Class 1 (unique) tumor antigens of human melanoma: Identification of unique and common epitopes on a 90-kDa glycoprotein

(cell-surface antigens/mouse monoclonal antibodies/tumor immunology)

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Contributed by Lloyd J. Old, December 28, 1987

ABSTRACT Antibodies present in the serum of melanoma patient FD detect an antigenic determinant (FD) restricted to the autologous melanoma cell line SK-MEL-131. This cell-surface determinant is carried on a glycoprotein of 90 kDa, designated gp90. Mice were immunized with a partially purified preparation of gp90 derived from SK-MEL-131 clone 1.5, and three murine hybridomas (KF23, KF26, and KF104) secreting monoclonal antibodies (mAbs) detecting this antigen have been generated. Sequential immunoprecipitation experiments demonstrate that the three mAbs and human FD serum react with the same gp90 species. The mAbs, in contrast to FD serum, react with a broad range of cultured cells in assays for cell-surface antigens and immunoprecipitate a gp90 component from radiolabeled extracts of these cells, including autologous FD B cells. We conclude that gp90 from SK-MEL-131 has two types of determinants: restricted (detected by FD serum) and common (detected by the mouse mAbs). gp90 molecules from cell lines other than SK-MEL-131 carry only the common determinant(s). Immunoperoxidase analysis of frozen tissue sections with mAb KF23 demonstrated a restricted gp90 expression in normal tissues (capillary endothelial cells, duct epithelium in sweat glands, breast, and pancreas). Melanomas and sarcomas showed strong gp90 expression, suggesting up-regulation of gp90 synthesis in certain human cancers.

The nature, specificity, and importance of immune recognition of cancer remain critical issues in tumor immunology. An important objective of work in this field is to distinguish immune responses that have specificity for cancer cells from responses that are not relevant to anti-tumor immunity. Analysis of the humoral immune response of melanoma patients to cell-surface antigens of their own tumor cells has been extensively pursued, initially by conventional serological techniques (1) and more recently by dissecting the B-cell response at the clonal level (2–4). Two categories of autoimmunogenic antigens have been defined in these studies: ganglioside antigens, such as GM2 and GD2 (4–7), and antigens that are restricted to autologous melanoma cells and are not detected on any other normal or allogeneic cell type (4, 8–12). These highly restricted melanoma cell-surface antigens have been referred to as class 1 (unique) tumor antigens and have the properties of glycoproteins (12–14). Although several examples of class 1 melanoma antigens have been defined by cell-surface rosetting assays, their biochemical characterization has been difficult because antibodies recognizing these antigens have not been of sufficient titer or affinity to immunoprecipitate labeled antigen. However, we recently identified a melanoma patient (FD) whose serum contained antibodies that define a class 1

melanoma antigen and also immunoprecipitate a specific glycoprotein of 90 kDa (gp90) from radiolabeled autologous SK-MEL-131 melanoma extracts (12, 13). Several lines of evidence, including cotyping of expression on a panel of cultured cells, led to the conclusion that the class 1 determinant is carried on this gp90 molecule. gp90 from autologous melanoma cells was partially purified and used to produce a mouse polyclonal antiserum (13). This antiserum immunoprecipitates gp90 from the autologous melanoma cells, and its reactivity is absorbed by other cell lines, indicating that cells lacking the FD class 1 determinant express a related molecule.

In the present study, we have extended our analysis of the FD antigenic system by producing three mouse monoclonal antibodies (mAbs) reactive with gp90 and analyzing their reactivity with cultured cells and fresh tissues.

MATERIALS AND METHODS

Cell Culture. Cell lines were obtained from the tumor cell bank of the Human Cancer Immunology Laboratory (Sloan-Kettering Institute, New York). The establishment of the SK-MEL-131 cell line and the FD⁺ and FD[−] cloned cell lines derived from it has been described (12); the three FD⁺ clones used in this study were 1.36-1-5, 1.36-1-9, and 1.36-1-13 (1-5, 1-9, and 1-13); the three FD[−] clones were 2.42, 3.44, and 3.45. Cultures were maintained as described (8). All cultures were tested for mycoplasma contamination; contaminated cultures were discarded.

Production of Mouse Anti-gp90 mAbs. Splenocytes from a BALB/c mouse immunized as described (13) were fused with NS-1 cells at a ratio of 5:1. Culture supernatants from wells containing hybridoma colonies were screened 2–3 weeks after the fusion. Cells from selected wells were subcloned three times by limiting dilution. High titer sera were obtained from hybridoma-bearing nu/nu mice.

Hemadsorption Assays. The anti-mouse immunoglobulin and protein A assays for cell-surface antigens were performed as described (15).

Immunohistochemistry. The reactivity of mAbKF23 with frozen sections of normal tissues and tumor tissues was tested with purified antibody (20 µg/ml) and the avidin-biotin complex immunoperoxidase method (16).

Radiolabeling, Immunoprecipitation, and Electrophoresis. Cells were cultured in methionine-free modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum and [³⁵S]methionine (New England Nuclear) (50 µCi/ml; 1 Ci = 37 GBq) for 24 hr, or [³H]GlcN (New England Nuclear) (50 µCi/ml) for 48 hr. Cell lysates were fractionated on Con A-Sepharose as reported (12, 13). A partially purified preparation of gp90 from the FD-expressing clone 1.5 was obtained as described (13) and was labeled with ¹²⁵I by the

chloramine-T method. For radioimmunoprecipitation experiments, $1-2 \times 10^6$ [^{35} S]Methionine or [^{3} H]GlcN cpm per sample or 5×10^5 [^{125} I] cpm per sample were used. Immunoprecipitation procedures using protein A-Sepharose, sequential immunoprecipitation, and analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis have been described (12, 13).

RESULTS

Production and Properties of mAbs Detecting gp90. Mice were immunized with gp90 antigen partially purified from clone SK-MEL-131 1-5 as described (13). The serum of the mouse selected as the source of splenocytes contained antibodies reactive with the gp90 molecule as detected in sequential immunoprecipitation experiments (13). Splenocytes (1×10^8) were fused with NS-1 cells and seeded in 192 wells. Hybridoma colonies were detected in 175 wells (90%). Hybridoma culture supernatants were assayed for cell-surface reactivity in rosetting assays with a panel of three FD⁺ and three FD⁻ clones derived from SK-MEL-131 and for their ability to immunoprecipitate a 90-kDa molecule comigrating with gp90. Six of 158 supernatants (4%) were reactive with the three FD⁺ clones and unreactive with the three FD⁻ clones, and 11 of 175 (6%) supernatants immunoprecipitated FD antigen. Cells from 11 wells producing these antibodies were selected for subcloning. Three hybridomas, designated KF23 (IgG2b), KF26 (IgG1), and KF104 (IgG1), were subcloned and maintained stable antibody production. In tests on a panel of 16 clones derived from SK-MEL-131, there was total concordance between reactivity with FD serum and the three mAbs (Fig. 1).

Identity of the Antigen Precipitated by Mouse mAbs and Human FD Serum. mAbs KF23, KF26, and KF104 and human FD serum all immunoprecipitated a component of 90 kDa from [^{125} I]-labeled partially purified FD antigen preparation from SK-MEL-131 clone 1-5 (Fig. 2). To demonstrate the identity of molecules immunoprecipitated by the mouse mAbs and FD serum, sequential immunodepletion experiments were performed. Each of the mAbs specifically removed antigen immunoprecipitated by the other two mAbs from the labeled samples. Moreover, mAbKF26 depleted the labeled sample of antigen reactive with FD serum. In contrast, FD serum only partially immunodepleted the molecules reactive with mAbKF26 (Fig. 2). These results demonstrate that mAbs KF23, KF26, and KF104 react with

Fig. 1. Cotyping of FD serum and mouse anti-gp90 mAbs (KF23, KF26, and KF104) on a panel of three FD⁻ (2.42, 3.44, and 3.45) and three FD⁺ (1-1, 1-5, and 1-13) cloned lines derived from SK-MEL-131. Solid bars indicate reactivity with FD serum (starting dilution, 1:10, followed by 1:2 dilutions); open bars indicate, from left to right, reactivity with mAbs KF23, KF26, and KF104 (hybridoma culture supernatant undiluted, followed by 1:6 dilutions). Serological assays: protein A (FD serum) and rabbit anti-mouse immunoglobulin (mouse mAbs) hemadsorption.

Fig. 2. Direct and sequential radioimmunoprecipitation (RIP) tests demonstrating the identity of antigens reactive with FD serum and with mAbKF26. Samples of [^{125} I]-labeled partially purified FD antigen were incubated twice with the first antibody and protein A-Sepharose; unbound molecules were then incubated with a second antibody and protein A-Sepharose. Immunoprecipitated molecules were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. (Left) Direct radioimmunoprecipitation assays with normal human serum (NHS) (lane 1), FD serum (lane 2), or mAbKF26 (lane 3). (Right) Sequential immunoprecipitation experiments with samples precleared with normal human serum (NHS), FD serum, or mAbKF26 and subsequently immunoprecipitated with NHS (lanes 4, 7, and 10), FD serum (lanes 5, 8, and 11), or mAbKF26 (lanes 6, 9, and 12).

epitopes present on the same molecule (gp90) and that this species also carries the epitope recognized by FD serum. The partial clearing observed with FD serum may be due to the lower affinity of antibodies present in FD serum or to the fact that not all gp90 molecules carry the FD determinant.

Expression of gp90 in a Panel of Cultured Cells. mAbs KF23, KF26, and KF104 showed an identical pattern of reactivity with the cultured cell panel in cell-surface rosetting assays. In contrast to the restricted reactivity of FD serum, the anti-gp90 mAbs reacted with most tumor cell lines tested (Table 1). Among cultured normal cells, mAbKF26 reacted with newborn skin melanocytes (4/4), normal kidney epithelium (2/2), and normal adult skin fibroblasts (6/6, only a proportion of the cells being reactive). No reactivity was detected with fibroblasts from normal fetal tissues (0/3). mAbKF26 had similar titration endpoints on cultured normal cells and tumor cell lines (Fig. 3).

Biochemical Characteristics of gp90. FD serum failed to immunoprecipitate a specific component from [^{35} S]methionine-labeled FD⁻ melanoma and nonmelanoma cell lines, whereas mAbKF23 immunoprecipitated gp90 from these sources (Fig. 4). With FD⁺ SK-MEL-131 cell lysates, both FD serum and mAbs KF23, KF26, and KF104 immunoprecipitated a component of 90 kDa (Fig. 4). This component was present in the eluate fraction, but not in the effluent, from lysates fractionated on Con A-Sepharose. Under non-reducing conditions the molecular mass was 85 kDa. gp90 could also be immunoprecipitated from [^{3} H]GlcN-labeled SK-MEL-131 clone 1-5 cells. FD serum and the three mouse mAbs did not immunoprecipitate any molecules from extracts of [^{35} S]methionine-labeled FD⁻ cell variants from SK-MEL-131 (clone 3.44) (Fig. 4). These results are in agreement with prior studies using mouse polyclonal sera

Table 1. Reactivity of mouse anti-gp90 mAbKF23 with cultured normal and tumor cells

Tumor cells	
Positive	
Titer: 10^{-6}	Melanoma: SK-MEL-28, -30, -37, -73, -93-II, -178, -188, -210-I, -210-II, -215-I, -215-II, -223 Astrocytoma: SK-MG-1, -12, -13, -14 Kidney cancer: SK-RC-2, -6, -9, -17, -18, -21, -29, -35, -39, -41, -42, -47, Caki-I Colon cancer: SK-CO-15, LoVo, COLO 320HSR Breast cancer: MDA-MB-231, SK-BR-7, MCF-7 Lung cancer: SK-LC-8, -10 Other carcinomas: T24 (bladder), A431 (vulva) Sarcoma: U205, HT1080, A204, TE85 Epstein-Barr virus-transformed B cells: AG, AV, BD, CZ, DS, DX, FD T cells: HSB-2, HPB-ALL Other hematopoietic lines: K562 (erythroleukemia), U937 (myelomonocytic).
Titer: 10^{-3} – 10^{-6}	Melanoma: SK-MEL-23-II, -133, -211, -212-II, -213, MeWo, SW843, 5441 Astrocytoma: SK-MG-4, SK-MG-21 Kidney cancer: SK-RC-48, -52, -53 Colon cancer: SK-CO-1, -17, HCT-15, HCT-116, Caco-2, HT-29, SW480, SW620, SW707, SW802, SW1116 Pancreas cancer: Capan-1, ASPC-1 Breast cancer: BT-20 Lung cancer: SK-LC-1, -9, -13, -14, -17, -21, Calu-B Other carcinomas: SK-OV-3 (ovary) Sarcoma: SA-OS-2, SW872 Epstein-Barr virus-transformed B cells: AX B-cell leukemia: ARA-10.
Negative	
Titer: $>10^{-2}$	Astrocytoma: SK-MG-5, -6, -7, -15 Kidney cancer: SK-RC-1, -7, -10, -38 Colon cancer: COLO 205, COLO 571, DLD-1, SW403 Pancreas cancer: Capan-2 Breast cancer: BT-00474, ZR-75-1, Cama Lung cancer: SK-LC-2, -6 T-cell leukemia: HT-1, P12, HTLV-102 Other hematopoietic lines: RPMI 8226 (myeloma), HL-60 (promyelocytic)
Normal cells	
Positive	
Titer: 10^{-3} – 10^{-6}	Melanocytes: FS105, FS120, FS123, MCT 1404 Normal kidney epithelium: NHK-1, NHK-2 Adult skin fibroblast: 7, 18, 26, 125, 127, 155
Negative	
Titer: $>10^{-2}$	Fetal fibroblast: FF-1, HS-74, FLF

Results were determined by using hybridoma supernatant as the source of antibody and rabbit anti-mouse immunoglobulin hemadsorption assays. Titer refers to the highest antibody dilution giving 10% positive cells.

(13) and demonstrate that FD[−] clones derived from the parental SK-MEL-131 cell line lack expression of both unique (FD) and shared gp90 epitopes (KF23, KF26, KF104).

To determine the biochemical characteristics of gp90 in other cultured cell types, established cell lines derived from four melanomas, two colon cancers, one renal cancer, and Epstein-Barr virus-transformed lymphocytes from two pa-

tients were labeled with [³⁵S]methionine and extracts were fractionated on Con A-Sepharose before radioimmunoprecipitation. In all cases, mAbKF23 immunoprecipitated a 90-kDa component from Con A eluates but not from Con A effluents. gp90 was also immunoprecipitated from Epstein-Barr virus-transformed lymphocytes from patient FD (Fig. 4). These results indicate that gp90 molecules expressed by

FIG. 3. Reactivity of mouse anti-gp90 mAbKF26 with cultured tumor cells and normal cells. (A) Melanomas (●, SK-MEL-28; ▲, SK-MEL-23-II) and melanocytes (○, FS105; △, MCT 1404). (B) Renal cancers (●, SK-RC-9; ▲, SK-RC-42) and normal kidney epithelium (○, NHK-1). (C) Sarcomas (●, U205; ▲, TE85) and fetal fibroblasts (○, HS-74; △, FLF). Serological assay: rabbit anti-mouse immunoglobulin hemadsorption.

FIG. 4. Autoradiogram of immunoprecipitates from FD⁻ and FD⁺ cell lines using FD serum and mouse anti-gp90 mAbKF23. Cells were labeled with [³⁵S]methionine and cell lysates were fractionated on Con A-Sepharose. Samples eluted from the lectin column were incubated with antibody and immunoprecipitates were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. (A) Normal human serum (lanes 1, 3, 5, and 7); FD serum (lanes 2, 4, 6, and 8). (B) Control mouse mAb (lanes 9, 11, 13, 15, and 17); mAbKF23 (lanes 10, 12, 14, 16, and 18). SK-MEL-131 clone 1-5 (lanes 1, 2, 9, and 10); SK-MEL-31 melanoma (lanes 3, 4, 11, and 12); SK-RC-42 renal cancer (lanes 5, 6, 13, and 14); Epstein-Barr virus-transformed FD-B cells (lanes 7, 8, 15, and 16); FD⁻ variant SK-MEL-131 clone 3.44 (lanes 17 and 18).

SK-MEL-131 and other cultured cells have similar biochemical characteristics.

Distribution of gp90 in Tissues. The distribution of gp90 in normal fetal and adult tissues and in tumors was determined by using mAbKF23 on frozen sections and the ABC immunoperoxidase technique; no reactivity was noted on paraffin-embedded tissue sections. Only a few normal cell types showed strong reactivity: sweat glands and the capillary endothelium in some tissues. Weaker and heterogeneous staining was observed in the myometrium and in ductal and acinar cells of the breast and pancreas. No reactivity was found with other normal cell types, including skin melanocytes and connective tissue fibroblasts. Among tumors, melanomas (9/10) and sarcomas (7/8) showed strong and homogeneous reactivity; other tumor types, including colon cancer (3/5), breast cancer (4/8), renal cancer (2/5), lung cancer (0/4), and bladder cancer (0/5) showed weaker and more heterogeneous reactivity.

DISCUSSION

Since the recognition of class 1 (unique) tumor antigens, an important question has been whether these antigens represent unique molecules or unique determinants on more widely expressed molecules. The identification of the gp90 system by antibodies from patient FD permits biochemical insight into the nature of these antigens. The generation of mouse mAbs to gp90 provides reagents to characterize these molecules further and to define other epitopes on gp90. The results indicate that the mouse mAbs recognize common epitopes on gp90 molecules expressed on a wide variety of cell types. In contrast, FD melanoma SK-MEL-131 carries two types of gp90 determinants: common determinants (detected by the mouse mAbs) and a unique determinant (detected by autologous FD serum). Other cell types, including Epstein-Barr virus-transformed lymphocytes from patient FD, express only the common determinants.

The biochemical nature of the two types of gp90 epitopes is not known. Results of endoglycosidase treatment of gp90 from SK-MEL-131 indicate that it contains predominantly high-mannose carbohydrate chains, raising the possibility that the FD epitope arises as a consequence of this less common glycosylation characteristic (13). On the other hand, the FD epitope is heat labile and protease sensitive, consist-

ent with a protein determinant (12). This idea is supported by the identical molecular masses and lectin-binding properties of FD⁺ gp90 molecules from SK-MEL-131 and gp90 molecules from FD⁻ cells. Understanding the genetic basis for generating a unique determinant on a broadly expressed molecule will require a molecular definition of the FD antigen. An obvious possibility is a point mutation in the coding gene for gp90 in FD melanoma. The fact that FD⁻ variants can be derived from FD⁺ melanoma cells indicates that FD expression is not required for maintenance of the transformed phenotype. In addition, the FD⁻ variants lack not only the unique determinant but also the common determinant(s), and this could be explained by loss of both gp90 structural alleles or changes in genetic elements that regulate gp90 expression.

In contrast to the broad expression of gp90 in cultured cells, gp90 can be detected in only a limited range of normal cell types in tissue sections. No proliferation-related or differentiation-related patterns of gp90 expression could be discerned in normal tissues. Strong gp90 expression was found in specimens of melanoma and sarcoma, whereas normal melanocytes and connective tissue fibroblasts lacked detectable levels of gp90. Although these results on the distribution in normal and malignant tissues need to be extended, there appears to be a marked up-regulation of gp90 expression in certain human cancers.

As yet, FD is the only class 1 (unique) antigen shown to reside on gp90. Five other class 1 (unique) antigens on melanoma cells have been identified by using autologous serum (8-11) and mAbs detecting common determinants immunoprecipitate gp90 molecules from each of the five melanoma cell lines that express these unique antigens. Since the human sera identifying these other unique specificities do not have immunoprecipitating activities, additional methods will be needed to determine whether all class 1 (unique) antigens belong to the gp90 family or whether unique determinants occur on other molecular species. With regard to tumor antigens in experimental systems, the FD gp90 antigen bears some resemblance to the recently identified family of 96-kDa glycoproteins (gp96) on the surface of chemically induced sarcomas of inbred mice (17). These tumors have long been known to express transplantation rejection antigens that make it possible to immunize syngeneic hosts against tumor challenge. A remarkable feature of these

transplantation antigens is their extensive polymorphism; each tumor, even two or more tumors induced in the same mouse, appears to have an individually distinct antigen. Srivastava *et al.* (17) have shown that the transplantation rejection antigens of two of these tumors are carried by a gp96 molecule. Rabbit antisera recognizing common gp96 determinants detect antigens related to gp96 in normal tissues. Thus, like the FD antigen, gp96 rejection antigens on chemically induced tumors are unique determinants found on molecules that are widely expressed. Although these similarities in molecular mass and other characteristics of gp90 and gp96 are provocative and might suggest a relation between the two systems, no cross-reactivity has been detected using currently available serological reagents.

This work is supported by grants from the National Cancer Institute (CA-08748, CA-33049, and CA-21445).

1. Old, L. J. (1981) *Cancer Res.* **41**, 361–375.
2. Houghton, A. N., Brooks, H., Cote, R. J., Taormina, M. C., Oettgen, H. F. & Old, L. J. (1983) *J. Exp. Med.* **158**, 53–65.
3. Irie, R. F., Sze, L. L. & Saxton, R. E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5666–5670.
4. Yamaguchi, H., Furukawa, K., Fortunato, S. R., Livingston, P. O., Lloyd, K. O., Oettgen, H. F. & Old, L. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2416–2420.
5. Watanabe, T., Pukel, C. S., Takeyama, H., Lloyd, K. O., Shiku, H., Li, L. T. C., Travassos, L. R., Oettgen, H. F. & Old, L. J. (1982) *J. Exp. Med.* **156**, 1884–1889.
6. Tai, T., Paulson, J. C., Cahan, L. D. & Irie, R. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5392–5396.
7. Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A. & Paulson, J. C. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7629–7633.
8. Shiku, H., Takahashi, T., Oettgen, H. F. & Old, L. J. (1976) *J. Exp. Med.* **144**, 873–881.
9. Carey, T. E., Takahashi, T., Resnick, L. A., Oettgen, H. F. & Old, L. J. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3278–3282.
10. Takeyama, H., Shiku, H., Resnick, L. A., Houghton, A. N., Albino, A. P., Oettgen, H. F. & Old, L. J. (1981) *Proc. Am. Assoc. Cancer Res.* **21**, 300 (abstr.).
11. Albino, A. P., Lloyd, K. O., Houghton, A. N., Oettgen, H. F. & Old, L. J. (1981) *J. Exp. Med.* **154**, 1764–1778.
12. Real, F. X., Mattes, M. J., Houghton, A. N., Oettgen, H. F., Lloyd, K. O. & Old, L. J. (1984) *J. Exp. Med.* **160**, 1219–1233.
13. Mattes, M. J., Real, F. X., Furukawa, K., Old, L. J. & Lloyd, K. O. (1988) *Cancer Res.* **47**, 6614–6619.
14. Carey, T. E., Lloyd, K. O., Takahashi, T., Travassos, L. & Old, L. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2898–2902.
15. Real, F. X., Oettgen, H. F. & Old, L. J. (1986) *Manual of Clinical Laboratory Immunology* (Am. Soc. Microbiol., Washington, DC), pp. 798–801.
16. Hsu, S. M., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29**, 577–580.
17. Srivastava, P. K., DeLeo, A. B. & Old, L. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3407–3411.