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Immunology

De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis

(expression vector/complement binding consensus sequence/interferon-γ)

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ABSTRACT **The 89-kDa cell surface glycoprotein, P3.58, is detectable on advanced human melanomas *in situ* but not on benign melanocytes or early melanomas. cDNA cloning of P3.58 from melanoma cells was accomplished by screening a λ zap expression vector library with monoclonal antibodies produced against the denatured antigen. Nucleotide sequencing of the clones revealed that P3.58 is identical to the intercellular-adhesion molecule 1. No qualitative differences in P3.58 mRNA species could be seen between melanoma cells and hematopoietic cells and no differences in gene organization were observed between peripheral blood leukocytes and melanoma cells. Inspection of the deduced amino acid sequence of P3.58 indicated the presence of the consensus sequence characteristic for complement-binding proteins. The acquisition of this cell-adhesion molecule during the process of tumor progression is speculated to contribute to the development of metastasis in melanoma.**

Immunohistochemical analysis of *in situ* phenotypic changes correlating with tumor progression in human cutaneous melanoma has led to the identification of molecules potentially involved in metastasis (1, 2). One of these is the melanoma-associated antigen P3.58, an 89-kDa cell surface glycoprotein (3), that is not detectable on quiescent melanocytes and is only sporadically found on proliferative benign melanocytic lesions (i.e., nevi). On melanomas the frequency with which this antigen is found increases with increasing tumor thickness (2). Among normal tissues, P3.58 expression is limited to vascular endothelia, lymphoid follicle germinal centers, and tissue macrophages (4). It is expressed by adherent monocytes and Epstein–Barr virus-transformed B-cell lines and can be modulated *in vitro* by interferon γ and tumor necrosis factor type α (2, 4).

Here we report the isolation of cDNA clones encoding the P3.58 antigen from melanoma cells. Nucleotide sequencing of these clones revealed that the P3.58 antigen is identical to the intercellular adhesion molecule 1 (ICAM-1), a cell adhesion molecule (CAM) mediating leukocyte adhesion through the lymphocyte function-associated molecule 1 (LFA-1) (5). CAMs mediating homotypic and heterotypic cell contacts are essential for the assembly and maintenance of the cellular architecture of complex tissues (6, 7). The perturbations of normal cell interactions that characterize invasive and metastatic malignant cells might, therefore, be expected to reflect changes in CAM function. Although the highly malignant Wilms tumor was shown (8) to express a low-adhesive form of the neural adhesion molecule, a direct association between the expression of CAMs and behavior of tumors *in vivo* has not been observed. We speculate that the *de novo* expression of ICAM-1 by melanomas leads to heterotypic adhesion between melanoma cells and LFA-1-bearing leukocytes and thereby contributes to dissemination of the cells from the primary tumor.

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MATERIALS AND METHODS

Immunohistochemistry. Tissues were quickly frozen in liquid nitrogen and stored at −70°C. Indirect immunoperoxidase staining was performed as described (2) by using 3-amino-9-ethylcarbazole (0.25 mg/ml in 0.1 M sodium acetate, pH 4.9) plus 0.003% H_2O_2. Antibodies P3.58^a (IgM) and P3.58^b (IgG2a) were used to evaluate P3.58 expression, and UPC10 (IgG2a myeloma) and 141.11 [IgM anti-H2-K^b, -K^k, and -D^k antigens, obtained from G. Hämmerling (German Cancer Center, Heidelberg)] were used as isotype controls. Monoclonal antibody Gap8.3 [anti-CD45 (American Type Culture Collection)] was used to identify infiltrating leukocytes. Tissues were regarded as positive when ≥5% cells were stained. The following numbers of lesions were examined: 54 melanocytic nevi, 29 dysplastic nevi, 28 primary tumors ≤0.75 mm in diameter, 18 primary tumors 0.76–1.5 mm, 24 primary tumors 1.6–3.0 mm, 23 primary tumors 3.0 mm, and 46 metastases.

Antibodies and Cells. Antibodies P3.58^a and P3.58^b were isolated after immunization with fresh melanoma tumor material and were selected for their discrimination between malignant melanoma and benign nevi on frozen tissue sections (3, 4). Eleven monoclonal antibodies were produced against denatured P3.58 antigen isolated by affinity chromatography from 3×10^9 interferon-γ-stimulated Mel JuSo cells. The antibodies also reacted with native P3.58 antigen and precipitated only this molecule from radiolabeled melanoma cells.

The L89 transfectant cell line was obtained after cotransfection of murine Ltk$^-$ cells with Mel JuSo DNA and the thymidine kinase gene (ptk-5) as described (4). Human cell lines were established in our laboratory, or obtained from the American Type Culture Collection, and were cultured in RPMI 1640 containing 10% (vol/vol) fetal calf serum, 1 mM sodium pyruvate, and antibiotics. Interferon-stimulated cells were cultured for 48 hr in recombinant interferon γ at 100 units/ml (Bioferon, Laupheim, F.R.G.).

cDNA Library Screening and DNA Sequencing. A λ zap (Stratagene, San Diego, CA) cDNA expression library produced from interferon-stimulated Mel JuSo cells was kindly provided by J. Lehmann (at this institute), and a λgt11 melanoma cDNA library was obtained from Clontech (Palo Alto, CA). The λ zap library was screened with a pool of four antibodies directed to denatured P3.58 antigen (P3.58BA 12, 14, 17, and 18) by using an immunoperoxidase-coupled rabbit anti-mouse immunoglobulin (Dakopatts, Hamburg, F.R.G.)

Abbreviations: CAM, cell-adhesion molecule; ICAM-1, intercellular-adhesion molecule 1; LFA-1, lymphocyte function-associated molecule 1.

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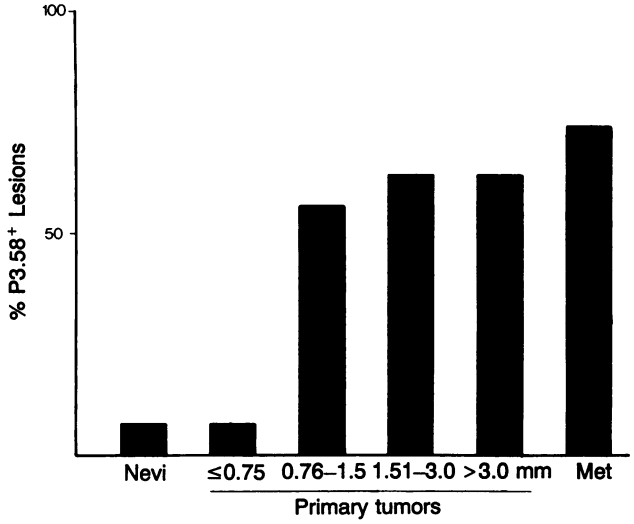

FIG. 1. P3.58 expression on benign and malignant melanocytes *in situ*. Reactivity was determined by immunoperoxidase staining of frozen tissue sections using monoclonal antibodies P3.58[a] and P3.58[b]. Serial sections were stained with isotype controls and antibody directed to CD45. Positive lesions contained ≥5% stained cells. Met, metastasis.

and a substrate consisting of 1.26 mM *o*-dianisidine hydrochloride, 15.3 mM sodium nitroferricyanide, and 0.007% H_2O_2. For DNA sequencing, suitable restriction fragments of λ89-1, λ89-48, and λ89-49 were subcloned into the plasmid pUC18. Double-stranded plasmid DNA was sequenced using a modification of the dideoxy chain-termination method (ref. 9; Sequenase, United States Biochemical, Cleveland).

DNA and RNA Analysis. RNA and DNA analysis were carried out essentially as described (10). Genomic DNA (15 μg) was digested with restriction enzymes, electrophoresed in 0.7% agarose gel, and transferred to nylon membranes (Hybond, Amersham). For RNA analysis, 20 μg total RNA was denatured in formaldehyde, electrophoresed, and transferred to nylon membranes. Membranes were hybridized at 65°C in 6× SSC/5× Denhardt's solution/0.5% sodium dodecyl sulfate (SDS)/salmon sperm DNA at 20 μg/ml. Wash conditions were 3× SSC/0.1% SDS, 1× SSC/0.1% SDS, 0.3× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS at 65°C. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.)

RESULTS AND DISCUSSION

Expression of P3.58 Is an Early Event in Melanoma Tumor Progression. The vertical thickness of primary tumors is a measure of progression in melanoma and is directly correlated with the probability of metastatic disease (11). The frequency of P3.58 expression on malignant melanomas *in situ* increases with increasing tumor thickness (2). The finding that P3.58 is a leukocyte activation antigen primarily expressed on monocytes and macrophages (4) prompted an examination of additional tumors and staining of serial sections with an antibody specific for CD45 to identify infiltrating leukocytes. These results confirmed our initial observations but revealed a nonlinear correlation with tumor thickness (Fig. 1). As reported (2), P3.58 expression is no more frequent on thin melanomas (i.e., ≤0.75 mm) than on benign nevi. However, it is strongly expressed on ≈70% of all tumors with a vertical thickness of 1 mm or greater and, thereafter, shows only a

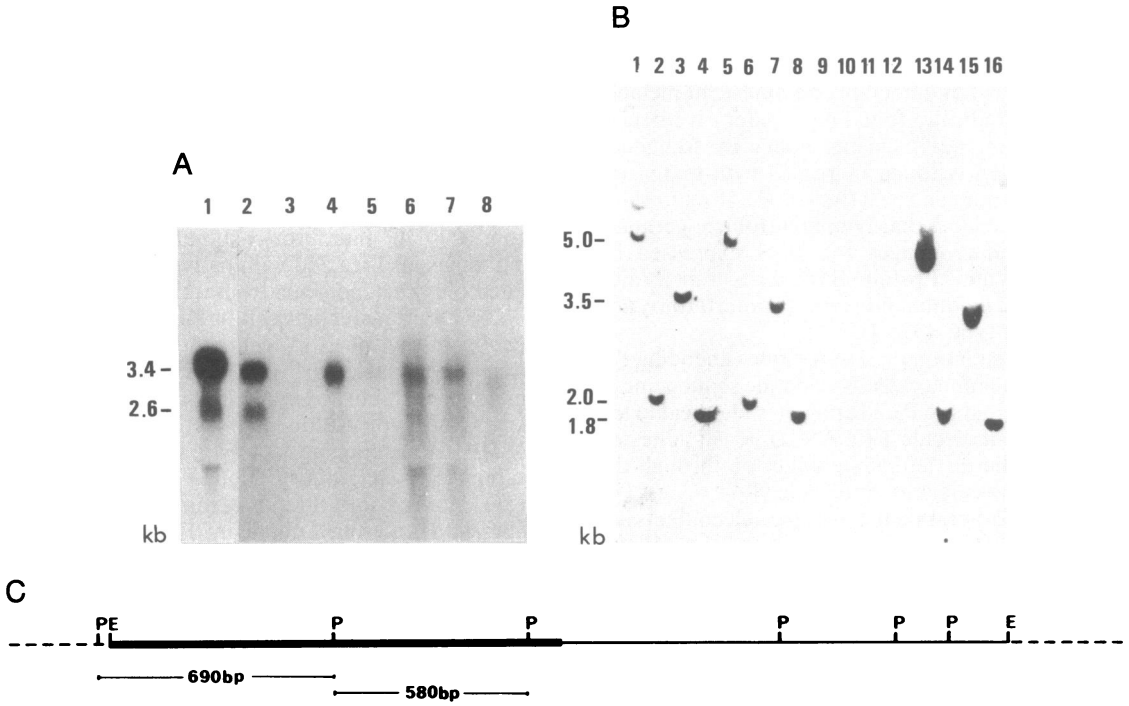

FIG. 2. Expression and structure of P3.58 gene in melanomas and hematopoietic cells. (*A*) Analysis of the following P3.58/ICAM-1 mRNAs hybridized with the cDNA clone λ89-1. Lanes: 1, SkMel 28 (melanoma cell); 2, L89 (P3.58[+] mouse L-cell transfectant); 3, Ltk[−] (untransfected mouse L cell); 4, interferon-γ-stimulated A375 (melanoma cell); 5, unstimulated A375; 6, U937 (monoblast-like cell); 7, Daudi (Burkitt lymphoma); 8, CoJä (Epstein–Barr virus-transformed B cell). (*B*) Analysis of the P3.58/ICAM-1 gene. The following genomic DNAs were hybridized with a 690-base-pair (bp) *Pst* I fragment of λ89-1. Lanes: 1–4, peripheral blood lymphocytes; 5–8, L89; 9–12, Ltk[−]; 13–16, Mel JuSo (melanoma cell). The following restriction enzymes were used: Lanes: 1, 5, 9, and 13, *Eco*RI; 2, 6, 10, and 14, *Taq* I; 3, 7, 11, and 15, *Pvu* II; 4, 8, 12, and 16, *Bgl* II. (*C*) Restriction map of the 2675-bp cDNA clone λ89-1 subcloned in the plasmid vector pUC18. The bold line indicates the coding region (1343 bp) and the thin line the untranslated region (1332 bp) of λ89-1. The broken line indicates vector sequences. Both the 690-bp and the 580-bp *Pst* I fragments were used as probes in Southern analyses. P, *Pst* I; E, *Eco*RI.

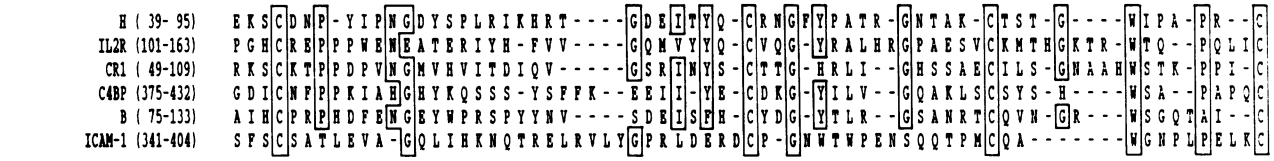

FIG. 3. Relationship of P3.58/ICAM-1 to the C3/C4-binding protein superfamily. Amino acids that are conserved in >50% of the members of this family (ref. 16) are boxed. The sequence data are from the references below and the residue numbers of the amino acids involved are indicated in the figure: H, factor H (18); CR1, C3b/C4b receptor (17); C4BP, C4b-binding protein (19); B, factor B (20); IL2R, interleukin 2 receptor (21); and ICAM-1 (14).

slight increase in incidence with increasing tumor thickness. The association between P3.58 expression and tumor thickness suggests that the appearance of this molecule reflects a change in the tumor cells that may contribute to the development of metastatic capacity. The abrupt increase in incidence of P3.58 expression on tumors of 1 mm is consistent with the hypothesis, derived primarily from experimentally induced tumors, that the progression of malignant tumors occurs by a series of discontinuous steps, each of which is delineated by the sudden appearance of new characteristics (12).

Identification of cDNA Clones Encoding P3.58. Approximately 360,000 phage from a melanoma cDNA λ zap expression library were screened with a panel of four monoclonal antibodies produced against denatured P3.58 antigen. A single clone, λ89-1 was isolated. This clone contained a 2.7-kilobase (kb) insert encoding a product reactive with six antibodies. Two additional clones (λ89-48 of 1 kb and λ89-49 of 1.6 kb) were isolated from a λgt11 cDNA melanoma library by hybridization with λ89-1. To confirm that λ89-1 contained P3.58 cDNA, it was hybridized to DNA and RNA isolated from L89, a P3.58-expressing L cell obtained after transfection with DNA derived from the melanoma cell line Mel JuSo (5). Identical mRNA species and genomic fragments were observed in L89 and melanoma cells (Fig. 2*A*, lanes 1 and 2, and *B*, lanes 5–8 and 13–16). No hybridization was seen with RNA or DNA isolated from the untransfected Ltk^- cells (Fig. 2*A*, lane 3, and *B*, lanes 9–12). Furthermore, in A375 melanoma cells mRNA hybridizing with λ89-1 was detected only after interferon-γ stimulation (Fig. 2*A*, lanes 4 and 5), which is also required to obtain expression of the P3.58 antigen (2).

Clones λ89-1, λ89-48, and λ89-49 were subcloned into the vector pUC18 and sequenced by using the dideoxy chain-termination method (9). The sequence obtained was identical to that of ICAM-1 isolated from myeloid and endothelial cells (refs. 13 and 14; data not shown).

Characterization of P3.58 mRNA and Genomic Organization. Comparison of melanoma and hematopoietic cells revealed no qualitative differences in P3.58/ICAM-1 mRNA species or gene organization. Melanoma, monoblast, and B-cell lines expressed two species of mRNA (3.4 and 2.6 kb) hybridizing with λ89-1 (Fig. 2*A*, lanes 1 and 6–8). Restriction analyses of genomic DNA isolated from melanoma cells and peripheral blood mononuclear cells showed one major band, consistent with a single-copy gene (Fig. 2*B*, lanes 1–4 and 13–16). Identical restriction fragments were observed in all cells analyzed, including four autologous melanoma/B-cell pairs. The P3.58/ICAM-1 gene, therefore, does not seem to be polymorphic or to be rearranged in melanomas. A second band with a considerably weaker hybridization signal (visible in lane 1) was observed in all digests. This band, seen with both probes, was also present in L89, suggesting that there is a second closely related and possibly linked sequence.

Presence of a Consensus Sequence for Complement Component C3/C4-Binding Proteins in P3.58/ICAM-1. Antibodies binding the P3.58 antigen have been reported to inhibit attachment of the complement component C3b to B-cell lines (15). C3- and C4-binding proteins are characterized by a consensus sequence of ≈60 amino acids containing a framework of highly conserved residues (16, 17). Inspection of the deduced amino acid sequence revealed the presence of the C3/C4-binding protein consensus sequence between residues 341 and 404 (Fig. 3). This region, which spans the junction between the putative fourth and fifth immunoglobulin-like domains of ICAM-1, lacks several of their typical features (14) and thus might form the loop predicted for the C3/C4-binding proteins (17). The presence of this consensus sequence together with the antibody-blocking studies suggest that ICAM-1 may be involved in C3b binding. Since C3b-binding proteins generally act to accelerate the dissociation of active complement complexes (16), the expression of P3.58/ICAM-1 may help the tumor escape immune destruction, providing an important property needed for successful metastasis. In support of this proposal is the observation that melanoma cell lines have been shown to escape complement-dependent cytotoxicity *in vitro* through the rapid inactivation of cell-bound C3b (22).

The expression of ICAM-1 may contribute to the metastatic capability of melanoma cells through several mechanisms. ICAM-1 has been shown to directly bind to LFA-1 (5), an adhesion glycoprotein broadly expressed on cells of the hematopoietic lineage (23, 24). Since LFA-1-negative melanoma cells nevertheless participate in LFA-1-dependent monocyte adhesion (25), the melanoma P3.58/ICAM-1 molecule may also function as a LFA-1 ligand. Through this interaction, melanoma cells could establish heterotypic cell contacts with leukocytes present in the tumor infiltrate. This might lead to a reduction in homotypic adhesion between melanoma cells while enhancing tumor cell adhesion to migratory and invasive leukocytes enabling individual cells to dissociate from the primary tumor.

CAMs play an important role in organogenesis and tissue regeneration, where their expression is regulated by microenvironmental signals (6). In this context it is of interest that P3.58 expression in primary melanomas correlates with the local presence of leukocytes containing its inducer, interferon γ (26). Such observations suggest that the role of the mononuclear cell infiltrate in melanomas may be two sided. Infiltrating leukocytes have been shown to include cells capable of specifically killing the tumor cells *in vitro* (27). However, by regulating gene expression in the tumor cells, the mononuclear cell infiltrate may also play a direct role in the acquisition of properties that contribute to progression of the tumor toward metastatic disease.

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