Rapid Publication

HLA-DR Histocompatibility Leukocyte Antigens Permit Cultured Human Melanoma Cells from Early But Not Advanced Disease to Stimulate Autologous Lymphocytes

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Abstract. HLA-DR histocompatibility antigens are commonly expressed by the melanocytes of melanoma and its precursors, but not by the melanocyte of normal skin. Further, the primary lesion of biologically early melanoma is commonly infiltrated with host T cells. Advanced disease is characterized by a paucity of such cells. To investigate the interaction of melanoma cells and autologous lymphocytes and its dependence on HLA-DR expression, we have established cell lines from biologically early (4 lines) and advanced disease (11 lines) and examined their capacity to stimulate blastogenesis of autologous T cells in vitro. Melanocytes from early disease expressed HLA-DR antigens and stimulated autologous T cells. Those from advanced disease, irrespective of DR expression, were nonstimulatory. To determine whether expression of DR was required for melanoma cells to be stimulatory, we first treated a stimulating cell line of DR3

allospecificity with anti-DR3-specific serum and demonstrated marked inhibition of its capacity to provoke blastogenesis. Next we used fluorescence-activated flow cytometry to sort a stimulating line heterogeneous for DR expression into DR-enriched and -depleted populations. When such cells were examined in the lymphocyte proliferation assay, their stimulatory capacity was proportional to their quantitative expression of HLA-DR. These studies indicate that cell lines may reflect important biological differences between early and advanced melanoma. HLA-DR expression may be an early event in neoplasia of melanocytes. These antigens are able to interact directly with autologous T cells; and their expression is necessary, but not sufficient, for melanoma cells to induce lymphocyte proliferation.

Introduction

In common with other malignancies, melanoma evolves in stepwise fashion. Qualitatively different steps in tumor progression include: a variety of precursor melanocytic lesions; a cutaneous plaque of primary tumor comprised of invasive melanocytes with no competence for metastasis ("the radial growth phase"); a complex tumor additionally containing a population(s) of cells capable of discontinuous (metastatic) growth ("the vertical growth phase"); and metastatic disease itself (1–3). The histopathology of primary melanoma in radial growth phase is characterized by a cellular infiltrate largely made up of thymus-

derived (T) lymphocytes (4–6). In contrast, vertical growth phase and metastatic melanoma commonly evoke a minimal host cellular response (1). Although these and other data suggest that host immunity modulates tumor progression, the nature of the interaction between melanoma and responding T cells is largely unexplored, as are the antigens involved in provoking this putatively immune response.

DR antigens are cell-surface glycoproteins encoded by genes mapping into the HLA-D histocompatibility region. These Class II HLA antigens have a limited tissue distribution, are homologous to certain murine Ia molecules (I-E molecules), and function as critical elements in cell-cell interaction in the immune system (7). Recent data indicate that melanoma cells, whether in situ or in tissue culture, commonly express HLA-DR antigens (8–10). Additionally, DR antigens have been identified on the melanocytes of common and dysplastic nevi (11), precursors of melanoma (1). The melanocytes of normal skin do not display such antigens (10–12). Thus, expression of HLA-DR might be viewed as reflecting a qualitative step in the evolution of neoplasia of melanocytes. The function of these antigens, as newly displayed on melanocytes, is unexplored, but they are candidate participants in the immune response to melanoma.

To investigate the apparent difference in cellular immune response to early primary vs. late primary and metastatic melanoma, we asked whether cell lines established from radial growth phase, vertical growth phase, and metastatic disease differ in their capacity to stimulate proliferation of autologous T lymphocytes. Further, we asked whether HLA-DR antigens, displayed on the cell surface of malignant melanocytes, participate in stimulating such lymphocytes.

Methods

Cell lines. Melanoma cell lines (Table I) were established by previously described methods (13) from two primaries exclusively in radial growth phase, from areas of radial growth phase of two complex primaries, from a primary exhibiting only vertical growth phase and a subsequent metastasis, and from nine metastatic lesions (in nine additional patients). The fidelity of cell lines was assessed by karyotype analysis and HLA typing. B cell lines were established by infection with Epstein-Barr virus by standard methods (14).

Anti-DR monoclonal antibody, allospecific anti-DR serum, and determination of cellular expression of HLA-DR. Antibody WI691-13-17 is a well-characterized murine monoclonal antibody that recognizes an epitope common to HLA-DR antigens (15–17). P3 is a monoclonal immunoglobulin with no specificity for melanoma cells and is secreted by mouse myeloma cells (P3 $\times$ 63Ag8) used in the production of the anti-DR hybridoma. Qualitative expression of HLA-DR by melanoma cell lines (Table I) was determined in a mixed hemadsorption assay using WI691-13-17 (13, 18). Relative quantitative expression of cell surface HLA-DR (Fig. 1) was measured as in Goldstein et al.'s radioimmunoassay (19), modified here using WI691-13-17 and polyclonal, ^{125}I rabbit anti-mouse IgG. The allospecificity of the human anti-DR serum used to block stimulation of melanoma cells of DR3 specificity was demonstrated by microcytotoxicity assay (20) against three separate panels of cells previously typed with sera from both International and American Workshops. These panels represent all major and most workshop-defined

antigens of HLA-A, B, C, and DR. An identically characterized control antiserum had no anti-DR activity, having only an irrelevant HLA-B specificity. Melanoma and B cell lines were typed for DR allospecificity using a microcytotoxicity assay and a serum panel which identifies the World Health Organization-recognized DR specificities (16).

Lymphocyte blastogenesis. For lymphocyte stimulation studies, peripheral blood T cells were obtained by standard methods from the mononuclear cell population of heparinized whole blood after density-gradient centrifugation, plastic adherence, and rosetting with sheep erythrocytes. Greater than 95% of these cells were viable (erythrosin B exclusion) and greater than 98% bound sheep erythrocytes upon rerosetting. Lymphocyte proliferation in response to melanoma was sought by culturing T cells with melanoma lines treated with mitomycin-C (Sigma Chemical Co., St. Louis, MO) to block their DNA synthesis (Table I).

Results

As shown in Table I, all melanoma cell lines established from primary disease expressed HLA-DR, whereas approximately half of metastatic lines did not. Cell lines established from biologically early malignant disease (lines 1–4) stimulated proliferation of autologous T cells. In sharp contrast, cell lines derived from vertical growth phase (line 5) and metastatic disease (lines 6–15) did not evoke a blastogenic response. A variety of manipulations failed to cause melanoma cells from biologically late disease to stimulate autologous lymphocytes. These included altering the ratio of melanoma cells to T cells from 0.04–10:1; incubating in autologous serum, allogeneic serum, or fetal calf serum; and assessing blastogenesis after 3, 5, 7, 10, or 14 d of incubation. Melanoma cells from biologically late disease were not inert as stimulators, but were frequently capable of provoking blastogenesis when cultured with allogeneic T cells (21 and Table I, line Nos. 6–9). Moreover, their ability to do so was dependent on their expressing HLA-DR. Additional experiments demonstrated that T cells unresponsive to autologous melanoma could proliferate in response to a variety of allogeneic stimuli (including melanoma lines, normal peripheral blood B cells, and the malignant B lymphoblast, Raji) as well as autologous stimuli, such as peripheral blood non-T cells and B cells transformed by Epstein-Barr virus (data not shown).

Since no stimulation of lymphocytes (autologous or allogeneic) was seen with any melanoma not expressing DR antigens, and since not all DR-positive lines were stimulatory, we postulated that such cell-associated antigens were necessary, but insufficient, for lymphocyte recognition and response. To test this hypothesis, we first sought to block the stimulation of autologous T cells by preincubating a melanoma line of DR3 allospecificity with human anti-DR3 serum. As shown in Table II, treatment of these melanoma cells with the allospecific antiserum efficiently abrogated their capacity to stimulate autologous T cells. Virus-transformed autologous B cells were definitely, but less efficiently, inhibited (likely because our experimental design involved washing the stimulating cells before co-culture with T cells). As expected, B cells of a different allospecificity (DR 7/2) were not significantly inhibited.

Cell line (HLA-DR [+] or [−])	Stimulation index (mean of [n] experiments) of responding T cells	
	Autologous	Allogeneic
Primary melanoma lines		
Radial growth phase		
1. WM 35 (+)	29.9 (2)	14.2 (1)
2. WM 245 (+)	43.0 (2)	105.3 (2)
Complex Primaries		
3. WM 75 (+)	11.7 (3)	36.2 (4)
4. WM 98 (+)	21.8 (4)	101.3 (1)
Vertical growth phase		
5. WM 115 (+)	1.4 (4)	18.7 (3)
Metastatic lines		
6. WM 9 (+)	1.6 (4)	78.9 (4)
7. WM 28 (+)	1.3 (3)	163.0 (8)
8. WM 47 (+)	ND‡	25.6 (4)
9. WM 239A (+)*	1.3 (2)	44.5 (1)
10. WM 262 (+)	1.0 (1)	1.2 (1)
11. WM 8 (−)	0.1 (1)	0.5 (1)
12. WM 46 (−)	1.4 (1)	0.9 (1)
13. WM 164 (−)	1.1 (2)	1.3 (2)
14. WM 247 (−)	1.3 (1)	1.8 (1)
15. CCM 2b (−)	1.6 (2)	1.2 (2)

Cell lines were derived from biologically early disease, radial growth phase (lines 1–4), or biologically late disease, vertical growth phase (line 5) and metastatic melanoma (lines 6–15). HLA-DR expression was determined by mixed hemadasorption assay. T cells (2×10^5) were cultured alone or with an equal number of mitomycin C-treated melanoma cells in triplicate wells of a microtest plate (Linbro, Hamden, CT). As a measure of lymphocyte proliferation at 5 d, [^{3}H]thymidine (New England Nuclear, Boston, MA) incorporation was recorded as counts per minute in a scintillation counter (Searle Radiographics, Inc., Des Plaines, IL). Data are expressed as a stimulation index (SI), where SI = mean cpm in test wells/mean cpm of T cells alone. The mean counts per minute of melanoma cells treated with mitomycin was always <100 and the mean counts per minute of T cells alone was generally <100 and never >350. We take an SI to be significant when >2.5. In this table the mean SI and the number of experiments (n) are given for each cell line. The data were analyzed using the two-tailed Wilcoxon's Rank Sum Test. The results of this analysis were that the SI's of T cells autologous to lines 1–4 were greater than those to lines 5–15 ($P < 0.00005$). The allogeneic response to DR (+) lines (lines 1–10) also differed from that to DR (−) lines ($P = 0.0015$).
* Line derived from a subsequent metastasis of the vertical growth phase primary from which line 5 (WM 115) was derived.
‡ ND, not done.

It is possible that antibodies mediating blocking recognize different Class II antigens than those mediating cytotoxicity. To refute this hypothesis and to further test whether it is HLA-DR

on the melanoma cell (rather than on host accessory cells or T cells [22]) which participates in stimulation, we used a fluorescence-activated cell sorter and a monoclonal antibody that recognizes an invariant epitope of DR to fractionate the cells of a melanoma line heterogeneous for DR expression (Fig. 1). This resulted in three populations of melanoma cells (DR-enriched, unsorted, and DR-depleted) whose relative capacity as autologous stimulators was examined. As shown in Fig. 1, the stimulation of autologous T cells by the DR-enriched population of line 4 melanoma was ninefold that of the population depleted of HLA-DR. Unsorted melanoma cells, intermediate in DR expression, were intermediate in stimulation.

Discussion

These studies indicate that cell lines derived from biologically early and late melanomas differ in their capacity to provoke a proliferative response of autologous T cells. Failure of lymphocyte proliferation in patients with established metastatic disease does not appear to reflect an inhibitory effect of patient serum or a general defect in T cell responsiveness. An overall defect in autologous responsiveness is made unlikely by the demonstration of preserved proliferation to autologous non-T cells, the so-called "autologous mixed lymphocyte response" (23).

Stimulator (DR allotype)	Experiment No.	Pretreatment		% inhibition	Mean % inhibition
		Control serum	Anti-DR3 serum		
Line 4 (DR3)	1	635	94	85	
	2	1,440	288	80	87
	3	16,322	853	95	
Line 4-EBV	1	17,739	2,931	85	
	2	27,150	16,267	40	62
	3	69,709	27,553	60	
Allogeneic EBV (DR 7/2)	1	74,414	75,061	0	
	2	48,717	38,193	22	12
	3	16,920	14,784	13	

The response (counts per minute) of T cells from patient 4 to autologous melanoma cells (line 4), autologous (line 4-EBV), and allogeneic peripheral blood B cells transformed by Epstein-Barr virus was measured. Stimulating cells were first incubated (37°C, 30 min) with heated (56°C, 45 min) control serum having no anti-DR activity or with heated anti-DR3 serum. After extensive washing to lessen the likelihood of significant amounts of anti-DR antibody binding to T cells, melanoma and B cells were tested as stimulators as described in the legend to Table I.

Percent inhibition = $100 - 100$

$$\times \frac{\text{cpm in wells with stimulators preincubated with anti-DR serum}}{\text{cpm in wells with stimulators preincubated with control serum}} .$$
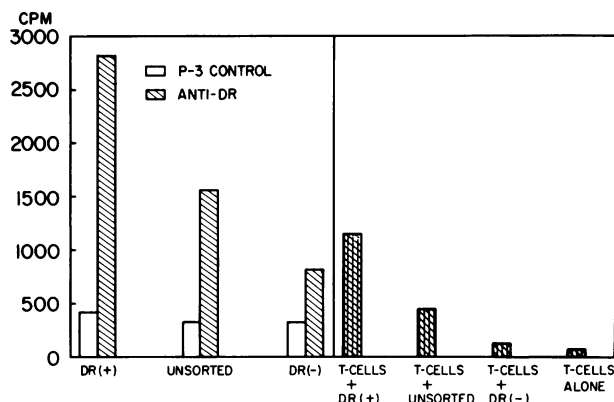
Figure 1. Quantitative relationship of HLA-DR expression and lymphocyte stimulation by line 4 melanoma cells sorted into DR-enriched and depleted populations. Melanoma cells were incubated with monoclonal anti-HLA-DR (WI691-13-17), washed, and incubated with fluorescein isothiocyanate-conjugated rabbit IgG anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Cell populations enriched and depleted of HLA-DR were segregated by a fluorescence-activated cell sorter (Beckton-Dickenson FACS IV Cell Sorter, Sunnyvale, CA) and placed back into culture to allow for elution of antibodies and for regrowth of sufficient cell numbers for analysis and use as stimulators. Relative HLA-DR expression by cells enriched in DR (DR[+]), unsorted, and depleted in DR (DR[−]) was then measured in radioimmunoassay (*Left*). In this assay, melanoma cells were incubated with P3 or the anti-DR monoclonal antibody WI691-13-17. Washed cells were then incubated with ^{125}I-sheep F(ab')$_2$ anti-mouse IgG (New England Nuclear), rewashed, harvested, and their gamma emission quantitated as counts per minute. Simultaneously, each melanoma cell population was assessed as in Table I for its capacity to stimulate autologous lymphocytes (*Right*, T cell stimulation: counts per minute in "autologous melanoma mixed lymphocyte reaction").

The failure of DR-negative melanoma cells to stimulate either autologous or allogeneic lymphocytes, the inhibition by anti-DR serum of stimulation by DR-positive primary melanoma, and the quantitative relationship between DR expression by melanoma cells and lymphocyte stimulation all suggest a role for tumor cell-associated HLA-DR antigens in cellular immunity to melanoma. Such antigens, as in the case of virus-transformed B lymphoblastoid cells (24), are apparently capable of directly interacting with host T cells and are necessary to provoke autologous lymphocyte proliferation. These data are the first to demonstrate a direct and functional role for HLA-DR in autologous lymphocyte reactivity to melanoma. That the expression of HLA-DR may have a general role in the interaction of tumor cells and host is suggested by studies reporting the expression of DR by a number of nonmelanocytic malignancies (11, 25).

The inability of DR-positive melanoma lines from advanced disease to stimulate autologous lymphocytes is of considerable interest. As we and others (8–10, 17) have not found significant differences in the kind or quantity (in quantitative radioim-munoassay) of HLA-DR expressed on or shed by DR-positive but nonstimulating lines (data not shown), we hypothesize that such lines have lost a "melanoma associated antigen" responsible for lymphocyte stimulation. Thus, a reasonable hypothesis is that an early, qualitative step in tumor progression in the melanocytic system is marked by cell surface display of HLA-DR antigens. When a "melanoma associated" epitope is additionally expressed and seen in the context of DR, a host T cell response ensues. Achievement by the tumor of the capacity to metastasize (a late, qualitative step in tumor evolution) may be realized in a variety of ways. One of these is to fail to express HLA-DR or the postulated "melanoma associated antigen", and so escape immune detection. That the former mechanism is operative is additionally suggested by Natali et al. (25) who found that the proportion of melanoma cells expressing HLA-DR was significantly decreased in advanced primary disease and metastases as compared with early primary melanoma.

Acknowledgments

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