequent in metastatic melanomas (Kornstein *et al.*, 1987). Using the same antibody, the investigators found later that a staining reaction was obtained exclusively with malignant cells in the last two stages of the tumor progression—the vertical growth phase primary melanoma and the metastatic tumor (Kornstein *et al.*, 1987).

To understand further the role of NK cells in response to melanocytic tumors, we aimed to investigate the expression of ligands to NKp46 by the different melanocytic tumors. We employed recombinant NKp46, in which the extracellular domains are fused to the Fc backbone of human IgG1, to stain sections of formalin-fixed, paraffin-embedded nevi and melanomas. Thus, expression pattern of all ligands to NKp46 on primary cancerous and interstitial cells in these primary tissues could be analyzed.

RESULTS

We previously published that recombinant NKp46-Ig binds to cellular ligands expressed on tumor cells (Arnon *et al.*, 2001; Mandelboim and Porgador, 2001; Mandelboim *et al.*, 2001). We further showed that the membrane-proximal domain of NKp46 (NKp46D2), but not the membrane-distal domain (NKp46D1), retained the binding of NKp46 to tumoral and viral ligands (Arnon *et al.*, 2004; Bloushtain *et al.*, 2004; Zilka *et al.*, 2005). Similarly, others have shown that NKp46 and NKp46D2, but not NKp46D1, bind to Duffy binding-like-1α domain of *Plasmodium falciparum* erythrocyte membrane protein-1 expressed on parasitized erythrocytes (Mavoungou *et al.*, 2007). In these studies, NKp46 and NKp46D2 had identical binding phenotype, yet NKp46D2 manifested better binding capacity. Thus, in this study we used the NKp46D2-Ig for detecting NKp46 ligands expressed by normal and transformed melanocytes. 1106mel and A-375 are human melanoma cell lines derived from metastatic lesions. We previously published that 1106mel cells express ligands to NKp46 (Arnon *et al.*, 2001; Mandelboim *et al.*, 2001). We now compared expression of ligands to NKp46 between 1106mel and A-375 employing the NKp46D2-Ig. A-375 cells are stained positively with NKp46D2-Ig, yet to a lesser extent compared to 1106mel cells (Figure 1a, representative experiment). Both cells were negative for staining with NKp46D1-Ig (Figure 1b for A-375). Therefore, both metastatic melanoma cell lines express membrane-associated ligands to NKp46, yet they vary in expression densities.

We next studied whether the ligands are expressed within intracellular compartments. Permeabilization of A-375 cells significantly enhanced the binding of NKp46D2-Ig, while NKp46D1-Ig binding was null indicating the specificity of the NKp46D2-Ig binding to intracellular ligands (Figure 1b). Confocal analysis of A-375 cells stained with NKp46D2-Ig further revealed the intracellular localization of NKp46 ligands, and showed their abundance in the perinuclear zone (Figure 1c, arrow-marked). Again, NKp46D1-Ig did not stain the cells (Figure 1c).

We further investigated primary human malignant and non-malignant melanocytes hyperplasia. We stained with NKp46D2-Ig to detect the expression of NKp46 ligands and employed NKp46D1-Ig as a negative staining control. Staining with NKp46 fusion proteins was performed on different kinds of benign and malignant melanocytic lesions (detailed in Table 1). Nevi were represented by one junctional nevus, seven IN, twelve compound nevi (CN), and nine DysN. The patients were 12 females and 17 males (range 8–63 years). The nevi were located mostly on the back (12/29). The malignant melanocytic lesions included two lentigo maligna, eleven superficial spreading malignant melanomas (SSMMs), and nine nodular melanomas. The patients were 11 females and 11 males (range 23–83 years). The malignant lesions were biopsied mostly from the back (4/22), arm (4/22), leg (3/22), and cheek (3/22). One of the lentigo maligna was *in situ*, the other showed invasion into the papillary dermis (Clark level II). Clinical staging of the SSMMs varied between Clark levels II, III (7/11), and IV, and Breslow (tumor thickness) between 0.20 and 1.8 mm. The cases of nodular melanomas showed Clark levels III, IV (7/9), and V, and Breslow thicknesses from 1.30 mm to 1.8 cm.

Table 2 summarizes the staining results of all melanocytic lesions. NKp46D1-Ig immunostains were negative in all benign and malignant lesions in melanocytes as well as in melanophages and in normal skin structures. This is expected since we and others showed that in contrast to NKp46D2, NKp46D1 is not involved in NKp46 binding to cellular/pathogen ligands (Arnon *et al.*, 2004; Bloushtain *et al.*, 2004; Mavoungou *et al.*, 2007). Since staining with NKp46D1-Ig was negative, positive staining results with NKp46D2-Ig cannot be attributed to staining artifacts such as binding of the Fc portion of the fusion protein to Fc receptors expressed by melanocytes or melanophages; rather, positive staining with NKp46D2-Ig indicates binding to NKp46 ligands.

Results of staining with NKp46D2-Ig were as follows:

(i) Dermoepidermal junction (DEJ): NKp46D2-Ig immunostains were positive in the melanocytes of the DEJ in benign as well as in malignant lesions but the intensity

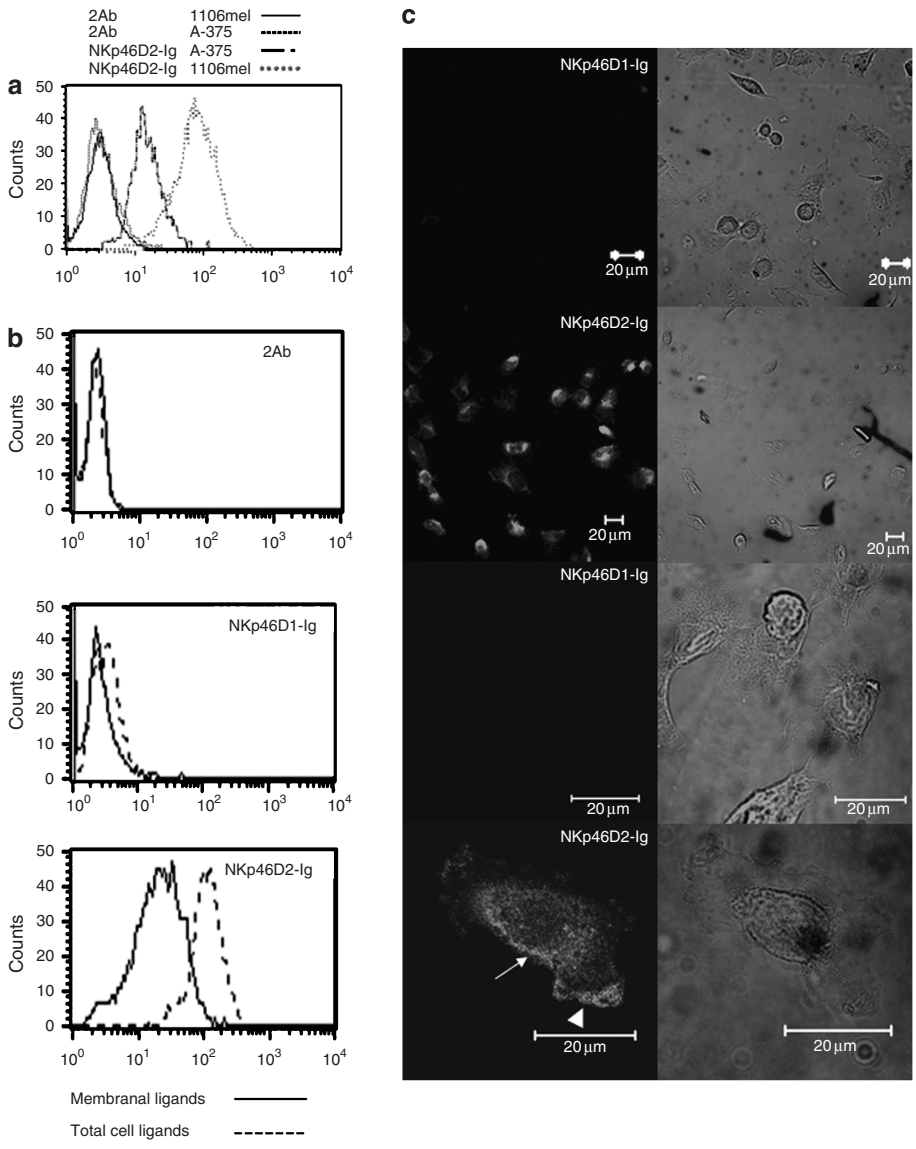

Figure 1. Binding of NKp46-Ig to melanoma cell lines. (**a**) Staining of membrane-associated ligands. Human melanoma cells were incubated with NKp46D2-Ig, washed, and stained with FITC anti-human Fc second antibody. PI was added to exclude dead cells. (**b**) Comparison between membrane-associated ligands and total cell ligands to NKp46. A-375 cells were stained as above for membrane-associated ligands. For total cell ligands, cells were prefixed and permeabilized with BD Cytofix/Cytoperm for staining with either NKp46D2-Ig or NKp46D1-Ig. (**c**) A-375 cells were grown on slides, fixed, and stained with NKp46D2-Ig and NKp46D1-Ig for confocal immunofluorescence analysis. The arrow points to perinuclear staining and the arrowhead marks membrane-associated staining. Bar = 20 μm.

was stronger in DysN, SSMM, and lentigo maligna (Table 2 and Figure 2).

(ii) Reticular dermis: the melanocytes present in the reticular dermis were mostly negative in junctional nevus, CN, and IN types (Table 2 and Figure 2); we observed focal staining (intensity 1) with NKp46D2-Ig only in 1/20 cases of junctional nevus, CN, and IN. For DysN, 3/9 samples manifested focal-positive staining of reticular melanocytes (intensity 1), whereas 6/9 were negative. In contrast, in the malignant melanocytic lesions, the deeper melanocytes were focally positive (Table 2 and Figure 2).

(iii) Melanophages and background: in all the different types of lesions, the melanophages showed a markedly positive staining with NKp46D2-Ig (Table 2 and Figure 2). The background represented by collagen, adnexa, and normal squamous epithelium was stained slightly. Yet, we assume that it may represent nonspecific staining.

(iv) Normal melanocytes: the edges of most samples (benign and malignant) contained normal melanocytes. In all cases, normal melanocytes were negative for NKp46D2-Ig staining. Figure 2h shows normal melanocytes (NKp46D2-Ig negative, marked with arrow) adjacent to atypical and nested melanocytes (NKp46D2-Ig positive) at the edge of SSMM, and Figure 2i shows normal melanocytes at the edge of DysN.

Table 1. Features of melanocytic lesions

Lesion	Sex	Age range (years)	Place	Clinical staging
JN	F	46	Foot	
CN	F (4) M (8)	8–54	Back (7), cheek (2), neck, l, thigh	
IN	F (5) M (2)	22–63	Back, chest, chin, face, auricle (2), leg	
DysN	F (2) M (7)	12–61	Back (4), chest, abdomen (2), ankle, flank	
LM	M (2)	76–79	Cheek (2)	Clark II, *in situ*
SSMM	F (6) M (5)	23–79	Back (3), ankle, flank, arm, forearm, leg (3), shoulder	Clark II (2), Clark III (7), Clark IV(2)
NM	F (5) M (4)	47–83	Back, chest (2), head, arm (3), shoulder, cheek	Clark IV (7), Clark V (2)

CN, compound nevus; DysN, dysplastic nevus; F, female; IN, intradermal nevus; JN, junctional nevus; LM, lentigo maligna; M, male; NM, nodular melanoma; SSMM, superficial spreading malignant melanoma.
The number of samples are indicated in parenthesis. .

Table 2. Staining of different melanocytic lesions with NKp46D2-Ig and NKp46D1-Ig

Lesion type	*No.*	Mean D2 intensity Melanocytes in DEJ	Mean D2 intensity Melanocytes in reticular dermis	Mean D2 intensity Melanophages	D1 intensity Melanocytes/ melanophages
J N	1	1.0	0.0	3.0	0.0
C N	12	1.4±0.5	0.0±0.0	2.7±0.5	0.0±0.0
I N	7	NE	0.1±0.4	2.3±1.0	0.0±0.0
DysN	9	2.0±0.7	0.3±0.5	3.0±0.0	0.0±0.0
LM	2	2.0±0.0	1.0±0.0	3.0±0.0	0.0±0.0
SSMM	11	2.1±0.3	1.0±0.9	2.7±0.5	0.0±0.0
NM	9	1.7±0.5[1]	1.1±0.6	2.6±0.5	0.0±0.0

CN, compound nevus; DEJ, dermoepidermal junction; DysN, dysplastic nevus; IN, intradermal nevus; JN, junctional nevus; LM, lentigo maligna; NE, not exist; NM, nodular melanoma; SSMM, superficial spreading malignant melanoma.
Analysis of staining intensity and percentage of stained tumor cells (0, 1, 2, and 3) was performed as follows: 0: nil; 1: 10–20% strongly positive or 30–80% weakly positive cells; 2: 20–50% strongly positive or >80% weakly positive; and 3: >50% strongly positive.
[1]DEJ results are for 7/9 samples, since DEJ did not exist in 2/9 samples.

Two cases of metastatic melanomas on lymph nodes were also submitted for immunostains with NKp46D1-Ig and NKp46D2-Ig; a mild-to-moderate positivity was observed in atypical melanocytes and a stronger positivity in surrounding macrophages. NKp46D1-Ig staining was negative also in these sections. We further stained several samples with NKp46-Ig; as expected, NKp46 staining phenotype followed the NKp46D2 phenotype (data not shown).

The results obtained from the immunohistochemistry studies indicated that normal melanocytes do not express ligands for NKp46, whereas transformed melanocytes (both benign and malignant) located in the active proliferation zone (the DEJ) express NKp46 ligands. To verify these results, we employed flow cytometry analysis on normal fresh human melanocytes and on Mel-STR. Mel-STR were derived from human melanocytes transformed to proliferate with complementary DNAs encoding SV40ER, hTERT, and oncogenic RasG12V (Gupta *et al.*, 2005). Transformed human melanocytes (Mel-STR) expressed ligands to NKp46 in levels comparable to A-375 (Figure 3a). In contrast, fresh normal human melanocytes were negative as compared to A-375 cells (Figure 3b).

DISCUSSION

A number of researchers, including our group, have shown that the NKp46 NCR recognizes cellular ligands expressed on a wide variety of tumor cell lines including melanoma cell lines (Moretta *et al.*, 2000; Arnon *et al.*, 2001, 2004; Biassoni *et al.*, 2001; Mandelboim *et al.*, 2001). The immunohistochemical analysis of ligands for NKp46 on primary human melanoma cells has never been performed. To explore the expression of NKp46 ligands on primary human nevi and melanomas, we employed the NKp46D2-Ig that stains cellular ligands to NKp46 expressed by human cancer cells

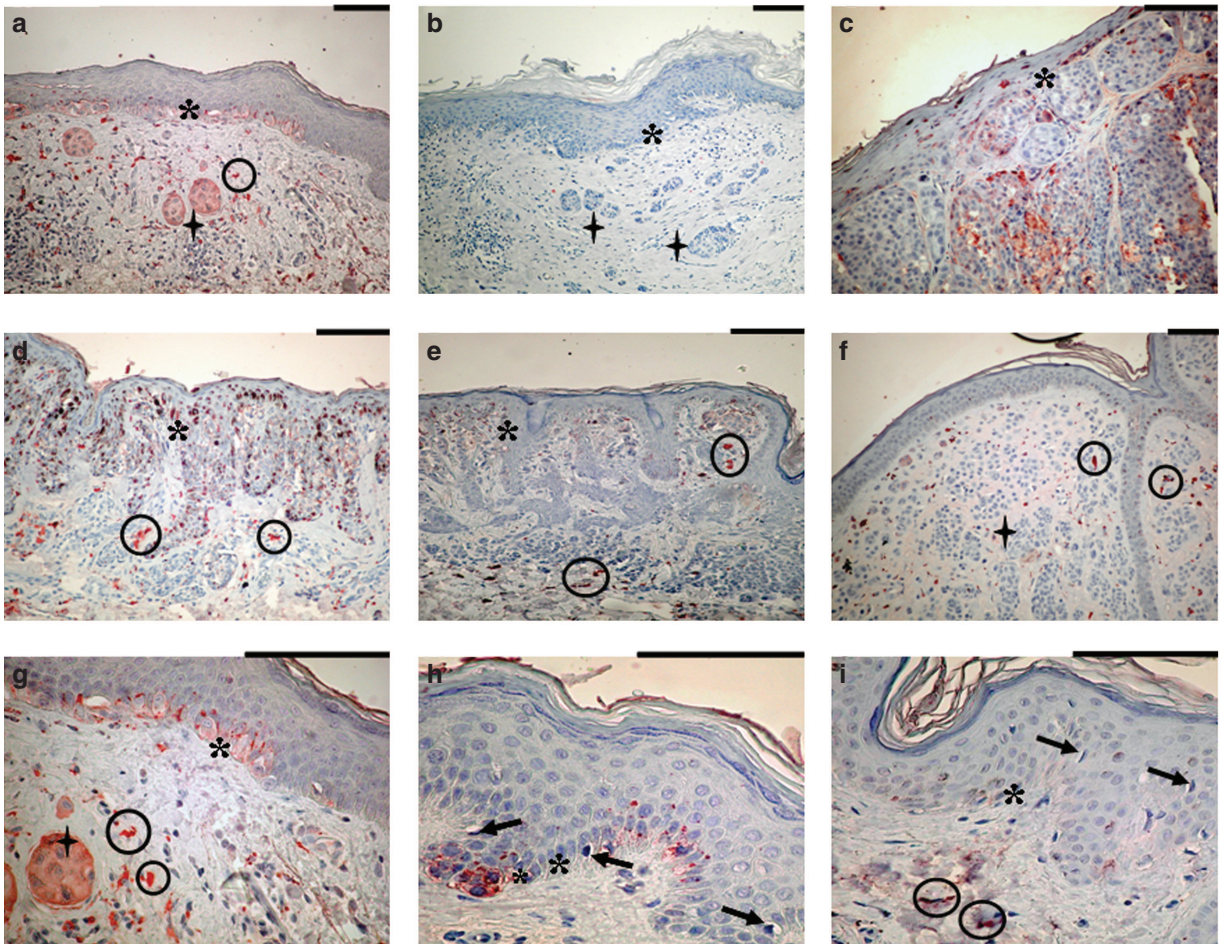

Figure 2. Immunohistochemical staining of ligands to NKp46 on primary human melanocytic lesions. Staining procedure: following antigen retrieval, sections were then stained with NKp46D2-Ig or with negative control NKp46D1-Ig, followed by biotinylated-goat anti-human Fcγ and avidin-biotin horseradish peroxidase complex. Substrate for horseradish peroxidase was aminoethyl carbazole (red color) and slides were counter-stained with hematoxylin. Sections from SSMM were stained with (**a**) NKp46D2-Ig or with (**b**) NKp46D1-Ig. Sections from (**c**) nodular melanomas, (**d**) DysN, (**e**) CN, (**f**) and IN were stained with NKp46D2-Ig. Sections from (**g**) SSMM, (**h**) SSMM edge, and (**i**) DysN edge were stained with NKp46D2-Ig. Melanophages are circled; arrows point to normal epidermal melanocytes; DEJ is marked with asterisk; cross-marks nested melanocytes in reticular dermis. Bar = 50 μm.

(Arnon *et al.*, 2004; Bloushtain *et al.*, 2004). We provide early evidence that ligands to NKp46 are expressed on human primary melanocytic lesions. The NKp46 ligands expression by the transformed melanocytes was mostly evident in melanocytes proliferating in the DEJ zone (Table 2 and Figure 2). This positive immunostaining in the DEJ was evident in both benign and malignant melanocytic lesions. Therefore, expression of NKp46 ligands could be a marker of actively proliferating melanocytes. This observation correlates with the report that NKp46 is involved in the recognition of mitotic cells by NK (Nolte-'t Hoen *et al.*, 2007). Normal epidermal melanocytes did not express NKp46 ligands (Figure 2h and i). The melanocytes present in the reticular dermis were mostly negative in the following nevi types: junctional nevus, CN, and IN (only 1/20 positive). Reticular melanocytes in 3/9 DysN samples manifested focal positive staining. In contrast, in most of the malignant melanocytic lesions, the deeper melanocytes were focally positive (Table 2 and Figure 2). Therefore, the expression of NKp46 ligands by reticular dermis' melanocytes could be also related to the grade of malignancy.

The strong expression of NKp46 ligands by melanophages, in melanocytic lesions of all types and within lymph node metastasis, but not in normal tissues (data not shown), suggests that transformed melanocytes induce NKp46 ligands expression on adjacent melanophages. Indeed, transformed cell-mediated induction of different ligands and receptors on tumor-adjacent cells is well established (Mueller and Fusenig, 2002). Ligands to NCRs in general, and NKp46 in particular, may be expressed primarily as a consequence of cellular stress, activation, viral infection, or tumor transformation (Moretta and Moretta, 2004; Moretta *et al.*, 2005). Therefore, their expression is not unique to tumor cells and the overexpression of NKp46 ligands by melanoma-adjacent melanophages could represent the cellular stress imposed on the melanophages by adjacent malignant melanocytes. Yet, regulatory function in NK–dendritic cells interactions was reported for the interaction between NK-expressed

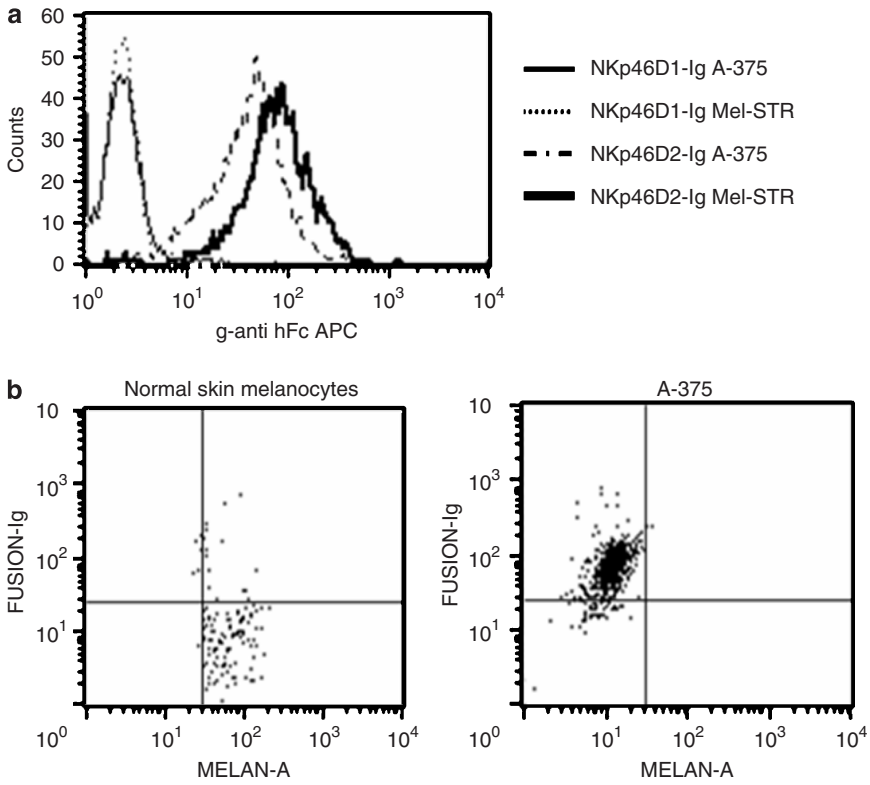

Figure 3. Binding of NKp46-Ig to normal and transformed melanocytes. (**a**) Staining of membrane-associated ligands. Human A-375 melanoma cells and human Mel-STR-transformed melanocytes were incubated with NKp46D1-Ig or NKp46D2-Ig, washed and stained with antigen-presenting cell anti-human Fc second antibody. PI was added to exclude dead cells. (**b**) Intracellular co-staining with anti-Melan-A and NKp46D2-Ig of normal human skin-derived cells (left, Melan-A$^+$ gated) and A-375 cells (right). Cells from trypsin-digested normal human skin were left to recover for 1 hour in 37°C with complete medium. A-375 and normal human skin cells were then prefixed, permeabilized, and co-stained with anti-Melan-A and NKp46D2-Ig followed by appropriate secondary antibody.

NCR (NKp30) and its dendritic cell-expressed ligand. Therefore, the NKp46 ligand induced on the melanophages could be employed for NK–melanophages regulatory interactions.

The nature of NKp46 cellular ligands is not yet fully understood. Previous reports suggested that membranal heparan sulfate proteoglycans or membranal vimentin could be involved in the binding of NKp46 to its cellular ligands (Bloushtain *et al.*, 2004; Zilka *et al.*, 2005; Garg *et al.*, 2006). Previous immunohistochemical study revealed that anti-vimentin Ab stained all neoplastic cells in all melanocytic lesions (Puches *et al.*, 1991), while we observed specific pattern of staining (Table 2 and Figure 2). Contrary to vimentin, heparan sulfate molecules are composed from numerous different epitopes with differential expression in different tissues. Previous immunohistochemical study of human cutaneous melanocytic lesions with a panel of phage display-derived anti-heparin/heparan sulfate antibodies identified three Abs (EW4E1, EW4G2, and EW4B7) that recognize melanoma-associated heparan sulfate epitopes. In particular, EW4E1 stained melanoma sections in the dermis and DEJ, yet recognized only the DEJ zone in sections of atypical and normal nevi (Smetsers *et al.*, 2003). Therefore, EW4E1 antibody manifested staining pattern similar to that shown for NKp46D2-Ig (Table 2 and Figure 2).

NKp46 interaction with its tumoral ligands is essential for lysis of tumor cell by NK cells as was previously shown for NKp46 ligands expressed by melanoma cell lines (Moretta *et al.*, 2000; Pende *et al.*, 2002). Yet, the infrequent presence of NK within DysN and primary melanoma (Kornstein *et al.*, 1987; Vetter *et al.*, 2000) precludes the expected lysis of melanocytic cells due to the expression of ligands for NKp46. Similarly, we observed rare NK filtration in our samples and no correlation to intensity of ligands to NKp46 (data not shown). The interaction between NKp46 on NK cells and their putative ligands on tumor target cells led to NK cell apoptosis, and this event was abolished by blocking NKp46/NKp46-ligand interaction by anti-NKp46-specific mAbs (Poggi *et al.*, 2005). Therefore, for NK, which do penetrate into melanocytic lesions, the expected outcome of tumor lysis could be hindered due to tumor-induced apoptosis of NK cells mediated by NKp46 engagement.

It is clear that the major physiological function of tumoral-expressed ligands to NK-activating receptors (NCRs and NKG2D) is not to serve as target molecules for NK. Rather the evolving tumor is acquiring these ligands during the selection of tumor cells capable of expansion and proliferation. The price that the tumor should pay by being more sensitive to NK is easily compensated by the positive gains and by other means that evolving tumor can acquire to inhibit NK.

Heparan sulfate is involved in angiogenesis and cell proliferation, and melanoma- and nevi-associated heparan sulfate was reported (Smetsers *et al.*, 2003). We previously reported that tumor membrane-associated heparan sulfate is involved in NKp46 recognition; the staining pattern we observed for NKp46D2-Ig (Table 2 and Figure 2) correlates with the pattern observed for EW4E1 Ab recognizing melanoma- and nevi-associated heparan sulfate (Smetsers *et al.*, 2003; Bloushtain *et al.*, 2004). Yet, the physiological role of NKp46 ligands in the progression of malignancy within melanocytic lesions should be further explored.

MATERIALS AND METHODS

Cells

Human melanoma cell lines used in this study are 1106mel (Porgador *et al.*, 1997) and A-375 (ATCC no. CRL-1619).

Ig-fusion proteins

The generation of NKp46D1-Ig and NKp46D2-Ig fusion protein has been described previously (Arnon *et al.*, 2001; Bloushtain *et al.*, 2004). Briefly, truncated fusion proteins of NKp46D1-Ig (including the leader peptide 1–21 and residues 1–100) and NKp46D2-Ig (residues 101–235) were generated by PCR amplification and cloned into a mammalian expression vector containing the Fc portion of human IgG1 as described previously. To allow expression of NKp46D2-Ig, which lacks its original leader peptide sequence, we added a methionine start codon and cloned the PCR-amplified fragment of NKp46D2 in frame with the leader peptide of CD5. Sequencing of the constructs revealed that all complementary DNAs were in frame with the human Fc genomic DNA and were identical to the reported sequences. For the production of fusion-Ig in Chinese hamster ovary cells, the corresponding fragment was cloned into the pcDNA 3.1 vector. After recloning, the highest protein-producing clone was adapted for special serum-free medium (CHO-SFM II; Gibco, Grand Island, NY), followed by optimization for growth in large-scale cultures. Supernatants were collected and purified on protein-G columns using fast-protein liquid chromatography.

Flow cytometry

Staining of melanoma cell lines using NCR-fusion protein, for expression of membrane-associated ligands to NCR, was carried out as described previously (Arnon *et al.*, 2004; Bloushtain *et al.*, 2004; Hershkovitz *et al.*, 2007). Briefly, cells were incubated with the fusion-Ig for 2 hours at 4°C, washed, and stained with FITC- or antigen-presenting cell-conjugated $F(ab')_2$ goat anti-human-IgG Fc (Jackson ImmunoResearch, West Grove, PA). For staining of total cell ligands, harvested cells were prefixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Diego, CA), washed with Perm/Wash buffer (BD Biosciences), and stained with the fusion-Ig, followed by secondary antibody as above. For staining of fresh melanocytes, cells from trypsin-digested normal human skin were recovered for 1 hour in 37°C with complete medium. Cells were then prefixed, permeabilized, and washed as above. Anti-Melan-A (10 µl, BioGenex, San Ramon, CA) and fusion-Ig were co-added. Following incubation and wash, secondary antibodies were added (FITC-conjugated $F(ab')_2$ goat anti-mouse IgG for the anti-Melan-A and antigen-presenting cell goat anti-human as above for the fusion-Ig). Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Confocal immunofluorescence analysis

Cells were allowed to settle on glass cover slides overnight at 37°C before fixation with ice-cold acetone/ethanol (1:1) for 10 minutes. Specimens were then blocked with phosphate-buffered saline (PBS)/10% FBS for 1 hour at room temperature to saturated nonspecific sites. Cells were then incubated with 10 µg ml^{-1} of fusion-Ig proteins in PBS/10% FBS for 1 and a half hours, washed with PBS, and stained with Cy3-conjugated $F(ab')_2$ goat anti-human IgG secondary antibodies (Jackson ImmunoResearch) in PBS/10% FBS for 1 hour at room temperature. Specimens were washed with PBS and mounted in the dark using mounting medium (Shandon, Pittsburgh, PA). Confocal images were acquired using a CarlZeiss LSM510 microscope and processed using the CarlZeiss LSM510 software (CarlZeiss, Jena, Germany).

Immunohistochemistry

Immunohistochemistry was preformed on archival sections of formalin-fixed, paraffin-embedded nevi and melanomas. After deparaffinization with xylol and hydration with decreasing concentration of alcohol, endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 15 minutes. The sections were then washed with ddH_2O and antigen retrieval was achieved by warming the sections for 15 minutes in commercial citrate buffer (DAKO, Glostrup, Denmark) using microwave. The sections were then washed with PBS and incubated for 20 minutes with normal goat serum (Jackson ImmunoResearch) diluted 1:300 with PBS (NGS stock). Fusion proteins diluted with NGS stock to a final concentration of 8 µg ml^{-1} were added to the sections. After 60 minutes incubation, the sections were washed three times with PBS and incubated with biotin-goat anti-human Fcγ (Jackson ImmunoResearch) diluted 1:2,000 in NGS stock for 30 minutes. Sections were washed again and specific interaction was detected by incubating the sections for 30 minutes with Elite ABC (Vector, Burlingame, CA). Red chromogen dye (Zymed Labs, San Francisco, CA) substrate was applied to the sections, incubated for 5 minutes, and finally washed in water. Tissue sections were then counterstained with hematoxylin and examined using light microscopy. Staining intensities are as follows: 0: nil; 1: 10–20% strongly positive or 30–80% weakly positive cells; 2: 20–50% strongly positive or >80% weakly positive; 3: >50% strongly positive.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by grants from the Ministry of Health (MOH) and the Israel Cancer Research Foundation (ICRF). This work was also supported by the Cooperation Program of the Deutsches Krebsforschungszentrum (DKFZ) and Israel's Ministry of Science (MOS). We thank Ms Eugenia Mejinovsky for assistance in staining of the nevi and melanoma sections. We also thank Dr Robert A. Weinberg (MIT, Cambridge, MA) and Dr Riki Perlman (Hadassah Medical Center, Jerusalem, Israel) for the Mel-STR cells. We thank Dr Chaim Rubinovitz (Soroka Skin Bank) for technical assistance. This study was carried out in Ben-Gurion University, Beer-Sheva, Israel.

REFERENCES

Arnon TI, Achdout H, Lieberman N, Gazit R, Gonen-Gross T, Katz G *et al.* (2004) The mechanisms controlling the recognition of tumor and virus infected cells by NKp46. *Blood* 103:664–72

Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* 31:2680–9

Bakker AB, Wu J, Phillips JH, Lanier LL (2000) NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals. *Hum Immunol* 61:18–27

Biassoni R, Cantoni C, Pende D, Sivori S, Parolini S, Vitale M *et al.* (2001) Human natural killer cell receptors and co-receptors. *Immunol Rev* 181:203–14

Bloushtain N, Qimron U, Bar-Ilan A, Hershkovitz O, Gazit R, Fima E *et al.* (2004) Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J Immunol* 173:2392–401

Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH *et al.* (2000) Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 12:721–7

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3:991–8

Garg A, Barnes PF, Porgador A, Roy S, Wu S, Nanda JS *et al.* (2006) Vimentin expressed on *Mycobacterium tuberculosis*-infected human monocytes is involved in binding to the NKp46 receptor. *J Immunol* 177:6192–8

Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW *et al.* (2005) The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* 37:1047–54

Hershkovitz O, Jivov S, Bloushtain N, Zilka A, Landau G, Bar-Ilan A *et al.* (2007) Characterization of the recognition of tumor cells by the natural cytotoxicity receptor, NKp44. *Biochemistry* 46:7426–36

Karre K (2002) NK cells, MHC class I molecules and the missing self. *Scand J Immunol* 55:221–8

Kornstein MJ, Stewart R, Elder DE (1987) Natural killer cells in the host response to melanoma. *Cancer Res* 47:1411–2

Mandelboim O, Porgador A (2001) NKp46. *Int J Biochem Cell Biol* 33:1147–50

Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y *et al.* (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409:1055–60

Mavoungou E, Held J, Mewono L, Kremsner PG (2007) A duffy binding-like domain is involved in the NKp30-mediated recognition of *Plasmodium falciparum*-parasitized erythrocytes by natural killer cells. *J Infect Dis* 195:1521–31

McQueen KL, Parham P (2002) Variable receptors controlling activation and inhibition of NK cells. *Curr Opin Immunol* 14:615–21

Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L (2000) Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunol Today* 21:228–34

Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC *et al.* (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* 19:197–223

Moretta L, Moretta A (2004) Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J* 23:255–9

Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A (2005) Human natural killer cells: molecular mechanisms controlling NK cell activation and tumor cell lysis. *Immunol Lett* 100:7–13

Mueller MM, Fusenig NE (2002) Tumor-stroma interactions directing phenotype and progression of epithelial skin tumor cells. *Differentiation* 70:486–97

Nolte-'t Hoen EN, Almeida CR, Cohen NR, Nedvetzki S, Yarwood H, Davis DM (2007) Increased surveillance of cells in mitosis by human NK cells suggests a novel strategy for limiting tumor growth and viral replication. *Blood* 109:670–3

Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R *et al.* (2002) Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res* 62:6178–86

Poggi A, Massaro AM, Negrini S, Contini P, Zocchi MR (2005) Tumor-induced apoptosis of human IL-2-activated NK cells: role of natural cytotoxicity receptors. *J Immunol* 174:2653–60

Porgador A, Mandelboim O, Restifo NP, Strominger JL (1997) Natural killer cell lines kill autologous beta2-microglobulin-deficient melanoma cells: implications for cancer immunotherapy. *Proc Natl Acad Sci USA* 94:13140–5

Puches R, Smolle J, Rieger E, Soyer HP, Kerl H (1991) Expression of cytoskeletal components in melanocytic skin lesions. An immunohistochemical study. *Am J Dermatopathol* 13:137–44

Smetsers TF, van de Westerlo EM, ten Dam GB, Clarijs R, Versteeg EM, van Geloof WL *et al.* (2003) Localization and characterization of melanoma-associated glycosaminoglycans: differential expression of chondroitin and heparan sulfate epitopes in melanoma. *Cancer Res* 63:2965–70

Vetter CS, Straten PT, Terheyden P, Zeuthen J, Brocker EB, Becker JC (2000) Expression of CD94/NKG2 subtypes on tumor-infiltrating lymphocytes in primary and metastatic melanoma. *J Invest Dermatol* 114:941–7

Wick MR, Patterson JW (2005) Cutaneous melanocytic lesions: selected problem areas. *Am J Clin Pathol* 124 (Suppl):S52–83

Zilka A, Landau G, Hershkovitz O, Bloushtain N, Bar-Ilan A, Benchetrit F *et al.* (2005) Characterization of the heparin/heparan sulfate binding site of the natural cytotoxicity receptor NKp46. *Biochemistry* 44:14477–85