[CANCER RESEARCH 63, 6501–6505, October 1, 2003]

B7-H1 Blockade Augments Adoptive T-Cell Immunotherapy for Squamous Cell Carcinoma[1]

Scott E. Strome,[2,3] Haidong Dong,[2] Hideto Tamura, Stephen G. Voss, Dallas B. Flies, Koji Tamada, Diva Salomao, John Cheville, Fumiya Hirano, Wei Lin, Jan L. Kasperbauer, Karla V. Ballman, and Lieping Chen

Departments of Otolaryngology [S. E. S., S. G. V., W. L., J. L. K.], Immunology [H. D., H. T., D. B. F., K. T., F. H., L. C.], Laboratory Medicine and Pathology [D. S., J. C.], and Biostatistics [K. V. B.], Mayo Clinic, Rochester, Minnesota 55905

ABSTRACT

In this report, we demonstrate that B7-H1, a B7 family molecule implicated in tumor immune evasion, is constitutively expressed on 66% of freshly isolated squamous cell carcinomas of the head and neck (SCCHN). To define the potential impact of tumor-associated B7-H1 on immunotherapy, the B7-H1-negative mouse SCC line, SCCVII, was transfected to express B7-H1. Although all of the animals succumbed to B7-H1/SCCVII tumors even after adoptive T-cell immunotherapy, the infusion of B7-H1 blocking monoclonal antibody with activated T cells cured 60% of animals. These data support B7-H1 blockade as a new approach to enhance the efficacy of T-cell immunotherapy.

INTRODUCTION

The means by which SCCHN[4] avoid detection and destruction by tumor-reactive T cells remain poorly defined (1, 2). Recently, we have demonstrated that tumor-associated B7-H1, a B7 family molecule found on stimulated monocytes/macrophages, DCs, and T cells, as well as some endothelial and epithelial cell lines, induces apoptotic death of activated tumor-reactive T cells (3, 4). The functional effects of B7-H1 ligation to counter receptors present on the T-cell surface depends on the cellular activation state at the time of exposure. Specifically, although B7-H1 ligation to naïve or early primed T cells costimulates proliferation and production of IFN-γ and IL-10, binding to activated T cells promotes apoptotic cell death (4) or cell cycle inhibition (5). Although some data suggest that PD-1 is a counter-receptor for B7-H1, which may mediate the inhibition of T-cell response (5), recent studies support the hypothesis that costimulation observed in T cells may be mediated by a receptor other than PD-1 (4, 6). On the basis of the ability of tumor-associated B7-H1 to mediate activated T-cell death, it is likely that manipulation of the B7-H1 pathway at defined time points during the development of the T-cell antitumor immune response can enhance the efficacy of T-cell-based immunotherapy. Specifically, the timing of such manipulation would ideally not block the immunostimulatory effects of B7-H1 ligation to naïve T cells but would prevent the apoptotic T-cell death observed after the binding of tumor-associated B7-H1 to tumor-reactive CTLs. In this study, we investigated the patterns of B7-H1 expression on SCCHN and defined a blocking strategy to manipulate the B7-H1 T-cell interaction to enhance the therapeutic efficacy of tumor-reactive CTLs. We show here that blockade of B7-H1 by neutralizing antibody augments the therapeutic antitumor effects of transferred T cells.

Received 4/15/03; revised 6/25/03; accepted 7/8/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grants DE00459 and CA97085, and by Mayo Foundation.

[2] S. E. S. and H. D. contributed equally to this article.

[3] To whom requests for reprints should be addressed, at Mayo Clinic, Department of Otolaryngology, 200 First Street SW, Rochester, MN 55905. Phone: (507) 284-3853; Fax: (507) 284-8855; E-mail: strome.scott@mayo.edu.

[4] The abbreviations used are: SCCHN, squamous cell carcinoma(s) of the head and neck; PD-1, programmed death 1; DC, dendritic cell; IL, interleukin; mAb, monoclonal antibody.

MATERIALS AND METHODS

Animals and Cell Lines. C3H/HeN mice were purchased from Charles River Laboratories. Mice were housed in a specific pathogen-free environment and treated in accordance with the guidelines established by the Animal Care and Use Committee of the Mayo Clinic. The SCCVII cell line was maintained in complete medium containing RPMI 1640 (Cellgro, Inc.), Ten % heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), penicillin and streptomycin (100 μg/ml), L-Glutamine (2 mM), and HEPES (10 mM). SCCVII cells were transfected with a pcDNA vector containing mouse B7-H1 cDNA (7). After transfection, cells were selected in complete medium containing 0.25 μg/ml G418 and were cloned by limiting dilution. Clones were analyzed by flow cytometry using antimouse B7-H1 mAb, 10B5 (4). All of the experiments in this study used the 1A4 clone, which expressed the highest levels of B7-H1. Mock-transfected cells served as controls for all of these experiments.

mAbs to Human and Mouse B7-H1. 10B5 is an antimouse mAb against mouse B7-H1 and was generated by immunizing an Armenian hamster with mouse B7-H1Ig fusion protein as described previously (4). The mAb was purified from the supernatant using a 5-ml HiTrap protein G affinity column (Amersham Biosciences, Uppsala, Sweden) and a BioLogic LP purification system (Bio-Rad, Hercules, CA). Purified 10B5 was dialyzed in LPS-free PBS using a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). Mouse antihuman B7-H1 mAbs (clones 5H1) were generated and were prepared as described previously (3).

Detection of B7-H1 Expression. After appropriate Institutional Review Board approval, fresh SCCHN samples were obtained from the Mayo Clinic Department of Pathology. Two of the samples were classified as basalosquamous variants. Frozen tissues were sectioned and stained by 5H1 (4) and isotype-matched control antibody (mIgG1). All of the samples were analyzed by two surgical pathologists (D. S. and J. C.) to determine the presence or absence of B7-H1 staining, the pattern of staining and staining intensity. Pathologists were initially blinded to each other's results and subsequently evaluated discrepancies simultaneously to reach a consensus. The variance for the presence or absence of B7-H1 is reported. The human SCC-012 tumor cell line was purchased from the American Type Culture Collection, and SCC-WMM and SCC-15 were generously provided by Dr. Suyu Shu (Cleveland Clinic Foundation, Cleveland, OH), and Bert O'Malley, Jr. (University of Maryland, Baltimore, MD), respectively. Cells were cultured in either medium alone or medium containing 1000 IU/ml IFN-γ, for 24–48 h. Cells were incubated with anti-B7-H1 mAbs (2 μg/sample) at 4°C. After 30 min, the cells were washed and were further incubated with FITC- or phycoerythrin-conjugated (Biosource, Camarillo, CA) goat antimouse IgG $F(ab')_2$ for 30 min at 4°C. Cells were analyzed on a FACScan flow cytometry (Becton Dickinson, Sunnyvale, CA).

Adoptive Therapy by Tumor-Reactive T-Cells. To generate T-cells against SCCVII, we first prepared DCs from bone marrow as described previously (8). Bone marrow-derived DCs (1×10^6) were cultured overnight with 3×10^6 irradiated SCCVII cells, were resuspended in 0.05 ml of HBSS), and were injected intradermally into the flank of C3H/HeN mice. Seven days after vaccination, draining lymph nodes were harvested and stimulated *in vitro* for 2 days with 5 μg/ml anti-CD3 followed by 3-day expansion in 10 IU/ml human IL-2 (9, 10). These activated T-cells were washed in HBSS, and 5×10^6 cells were injected i.p. in 0.5 ml of HBSS. Three days before T-cell inoculation, mice were given i.p. injections of 2.5×10^6 B7-H1/SCCVII cells in 0.5 ml of HBSS (11). After T-cell transfer, one group of animals was treated with 100 μg of 10B5, and the remaining animals were treated with control

hamster IgG. Animals were evaluated on a daily basis and sacrificed if they developed external tumors that exceeded an estimated 10% of body weight, when they could not access food or water, or were deemed in a premorbid condition.

Statistical Analysis. The Kaplan-Meier estimator was used to generate survival curves for the animals treated with CD8+ T cells and anti-B7-H1 mAbs and the animals treated with CD8+ T cells and control IgG for each of two experiments. A comparison of the survival experience between the two treatment groups was made with a log-rank test, separately for both experiments. The data were then combined, and Kaplan-Meier curves were generated for the two treatment groups (10 animals in each group), and these were compared with a log-rank test. Finally, the proportion of animals alive at 6 weeks posttreatment was compared between the two treatments for the combined data using the Fisher's exact test.

RESULTS

Expression of B7-H1 on Human Head and Neck SCCs. We examined expression patterns of B7-H1 on frozen sections of SCCHN by immunohistochemistry using 5H1 mAbs as described previously (4). As shown in Fig. 1, 66% of specimens (16 of 24 patients) demonstrated membrane and/or intracytoplasmic expression of the B7-H1 molecule (79% agreement between pathologists on initial review; Fig. 1). Eleven of the 24 specimens examined had an intracytoplasmic staining pattern, whereas 11 tumors had evidence of surface reactivity. Ten tumors had evidence of B7-H1 in both the membrane and the cytoplasm. The majority of expressing tumors (11 of 16) had either a 2+ or 3+ staining intensity, indicating that the

A

Primary site	Total	Reactivity		Pattern		Strength*		
		Negative	Positive	Cytopl	Membr	1+	2+	3+
Lip/Oral Cavity	7	2	5	3	5	1	2	2
Hypopharynx	1	0	1	1	0	1	0	0
Larynx	7	3	4	4	3	1	1	2
Lymph node	6	1	5	2	5	2	1	2
Skin	1	1	0	0	0	0	0	0
Paranasal sinus	2	1	1	1	1	0	1	0
Total #(%)	*24*	*8(33)*	*16(66)*	*11(46)*	*11(46)*	*5(21)*	*5(21)*	*6(25)*

B

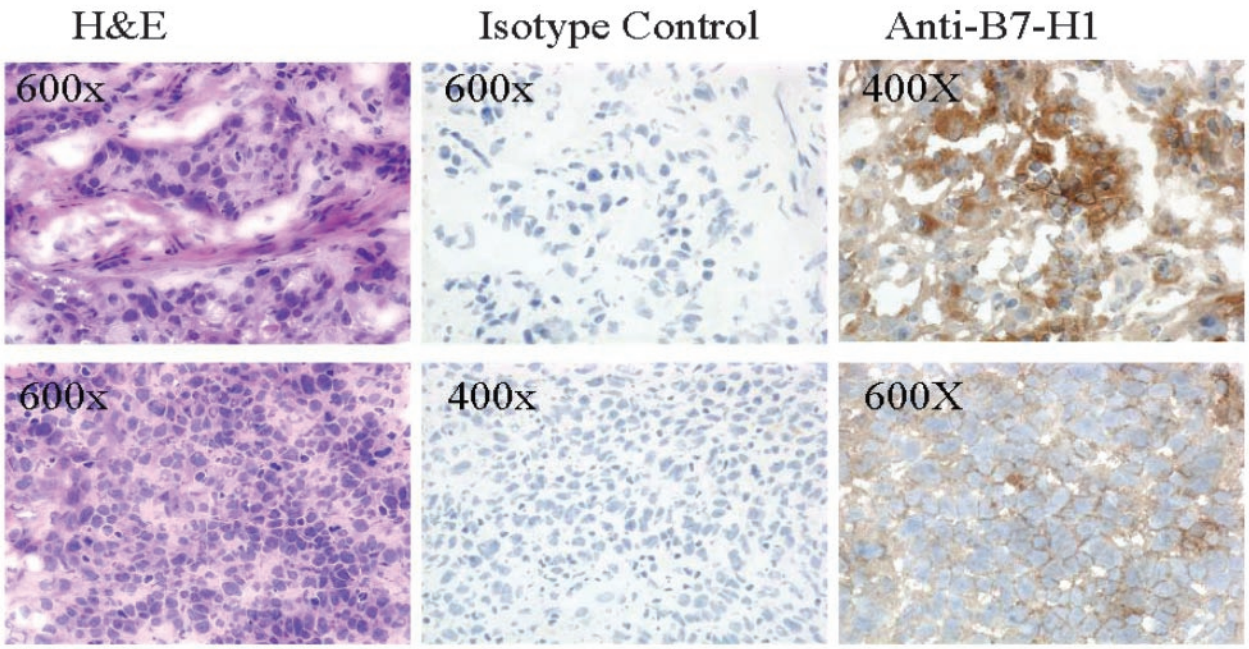

Fig. 1. B7-H1 expression on SCCHN. *A*, frozen human SCCHN were sectioned and stained by anti-human B7-H1 mAbs (5H1) or isotype control antibody (mIgG1) by standard immunohistochemistry. Staining intensity was determined semiquantitatively: −, negative; 1+, expression in 10–40% of the cancer cells; 2+, expression in 40–80% of the cancer cells, 3+, diffuse expression in >80% of the cancer cells. The numbers in the Table indicate the samples tested. *Cytopl*, cytoplasmic staining; *Membr*, membrane staining. *B, top panel*, staining of a moderately differentiated, keratinizing, invasive SCC of the larynx, by H&E, isotype control mAb, and anti-B7-H1 mAb. *Bottom panel*, staining pattern of a poorly differentiated, nonkeratinizing, metastatic SCC to a cervical lymph node. Magnification is also indicated in each panel.

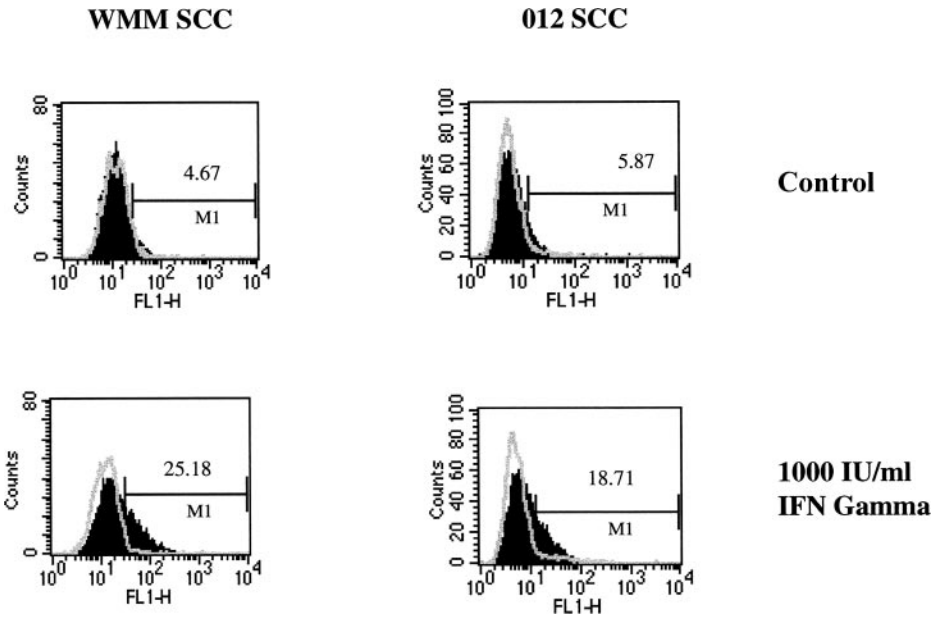

Fig. 2. Induction of B7-H1 on SCCHN cell lines by IFN-γ. Two SCCHN cell lines, WMM SCC and 012 SCC, were stimulated without (*Control*) or with the indicated doses of IFN-γ for 24–48 h. After culture, cells were stained with 5H1 mAb (filled lines), and expression was analyzed by flow cytometry. *The numbers above the bars*, the percentage positive staining of B7-H1.

majority of tumor cells stained positive. No expression was identified in specimens stained with isotype control antibodies. These data demonstrate that B7-H1 is present on the majority of SCCHN.

Three established SCCHN cell lines were stained with 5H1 mAbs by flow cytometry. B7-H1 expression was absent on the surface of these lines. However, B7-H1 expression was detected in two of three cell lines after exposure to IFN-γ (Fig. 2 and data not shown). These data demonstrate that cell surface expression of B7-H1 can be increased in the presence of IFN-γ and suggest an inducible nature of B7-H1 expression on SCCHN cells.

Expression of B7-H1 by Transfection of Mouse SCCVII Tumor Line. To establish a mouse model to define the role of tumor-associated B7-H1 on immunotherapy, we first examined the expression of B7-H1 on SCCVII, a mouse SCC line (11). As shown in Fig. 3, SCCVII does not express B7-H1, even after treatment by IFN-γ. We subsequently transfected a plasmid-encoding mouse B7-H1 into a SCCVII line using previously described methods (7). A SCCVII clone, 1A4, was selected with high levels of B7-H1 surface expression for further study.

Blockade of B7-H1 by mAbs Increases Efficacy of Adoptive T-Cell Therapy. We examined the effect of 10B5, a hamster mAb specifically against mouse B7-H1 (4) on adoptive T-cell therapy for SCCVII tumor (8–10). The1A4 cells were inoculated i.p. to establish progressively growing tumor. Activated T-cells were prepared from the mice that were preimmunized with SCCVII-pulsed DCs (8) and expanded *in vitro* by anti-CD3 and IL-2 (9, 10). As shown in Fig. 4*A*, all of the mice treated with such T cells and control IgG rapidly developed tumors and died within 40 days after 1A4 inoculation, indicating that T-cell therapy in this setting is not effective. In contrast, the majority of animals treated with T cells and 10B5 remained alive more than 80 days. The survival for animals treated with T cells and anti-B7-H1 mAb was superior to animals treated with T-cells and control IgG (log-rank $P = 0.003$; proportion alive at 6 weeks $P = 0.020$) for the combined data from two experiments. The data

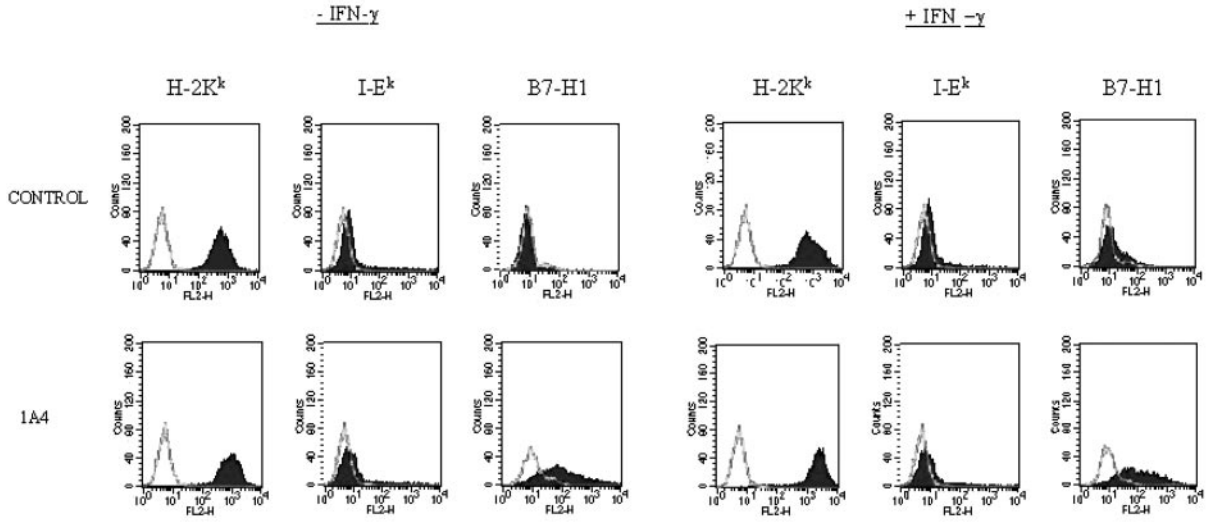

Fig. 3. Expression of B7-H1 on mouse SCCVII lines. Mouse SCCVII cell line was transfected with the vector containing full-length mouse B7-H1 or parental vector. Clones were selected in 0.25 μg/ml G418 and were cloned by limiting dilution. Mock transfectant (*CONTROL*) and a positive clone (*1A4*) were analyzed by flow cytometry using mAb against mouse H-2K^k (PharMingen), I-E^k (PharMingen), and B7-H1 (10B5). These lines were also treated either in the presence (+*IFN*-γ) or absence (−IFN-γ) of 1000 units/ml mouse (filled lines) IFN-γ for 24 h.

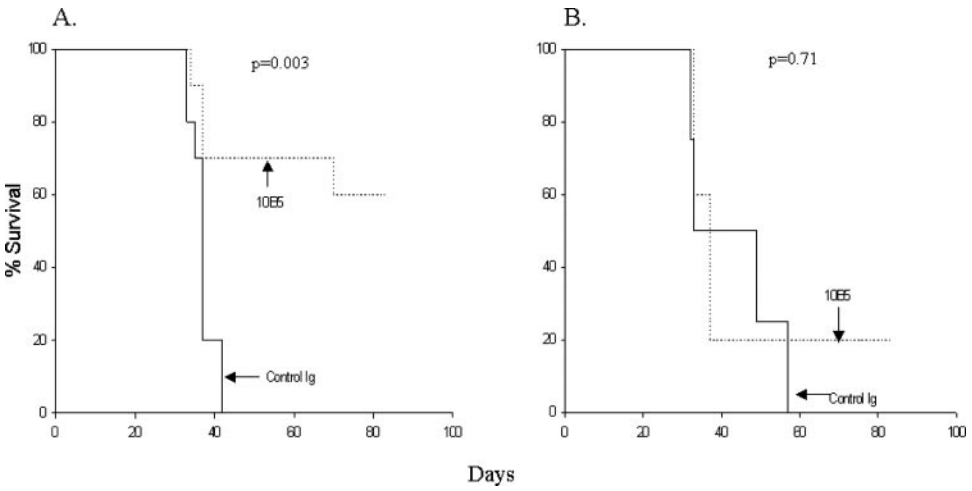

Fig. 4. Augmentation of T-cell adoptive immunotherapy by B7-H1 blockade. Five mice in each group were inoculated i.p. with 2.0×10^5 1A4, a B7-H1-transfected SCCVII line. Three days later, the mice were treated with (*A*) or without (*B*) activated T cells by i.p. infusion and were subsequently given injections with either control antibody (*Control Ig*) or anti-B7-H1 mAb (*10B5*) in the same day. The condition of the mice was monitored daily. The data shown in *A* are a summary of two identical experiments. The differences between the two groups were calculated by the log-rank test.

were combined because similar survival experience was seen in the two separate experiments. Without transfer of activated T cells, however, only 1 of 5 animals treated with anti-B7-H1 mAb survived more than 80 days, which was not statistically significantly different from the IgG control group (log-rank $P = 0.71$; proportion alive at 6 weeks, $P = 1.00$). These data support that B7-H1 blockade augments the antitumor immune response of adoptively transferred tumor-reactive T cells.

DISCUSSION

In this report, we demonstrate that 66% of freshly isolated SCCHN expressed B7-H1. Despite the absence of B7-H1 on SCCHN cell lines cultured in medium alone, up-regulation was observed in two of three samples after exposure to the proinflammatory cytokine IFN-γ. In a murine model of SCC, SCCVII, mAb blockade of tumor-associated B7-H1 enhanced the therapeutic efficacy of adoptively transferred cells. These observations likely have clinical relevance, because they suggest that the presence of B7-H1 on SCCHN contributes to tumor escape from immune system destruction.

Previously, we and others found that blockade of B7-H1 by neutralizing antibodies could protect CD8+ T-cells from apoptotic cell death *in vitro* and in a P815 tumor mouse model (4, 12). In the current investigation, we extend these findings and determined that blockade of the B7-H1-mediated counterattack by tumor cells enhanced the therapeutic efficacy of adoptively transferred tumor-reactive CTLs. Compared with unprotected tumor-specific T cells, which could not cure tumor-bearing animals, the blockade of B7-H1 by neutralizing mAbs resulted in enhanced overall survival. These results correlate well with data from Iwai *et al.* (12), which demonstrated that antibody blockade of B7-H1 inhibits the growth of B7-H1-positive P815 tumor *in vivo*. Our results, however, are different from this study in several important aspects. First, our results indicate that the expression of B7-H1 on SCCVII tumor did not enhance its growth in unimmunized syngeneic mice (data not shown). In addition, the blockade of B7-H1 without adoptive transfer of activated T cells did not have a significant effect on the progression of B7-H1-transfected SCCVII (Fig. 4*B*). Our results, thus, support that expression of B7-H1 *per se* does not promote tumor growth in the absence of tumor immunity. This result is consistent with our previous observation that SCCVII is a poorly immunogenic tumor. Iwai *et al.* (12), however, used a highly immunogenic mutant of P815 tumor, which regresses spontaneously in unimmunized syngeneic mice. Expression of B7-H1 on this mutant P815 appears to prevent the induction of immunity in their system rather than to confer resistance against immunotherapy. In this regard, we demonstrate that adoptive transfer of preactivated T cells could be less effective in the treatment of B7-H1-positive SCCVII tumors and that the blockade of B7-H1 augments the efficacy of this therapy. Our results thus support the role of B7-H1 in the evasion of the effector phase rather than the induction phase of T-cell immunity.

Although blockade of tumor-associated B7-H1 by antibodies is a simple and effective means to enhance the therapeutic efficacy of adoptive transfer strategies in animal models, several practical and conceptual matters needs to be addressed before serious consideration of B7-H1 blockade for clinical application. Specifically, additional studies are needed to identify the regulation of B7-H1 expression in tumor cells. Although the expression of B7-H1 is found in the majority of cancer patients, the expression of B7-H1 is often not present on all cells. Moreover, several SCCHN lines do not constitutively express B7-H1, but can be induced to express this molecule in the presence of IFN-γ. Taken together with our previous findings, our results support the conclusion that B7-H1 expression on cancers may be a highly regulated event. Understanding regulatory mechanisms of B7-H1 may thus facilitate design for better combination therapy with other therapeutic regimes; for example, cytokines that inhibit the expression of B7-H1 on tumor cells, to further enhance the efficacy of B7-H1 blockade. Additionally, it is important to note that because of the complexity of the B7-H1 pathway and the potential existence of additional receptors other than PD-1, we currently do not know whether the effect of enhanced tumor immunity by anti-B7-H1 is entirely attributable to a blockade of the interaction between B7-H1 and PD-1. Functional differences that are associated with B7-H1 binding to counter receptors will require the identification and physiological assessment of these molecules.

To the best of our knowledge, this is the first report that characterizes the expression patterns of B7-H1 on human SCCHN. More importantly, this study identifies a potential mechanism for B7-H1-mediated tumor evasion of the immune response and defines a strategy to manipulate the B7-H1 pathway to enhance the therapeutic effects of T-cell-based immunotherapy for SCCHN, with the potential for future clinical application.

REFERENCES

1. Byers, R. M. Dr. Martin: how are we doing in 2000? Arch. Otolaryngol-Head Neck Surg., *127:* 759–765, 2001.
2. Johansen, L. V., Grau, C., and Overgaard, J. Supraglottic carcinoma: patterns of failure and salvage treatment after curatively intended radiotherapy in 410 consecutive patients. Int. J. Radiat. Oncol. Biol. Phys., *53:* 948–958, 2002.

3. Dong, H., Zhu, G., Tamada, K., and Chen, L. B7-H1, a third member of the B7 family, costimulates T-cell proliferation and interleukin-10 secretion. Nat. Med., *5:* 1365–1369, 1999.
4. Dong, H., Strome, S. E., Salomao, D. R., Tamura, H., Hirano, F., Flies, D. B., Roche, P. C., Lu, J., Zhu, G., Tamada, K., Lennon, V. A., Celis, E., and Chen, L. Tumor-associated B7-H1 promotes T-cell apoptosis. A potential mechanism of immune evasion. Nat. Med., *8:* 793–800, 2002.
5. Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L. J., Malenkovich, N., Okazaki, T., Byrne, M. C., Horton, H. F., Fouser, L., Carter, L., Ling, V., Bowman, M. R., Carreno, B. M., Collins, M., Wood, C. R., and Honjo, T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp Med., *192:* 1027–1034, 2000.
6. Wang, S., Bajorath, J., Flies, D. B., Dong, H., Honjo, T., and Chen, L. Molelcular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. J. Exp. Med, *197:* 1083–1091, 2003.
7. Tamura, H., Dong, H., Zhu, G., Sica, G. L., Flies, D. B., Tamada, K., and Chen, L. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. Blood, *97:* 1809–1816, 2001.
8. Strome, S. E., Voss, S., Wilcox, R., Wakefield, T. L., Tamada, K., Flies, D., Chapoval, A., Lu, J., Kasperbauer, J. L., Padley, D., Vile, R., Gastineau, D., Wettstein, P., and Chen, L. Strategies for antigen loading of dendritic cells to enhance the antitumor immune response. Cancer Res., *62:* 1884–1889, 2002.
9. Strome, S. E., Krauss, J. C., Chang, A. E., and Shu, S. Strategies of lymphocyte activation for the adoptive immunotherapy of metastatic cancer: a review. J. Hematother., *2:* 63–73, 1993.
10. Strome, S. E., Martin, B., Flies, D., Tamada, K., Chapoval, A. I., Sargent, D. J., Shu, S., and Chen, L. Enhanced therapeutic potential of adoptive immunotherapy by *in vitro* CD28/4-1BB costimulation of tumor-reactive T cells against a poorly immunogenic, major histocompatibility complex class I-negative A9P melanoma. J. Immunother., *23:* 430–437, 2000.
11. Khurana, D., Martin, E., O'Malley, B., Chen, L., and Strome, S. Characterization of a spontaneously arising murine squamous cell carcinoma (SCC VII) as a prerequisite for head and neck cancer immunotherapy. Head Neck, *23:* 332–339, 2001.
12. Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., and Minato, N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc. Natl. Acad. Sci. USA, *99:* 12293–12297, 2002.

B7-H1 Blockade Augments Adoptive T-Cell Immunotherapy for Squamous Cell Carcinoma

Scott E. Strome, Haidong Dong, Hideto Tamura, et al.

Cancer Res 2003;63:6501-6505.