Loss of AP-2 Results in Up-regulation of MCAM/MUC18 and an Increase in Tumor Growth and Metastasis of Human Melanoma Cells*

(Received for publication, March 4, 1998, and in revised form, April 8, 1998)

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MCAM/MUC18 is a cell-surface glycoprotein of 113 kDa, originally identified as a melanoma antigen, whose expression is associated with tumor progression and the development of metastatic potential. We have previously shown that enforced expression of MCAM/MUC18 in primary cutaneous melanoma led to increased tumor growth and metastatic potential in nude mice. The mechanism for up-regulation of MCAM/MUC18 during melanoma progression is unknown. Here we show that up-regulation of MCAM/MUC18 expression in highly metastatic cells correlates with loss of expression of the transcription factor AP-2. The MCAM/MUC18 promoter contains four binding sites for AP-2, and electrophoretic mobility shift assay gels demonstrated that the AP-2 protein bound directly to the MCAM/MUC18 promoter. Transfection of AP-2 into highly metastatic A375SM melanoma cells (AP-2-negative and MCAM/MUC18-positive) inhibited MCAM/MUC18 promoter-driven chloramphenicol acetyltransferase reporter gene in a dose-dependent manner. MCAM/MUC18 mRNA and protein expression were down-regulated in AP-2-transfected but not in control cells. In addition, re-expression of AP-2 in A375SM cells inhibited their tumorigenicity and metastatic potential in nude mice. These results indicate that the expression of MCAM/MUC18 is regulated by AP-2 and that enforced AP-2 expression suppresses tumorigenicity and metastatic potential of human melanoma cells, possibly by down-regulating MCAM/MUC18 gene expression. Since AP-2 also regulates other genes that are involved in the progression of human melanoma such as c-KIT, E-cadherin, MMP-2, and p21WAF-1, we propose that loss of AP-2 is a crucial event in the development of malignant melanoma.

The ability of tumor cells to detach from the primary site and produce metastases in a distant organ is due to the survival and growth of a unique subpopulation of cells with metastatic properties (1, 2). One tumor cell property that is essential for metastasis is the expression of cell adhesion molecules, which mediate cell-to-cell or cell-to-matrix interactions. The cell-surface adhesion molecule MCAM/MUC18 is strongly expressed by advanced primary and metastatic melanomas but is weaker and less frequent in nevus cells (3, 4). Approximately 70% of melanoma metastases express this molecule, and among primary tumors, expression increases with increasing vertical thickness, an important predictor of metastatic disease (5, 6). Indeed, we have demonstrated that expression of MCAM/MUC18 by human melanoma cell lines correlates with their ability to grow and to produce metastases in nude mice (7), suggesting that MCAM/MUC18 may play a pivotal role in the development of malignant melanoma. This hypothesis is supported by the observation that the production of tumorigenic variants from a non-tumorigenic melanoma cell line is accompanied by MCAM/MUC18 up-regulation (8) and by our recent observation that enforced MCAM expression in primary cutaneous melanoma leads to increased tumor growth and metastasis in vivo (9). The transfected cells displayed increased homotypic adhesion, increased attachment to human endothelial cells, decreased ability to adhere to laminin, and increased invasiveness through Matrigel-coated filters due to up-regulation in the expression of MMP-2 (9). The above changes in the function attributed to the expression of MCAM may underlie the contribution of MCAM/MUC18 to the malignant phenotype.

MCAM/MUC18 is a member of the immunoglobulin superfamily (10) that shares homology with gicerin (33% amino and identity), a molecule mediating intercellular adhesion in the developing nervous system (11). It contains five immunoglobulin-like domains, and its cytoplasmic domain contains several protein kinase recognition motifs, suggesting the involvement of MCAM/MUC18 in cell signaling (12). MCAM/MUC18 can mediate homotypic interaction either through the MCAM antigen (9, 13) or via heterophilic ligand (14, 15).

The mechanisms for up-regulation of MCAM/MUC18 gene expression during melanoma progression are unknown. We have previously reported that overexpression of MCAM/MUC18 in highly metastatic melanoma cells is not due to amplification or to rearrangement of the gene (7). The promoter of MCAM/MUC18 has been cloned and sequenced (12). It is a G + C-rich promoter lacking the conventional TATA and CAAT boxes, but strikingly, it contains four putative AP-2-binding elements (12). These observations coupled with our previous finding that the vast majority of highly metastatic human melanoma cells do not produce the AP-2 transcription factor (16) led us to hypothesize that MCAM/MUC18 expression is regulated at the transcriptional level, and in particular AP-2 regulates MCAM/MUC18 expression in human melanoma cells. AP-2, a 52-kDa protein, was first purified from HeLa cells. Partial peptide sequences led to the isolation of the cDNA from...
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a HeLa cell culture (17), and the gene was mapped to a region on the short arm of chromosome 6 near the HLA locus (18, 19). The AP-2 protein binds to a consensus palindromic core recognition element with the sequence 5′-GCCNNNGGC-3′ (17). Functional AP-2-binding sites have been identified in the enhancer regions of viral genes such as simian virus 40 (SV40) (20), human T-cell leukemia virus type I (21), and cellular genes such as those for murine major histocompatibility complex (H-2Kb), human metallothionine-IIA (huMTIIa), human proenkephalin, human keratin K14, c-ERBB-2, plasminogen activator type I (PAI-1), and insulin-like growth factor-binding protein-5 (22–28). The DNA-binding domain is located within the C-terminal half of the 52-kDa protein and consists of two putative amphipatic α-helices separated by an 82-amino acid intervening span that is both necessary and sufficient for homodimer formation (29). An alternatively spliced AP-2 protein, AP-2B, that differs in its C terminus and acts as dominant-negative to AP-2 has been recently cloned (30).

AP-2 activity is regulated through a number of signal transduction pathways. Phorbol esters and signals that enhance cAMP levels induce AP-2 activity independently of protein synthesis, whereas retinoic acid treatment of teratocarcinoma cell lines result in a transient induction of AP-2 mRNA levels on a transcriptional level (30, 31). AP-2 is involved in mediating programmed gene expression both during embryonic morphogenesis and adult cell differentiation. By using in situ hybridization, a restricted spatial and temporal expression pattern has been observed during murine embryogenesis. In particular, regulated AP-2 expression was observed in neural crest-derived cell lineages (from which melanocytes are derived) and in facial and limb bud mesenchyme (19). Two recent reports of AP-2-null mutant mice have demonstrated that AP-2 is important for development of the cranial region and for midline fusions. The AP-2-null mice died at birth (32, 33).

In this study, we provide the first evidence that (i) there is a direct correlation between expression of the AP-2 transcription factor and expression of MCAM/MUC18 in human melanoma cells; (ii) transfection of highly metastatic cells (MCAM-positive and AP-2-negative) with the AP-2 gene resulted in a decrease in endogenous MCAM/MUC18 mRNA and protein expression; and (iii) transfection of AP-2 into highly metastatic melanoma cells inhibited their tumor growth and metastatic potential in nude mice, possibly through down-regulation of endogenous MCAM/MUC18 mRNA and protein expression (34). The MCAM/MUC18 promoter segment was generated by the polymerase chain reaction using primers encompassing both ends of this domain and “sticky ends” of the MCAM/MUC18 promoter region spanning nucleotides −642 to +26 (12) was ligated upstream of the basic CAT expression vector. The MCAM/MUC18 promoter segment was confirmed by sequencing.

Western blot and Flow Cytometry—Melanoma cells (1.5 × 106) were seeded in 100-mm Petri dishes in 10 ml of complete medium and incubated overnight. The cells were scraped off and washed in 4 °C PBS, resuspended in 1 ml of 5% BSA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors in PBS, and counted. A total of 5 × 104 cells were used in subsequent experiments.

Electrophoretic Mobility Gel Shift Assay (EMSA)—The double-stranded DNA fragment corresponding to the MCAM/MUC18 promoter region of −642 to +26 was amplified by PCR using the basic CAT expression vector. The ERB-2 promoter-luciferase reporter plasmid, P42 (15), was used as a positive control. Anti-AP-2 and anti-CREB antibodies (Santa Cruz, CA) were used in the supershift analyses. Double-stranded oligonucleotides corresponding to the binding sites of CTF/NF1 and Oct1 were purchased from Stratagene (La Jolla, CA). AP-2 oligonucleotides were purchased from Santa Cruz (Santa Cruz, CA). Nuclear extracts were prepared as described by Dignam et al. (41). Protein concentrations were determined using the Bio-Rad reagent. The extracts were brought to 2 mg/mL, and gel retardation assays were performed using the Stratagene Gelshift kit. Binding reactions were initiated by incubating 5 μl of nuclear extract with 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech) for 15 min at room temperature. 32P-End-labeled double-stranded (ds) DNA was then added (0.1–0.5 ng, 1–4 × 105 cpm), and the incubation was continued for an additional 15 min. Nonlabeled competitor oligonucleotides, when used, were added at the same time as the labeled oligomers. Approximately 0.7 fmol of end-labeled dsDNA oligonucleotide was allowed to react with 0.525 μg of nuclear protein extract as determined by the BCA protein assay (Pierce). Proteins were allowed to incubate with DNA for 15 min prior to a 20-min room temperature incubation with antibodies. Complexes were resolved on a (29%) polyacrylamide gel in 0.5 M TBE (45 mM Tris·HCl (pH 8.0 at 20°C), 1 mM EDTA, and 292 mM boric acid, 1 mM EDTA, and 292 mM boric acid, 1 M Tris·HCl (pH 8.0 at 20°C), and 0.5 M NaOH) at 4°C, 200 V, and 2 h at room temperature. Gels were dried and exposed to x-ray film.

\[ \text{Western blot and Flow Cytometry—Melanoma cells (1.5} \times 10^6) \text{ were seeded in 100-mm Petri dishes in 10 ml of complete medium and incubated overnight.} \]
was separated by centrifugation at 15,000 \( \times g \) for 30 min at 4 °C. Protein concentration in the lysates was determined, and the samples were then diluted to 1 mg/ml in the lysis buffer. After boiling, 20 μg of the samples were loaded onto and separated on 7.5% SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The membrane was washed in a blocking buffer and incubated in a 1:5000 dilution of the first antibody (anti-MCAM monoclonal antibody BA3, 18.3 (10), or anti-AP-2 followed by treatment with a 1:3000 dilution of the second antibody (anti-immunoglobulin, horseradish peroxidase-linked Fab\(_2\) fragment from donkey). The probed proteins were detected with the enhanced chemiluminescence system (Amersham Pharmacia Bio tech, Piscataway, NJ) and 2.5 mg of purified mRNA was separated on 1% denaturing formaldehyde/agarose gels, electrotransferred at 0.6 A to Geneclean (Bio 101, La Jolla, CA), and radiolabeled by the random primer transcription method (Life Technologies, Inc.) and 2 μg of pSG5-Neo-A2 expression vector or control pSG5-Neo vector. Transfections were carried out according to the manufacturer's instructions. Fourteen days later, the DNA probes used in these analyses were a 1.9-kilobase pair AP-2 cDNA fragment digested with EcoRI from the pINAP2 plasmid (38) and a 1.3-kilobase pair PstI cDNA fragment corresponding to rat glyceroldehyde 3-phosphate dehydrogenase (GAPDH) (43). Each DNA fragment was purified by agarose gel electrophoresis, recovered using Geneclean (Bio 101, La Jolla, CA), and radiolabeled by the random primer technique using \( ^{32}P \)-dideoxyribonucleotide triphosphates. Stable Transfection of Melanoma Cells with AP-2—5 × 10^5 A375SM cells were transfected using 15 μl of Lipofectin reagent (Life Technologies, Inc.) and 2 μg of pSG5-Neo-A2 expression vector or control pSG5-Neo vector. Transfections were carried out according to the manufacturer's instructions. At 10 h following transfection, the medium was changed to serum-containing complete medium, and the cells were then further incubated for 48 h at 37 °C. Cells were selected after 48 h with standard medium containing G418 500 μg/ml. Fourteen days later, neo-resistant colonies were isolated by trypsinization and established in culture. Tumor Cell Injections—To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to 0.25% trypsin, 0.02% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells, and supplemented medium was added. The cell suspension was used to produce a single-cell suspension. The cells were washed and resuspended in Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of more than 90% viability were used. Subcutaneous tumors were produced by injecting 1 × 10^6 tumor cells in 0.2 ml of HBSS over the right scapular region. Growth of subcutaneous tumors was monitored by examination of the mice every day and weekly measurement of tumors with callipers. The mice were killed 49 days after injection, and tumors were processed for hematoxylin and eosin staining.

For experimental lung metastasis, 1 × 10^6 tumor cells in 0.2 ml of HBSS were injected into the lateral tail vein of nude mice. The mice were killed after 60 days, and the lungs were removed, washed in water, and fixed with Bouin's solution for 24 h to facilitate counting of tumor nodules as we described previously (44). The number of surface tumor nodules was counted under a dissecting microscope. Sections of the lungs were stained with hematoxylin and eosin to confirm that the nodules were melanoma and to identify micrometastasis.

Statistics—The significance of the in vitro results was determined by the Student's t test (two-tailed); the significance of the in vivo metastasis results was determined by the Mann-Whitney test.

RESULTS

Direct Correlation between MCAM/MUC18 and AP-2 Expression in Human Melanoma Cell Lines—The cell-surface adhesion molecule MCAM/MUC18 is associated with tumor progression and the development of metastasis in human melanoma. Indeed, the majority of advanced and metastatic tumors strongly express the MCAM/MUC18 antigen, whereas its expression on thin tumors (<0.75 mm), which have only a low probability of metastasizing, and on benign nevi is weaker and less frequent (3, 4). Furthermore, expression of MCAM/MUC18 by human melanoma cell lines correlates with their ability to grow and produce metastases in nude mice (7). We have recently demonstrated that transfection of MCAM-negative primary cutaneous melanoma with MCAM cDNA led to increased tumor growth and metastatic potential in nude mice (9), suggesting that MCAM plays a pivotal role in the progression of human melanoma. The mechanism(s) for MCAM up-regulation in metastatic melanoma cells have, however, remained unknown. In an effort to determine the molecular basis for MCAM up-regulation in metastatic cells, we found that MCAM overexpression is not due to gene amplification or rearrangement (7). Moreover, sequencing of the entire MCAM promoter revealed no abnormalities that could account for MCAM overexpression in metastatic cells (data not shown). These observations suggest that MCAM expression might be regulated at the transcriptional level.

To test this hypothesis, the activity of the CAT reporter gene driven by the MCAM promoter (−642 to +26) (12) was analyzed in MCAM-positive and MCAM-negative melanoma cells. Strong CAT activity was observed in the highly metastatic cell line A375SM (Fig. 1A, lane 2) that express abundant levels of MCAM (7, 9), as compared with a background level of CAT activity in the non-metastatic MCAM-negative SB-2 melanoma cells (lane 3). The SB-3 cell line that was established from a skin metastasis from the same patient, 1 year later, produces few metastases in nude mice and expresses low levels of MCAM (7, 9); it exhibited low levels of CAT activity (Fig. 1A, lane 4). These results suggest that MCAM production is regulated at the transcriptional level in these melanoma cells. To determine whether there is differential production of transcription factor(s) between MCAM expressor and non-expressor cells, we prepared nuclear extracts from A375SM (MCAM-positive) and SB-2 cells (MCAM-negative) and reacted them with the MCAM promoter on EMSA gel. Fig. 2A shows that nuclear extracts from SB-2 cells yielded a pattern of four protein/DNA bands (lane 2), whereas nuclear extract from A375SM cells produced only the top three bands with the bottom band (arrowhead) missing (lane 4), suggesting that the two cell populations differed in the production of factor(s) bound to the MCAM promoter.

The 0.9-kilobase pair MCAM promoter lacks TATA or CAAT boxes, is highly G + C-rich, and contains binding sites for SP-1, CREB, MYB, and four AP-2-binding elements (12). The presence of four AP-2-binding sites within the essential region of the MCAM promoter suggested that AP-2 might regulate MCAM gene expression.

Fig. 2B is an EMSA gel demonstrating that the bottom of the four bands that were observed with nuclear extracts from the SB-2 cells could be eliminated with an excess of ds AP-2-binding motif (lane 5) but not by oligonucleotides corresponding to other transcription factors such as CTF/NF-1 (lanes 6 and 7) or Oct1 (lanes 8 and 9). More importantly, when recombinant human AP-2 protein (rh-AP-2) was added to nuclear extracts of A375SM cells, the missing bottom band (arrowhead) reappeared (lane 10). These observations led us to determine whether the progression of human melanoma was associated with changes in the expression of the AP-2 transcription factor via regulation of MCAM gene expression.

To study whether AP-2 plays a role in the regulation of MCAM expression in human melanoma cells, we first exam-
ined the status of AP-2 in MCAM-positive and MCAM-negative human melanoma cell lines. Fig. 3A demonstrates that AP-2 mRNA expression was observed in the nonmetastatic MCAM/MUC18-negative A375SM cell line (lane 3), whereas the MCAM-positive cell lines A375P, A375SM, and WM-2664, which are also metastatic in nude mice (7), did not transcribe AP-2 mRNA. The SB-3 cell line, which produces low levels of MCAM (7), expressed only one of the AP-2 transcripts (lane 3). Expression of AP-2 in SB-2 cells and its lack in A375SM cells was also confirmed by Western blot analysis (Fig. 3B, lanes 1 and 3, respectively). The SB-3 cell line that expresses low levels of MCAM produced a faint band of AP-2 (Fig. 3B, lane 2). These results suggest that overexpression of MCAM in metastatic melanoma cells correlated with a deficiency in the level of AP-2 transcription factor.

**Down-regulation of MCAM Promoter by AP-2 in A375SM Cells**—To assess further the effect of AP-2 on MCAM transcription, the MCAM promoter-CAT construct was co-transfected into A375SM cells with increasing concentrations of an expression vector encoding AP-2 (pSG5-AP-2) or with a control vector lacking the AP-2 cDNA (pSG5). Fig. 1B shows that CAT activity driven by the MCAM/MUC18 promoter was inhibited by AP-2 in
A375SM cells in a dose-dependent manner. These experiments indicate the presence of functional AP-2 elements within the MCAM promoter, which regulate MCAM expression in human melanoma cells.

**Direct Interaction of AP-2 with the MCAM Promoter**—To determine if AP-2 down-regulation of the MCAM promoter was due to direct AP-2 binding to the MCAM promoter, we examined whether the MCAM promoter region (−642 to +26) that contains four AP-2-binding sites would react with recombinant human AP-2 (rh-AP-2) on an EMSA gel. Fig. 2C shows that rh-AP-2 bound directly to this fragment (lane 2, arrowhead). This binding was abrogated by an excess of unlabeled double-stranded AP-2-binding DNA sequences (Fig. 2C, lanes 4 and 5) but not by the AP-1-binding motif (lanes 6 and 7). Furthermore, the observed DNA-protein complex was supershifted by anti-AP-2 antibody (lane 3). These data demonstrate that AP-2 bound directly to a region of the MCAM promoter that is required for its transcription.

**Ectopic Expression of AP-2 in A375SM Human Melanoma Cells**—To assess the effect of the AP-2 transcription factor on MCAM expression and on the acquisition of the metastatic phenotype in human melanoma cells, we decided to re-express AP-2 in A375SM cells. A375SM cells are highly metastatic in nude mice (7, 9), overexpress MCAM (7, 9), and produce little or no endogenous AP-2 (Fig. 3, A-D). Following gene transfection with expression vector carrying a full-length human AP-2 cDNA or an empty vector, neo-resistant colonies were pooled and established in culture. Two independent transfections were performed. Therefore, two transfectants were obtained and designated A375SM-AP-2.T1 (or T1) and A375SM-AP-2.T2 (or T2), respectively. Northern blot analysis using AP-2 cDNA as a probe detected high levels of AP-2 mRNA transcripts in the two transfectants (Fig. 3C) but only residual levels in parental A375SM cells. Furthermore, nuclei extracts from parental A375SM cells yielded three strongly shifted bands similar to the pattern observed with nuclear extracts from the SB-2 cells (lanes 6 and 7). The upper bands (band 1) in the two transfectants were supershifted when reacted with anti-AP-2 antibody (lane 4) but not with anti-CREB antibody (lane 3). In contrast, nuclear extracts from parental A375SM cells yielded a strong band 2 and very faint bands 1 and 3 (lane 5), whereas nuclear extracts from the two AP-2 transfectants T1 and T2 yielded three strongly shifted bands similar to the pattern observed with nuclear extracts from the SB-2 cells (lanes 6 and 7). The upper bands (band 1) in the two transfectants were supershifted when reacted with anti-AP-2 antibody (lanes 8 and 9), but bands 1 and 3 were competed out by an excess of unlabeled ds oligonucleotide probe (lanes 10 and 11). Formation of band 2 was slightly competed out with a 100 molar excess of unlabeled AP-2 (lane 11).

To confirm further that the AP-2 in the transfected cells was transcriptionally active, we took advantage of the observation...
MCAM protein expression was observed in A375SM parental cells (AP-2 stably transfected human melanoma cells). The reporter constructs were transfected into control or A375SM-AP-2 transfectants either did not express MCAM (A375SM-AP-2,T1, lane 5) or expressed a faint band (A375SM-AP-2,T2, lane 6). The non-metastatic SB-2 melanoma cells that do not express MCAM served as a negative control (lane 1). The same membrane was probed with β-actin to show equal protein loading.

that AP-2 transactivates the metallothionein promoter (45). To that end, we constructed a luciferase reporter gene expression vector driven by three AP-2 consensus response elements from the human metallothionein gene IIA ligated in front of a minimal thymidine kinase promoter. The reporter constructs were transfected into control or AP-2-transfected A375SM cells, together with the β-actin-RL plasmid that served as an internal control for transfection efficiency. The luciferase activity was 5- and 8-fold higher in the AP-2 transfectants A375SM-AP-2,T1 and A375SM-AP-2,T2, respectively, compared with the neo-control cells (data not shown). These data demonstrate that the two AP-2 stably transfected T1 and T2 clones produced high levels of active AP-2.

**Down-regulation of MCAM Gene Expression in A375SM Cells Transfected with AP-2**—Our promoter analyses (Fig. 1B) indicated that AP-2 is an important regulator of MCAM gene expression. We therefore next examined the effect of AP-2 transfection into A375SM cells on MCAM gene expression. Expression of MCAM in the AP-2-transfected cells was analyzed by Western blot analyses (Fig. 5). Production of the 113-kDa MCAM protein was observed in parental A375SM cells (Fig. 5, lane 2) and in two neo-transfected cells (neo.a, lane 3; and neo.b, lane 4). In contrast, the AP-2 transfectants either did not express the MCAM protein (T1, lane 5) or expressed a faint band (T2, lane 6).

To provide further evidence that expression of AP-2 in A375SM cells down-regulated MCAM expression, we next examined MCAM expression by fluorescence-activated cell sorter analyses on the cell surface of parental A375SM, neo-transfected, and in AP-2-transfected cells (A375SM-AP-2,T1). By using specific monoclonal anti-MCAM antibody that recognizes the extracellular domain of MCAM (3, 10), we were able to demonstrate that MCAM expression occurred in 98.4% of A375SM parental cells, in 90.1% of control, neo-transfected cells, but in only 17.9% of the AP-2-transfected A375SM-AP-2,T1 cells.

**Tumorigenicity and Metastatic Potential of AP-2-transfected Melanoma Cells**—We have previously demonstrated that enforced MCAM/MUC18 expression in the primary cutaneous melanoma SB-2 cells increases tumor growth and metastatic potential in nude mice (9). To determine the tumorigenicity of the AP-2-transfected A375SM cells which exhibited down-regulation in MCAM expression, we injected 1 x 10⁶ cells subcutaneously into BALB/c nude mice and monitored tumor growth once a week for 50 days. Both A375SM parental and neo-transfected control cells grew in all mice (100% tumor uptake) and reached 1.0–1.4 cm in mean diameter within 6 weeks (Fig. 6). In contrast, A375SM AP-2-transfected cells did not begin to form palpable tumors until 3 weeks (T2) or 7 weeks (T1) after injection and produced smaller tumors (0.1 and 0.45 cm in mean diameter). Growth of the A375SM-AP-2,T1 cells differed significantly from parental and neo-control cells at all times (p < 0.01), whereas the difference between the A375SM-AP-2,T2 and parental or neo-transfected cells was statistically significant 5 and 6 weeks after injection (p < 0.05) (Fig. 6). These data suggest that transfection of AP-2 into A375SM cells suppressed their tumor growth in vivo.

In the next set of experiments, the metastatic potential of AP-2-transfected A375SM cells was determined in an experimental lung metastasis assay (40, 44). To that end, BALB/c nude mice were injected intravenously with 1 x 10⁶ A375SM AP-2-transfected, parental, or neo-control cells, and 60 days later, the number of lung metastases was counted. As shown in Table I, A375SM and A375SM-Neo (Neo.a and Neo.b) produced a high number of lung tumor colonies in all mice injected. In contrast, the A375SM-AP-2,T1-transfected cells did not metastasize to the lungs (T1) or produced only a few lung metastases in some mice (T2). To determine whether those metastatic revertants still overexpressed AP-2, five metastatic nodules were isolated and established as individual cell lines. AP-2 expression in these cell lines was analyzed by Northern blot. We could not detect increased levels of AP-2 mRNA in these cell lines as compared with parental and neo-control cells (data not shown). These data implied that the occasional gain of metastatic potential in A375SM-AP-2 cells was due to the loss of exogenous AP-2 expression.
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TABLE I
Metastatic potential of A375SM human melanoma cells transfected with transcription factor AP-2

| Cell line       | Incidence | Experimental lung metastasis | Median no. | Range          | p* |
|-----------------|-----------|-------------------------------|------------|----------------|----|
| A375SM-P        | 5/5       | 121                           | 74–200     | 0.05           |
| A375SM-Neo.a    | 5/5       | 153                           | 111–200    | >0.05          |
| A375SM-Neo.b    | 5/5       | 87                            | 54–143     | >0.05          |
| A375SM-AP-2.T1  | 0/5       | 0                             | All 0      | <0.01          |
| A375SM-AP-2.T2  | 3/5       | 19                            | 0–47       | <0.05          |

* Tumor cells (1 × 10⁶/mouse) were injected intravenously into nude mice; experimental metastasis was determined 60 days after tumor injection. The differences in metastatic potential between parental cells and transfected cells were analyzed by the Mann-Whitney U test.

To determine whether the inhibition of tumor growth and the decreased capacity to produce lung metastases by A375SM-AP2 cells in vivo was due to different growth rates in vitro, we compared their doubling time to control cells in cultures. When the cells were cultured in medium containing 0.5% FBS (limiting condition), the cell doubling times of A375SM parental and neo-control cells were indistinguishable from the A375SM-AP2 cells (45 h). In contrast, in medium containing 10% FBS, the doubling time for parental and neo-transfected cells was 24 h while the AP-2-transfected cells doubled every 36 h. In addition, in medium containing 10% FBS, the AP-2-transfected cells exhibited morphological changes; they were enlarged and flat and had more multinucleated cells (data not shown).

**DISCUSSION**

In this paper we demonstrate that the transcription factor AP-2 plays a key role in regulating tumor growth and metastasis of human melanoma. Loss of AP-2 results in up-regulation of MCAM/MUC18 and enhancement of melanoma tumorigenicity and metastasis.

MCAM/MUC18 is an adhesion molecule associated with melanoma development and progression. MCAM is expressed most strongly on metastatic lesions and advanced primary tumors and is only rarely detected in benign lesions (3, 4). Moreover, expression of MCAM correlates directly with the metastatic potential of human melanoma cell lines in nude mice (7). In addition, we have previously shown that enforced expression of the MCAM gene in MCAM-negative primary cutaneous melanoma cells rendered them highly tumorigenic and increased their metastatic potential in nude mice, thus providing direct evidence for the involvement of MCAM in tumor growth and metastasis of human melanoma (9). The mechanism by which malignant melanoma cells up-regulate MCAM expression was unclear. Here, we provide evidence for the first time that in metastatic melanoma cells overexpression of MCAM correlates with lack of expression of the AP-2 transcription factor and that in melanoma cells AP-2 serves as a negative regulator of MCAM gene expression. Indeed, re-expression of AP-2 in the highly metastatic A375SM cells resulted in down-regulation of MCAM expression and in turn in inhibition of tumor growth and metastasis.

In our previous studies (9), we demonstrated that melanoma cells expressing MCAM displayed increased homotypic adhesion, increased attachment to human endothelial cells (which points to the involvement of MCAM in angiogenesis), increased MMP-2 activity, and an increased invasiveness through Matrigel-coated filters (9). These changes in function attributed to the expression of MCAM underlie its contribution to the malignant phenotype. Therefore, down-regulation of MCAM expression in the AP-2-transfected cells provides a possible mechanism for the reduction in tumorigenicity and the ability of these cells to form lung metastasis in vivo. However, in other studies we have demonstrated that the tyrosine kinase receptor c-KIT, which plays a major role in the acquisition of the metastatic phenotype in human melanoma (46), is also highly regulated by the AP-2 transcription factor. Although metastatic cells do not express the c-KIT receptor, following AP-2 gene transfection, c-KIT is re-expressed in these cells. As c-KIT expression renders the cells susceptible to stem cell factor-induced apoptosis in vitro and in vivo (46), this may provide an alternative mechanism for the inhibition of tumor growth and metastasis of the AP-2-transfected cells. In addition, other genes that are involved in the progression of human melanoma, such as MMP-2 (36, 47), E-cadherin (48), p21WAF-1 (49, 50), HER-2 (51), plasminogen activator inhibitor type I (27), and BCL-2 (52, 53), have either already been shown to be regulated by AP-2 (26, 54) or represent likely targets for AP-2 gene regulation based on the existence of AP-2 elements in their promoters. Therefore, we propose that loss of AP-2 expression is the crucial event in the development of malignant melanoma. As such, AP-2 may act as a “master switch” in the progression of human melanoma. In our proposed model, AP-2 plays a pivotal role in regulating the expression of several genes whose products are involved in tumor growth and metastasis of melanoma. AP-2 regulates genes that are involved in proliferation (c-KIT and BCL-2), adhesion (MCAM/MUC18 and E-cadherin), and invasion/angiogenesis (MMP-2, plasminogen activator inhibitor type I, and MCAM/MUC18). These functional changes attributed to one transcription factor may underlie the contribution of AP-2 loss of expression to the malignant phenotype.

Besides containing four binding sites for AP-2, the promoter of the MCAM/MUC18 gene also contains a potential cAMP-responsive element (12). Indeed, recent studies have shown that agents that increase intracellular cAMP levels can also increase MCAM protein and mRNA levels, whereas exposure to phorbol ester, a direct activator of protein kinase C, can lead to down-regulation of MCAM gene expression (55). Although the natural triggers of these pathways remain unknown, recent studies have shown that cellular expression of MCAM is regulated by its microenvironment and that keratinocytes can modulate the expression of MCAM in melanocytes and nevus cells but not in melanoma cells from primary and metastatic lesions (56). It is possible that keratinocytes regulate MCAM expression via regulation of AP-2 gene expression. This hypothesis is currently being tested in our laboratory.

The mechanisms accounting for the lack of expression or down-regulation of AP-2 in malignant melanoma cells are currently unknown. One tempting possibility is to link the lack of AP-2 expression to cytogenetic evidence demonstrating that the majority of malignant melanoma cells exhibit deletion of the distal portion of the long arm of chromosome 6 (57, 58) or, most importantly, with abnormalities in the short arm of chromosome 6 (6p) near the HLA locus, to which the AP-2 gene is mapped (18). Indeed, deletions and LOH have been reported in this region in malignant melanoma (59–61). Moreover, reintroduction of chromosome 6 into metastatic melanoma cells inhibited their tumorigenicity and metastatic potential (57, 63). These studies suggest that inactivation of a tumor suppressor gene on chromosome 6 may be the critical event in the progression of melanoma. The mechanism for the lack of AP-2 expression in the metastatic melanoma cells used in our study is currently under investigation.

Another possibility is a link between UV irradiation and AP-2 expression. Epidemiological data suggest that exposure to...
UV radiation plays a major role in the development of at least some cutaneous melanomas (64, 65). The role of UV radiation in the development of human cutaneous melanoma to its metastatic state is not very well characterized. We have recently demonstrated that UV-B irradiation can promote tumorigenic and metastatic properties in primary cutaneous melanomas via induction of interleukin-8 (36, 47). A provocative idea will be to link abnormalities in the AP-2 gene with UV radiation that serves as a carcinogen for cutaneous melanoma. Indeed, two recent reports have shown that both UV-A and UV-B radiation can alter the expression and activity of the AP-2 transcription factor in human keratinocytes and epidermoid carcinoma cell line (A431) (66, 67). The question of whether UV radiation can affect AP-2 expression and AP-2 activity in human primary cutaneous melanoma is currently under investigation in our laboratory.

Finally, the diagnosis of melanoma is often made after the cancer has already metastasized to the regional and distant lymph nodes, brain, liver, lung, and central nervous system (68). Since current staging systems enable us to identify only some of the melanoma patients who will develop metastases, better prognostic markers need to be identified. AP-2 may serve as an excellent prognostic marker. Our analysis of AP-2 expression in human melanoma cell lines indicates that AP-2 is expressed only in cell lines derived from primary cutaneous melanoma (radial growth phase), but not in cell lines derived from metastatic lesions (16). It will be extremely important to establish at what stage in the progression of melanoma this event occurs. Currently, several hundred human melanoma specimens collected at the M. D. Anderson Cancer Center and representing different stages in the progression of human melanoma (from atypical nevi to metastatic lesions) are being evaluated for AP-2 expression. AP-2 may be useful not only as a new molecular staging marker but eventually as a common target for anti-tumor and anti-metastatic therapy.

Acknowledgments—We thank Dr. Isaiah J. Fidler for critical reading of the manuscript, Dr. Judith P. Johnson for providing anti-MCAM/UCH18 antibody, and Patherine Greenwood for expert preparation of this manuscript.

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