Tracking the Common Ancestry of Antigenically Distinct Cancer Variants[1]

Karin Schreiber,[2] Terry H. Wu,[2] W. Martin Kast, and Hans Schreiber[3]

Department of Pathology, The University of Chicago, Chicago, Illinois 60637 [K. S., T. H. W., H. S.], and Cancer Immunology Program, Cardinal Bernardin Cancer Center, Loyola University Chicago, Maywood, Illinois 60153 [W. M. K.]

Abstract

In the months and years after first diagnosis, cancers often show an increase in their malignancy such as faster growth, resistance to chemo- and/or hormonal therapy, and loss of antigens targeted by immunotherapy. Our objective was to develop a model in which one can track the changes occurring as a result of *in vivo* immune selection, such as the loss of antigen, the emergence of previously hidden antigens, or the acquisition of new tumor-specific antigens. In this study, we used the primary UV-induced murine tumor 8101, which consists predominantly of regressor tumor cells that express the immunodominant mutant p68 antigen, but this tumor also contains progressor variants that have lost this antigen. To search for tumor-specific antigens on the immune escape progressors, we raised CD8$^+$ T cells specific for these variants. We found that one of the escape variants expressed a previously unrecognized, unique tumor-specific antigen. However, this unique antigen was not readily detectable on any of the other 8101 lines we tested. To prove that these antigenically distinct cancer variants had indeed been derived from the same tumor and neither represented new tumors nor contaminations by other cell lines, we used unique tumor-specific p53 mutations as a lineage-specific marker to demonstrate that these antigenically distinct progressor variants were derived from the 8101 tumor. Because p53 mutations occur very early during UV carcinogenesis and vary from tumor to tumor, they provide convenient reliable markers for tracking the origin of cancers arising after immune selection or immunotherapy.

Introduction

Cancer cells are known to harbor numerous mutations (1, 2) and to be karyotypically unstable, but the exact stage of carcinogenesis at which the most critical mutations occur is still unclear. Many mutations are present already in the cancers at diagnosis and seem to occur earlier than assumed previously; for example, loss of heterozygosity has been reported to be present in morphologically normal tissue adjacent to premalignant lesions (3). However, new mutations may also be acquired by the cancer cells in later stages of cancer development after first diagnosis, and these later mutations may also help cancers to escape therapy (4).

Unique tumor-specific antigens are caused by tumor-specific somatic mutations (Ref. 5; reviewed in Refs. 6 and 7), and at least some of these mutations play a role in causing and/or increasing malignant behavior of the cancer cells (7). If these mutations were to arise after the clinical diagnosis of the original cancer, then the more malignant variants might acquire new tumor-specific antigens. Alternatively, mutant proteins could be present in the original primary tumor as immunorecessive antigens (8, 9), and subsequent selection for more aggressive variants might lead to the unmasking of tumor-specific antigens (9, 10). Because loss of target antigens during immunotherapy is a frequent occurrence (11), identification of such neoantigens or unmasked antigens in tumor variants would be important for immunotherapy because these additional targets could be exploited therapeutically (12). A *sine qua non* for analyzing the origin of the antigenic changes is the existence of lineage-specific markers that can be used to determine whether the variants indeed arise from a common precursor and are neither new tumors nor contaminations by other cell lines.

In this study, we examined progressor variants of the UV-induced regressor tumor 8101 (13) for previously unrecognized tumor-specific antigens. Indeed, we discovered a host-selected progressor variant displaying a tumor-specific antigen that we had not detected previously in clones of the original primary tumor. Using a unique tumor-specific p53 mutation as a lineage-specific marker, we could prove that all of the antigenically distinct variants had been derived from the same original tumor and that the previously unrecognized tumor-specific antigen was either immunorecessive in the original tumor, existent and now unmasked, or had newly arisen in the escape variant.

Materials and Methods

Mice. Normal and nude C57BL/6 mice were purchased from Frederick Cancer Research Institute (Frederick, MD) and maintained in a barrier facility free of specific pathogens at The University of Chicago.

Tumors. The 8101 tumor was induced in C57BL/6 mice by chronic UV irradiation in our laboratory as described (13). The primary tumor was frozen as fragments. The heart and lungs were removed *en bloc* and chopped into fragments to generate a nonmalignant heart-lung fibroblast cell line as control. The dominant CD8$^+$ T cell-recognized epitope on the 8101-RE tumor has been identified as an octameric peptide SNFVFAGI presented by K^b. This peptide is derived from the DEAD box protein p68 that carries a serine-to-phenylalanine substitution

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[2] These authors contributed equally to this paper.

[3] To whom requests for reprints should be addressed, at The University of Chicago, Department of Pathology, MC 3083, Chicago, IL 60637. Phone: (773) 702-9204; Fax: (773) 702-9224.

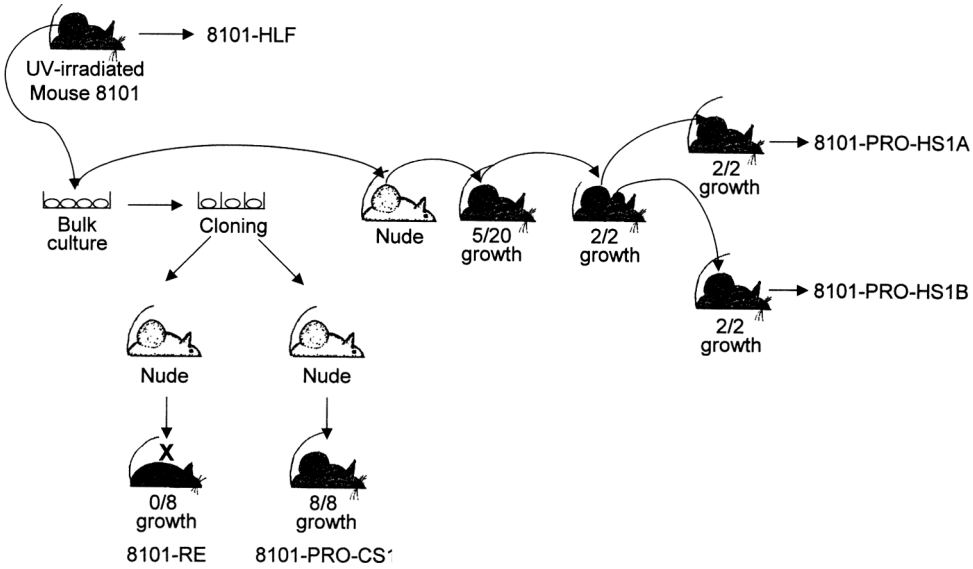

Fig. 1 Derivation of different 8101 tumor lines and the generation of progressor variants through selection in the normal immunocompetent host. Cells from a bulk culture derived from 8101 tumor fragments were injected into a C57BL/6 nude mouse, and fragments of the resulting tumor were injected into 20 syngeneic normal C57BL/6 mice. The tumor from one of the five normal mice that failed to reject the tumor was again transplanted into two normal C57BL/6 mice. Both of these mice developed tumors; one of these mice had two distinct nodules at the injection site. Each of these nodules grew when transplanted into two normal C57BL/6 mice. One tumor was named PRO-HS1A, and the other was named PRO-HS1B. The *in vitro* derivation of random clones from the 8101 bulk culture and the characterization of these clones as regressor or progressor tumor lines have been described previously (13). 8101-HLF refers to normal heart-lung fibroblasts isolated from the mouse of 8101 tumor origin as autochthonous nonmalignant control cells.

caused by a tumor-specific single C-to-T nucleotide substitution and providing, in position 5 of the peptide, a K^b-specific anchor residue.

Generation of CTLs. Spleen cells were removed from immunized animals, and 8×10^6 immune spleen cells were restimulated in 6-day mixed lymphocyte tumor cell cultures with 4×10^4 tumor cells as stimulators irradiated with 250 Gy in 3.5 ml of supplemented RPMI 1640 (Life Technologies, Inc., Grand Island, NV) in round-bottomed tissue culture tubes 3033 (Falcon Laboratories Becton Dickinson, Lincoln Park, NJ) using highly selected FCS as described (14). The establishment and maintenance of T cells has also been described previously in detail. However, we no longer routinely use 33% secondary mixed lymphocyte culture supernatants but use 3 IU/ml recombinant interleukin 2 (Hoffmann-LaRoche, Nutley, NJ) instead. The methods used for the derivation and characterization of the T-cell clone line has been described (14).

^{51}Cr-Release Assay. Cytolysis of target cells by T cells was analyzed essentially as described (14). Briefly, 5×10^3 ^{51}Cr-labeled target cells were incubated with varying numbers of effectors in 0.2 ml of complete RPMI for 4–5 h in flexible V-bottomed, 96-well plates in a humidified incubator. The supernatant was harvested, and the radioactivity was measured in a gamma counter (ICN). The percentage of lysis was calculated by the formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. For peptide loading, RMA-S cells were kept at room temperature to bring the "empty" MHC class I molecules to the cell surface before loading with peptides. Ninety μl of complete DMEM containing maximally 10^6 target cells, 10 μl of the peptide (10 ng/ml) to be loaded, and 50 μl of FCS were incubated for 1.5 h at 37°C; thereafter, 50 μl of the ^{51}Cr were added for an additional hour of incubation (final volume, 200 μl).

Sequencing of the *p53* Gene. DNA fragments containing the p53 sequence were generated by reverse transcription-PCR using the sense primer 5′-ATGCGAATTCTCCTCCCCT-CAATAAGCT-3′and the antisense primer 5′-TATAGGAAT-TCAGGGCAAAGGACTTCCT-3′ (Clontech, Palo Alto, CA). These primers amplify p53 sequences from exon 5 to the end of exon 8. The fragments were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced at The University of Chicago Cancer Research Center DNA Sequencing Facility (Chicago, IL).

Results

The Normal Host Selects for Progressor Tumor Variants. As explained in Fig. 1, tumor fragments of the original primary 8101 tumor were minced to establish a bulk culture. Cells from this culture were injected into one C57BL/6 nude mouse, and fragments of the tumor that developed in this nude mouse were injected into 20 normal syngeneic C57BL/6 mice. Although 15 of the 20 mice rejected the tumor fragments, 5 mice developed progressively growing cancers. The progressor tumor of one of these mice was reisolated, and the fragments were transplanted into 2 normal syngeneic mice, which in turn developed progressive tumors. One of these mice developed two distinct tumor nodules at the site of s.c. tumor challenge. Fragments of the smaller tumor (referred to as PRO-HS1A, where HS means host selected) and fragments of the larger nodule

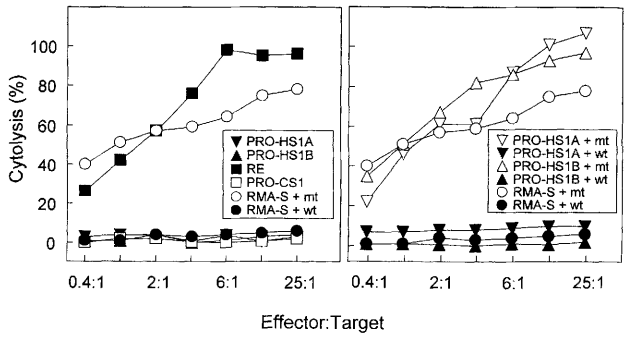

Fig. 2 *Left panel,* lack of expression of the mutant p68 target antigen by all of the progressor tumor variants. The specificity of the $CD8^+$ cytolytic T cell clone for the mutant p68 peptide SNFVFAGI (*mt*) is shown by lysis of RMA-S cells loaded with this peptide and resistance to lysis of RMA-S cells loaded with the control peptide SNFVSAGI (*wt*). *Right panel,* PRO-HS1A and PRO-HS1B are not resistant *per se* to lysis by the mutant p68-specific T-cell clone because these target cells can be lysed when loaded with the relevant mutant (*mt*) peptide. This strongly suggests that the loss of expression of the relevant antigen is responsible for the resistance of the PRO cell lines to the mutant p68-specific T cells.

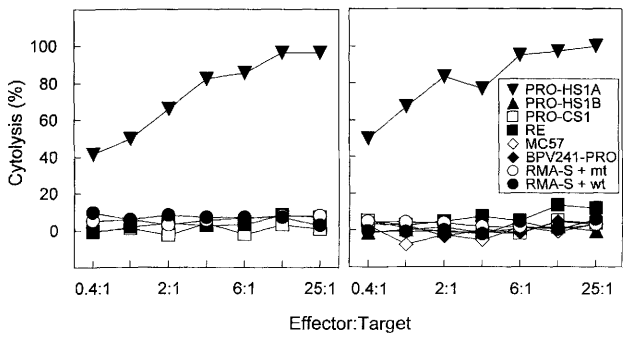

Fig. 3 The PRO-HS1A tumor expresses an antigen that is not shared by several other previously derived tumor cell lines. *Left panel,* PRO-HS1A cells are effectively lysed by a T-cell line established from immune spleen cells of a mouse that had rejected a lethal challenge of PRO-HS1A cells. Effector cells are from a mixed lymphocyte-tumor cell culture, followed by continued passing of the generated effector cells with irradiated PRO-HS1A cells as stimulators and recombinant IL-2. *Right panel,* a cytotoxic T-cell clone derived from the T cell line shown in the *left panel* has similar specificity and lytic capacity. None of the other presently derived cloned 8101 cell lines show any significant reactivity with the T-cell clone. Furthermore, the resistance of other C57BL/6 tumor cell lines to lysis by this T-cell clone suggests that the target antigen is a unique tumor-specific antigen.

(referred to as PRO-HS1B) were injected into 2 normal mice and grew in both of the two mice challenged, indicating a heritably stable progressor phenotype. Furthermore, 10^7 cultured cells of either 8101-HS1A or 8101-HS1B caused lethal tumors when injected into normal syngeneic mice. Fig. 1 shows that the bulk culture of the original 8101 tumor was cloned, as reported previously, and several clones were selected randomly (13). Tumor cells from one clone gave rise to tumors in nude mice that were regularly rejected by normal mice, and the line was therefore designated as 8101-RE. Tumor cells from a second clone regularly formed progressive lethal tumors, and this culture-selected cloned line was a progressor variant and therefore designated in the current study as 8101-PRO-CS1 (where CS means culture selected).

The Host-selected Progressor Variants Have Lost Expression of the Immunodominant Mutant p68 Antigen. Fig. 2 (*left panel*) shows that the two newly derived host-selected progressor variants PRO-HS1A and PRO-HS1B were not lysed by a $CD8^+$ cytolytic T cell clone (Fig. 2, *left panel*) we generated, *i.e.,* specific for the peptide SNFVFAGI of the mutant p68 antigen. The mutant p68 antigen was also no longer detected on the PRO-CS1 variant. The specificity of the T-cell clone is demonstrated by showing that RMA-S cells loaded with the corresponding wild-type sequence p68 peptide SNFVSAGI were not lysed, whereas RMA-S cells loaded with the mutant peptide were lysed. The host-selected progressor tumor variants were as susceptible as the RMA-S cells to lysis by the mutant p68-specific T cells when these target cells were preincubated with the mutant p68 peptide (Fig. 2, *right panel*). This sensitization of the PRO-HS1A and PRO-HS1B cells by incubation with the mutant peptide was specific because preincubation with the wild-type peptide rendered neither the RMA-S cells nor the progressor tumor cells susceptible to lysis by the mutant p68-specific T cells. This suggested that resistance of the PRO-HS1A and PRO-HS1B cells to lysis was attributable to loss of the p68 antigen and not attributable to an inherent resistance to T cell-mediated lysis *per se* or by the absence of the MHC class I-presenting molecules on the progressor tumor cell surface.

The Host-selected Progressor Variant PRO-HS1A Expresses a Previously Unrecognized, Unique Tumor-specific Antigen. To search for tumor-specific antigenic targets on the host-selected progressor variants, 10^7 PRO-HS1A or PRO-HS1B cultured cells were each injected into 3 mice that had previously rejected the bulk culture-derived nude mouse tumor fragments (*i.e.,* 6 mice from the group of 15 mice that rejected the fragments; see Fig. 1) and 2 normal mice as naïve controls. After transient growth, all of the PRO-HS1A variant tumor cells were rejected by the 3 mice that had previously rejected the bulk culture-derived nude mouse tumor fragments, whereas the 2 naïve mice failed to reject the same tumor challenge of 10^7 PRO-HS1A cells. By contrast, the PRO-HS1B tumor was not rejected by either the 2 naïve mice or the 3 mice that had previously rejected the bulk culture-derived tumor fragments. We then cultured the spleen cells from 1 of the mice that had rejected a lethal PRO-HS1A challenge using irradiated PRO-HS1A cells as stimulators. After 6 days of culture, the generated effector cells were used in a ^{51}Cr-release assay using the 8101-RE, the culture-selected PRO-CS1, and the two host-selected PRO-HS1A and PRO-HS1B as targets. Fig. 3 (*left panel*) shows that the effectors were highly specific for PRO-HS1A, *i.e.,* the progressor line that had been used for challenge. We, therefore, isolated T-cell clones from these effectors by limiting dilution cloning, and the *right panel* of Fig. 3 shows that these cloned T cells are also highly selective for the PRO-HS1A. The antigen seemed to be uniquely tumor specific because three other C57BL/6-derived tumor cell lines were not lysed by the T-cell clone. Surprisingly, the CTL-recognized antigen seemed also to

Source	191	238
WT	A P P Q H L I R V E G	Y M C N S S C M G G M
8101-Bulk	A P P Q H F I R V E G	Y M C N S A C M G G M
8101-RE	A P P Q H F I R V E G	Y M C N S A C M G G M
8101-PRO-CS1	A P P Q H F I R V E G	Y M C N S A C M G G M
8101-PRO-HS1A	A P P Q H F I R V E G	Y M C N S A C M G G M
8101-PRO-HS1B	A P P Q H F I R V E G	Y M C N S A C M G G M
4102-RE	A P P Q H L I R V E G	Y M C N S S C M G G M
6139B-PRO	A P P Q H L I R V E G	Y M C N S S C M G G M
6130-PRO	A P P Q H L I R V E G	Y M C N S F C M G G M
6132A-RE	A P P Q H F I R V E G	Y M C N S S C M G G M

Fig. 4 PRO-CS1, PRO-HS1A, PRO-HS1B, and RE cell lines (derivations depicted in Fig. 1) all share a unique single amino acid substitution (serine to alanine) at position 238. This strongly suggests that all four tumor lines are derived from single 8101 precursor cell. Furthermore, it is shown that four other UV-induced murine tumors that originated independently all carry unique p53 mutations different from the one observed in 8101 lineage tumors. All 8101 lineage tumors also share a leucine to phenylalanine mutation in position 191. This transition mutation occurs more frequently and is also found in 6132A-RE; however, 6132A-RE is distinguished by the lack of the codon 238 mutation and the presence of an arginine to cysteine mutation in codon 270 (not shown).

be absent on the other three cell lines derived from the same original 8101 bulk culture.

PRO-HS1A Shares an 8101 Lineage-specific p53 Mutation with the Other 8101-PRO Variants. Because we failed to detect the tumor-specific PRO-HS1A antigen on any of the other 8101 tumor lines and because the mutant p68 antigen expressed by the RE clone had been lost by the 2 *in vivo*-selected and the 1 *in vitro*-derived progressor variants, we searched for evidence that these tumors were indeed related to the 8101 tumor lineage. Because p53 mutations occur very early during UV-induced carcinogenesis (15) and because these mutations are individually distinct between different cancers (16, 17), we sequenced the *p53* gene of the various 8101 lineage tumors and found that all of them shared a single amino acid substitution at position 238 (Fig. 4), suggesting that the 8101-RE and the three progressor variants were indeed derived from a single common precursor. The mutation in amino acid position 238 consisted of a serine (S) to alanine (A) mutation caused by a T-to-G transversion at nucleotide 712 (TCC→GCC). The four other UV-induced tumors used as controls each showed either a different unique mutation at this position (*i.e.,* S→F in 6130-PRO) or expressed the wild-type amino acid at this position but a different mutation elsewhere: 4102-RE had a R→C mutation in amino acid position 245, 6139-PRO had a R→C mutation in position 210, and 6132A-RE had a R→C mutation in amino acid position 270. All of the 8101 lineage tumor cell lines also showed a second mutation in amino acid position 191 of p53. The mutation in amino acid position 191 consisted of a leucine (L) to phenylalanine (F) substitution caused by a C→T transition in nucleotide codon 571 (CTT→TTT). This is a "signature" mutation characteristic of UV damage to DNA. As could therefore be expected, one of the other tumors, 6132A, showed the same mutation in this codon, but the other *p53* allele of this tumor contained the R→C mutation in amino acid position 270, which was not found in 8101. None of the UV-induced tumors we examined revealed wild-type p53 sequences. Except for the 8101 bulk population, none of the *p53* gene sequences showed more than one mutation, *i.e.,* p53 sequences found in 8101 lineage tumors either showed a mutation in codon 191 or in codon 238.

Discussion

UV-induced murine tumors are excellent models for dissecting the antigenic makeup of cancer cells (13, 18, 19) and for analyzing changes during tumor progression (20). These cancers usually progress from less malignant cancer cells that are rejected by normal syngeneic immunocompetent mice to more malignant cancer variants that kill normal immunocompetent animals (13, 20). In this study, we found that the UV-induced skin cancer 8101 was rejected by the majority of normal syngeneic mice. However, tumors that grew progressively after tumor challenge were progressor variants because these tumors regularly failed to be rejected when further transplanted into naïve syngeneic mice. Both of the *in vivo*-derived progressor variants had lost expression of the mutant p68 antigen, as we had observed previously in the culture selected progressor variant. This is consistent with our previous suggestion that the immunodominant mutant p68 epitope represents a rejection antigen (14, 21).

Certain unique tumor-specific antigens that are retained during tumor development can be used as lineage-specific markers (20, 22). Because the loss of the mutant p68 epitope deprived us of a lineage-specific marker, we could not ascertain that the RE and the culture-selected and the host-selected progressor variants had been derived from a common precursor cell. Because p53 mutations are highly specific for individual tumors (16, 17) and because p53 mutations occur very early in UV-induced carcinogenesis (15), we explored the use of individual p53 mutations as lineage-specific markers. We found that all of the progressor variants as well as the mutant p68-positive regressor clone shared the same amino acid substitution at position 238. None of the four other tumors tested had this mutation. Furthermore, a review of the particular position and review of the types of amino acid substitutions in the *p53* gene in tumors from humans or mice (16, 17, 23, 24) suggested that the probability of tumors sharing the same amino acid substitution at position 238 is $<1\%$ ($P < 0.01$). However, basing the frequency of occurrences on the p53 mutations detected in all human and mouse tumors may be misleading, and the probability should be reexamined once sufficient numbers of UV-induced mutations in murine tumors have been analyzed and reported. Nevertheless, it appears from our data that p53 mutations can be a reliable indicator of tumor lineage. The usefulness of unique p53 mutations as lineage-specific markers depends upon the mutations occurring early, preferably in the premalignant lesions, as observed in UV-induced carcinogenesis (15, 25). For other cancers in which mutations in p53 may not occur or occur only at later stages, unique cancer-specific mutations in

other genes, such as the *FHIT* gene (26), may have to be used. Finally, we do not know whether certain CTL-recognized p53 epitopes (such as the wild-type p53 epitope 232–240 (27), defined by "reverse" immunology and based on sequence motifs), play a role in the natural immune response and immune selection occurring in mice growing or rejecting tumors, because these p53 epitopes seem to require immunization with the peptide to induce a CTL response.

Our study shows that a previously unrecognized antigen is expressed on the PRO-HS1A tumor. This antigen is detected by T cells from mice that had rejected a lethal challenge with this progressor tumor. The very effective lysis of these tumors at very low E:T cell ratios suggests that this antigen may be a powerful target for rejection of the progressor variant by T cells. Furthermore, the absence of the antigen in other syngeneic tumors suggests that it is a unique tumor-specific antigen. Such antigens are usually caused by somatic tumor-specific mutations (Ref. 5; reviewed in Refs. 6 and 7) and may therefore represent a powerful non-self antigen. We do not know at present whether this antigen arose during host selection for the progressor variant PRO-HS1A or whether this antigen already existed in some of the cells in the primary tumor.

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