Melanocyte Lineage-Specific Antigens Recognized by Monoclonal Antibodies NKI-beteb, HMB-50, and HMB-45 are Encoded by a Single cDNA

The glycoproteins recognized by monoclonal antibody (MAb) NKI-beteb are among the best diagnostic markers for human melanoma. MAb NKI-beteb reacts with melanoma cells throughout tumor development and does not cross-react with other tumor or normal cells, except for cells of the melanocytic lineage. Two other melanocyte lineage-specific MAbs, HMB-50 and HMB-45, show a specificity and staining pattern strikingly similar to the ones observed for MAb NKI-beteb. Herein, we demonstrate that all three MAbs recognize protein products encoded by a single cDNA. Expression of this cDNA in BLM cells results in immunoreactivity with all three MAbs. In addition, we demonstrate co-distribution of the RNA species detected by the cDNA with the proteins recognized by the MAbs in tissue sections. (Am J Pathol 1993, 143:1579–1585)
we report the isolation of a cDNA, which not only encodes the antigens recognized by MAb NKI-beteb, but also those recognized by MAbs HMB-45 and HMB-50.

Materials and Methods

Cells and Tissues

The melanoma cell lines MEL-2a, MEL-1, M14, MEL-57, MEWO, and BLM were described previously. Isolation of normal human melanocytes from breast or foreskin was performed by the method of Eisinger and Marko with modifications as described previously. Normal fetal epidermal tissue (approximately 15 weeks) and three primary cutaneous melanomas were obtained after surgery and immediately frozen in liquid nitrogen until use.

cDNA Library Construction, Screening, and Subcloning

A λ-gt11 cDNA expression library was constructed from poly(A) RNA isolated from the human melanoma cell line MEL-1 and screened with the rabbit polyclonal antiserum against the 95 kd glycoprotein using standard techniques. The cDNA insert of a positive clone, λ-gp100-c1, was removed as an EcoRI fragment, blunt-ended by filling in the ends with Klenow DNA polymerase and then ligated in the Sma I site of pRSV328A in either the coding (pRSVgp100+) or the noncoding (pRSVgp100-) orientation.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared by the guanidine thiocyanate procedure and centrifugation through a cushion of cesium chloride. RNA samples (5 to 30 µg) were size fractionated on a formaldehyde-agarose gel, transferred onto Hybond-N membranes (Amersham), and hybridized to α-[32P]-dATP-labeled probes (random primed DNA labeling kit, Boehringer Mannheim, Mannheim, Germany). As probes we have used the 5' EcoRl-Nco I fragment (±550 bp) or the 3' BelXI-EcoRl fragment (±450 bp) of gp100-c1 cDNA.

Transfection and Immunofluorescence

BLM cells were transfected with 20 µg of DNA by the calcium phosphate co-precipitation procedure using calcium phosphate transfection systems (Bethesda Research Laboratories, Bethesda, MD). Five hours after the addition of the precipitate cells were shocked with either dimethylsulfoxide (10% in medium for 90 seconds) or glycerol (10% in medium for 3 minutes). Forty-eight hours after transfection cells were prepared for immunofluorescence (fluorescein isothiocyanate) as described previously. MAbs NKI-beteb and HMB-50 were described previously. MAb HMB-45 was purchased from Enzo Biochem. Preparations were examined on a confocal laser scanning microscope at 488 nm (Bio-Rad MRC 600).

Immunocytochemistry and In Situ Hybridization

For immunocytochemistry, serial cryosections (5 µ) were fixed in acetone (10 minutes) then rehydrated and incubated with MAbs NKI-beteb (Zymed Laboratories, 1:150), HMB-45 (ENZO Biochem, 1:80), or HMB-50 ascites (1:1000) for 1 hour at 20°C. Subsequently, sections were washed then incubated with biotinylated horse anti-mouse antiserum (Vector, 1:200) for 30 minutes at 37°C. The immunohistochemical reaction was visualized with amino-ethyl-carbazole/H2O2. Finally, the sections were counterstained with hematein and embedded in Kaiser's glycerin. Cryosections not incubated with a primary MAb served as a control.

RNA in situ hybridization was performed on cryosections (8 µ) adjacent to those used for immunocytochemistry as described earlier. As a probe we used a α-[35S]-dCTP random primed labeled EcoRl-Nco I fragment (± 550 bp) of gp100-c1 cDNA. As a control, cryosections were hybridized to α-[32P] random primed labeled phage λ-DNA.

Results

Based on the immunohistochemical and biochemical data described above, we assumed that the gp100 antigen recognized by MAb NKI-beteb and the 95 kd glycoprotein recognized by MAb HMB-50 share a similar, if not identical protein backbone. Therefore, we used a rabbit polyclonal antiserum against the 95 kd glycoprotein to screen a λ-gt11 cDNA expression library prepared from poly(A) RNA isolated from human melanoma cells. A positive clone, λ-gp100-c1, containing a cDNA insert of approximately 2.2 kb was obtained and used for
Melanocyte Markers Encoded by a Single cDNA

Further analysis. To detect the RNA species from which the cDNA is derived, Northern blot analysis was performed using either the 5' part (± 550 bp) or the 3' part (± 450 bp) of gp100-c1 cDNA as a probe. Both probes specifically detected RNA species of 2.5 kb, 4.2 kb, and a high molecular weight RNA species (>15 kb) in total RNA isolated from adult and neonatal melanocytes as well as in total RNA isolated from four of five human melanoma cells (Figure 1 and results not shown). Note that no hybridizing RNA species were detected in RNA from BLM melanoma cells, consistent with the absence of reactivity with the MAbs (see below).

GP100-C1 cDNA Encodes a Protein that is Recognized by MAbs NKI-beteb, HMB-50, and HMB-45

To determine whether gp100-c1 cDNA encodes the antigen recognized by MAbs NKI-beteb and/or HMB-50, we cloned the cDNA into the eukaryotic expression vector pRSV328A15 in either the coding (pRSVgp100+) or the noncoding (pRSVgp100−) orientation and transfected these constructs in BLM melanoma cells. BLM cells were selected for the presence of reactivity with MAbs NKI-beteb, HMB-50, and HMB-45, because they do not react with MAbs NKI-beteb, HMB-50, or HMB-45 nor express the RNA species detected by the gp100-c1 cDNA probe (see above). As shown in Figure 2 B and H, BLM cells expressing the transcription unit containing the cDNA in the coding orientation reacted with MAb NKI-beteb and MAb HMB-50. The granular, cytoplasmic staining pattern observed with MAbs NKI-beteb and HMB-50 in the transfected cells is very similar to the one observed in MEWO melanoma cells, which express the antigens endogenously (Figure 2 A and G). No immunoreactivity to either MAb was observed when BLM cells were transfected with the construct containing the cDNA in the noncoding orientation (Figure 2 C and I).

The similar reactivity of MAbs NKI-beteb and HMB-45 to cells of the melanocytic lineage led us to investigate whether MAb HMB-45 reacts with the proteins encoded by gp100-c1 cDNA. As shown in Figure 2 E and F, only BLM cells transfected with pRSVgp100+ reacted with MAb HMB-45. The staining pattern observed with MAb HMB-45 in transfected BLM cells was similar to the one observed with MAbs NKI-beteb and HMB-50 and mimicked the one of HMB-45-stained MEWO melanoma cells (Figure 2 B, D, E, and H). We conclude from these data that a single cDNA encodes the melanocyte lineage-specific antigens recognized by MAbs NKI-beteb, HMB-50, and HMB-45.

In Situ Detection of GP100 RNA and Protein in Malignant Melanomas and Fetal Skin

Because of their specificity for cells of the melanocytic lineage MAbs NKI-beteb, HMB-45, and HMB-50 are important tools in the diagnosis of malignant melanoma. To compare the distribution of the proteins recognized by these MAbs with the expression pattern of the RNA species detected by gp100-c1 cDNA, we performed in situ hybridization in combination with immunocytochemistry on sequential cryosections of primary melanomas and fetal skin. In two primary melanomas co-distribution of gp100 RNA and the proteins recognized by each of the MAbs was observed (Figure 3). Beside melanocytes and/or melanoma cells, no other cells reacted with the cDNA probe or the MAbs. One other primary melanoma we selected as a control did not react with any of the MAbs nor with the cDNA probe (not shown). This result demonstrates that for the tumor biopsies the presence or absence of reactivity with all three MAbs and...
with gp100-c1 cDNA are mutually exclusive. Co-distribution of RNA and protein in fetal skin was most pronounced in melanocytes present in hair follicles (not shown). Hair follicles cut at a position devoid of melanocytes did not react with the cDNA probe or the MAbs (not shown). Taken together, these results clearly demonstrate specific co-distribution of RNA species detected by gp100-c1 cDNA with the proteins recognized by all three MAbs in cells of the melanocytic lineage.
Interestingly, we found that NK-1-deleted and HMB-50-interfering mice show high melanocyte infiltration to MAFs, which are negative for all three markers in BLM cells. This indicates that melanocyte infiltration may be a marker for high infiltration of MAFs.

**Discussion**

By using previously described markers, NK-1-deleted melanocytes infiltrate the skin in a single melanocyte-specific manner. These melanocytes are recognized as melanocytes by their expression of HMB-45 and HMB-50. In conclusion, our findings support the hypothesis that melanocyte infiltration may be a marker for high infiltration of MAFs.
MAb HMB-45, which is widely used in diagnostic pathology because of its specificity and sensitivity on paraffin-embedded tissue sections, also reacts with BLM cells expressing gp100-c1 cDNA. This result is consistent with previous data suggesting that MAbs NKI-beteb and HMB-45 recognize different epitopes on the same antigen.1

An RNA species of 2.5 kb is the major RNA product detected by the cDNA probe in melanoma cells as well as in adult and neonatal skin melanocytes. In these cells, additional RNA species of 4.2 and >15 kb were also discerned. The finding that the 5' part and the 3' part of gp100-c1 cDNA detect the same RNA species indicates that the cDNA isolated is derived from a single RNA species. The size of the major polyadenylated RNA species detected (2.5 kb) is also consistent with the size of gp100-c1 cDNA (2.2 kb). Using in situ hybridization in combination with immunocytochemistry on sequential sections of primary melanomas and fetal skin, we demonstrate co-distribution of gp100 RNA with proteins reacting to all three MAbs in cells of the melanocytic lineage. These findings provide additional evidence that gp100-c1 cDNA encodes the antigens recognized by all three MAbs. The notion that the melanocyte lineage-specific antigens recognized by MAbs NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA is also fully consistent with the finding that proteins of 100 and 10 kd are immunoprecipitated by MAbs NKI-beteb and HMB-50 from nonpigmented COS cells expressing gp100-c1 cDNA (G.J. A. et al, manuscript submitted).

Comparison of the reported reactivity of MAbs NKI-beteb, HMB-45, and HMB-50 to subpopulations of melanocytic cells reveals that all three MAbs react with melanoma cells and neonatal and fetal melanocytes, but that only MAB NKI-beteb recognizes resting adult melanocytes in skin.1,3,4 This observation led to the hypothesis that MAbs HMB-50 and HMB-45 detect oncofetal proteins.3,4 However, the finding that all three MAbs recognize the protein encoded by a single cDNA argues against this hypothesis and suggests a role for posttranslational protein modification. Aberrations in posttranslational modification (e.g., glycosylation) of proteins are known to frequently occur in tumor cells, including melanoma cells.20 Actually, differences in the posttranslational modification of the antigens detected by HMB-50 in melanoma cells compared with neonatal foreskin melanocytes have been reported.4 In addition, results of a recent study suggest that the epitope recognized by MAb HMB-45 is dependent on the presence of sialic glycoconjugates.21 Furthermore, we found that cultured adult melanocytes strongly react with all three MAbs (not shown).13 This latter result is consistent with previous data showing that beside neoplastic transformation, melanocyte stimulation also results in immunoreactivity to MAb HMB-45.22,23 Collectively, these data implicate that MAbs HMB-45 and HMB-50 recognize posttranslational modifications of a constitutively expressed melanocyte-specific protein, not a de novo synthesized oncofetal protein.

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