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Regulation of Gene Expression in Melanoma: New Approaches for Treatment

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Abstract The molecular changes associated with the transition of melanoma cells from radial growth phase (RGP) to vertical growth phase (VGP, metastatic phenotype) are not yet well defined. We have demonstrated that the progression of human melanoma is associated with loss of expression of the transcription factor AP-2. In metastatic melanoma cells, this loss resulted in overexpression of MCAM/MUC18, MMP-2, the thrombin receptor (PAR-1), and lack of c-KIT expression. The transition from RGP to VGP is also associated with overexpression of the angiogenic factor IL-8. Additionally, the transition of melanoma cells from RGP to VGP is associated with overexpression of the transcription factors CREB and ATF-1, both of which may act as survival factors for human melanoma cells. Inactivation of CREB/ATF-1 activities in metastatic melanoma cells by dominant-negative CREB or by anti-ATF-1 single chain antibody fragment (ScFv), resulted in deregulation of MMP-2 and MCAM/MUC18, increased the sensitivity of melanoma cells to apoptosis, and inhibition of their tumorigenicity and metastatic potential in vivo. In this prospect article, we summarize our data on the role of AP-2 and CREB/ATF-1 in the progression of human melanoma and report on the development of new fully human antibodies anti-MCAM/MUC18 and anti-IL-8 which could serve as new modalities for the treatment of melanoma. J. Cell. Biochem. 94: 25–38, 2005.

Key words: angiogenesis; gene expression; malignant melanoma; metastasis; transcription factor

MELANOMA PROGRESSION

As it progresses through a multi-step process, malignant melanoma in humans switches from melanocytes to nevi, to radial growth phase (RGP) and subsequently to vertical growth phase (VGP, metastatic phenotype, Fig. 1). These switches are associated with molecular and genetic changes [Clark et al., 1984; Bar-Eli, 1997; Luca and Bar-Eli, 1998]. The mechanisms, however, that mediate this differential expression of genes during melanoma progression remain largely unknown despite the fact that the worldwide incidence of melanoma is increasing more than any other neoplastic disease [Kopf et al., 1995]. There has been, however, some progress in our understanding the development of malignant melanoma. Of particular relevance to this prospect article are recent results concerning abnormalities in the expression of transcription factors, which in turn modulate and regulate several genes controlling tumor growth and metastasis of melanoma.

The prevalent working model for melanoma metastasis development defines it as a series of interrelated, sequential steps involving the survival and growth of a unique subpopulation of cells with metastatic properties that pre-existed within the parental neoplasm [Fidler, 1990]. By their ability to regulate the expression of several genes, transcription factors could play an important role during melanoma progression.

The first part of this prospect article will focus on our recent studies demonstrating that progression of human melanoma is associated with loss of expression of the transcription factor AP-2. In metastatic melanoma cells, this loss resulted in overexpression of MCAM/MUC18

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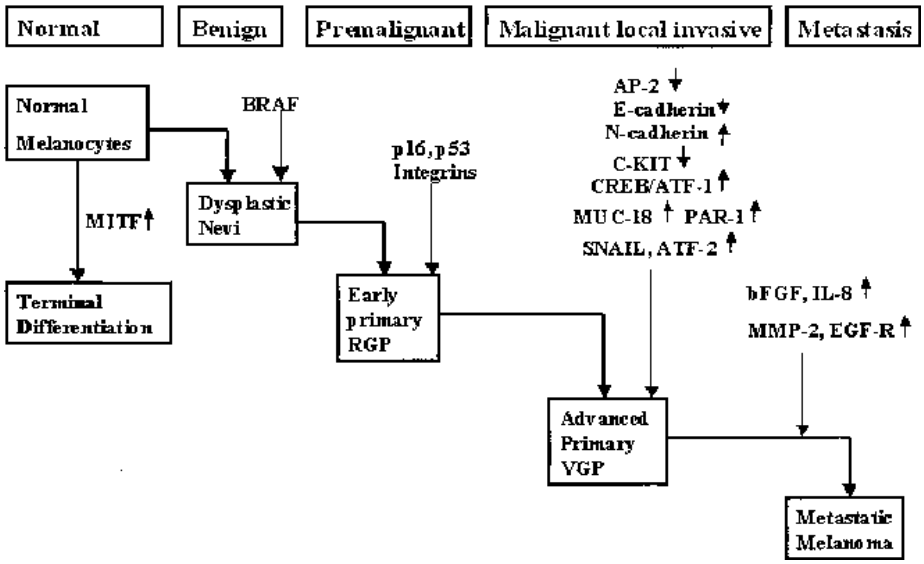

Fig. 1. Molecular changes associated with the progression of human melanoma. Mutations in BRAF have been observed in 70% of melanoma specimens and were observed as early as Dysplastic nevi. Abnormalities in the *p16/CDKN2* gene are usually an early event. Mutations in the *p53* gene are infrequent, but are observed in early stages. Abnormal functions of wild-type p53 were also observed. Transcription factor SNAIL normally functions in neural crest formation, but it also inhibits E-cadherin expression in melanoma cells. Alterations in c-KIT, MCAM/MUC-18, and N-cadherin, and integrins occur in the transition from RGP to VGP. The AP-2 transcription factor is not expressed in metastatic cells while the transcription factors CREB/ATF-1and ATF-2 are upregulated in these cells. The expression of genes involved in angiogenesis, invasion, and apoptosis such as bFGF, IL-8, EGF-R, PAR-1 correlates with higher metastatic potential of human melanoma cells.

and the matrix metalloproteinase (MMP)-2, and lack of expression of c-KIT. In further investigations, we inactivated AP-2 in SB-2 primary cutaneous melanoma cells by using a dominant-negative AP-2, the AP-2B gene. Expression of AP-2B in SB-2 cells augmented their tumorigenicity in nude mice and upregulated MMP-2 expression and activity. We have also recently demonstrated that loss of AP-2 expression in metastatic melanoma cells resulted in overproduction of the thrombin receptor, PAR-1. Other studies have shown that AP-2 regulates additional genes involved in melanoma development and progression, including E-cadherin, p21/WAF-1, HER2, Bcl-2, FAS/APO-1, IGF-R-1, and VEGF. We propose that the loss of AP-2 is crucial in the development of malignant melanoma. The transition of melanoma cells from RGP to VGP is also accompanied by upregulation of the angiogenic factor IL-8, which in turn upregulates the expression and activity of MMP-2. Additionally, the transition of melanoma cells from RGP to VGP is associated with overexpression of the two transcription factors, CREB and ATF-1. The expression of the transcription factors ATF-1 and CREB is upregulated in metastatic melanoma cells. However, how overexpression of ATF-1/CREB contributes to the acquisition of the metastatic phenotype is unclear. Previously we demonstrated that quenching of CREB activity in metastatic melanoma cells by means of a dominant-negative form of CREB (KCREB) led to a decrease in their tumorigenicity and metastatic potential in nude mice. We identified two mechanisms that explain how overexpression of CREB/ATF-1 contributes to the metastatic phenotype. The first is one in which CREB/ATF-1 play an essential role in invasion by regulating the CRE-dependent expression of MMP-2 and the adhesion molecule *MCAM/MUC18* genes. In the second mechanism, CREB and ATF-1 act as survival factors for human melanoma cells. Here, we will discuss the notion that the balance between AP-2 and CREB/ATF-1 expression determines the progression of melanoma cells towards the metastatic phenotype.

Based on our observations that the adhesion molecule MCAM/MUC18 and the angiogenic factor IL-8 play a major role in progression of human melanoma, the second part of this prospect article will summarize our data on the development of new fully humanized antibodies targeting these molecules and their potential usage as new modalities to treat melanoma patients.

ROLE OF AP-2 IN THE PROGRESSION OF MELANOMA

AP-2, a 52-kDa protein, was first purified from HeLa cells. Partial peptide sequences led to the isolation of cDNA from a HeLa cell library [Williams et al., 1988], and the gene was mapped to a region on the short arm of chromosome 6 near the HLA locus [Gaynor et al., 1991]. The AP-2 protein binds to a consensus palindromic core recognition element with the sequence 5′-GCCNNNGGC-3′ [Williams and Tjian, 1991]. Functional AP-2 binding sites have been identified in the enhancer regions of viral genes such as simian virus 40 (*SV40*) [Mitchell et al., 1987], and human T-cell leukemia virus type I, and cellular genes, such as those for a murine major histocompatibility complex (*H-2Kb*), human metallothionein-IIa (*huMTIIα*), human pro-enkephalin, human keratin K14, c-ERBB2, plasminogen activator type I (*PAI-I*), and insulin-like growth factor (*IGF*)-binding protein 5. The DNA-binding domain is located within the C-terminal half of the 52-kDa protein and consists of two putative amphipathic α-helices separated by a 92-amino acid intervening span that is both necessary and sufficient for homodimer formation [Williams and Tjian, 1991]. An alternatively spliced AP-2 gene, *AP-2B*, which encodes a protein that differs in its C terminus and acts as dominant-negative to AP-2, has been cloned [Buettner et al., 1993].

AP-2 is involved in mediating programmed gene expression during both embryonic morphogenesis and adult cell differentiation. With 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 [Mitchell et al., 1991]. Two 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 [Schorle et al., 1996; Zhang et al., 1996].

To understand the regulation of AP-2 expression itself, we recently demonstrated that AP-2 is downregulated by a post-transcriptional mechanism [Nyormoi et al., 2001]. We showed that in tumor necrosis factor alpha (TNFα)-induced apoptotic cells (1) AP-2 is cleaved by caspase 6 and to a lesser extent by caspases 1 and 3, (2) the cleavage site is 19 amino acids from the N-terminus at the sequence DRHD19, and (3) cleavage at this site renders AP-2 susceptible to degradation by proteasome, leading to loss of transcriptional activity. Collectively, these findings indicate that the first 19 amino acids of AP-2 play a critical role in the stability of AP-2. We propose that this might be a post-translational mechanism by which cells regulate the temporal and spatial expression of AP-2 during normal embryogenesis and tumor progression. It is conceivable that in the context of melanoma cells, this mechanism might be used to escape from the tumor suppressive activity of AP-2.

In the next sections, we will summarize our recent results, demonstrating that AP-2 is a major transcriptional regulator of genes involved in melanoma progression. Our data show that (1) there is a direct correlation between the expression of AP-2 and the expression of c-KIT and an inverse correlation between AP-2 and MCAM/MUC18 in human melanoma cells, (2) transfection of highly metastatic cells (c-KIT-negative, MCAM-positive- and AP-2-negative) with the *AP-2* gene resulted in downregulation of MCAM/MUC18 and re-expression of c-KIT; (3) transfection of AP-2 into highly metastatic melanoma cells inhibited their tumor growth and metastatic potential in nude mice, possibly through the regulation of c-KIT, MCAM/MUC18, and the thrombin receptor, PAR-1, and (4) expression of AP-2B in primary cutaneous melanoma cells augmented their tumorigenicity in nude mice, possibly through regulation of MMP-2, also known as type IV collagenase or gelatinase. Since AP-2 also regulates other genes involved in the progression of human melanoma such as E-cadherin, p21/WAF-1, IGF receptor-1 (IGFR-1), FAS/APO-1, Bcl-2, and VEGF, and since loss of AP-2 has now been observed in the vast majority of metastatic melanoma specimens [Karjalainen et al., 1998], our results support the notion that loss of AP-2 is a crucial event in the progression of melanoma from RGP to VGP.

AP-2 Regulates c-KIT and MCAM/MUC18 Expression

Studies from our laboratory and others have demonstrated that the transition of melanoma from RGP to VGP is associated with loss of expression of tyrosine-kinase receptor c-KIT,

and overexpression of the melanoma cell adhesion molecule (MCAM/MUC18) [Lehmann et al., 1989; Luca et al., 1993; Zakut et al., 1993; Bar-Eli, 1997, 1999; Huang et al., 1998; Jean et al., 1998a]. The mechanisms for upregulation of MCAM/MUC18 and loss of c-KIT gene expression during melanoma progression are unknown.

To determine the molecular basis for c-KIT's lack of expression in highly metastatic melanoma cells, we found that the c-KIT gene and its promoter in c-KIT-negative melanoma cells had no abnormalities (deletions, rearrangements, or mutations) that could account for the lack of c-KIT expression [Huang et al., 1996; Bar-Eli, 1999]. These observations suggest that c-KIT expression might be regulated at the transcriptional level. The 1.2-kb c-KIT promoter lacks TATA or CAAT boxes, is highly G + C rich, and contains binding sites for SP-1, myb, and GATA-1 plus three putative AP-2 sites [Yamamoto et al., 1993].

To assess the effect of AP-2 on c-KIT transcription, the c-KIT promoter-luciferase construct, pKLuc, was co-transfected into A375SM (c-kit-negative) cells with increasing concentrations of an expression vector encoding for wild-type AP-2α or with the control vector lacking AP-2α. Using the β-actin-renilla luciferase plasmid (pB-actin-RL) vector as a control to normalize for transfection efficiency, we found that the luciferase activity driven by the c-KIT promoter was activated by AP-2α in A375SM cells in a dose-dependent manner. A 9.5-fold stimulation was observed in cells co transfected with the plasmid expressing the AP-2α protein, which was not detected in transfections with the parent vector. Conversely, when the pKLuc construct was co-transfected into Mel-501 cells (which express high levels of c-KIT) with increasing concentrations of AP-2B (dominant-negative form of AP-2), luciferase activity was inhibited by 50% [Huang et al., 1998].

The contribution of the AP-2 transcription factor to c-KIT expression and to the acquisition of the metastatic phenotype in human melanoma cells was assessed by re-expressing AP-2 in A375SM cells. A375SM cells are highly metastatic in nude mice [Luca et al., 1995] are c-KIT-negative, and express negligible levels of endogenous AP-2. We were able to isolate two clones of A375SM cells (designated T1 and T2) that express high levels of functional AP-2. We found that c-KIT was expressed in the two AP-2 transfectants A375SM-AP-2.T1 and A375SM-AP-2.T2 but not in parental or neo-transfected control cells [Huang et al., 1998]. Collectively, these data demonstrate a direct correlation between AP-2 and c-KIT expression. These two clones exhibited decreased tumor growth and metastatic potential in nude mice (vide infra).

To determine the molecular basis for MCAM upregulation in metastatic cells, we found that MCAM overproduction is not due to gene amplification or rearrangement [Luca et al., 1993]. Moreover, sequencing of the entire MCAM promoter revealed no abnormalities that could account for overexpression of MCAM in metastatic cells. These observations suggest that MCAM might be regulated at the transcriptional level.

The 0.9-kb-pair MCAM promoter lacks TATA or CAAT boxes, is highly G + C-rich, and contains binding sites for transcription factors SP-1, CREB, MYB, and four binding sites for AP-2 [Sers et al., 1993]. The presence of four AP-2-binding sites within the essential region of the MCAM promoter suggests that AP-2 might regulate MCAM gene expression.

To assess 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 or with a control vector lacking the AP-2 cDNA. These experiments showed that CAT activity driven by the MCAM promoter was inhibited by AP-2 in A375SM cells in a dose-dependent manner. The downregulation of the MCAM promoter by AP-2 was due to a direct interaction of AP-2 with a region of the MCAM promoter that is required for its transcription [Jean et al., 1998a]. These experiments indicate the presence of functional AP-2 elements within the MCAM promoter that regulate MCAM expression in melanoma cells.

We next examined the effect of AP-2 transfection into A375SM cells on MCAM expression. MCAM expression was determined by fluorescence-activated cell sorter (FACS) analysis on the cell surface of parental A375SM cells, neo-transfected A375SM control cells, and AP-2-transfected A375SM cells. We were able to demonstrate that MCAM expression occurred in 98.4% of A375SM parental cells, and 90.1% of neo-transfected control cells, but in only 17.9% of the A375SM-AP-2.T1 cells [Jean et al., 1998a]. Downregulation of MCAM expression in the AP-2-transfected cells was also confirmed

by Western blot analysis [Jean et al., 1998a]. Collectively, these results indicate that expression of AP-2 in highly metastatic melanoma cells caused downregulation of MCAM gene expression. In addition, we and others have demonstrated that MCAM expression in melanoma cells is also regulated by the CREB family of transcription factors [Xie et al., 1997b; Karlen and Braathen, 2000]. The role of CREB/ATF-1 in the progression of human melanoma will be discussed later in this prospect article. Interestingly enough, loss of c-KIT can also influence the expression of MCAM in melanoma cells [Karlen and Braathen, 2000].

AP-2 Expression Inhibits Tumorigenicity and Metastasis in Metastatic Melanoma Cells

To determine the tumorigenicity of the AP-2-transfected A375SM cells that exhibited re-expression of c-KIT and downregulation in MCAM production, we injected 1×10^6 cells subcutaneously (s.c.) into BALB/c nude mice and monitored tumor growth twice a week for 56 days. Both parental A375SM and neo-transfected control cells grew in all mice (100% tumor take) and reached 1.0–1.4 cm in mean diameter within 6 weeks. In contrast, AP-2-transfected A375SM cells did not begin to form palpable tumors until 3 weeks (T2) or 7 weeks (T1) after injection and produced smaller tumors (0.2 and 0.5 cm in mean diameter, respectively; Table I).

In the next set of experiments, the metastatic potential of AP-2-transfected A375SM cells was determined in an experimental lung metastasis assay. 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-transfected cells did not metastasize to lungs (T1) or produced only a few lung metastases in some mice (T2). These data indicated that AP-2 exerts an inhibitory effect on experimental lung metastasis.

TABLE I. Tumor Growth and Metastatic Potential of AP-2-Transfected Cells

Cell type	Tumor diameter (cm)[a]	Median number of experimental lung metastases (range)[b]
A375SM-P	1.8	121 (74 to >200)
A375SM-Neo.a	2.0	153 (111 to >200)
A375SM-Neo.b	1.7	87 (54–143)
A375SM-AP-2.T1	0.2	0 (all 0)
A375SM-AP-2.T2	0.5	19 (0–47)

[a]Tumor cells (1×10^6) were injected subcutaneously (s.c.) into Balb/c mice, and the diameters of the tumors were measured twice weekly for 56 days.
[b]Tumor cells (1×10^6) were injected intravenously, and the number of lung colonies was determined after 60 days.

Regulation of the Thrombin Receptor PAR-1 by AP-2

Recent evidence suggests that the thrombin receptor, *PAR-1*, might be a crucial gene in the metastatic process of human melanoma [Nierodzik et al., 1992]; PAR-1 is a unique G-coupled protein receptor of the protease-activated receptor family. PAR-1 is activated when the N-terminus undergoes proteolytic cleavage by thrombin, resulting in irreversible activation that leads to downstream effector–response coupling events. In human melanoma cells, thrombin acts as a growth factor and is mitogenic, suggesting that signaling by PAR-1 is involved in the biological response of these cells [Maragoudakis et al., 2000]. PAR-1 was recently reported to be a rate-limiting factor in thrombin enhanced experimental pulmonary metastasis in a murine melanoma cell line, demonstrating the role of PAR-1 in the metastatic phenotype of melanoma. Activation of PAR-1 in cancer cells can lead to cell signaling and the upregulation of gene products involved in adhesion (alpha IIb beta 3 integrin), invasion (urokinase plasminogen activator, uPA, and MMP-2), and angiogenesis (interleukin 8, IL-8, vascular endothelial growth factor, VEGF, and platelet-derived growth factor, PDGF). This suggests that activation of PAR-1 may facilitate tumor progression through the induction of cell adhesion molecules and matrix-degrading proteases and through stimulation of the secretion of angiogenic factors, thus contributing to the metastatic phenotype of melanoma.

Despite the extensive data elucidating the cellular activation mechanisms mediated by this receptor, little is known about its regulation. Recently, the regulatory region of *PAR-1* gene has been identified. DNA sequence analysis indicated the presence of two AP-2/SP-1 complexes within the proximal 3′ region of the promoter. The AP-2/SP-1 complexes revealed the presence of four putative AP-2 consensus-binding sites, which strongly suggest that AP-2 potentially regulates the expression of this gene.

To further demonstrate that AP-2 is involved in the etiology of human melanoma, we

analyzed the role of AP-2 in transcriptional regulation of PAR-1 in human melanoma [Tellez and Bar-Eli, 2003]. Transfection of a metastatic AP-2-negative melanoma cell line with the *AP-2* gene reduced PAR-1 expression at the mRNA and protein levels and inhibited tumor growth in nude mice. In vivo analysis showed that SP-1 binds predominantly to the PAR-1 promoter in metastatic melanoma cells, whereas it is AP-2 that binds predominantly to the PAR-1 promoter in nonmetastatic melanoma cells. From these findings, we propose that loss of AP-2 increases the expression of PAR-1, which in turn upregulates the expression of several proteins, including, cell adhesion molecules, proteases, and angiogenic molecules, thus contributing to the metastatic phenotype of melanoma [Tellez et al., 2003].

Dominant-Negative AP-2, AP-2B, Augments Melanoma Tumor Growth In Vivo: Regulation of MMP-2

AP-2B protein contains the activation domain and part of the DNA-binding domain but lacks the dimerization domain, which is necessary for DNA binding [Buettner et al., 1993]. We further investigated the role of AP-2 in the progression of human melanoma by attempting to inactivate AP-2 in primary cutaneous melanoma using the dominant-negative *AP-2B* gene. Stable transfection of primary cutaneous melanoma SB-2 cells with the dominant-negative *AP-2B* gene was confirmed by RT-PCR and Northern blot analyses. EMSA using nuclear extracts from these cell lines demonstrated decreased functional binding of AP-2B-transfected cells to the AP-2 consensus binding sequence compared with neo-transfected control cells [Gershenwald et al., 2001]. In addition, CAT activity driven by a construct containing the AP-2 consensus binding sequence was downregulated in AP-2B transfected cells, indicating that AP-2 activity was quenched in the transfected cells. Orthotopic (s.c.) injection of AP-2B-transfected cells into nude mice increased their tumorigenicity compared with that in neo-transfected control cells. The AP-2B-transfected cells displayed increased MMP-2 expression (by Northern blot) and MMP-2 activity (by zymography), which resulted in an increase in invasiveness through Matrigel-coated filters [Gershenwald et al., 2001]. The AP-2B transfected tumors also displayed an increase in microvessel density and angiogenesis. Activation of MMP-2 may account for the increased tumorigenicity of AP-2B-transfected cells. These results demonstrate that inactivation of AP-2 contributes to the progression of melanoma by its ability to regulate the *MMP-2* gene.

How Loss of AP-2 Exerts its Metastatic Effect

Our studies provide evidence that lack of expression of AP-2 in metastatic melanoma cells can result in deregulation of the *c-KIT*, *MCAM*/*MUC18*, *MMP-2*, and *PAR-1* genes. All four of these genes are known to be involved in the progression of human melanoma [Luca et al., 1993, 1997; Huang et al., 1998; Jean et al., 1998a].

Other genes that are involved in the progression of human melanoma, such as *E-cadherin* [Cowley and Smith, 1996], *p21*/*WAF-1* [Jiang et al., 1995; Vidal et al., 1995], *HER2* [Natali et al., 1994], *PAI-1* [Descheemaeker et al., 1992], *Bcl-2* [van den Oord et al., 1994], *VEGF* [Gille et al., 1997; Silins et al., 1997], and *IGFR-1* [Werner et al., 1990], have either already been shown to be regulated by AP-2 or represent likely targets for *AP-2* gene regulation based on the existence of AP-2 elements in their promoters. Loss of AP-2 expression was also observed in advanced primary and metastatic melanoma lesions [Karjalainen et al., 1998]. Therefore, we propose that loss of AP-2 expression is a crucial event in the development of malignant melanoma. As such, AP-2 may act as a 'major regulator' in the progression of human melanoma. In our proposed model (Fig. 2), 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 cell proliferation (*HER2*), cycle regulation (*p21*/*WAF-1*), apoptosis (*c-KIT*, *Bcl-2*, *FAS*/*APO-1*), adhesion (*MCAM*/*MUC18*, *E-cadherin*), and invasion/angiogenesis (*MMP-2*, *PAI-1*, *VEGF*, and *PAR-1*). These functional changes attributed to one transcription factor may underlie the contribution of AP-2 loss of expression to the malignant phenotype.

ROLE OF CREB/ATF-1 IN MELANOMA PROGRESSION

A large body of data concerning the molecular control of melanoma progression has come from

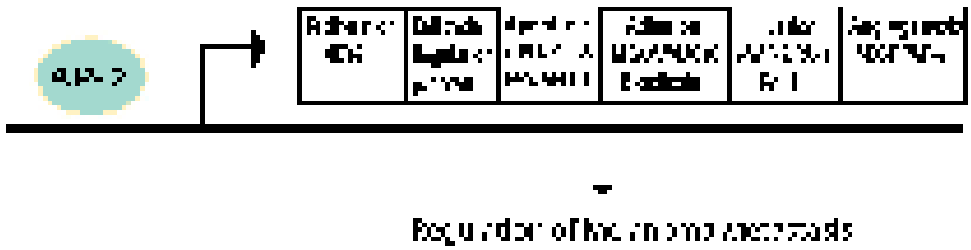

Fig. 2. A model for the role of AP-2 in the progression of human melanoma. AP-2 may act as a major regulator of several genes involved in tumor growth and metastasis of melanoma. These include genes involved in cell proliferation (HER2), cell cycle regulation (p21/^{WAF}), apoptosis (c-KIT, FAS/APO-1, and Bcl-2), adhesion (MCAM/MUC18 and E-cadherin), invasion (MMP-2, PAI-1, and KAI-1), and angiogenesis (VEGF and PAR-1). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

studies using mitogens. In culture, melanocytes synergistically respond to a number of growth factors, which, in combination with each other or with 12-*O*-tetradecanoylphorbol-13-acetate or cAMP stimulate not only proliferation but also pigmentation [Halaban et al., 1983]. These factors include several fibroblast growth factors, hepatocyte growth factor, and stem cell factor (also known as KIT ligand, MGF, and steel factor), all of which stimulate tyrosine kinase receptors. Because melanocyte proliferation and differentiation are positively regulated by agents that increase cAMP [Halaban et al., 1984], we have focused on the transcription factors CREB/ATF-1, which are known to be activated by cAMP, as possible mediators of tumor growth and metastasis of human melanoma.

ATF-1 and CREB are members of the large bZIP superfamily of transcription factors. Members of the CREB/ATF family bind to cAMP-responsive elements (CREs) within the promoter and enhancer sequences of many genes. ATF-1, CREB, and the cAMP-responsive element modulator protein (CREM) constitute the CREB/ATF subfamily within the bZIP superfamily. Members of this family are defined by their ability to heterodimerize with each other but not with members of other subfamilies [Meyer and Habener, 1993]. ATF-1, CREB, and CREM have similar structures and are highly homologous at the amino acid sequence level, especially within the bZIP region. Despite these similarities, members of the CREB multigene subfamily have distinct biological activities.

ATF-1, CREB, and CREM may act as either positive or negative regulators of transcription. Alternative mRNA splicing produces numerous isoforms of ATF-1 and CREB, which may account for this variability in transcriptional regulatory activities [Lee and Masson, 1993; Lemaigre et al., 1993]. In addition, each protein or isoform also possesses differing patterns of phosphorylation, and the specific patterns contribute to their ability as regulators of transcription [Meyer and Habener, 1993]. CREB and CREM have been shown to play important roles in basal and hormone-regulated transcription and differentiation, whereas the role of ATF-1 is less well defined. ATF-1 homodimers appear to be weaker transcriptional activators than either CREB or certain forms of CREM, since ATF-1-mediated activation is enhanced by heterodimerization with either CREB or CREM [Lemaigre et al., 1993]. In addition, it has been demonstrated that CREB can efficiently form heterodimers with ATF-1 rather than form the CREB homodimer [Hurst et al., 1991; Rehfuss et al., 1991].

Previous studies have demonstrated that CREB expression correlates directly with the metastatic potential of murine melanoma cells [Rutberg et al., 1994], and that ATF-1 is not detected in normal melanocytes but is easily found in metastatic melanoma cells [Bohm et al., 1995]. Whether these observations are causally related to tumorigenicity and metastasis of melanoma cells is not clear. In the next sections, we will summarize our current results describing how overexpression of the transcription factors CREB and ATF-1 contributes to the acquisition of the metastatic phenotype in human melanoma.

Effect of CREB/ATF-1 on Tumorigenicity and Metastasis of Human Melanoma Cells

To study the contribution of CREB and its associated proteins (mainly ATF-1) to tumor

growth and metastasis of human melanoma cells, we have taken the approach of using a dominant-negative form of CREB, KCREB. KCREB has a single base pair substitution in its DNA-binding domain that causes a change at position 287 from Arg to Leu. This mutation prevents the binding of KCREB to the CRE [Walton et al., 1992]; KCREB can associate with CREB and other proteins that interact with CREB, including members of the ATF and AP-1 transcription factor families and the co-activator CREB-binding protein (CBP). But, because of the mutation in the DNA-binding domain, heterodimers formed with KCREB do not possess the same degree of transcriptional activity as they would in the case of wild-type CREB. We reasoned that overexpression of KCREB in cells should quench CREB and its associated proteins. Indeed, the ability of KCREB to inhibit CRE-dependent transcription was demonstrated previously in F9 teratocarcinoma and CA-77 thyroid carcinoma cells [Walton et al., 1992; Woloshin et al., 1992].

To analyze the role of CREB in tumor growth and metastasis of melanoma cells, we have used MeWo human melanoma cells, which ectopically express KCREB. To determine the tumorigenicity of the KCREB-transfected cells, 1×10^6 cells were injected s.c. over the right scapular region of BALB/c nude mice, and the tumor diameter was determined 60 days after injection. We were able to demonstrate that, the two KCREB-transfected clones K-2 and K-10 formed smaller tumors than the control cells MeWo parental and neo-transfected cells did [Xie et al., 1997b; Jean and Bar-Eli, 2000].

Next, we analyzed the metastatic potential of KCREB-transfected cells in an experimental lung metastasis assay. To that end, 1×10^6 cells were injected into the lateral tail vein of BALB/c nude mice. The mice were sacrificed 80 days after the injection, and the lung tumor colonies were counted. Transfection of MeWo cells with KCREB significantly reduced their potential to produce lung metastasis. The median number of metastases was lower in mice injected with KCREB-transfected cells (12 and 4 for K2 and K10, respectively as compared with 22 and 27 for neo and parental control cells). The lower metastatic potential of K-10 cells as compared with that of K-2 cells could be explained by the higher level of KCREB activity in the K2 cells [Xie et al., 1997b; Jean and Bar-Eli, 2000].

Effect of CREB/ATF-1 on the Invasive Properties of Human Melanoma Cells

Several molecules involved in melanoma invasion, such as MMP-2 [Huhtala et al., 1990] and MCAM/MUC18 [Lehmann et al., 1989] have CRE in their promoters. As a result, these genes could be targeted for regulation by the CREB transcription factor. To test this possibility, we investigated the expression of MMP-2 and MCAM/MUC18 at the mRNA level in MeWo cells after transfection with KCREB. Northern blot analysis showed that KCREB-transfected cells displayed lower levels of the 3.1-kb MMP-2 transcript and the 3.3-kb MCAM/MUC18 transcript than control cells did. Expression of both MMP-2 and MCAM/MUC18 was inhibited by factors of 2.5 and 3.5 in K-2 and K-10 cells, respectively, as compared with that in MeWo-P and MeWo-neo cells [Xie et al., 1997b; Jean and Bar-Eli, 2000].

To verify that the downregulation of MMP-2 expression in KCREB-transfected cells was reflected in MMP-2 activity, we analyzed supernatants from control and KCREB-transfected cells for collagenase activity by zymography. The activity of the 72-kDa MMP-2 was significantly lower in K-2 and K-10 cells than it was in control MeWo-P and MeWo-Neo cells. It is possible that both the latent and activated forms of MMP-2 were reduced in the KCREB-transfected cells. The activity of the 92-kDa collagenase, MMP-9, which does not contain CRE in its promoter, remained unchanged and served as an internal control for equal loading [Xie et al., 1997b; Jean and Bar-Eli, 2000].

CAT activity driven by the MMP-2 promoter was inhibited by factors of 14 and 45 in KCREB-transfected K-10 and K-2 cells, respectively, compared with that in control cells, suggesting that KCREB's regulation of MMP-2 expression and activity occurred at the transcriptional level [Xie et al., 1997b; Jean and Bar-Eli, 2000].

Because we observed this downregulation of MMP-2 and MCAM/MUC18 expression in KCREB-transfected cells, we next analyzed the effect of KCREB expression on the invasive properties of melanoma cells. To that end, KCREB-transfected cells were assayed for their potential to penetrate through the basement membrane, an important component in the process of tumor invasion and metastasis. Migration of cells through Matrigel-coated filters was monitored by using fibroblast-conditioned

medium as a source of chemoattractant. We observed a 10- to 20-fold decrease in the number of the cells that invaded Matrigel-coated filters in K-2 and K-10 cells as compared with MeWo-P and MeWo-Neo cells. These results show that expression of KCREB inhibited the ability of melanoma cells to migrate and invade the basement membrane, possibly by decreasing MMP-2 and MCAM/MUC18 expression, and suggest that CREB plays an important role in the regulation of the invasive properties of melanoma cells [Xie et al., 1997b; Jean and Bar-Eli, 2000].

CREB and AFT-1 Act as Survival Factors for Human Melanoma Cells

Resistance to apoptosis induced by exogenous signals is an important property of tumor cells. Previous studies demonstrated that the induction of apoptosis by diverse exogenous signals depends on an increase in the level of cytosolic Ca^{2+} [McConkey et al., 1989; McConkey and Orrenius, 1996]. Because CREB mediates both cAMP and Ca^{2+} transcriptional responses [Gonzalez and Montminy, 1989; Sheng et al., 1991], we investigated the role of CREB in the resistance of melanoma cells to apoptosis induction. To induce apoptosis, we used thapsigargin (Tg), which inhibits endoplasmic reticulum-dependent Ca^{2+}-ATPase and thereby increases cytosolic Ca^{2+} levels [Thastrup et al., 1990]. Tg has also been shown to trigger apoptosis in melanoma cells [Muthukkumar et al., 1995].

The effect of Tg treatment on MeWo-P, MeWo-Neo, and the two KCREB-transfected clones (K-2 and K-10) was analyzed by flow cytometry after propidium iodide staining. After treatment with 1 μM Tg for 48 h, KCREB-transfected cells showed a higher percentage of hypodiploid cells than control cells did. Cells with hypodiploid DNA content, which is indicative of apoptotic cells, were increased by factors of 2.2 and 3.3 in K-2 and K-10 cells, respectively, as compared with that in MeWo-P and MeWo-Neo cells [Jean et al., 1998b; Jean and Bar-Eli, 2000].

To further confirm that Tg-treatment induced apoptosis in KCREB-transfected cells, we analyzed the cellular morphologic characteristics of K-10 cells. Transmission electron microscopic analysis of K-10 cells after treatment with 1 μM Tg for 48 h showed typical apoptotic morphology, with plasma and nuclear membrane blebbing, chromatin condensation, and cytoplasmic vascularization. In addition, nuclear changes consistent with apoptosis, such as condensation and segregation of chromatin into compact masses aligning with the inner side of the nuclear membrane, were apparent. Control cells did not show these changes. These results demonstrated that KCREB expression in melanoma cells decreased their resistance to Tg-induced apoptosis.

Collectively, these data show that CREB and ATF-1 are involved in the resistance of MeWo cells to Tg-induced apoptosis and suggest that they act as survival factors for human melanoma cells [Jean et al., 1998b; Jean and Bar-Eli, 2000].

Targeting CREB/ATF-1 by Single-Chain Fv Fragment Anti-ATF-1

To further investigate the cellular role of ATF-1 in melanoma progression, we used current advances in antibody engineering that have made possible the cloning of small single-chain Fv (ScFv) fragments. ScFv fragments contain the antigen binding variable domains of the light and heavy chains connected by a peptide spacer [Winter and Milstein, 1991; Raag and Whitlow, 1995]. Constructed in this manner, a single RNA transcript can be expressed and translated into an active protein that has the potential to interfere with the activity of targeted intracellular proteins.

To investigate the role of CREB/ATF-1 in tumor growth and metastasis of human melanoma, we attempted to quench their transcriptional activities by using ScFv anti-ATF-1 that was derived from a monoclonal antibody (mAb) anti-ATF-1, mAb 41.4, which has been shown to inhibit ATF-1 binding and transcriptional activation from CRE-dependent promoters in vitro [Orten et al., 1994]. We reasoned that expression of ScFv anti-ATF-1 in melanoma cells would bind ATF-1 and inactivate the transcriptional activation of both CREB/ATF-1.

We found that expression of ScFv anti-ATF-1 in MeWo melanoma cells inhibited their tumorigenicity and metastatic potential in nude mice. Intracellular expression of ScFv anti-ATF-1 rendered the melanoma cells susceptible to apoptosis in vivo, compared with untransfected control cells. These studies demonstrate that intracellular ScFv anti-ATF-1 can be used to quench ATF-1 activity not only as a method to explore its function but also as a modality for cancer therapy [Jean et al., 2000; Jean and Bar-Eli, 2001].

FULLY HUMAN ANTIBODIES TO MCAM/MUC18 INHIBIT TUMOR GROWTH AND METASTASIS OF HUMAN MELANOMA

MCAM/MUC18 expression correlates with tumor thickness and metastatic potential of human melanoma cells in nude mice. Moreover, ectopic expression of MUC18 in primary cutaneous melanoma cells leads to increased tumor growth and metastasis in vivo. Primary cutaneous melanoma SB-2 cells (MUC18-negative) were transfected with the *MUC18* gene. The MUC18-transfected cells displayed increased homotypic adhesion, increased attachment to human endothelial cells, upregulation of MMP-2, and increased invasiveness through Matrigel-coated filters [Xie et al., 1997a]. Moreover, it has recently been shown that the production of tumorigenic variants from a nontumorigenic melanoma cell line is accompanied by MCAM upregulation [Bani et al., 1996].

These observations have established MCAM/MUC18 as a candidate mediator of tumor growth, angiogenesis, and metastasis in human melanoma and lend credence to the rationale that blockade of MCAM/MUC18 might be a potential target for immunotherapy against human melanoma. To that end, we recently used a fully human anti-MCAM/MUC18 Ab (ABX-MA1; produced by Abgenix, Inc., Fremont, CA) to block the MCAM/MUC18 adhesion molecule on melanoma cells and analyzed its effect on tumor growth, angiogenesis, and metastasis of human melanoma.

ABX-MA1 had no effect on melanoma cell proliferation rate in vitro. However, when cells of the metastatic melanoma lines A375SM and WM2664 (which express high levels of MUC18) were injected s.c. into nude mice and treated with ABX-MA1 (100 μg, weekly, i.p. for 5 weeks), tumor growth was significantly inhibited compared with control IgG-treated mice. ABX-MA1 treatment also suppressed experimental lung metastasis of these melanoma cells. ABX-MA1 disrupted spheroid formation by melanoma cells expressing MUC18 (homotypic interaction) and the ability of these cells to attach to human umbilical vein endothelial cells [HUVECs (MUC18 positive)] in vitro. ABX-MA1 treatment of melanoma cells in vitro significantly inhibited the promoter and collagenase activity of MMP-2, resulting in decreased invasion through Matrigel-coated filters. Decreased expression of MMP-2 was also observed in the implanted tumors in vivo. Moreover, because HUVECs also express MUC18, ABX-MA1 directly disrupted the tube-like formation by HUVECs in an in vitro vessel formation assay. Collectively, these results point to usefulness of ABX-MA1 as a modality to treat melanoma either alone or in combination with conventional chemotherapy or other antitumor agents [Mills et al., 2002].

FULLY HUMANIZED NEUTRALIZING ANTIBODIES TO INTERLEUKIN-8 (ABX-IL8) INHIBIT ANGIOGENESIS, TUMOR GROWTH, AND METASTASIS OF HUMAN MELANOMA

The role of IL-8 in melanoma progression has previously been primarily attributed to its ability to act as an autocrine growth factor for melanoma cells [Schadendorf et al., 1993] and to induce haptotatic migration [Wang et al., 1990]. More recently, it has been shown that IL-8 also exhibits potent angiogenic activities both in vitro and in vivo [Koch et al., 1992; Hu et al., 1993; Smith et al., 1994; Szekanecz et al., 1994]. The angiogenic activity of IL-8 produced by monocytes and macrophages was first demonstrated by Koch et al. [1992]. They found that human recombinant IL-8 was potently angiogenic when implanted in a rat cornea and induced proliferation and chemotaxis of HUVECs. The involvement of IL-8 in tumor angiogenesis was first demonstrated in human bronchogenic carcinoma [Smith et al., 1994]. Tumor cell-derived IL-8 induces endothelial cell chemotaxis in vitro and corneal neovascularization in vivo. These observations have been confirmed in many other types of human tumors including melanomas [Westphal et al., 2000]. Now IL-8 is considered to be one of the most potent angiogenic factors secreted by melanoma cells. The question of how IL-8 exerts its angiogenic activity, however, remains unknown. We have recently demonstrated that metastatic melanoma cells producing IL-8 or primary cutaneous melanoma (IL-8-negative) transfected with the IL-8 gene displayed upregulation of MMP-2 expression and activity and increased invasiveness through Matrigel-coated filters [Luca et al., 1997]. Activation of MMP-2 by IL-8 can enhance the invasion of host stroma by tumor cells and increase angiogenesis and, hence, metastasis. In addition, IL-8 has been shown recently to act directly on vascular endothelial cells and to serve as a survival factor [Yoshida

et al., 1997]. Thus, multiple mechanisms seem to be involved in IL-8 action, including direct effects on tumor and vascular endothelial cell proliferation, angiogenesis, and migration. These observations suggest that IL-8 could be a mediator of angiogenesis, tumor growth, and metastasis in melanoma and offered a potential target for immunotherapies against human melanomas.

Recently, we used a fully human anti-IL-8 antibody (ABX-IL8, obtained from Abgenix) to neutralize the IL-8 secreted by melanoma cells and examine its effect on tumor growth. ABX-IL8 did not inhibit the proliferation of melanoma cells in vitro. However, ABX-IL8 suppresses the tumorigenicity and metastatic potential of metastatic human melanoma A375SM and TXM-13 cells in vivo. ABX-IL8 displayed potent inhibition of MMP-2 activity in melanoma cells, and inhibited the invasion of tumor cells through basement membrane in vitro. Inhibition of tumor growth and metastasis by ABX-IL8 in vivo correlated with decreased vascularization of melanomas in nude mice that was at least partially because of decreased MMP-2 expression. These results suggest that blocking of IL-8 by ABX-IL8 suppresses angiogenesis and metastasis of human melanoma. Thus, the human IL-8 neutralizing antibody ABX-IL8 may be beneficial for melanoma therapy either alone or in combination with other chemotherapeutic or anti-angiogenic agents [Huang et al., 2002].

SUMMARY AND PROSPECTS

In this article, we have summarized the results of our recent studies demonstrating that the progression of human melanoma is associated with the loss of AP-2 expression, on one hand, and overexpression of CREB/ATF-1 transcription factors on the other. Changes in these transcription factors modulate and regulate several genes involved in the development of malignant melanoma. Other transcription factors such as ATF-2, SNAIL, MITF, and NFκB, are also involved in the same or other stages of the progression of human melanoma.

Interestingly enough, some of the genes regulated by AP-2, such as *MCAM*/*MUC18*, *MMP-2*, *FAS*/*APO-1*, *HER2*, and *Bcl-2* are also regulated by the CREB/ATF-1 family of transcription factors. It is, therefore, feasible that the outcome of the metastatic phenotype in melanoma may well depend on the delicate balance between the expression of AP-2 and the CREB/ATF-1 transcription factors (Fig. 3). Loss of AP-2 and overexpression or hyperactivity of CREB/ATF-1, ATF-2, SNAIL, MITF, and NFκB in metastatic melanoma cells may work in concert to regulate several genes contributing to the development of the malignant phenotype.

Based on our observations that MCAM/MUC18 and IL-8 play a major role in the acquisition of the metastatic phenotype in human melanoma, we have developed two fully human antibodies targeting these molecules. In this prospect article, we have presented evidence that these antibodies inhibited tumor growth, invasion, angiogenesis, and metastasis of melanoma in animal models. Currently, both of these antibodies are being evaluated in clinical trials. The therapeutic modalities to control tumor growth and metastasis of human melanoma are very limited. The idea of using fully humanized Abs to block MUC18 and IL-8 is

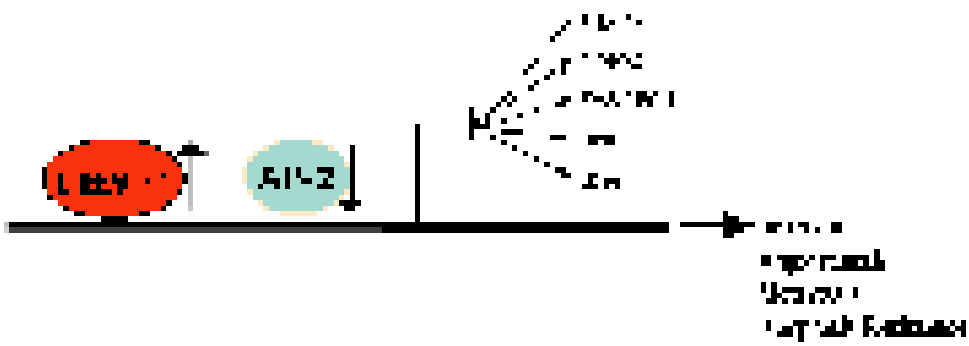

Fig. 3. Schematic of melanoma cells invasion, angiogenesis, metastasis, and apoptosis resistance controlled by the transcription factors AP-2 and CREB/ATF-1. Deregulation in the expression of the above mentioned genes require lack of AP-2 expression and overproduction of CREB/ATF-1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

especially appealing because multiple dose regimens of the Ab could be administered to the patients with little risk of mounting an immune reaction. Our studies should promote serious consideration for initiating a Phase I/II clinical trail with ABX-MA1 and ABX-IL8 in patients with metastatic melanoma. These Abs can be used either alone or in combination with chemotherapy or other anticancer agents to increase the efficacy of the treatment.

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