Effect of Human Leukocyte Antigen Class I Expression of Tumor Cells on Outcome of Intravesical Instillation of Bacillus Calmette-Guerin Immunotherapy for Bladder Cancer

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Abstract

Purpose: Various immune systems play important roles in the clinical efficacy of intravesical Bacillus Calmette-Guerin (BCG) instillation for bladder cancer. However, human leukocyte antigen (HLA) class I molecules on tumor cells and various immune system cells infiltrating to/around the tumor have not been evaluated, although many prognostic factors, including clinical, pathologic, and molecular ones, have been investigated. The aim of this study was to determine immunologic prognostic factors of BCG immunotherapy for bladder cancer.

Experimental Design: Immunohistochemical staining for HLA class I, CD4, CD8, CD20, CD68, TIA-1, S-100, and FOXP3 was carried out on specimens from 30 patients who underwent BCG immunotherapy from whom both pretreatment and posttreatment specimens were obtained. We did univariate and multivariate analyses of factors affecting recurrence-free survival. The positive, weakly positive, and negative groups of cells that infiltrated to/around the tumor were compared with recurrence-free survival using the Kaplan-Meier method and log-rank test.

Results: HLA class I was a significant prognostic factor both in univariate and multivariate analyses. The 5-year recurrence-free survivals of the patients with HLA class I – positive tumors and those with HLA class I – negative tumors were 55.7% and 19.1%, respectively ($P = 0.019$). There was a significant association between infiltration of CD8, CD20, and CD68-positive cells after BCG therapy and therapeutic effects.

Conclusions: Our data show that expression of HLA class I molecules on tumor cells contributes significantly to the therapeutic effect of BCG immunotherapy for bladder cancer. It is suggested that CTLs may be one of main effectors in this therapy.

Intravesical instillation of Bacillus Calmette-Guerin (BCG) is used for the treatment of superficial bladder cancer, both to reduce the recurrence rate of the cancer and to diminish the risk of progression (1). BCG therapy remains the most effective treatment for eradication and prophylaxis of recurrence of superficial bladder cancer, including carcinoma *in situ* and residual papillary tumors, after transurethral resection of bladder tumors (2, 3). However, 30% to 50% of cancers either fail to respond to or relapse from the therapy within the first 5 years of treatment. Although there have been many reports about factors predicting the response to intravesical BCG therapy for bladder cancer, no independent prognostic factor for the bladder cancer response to BCG has yet been identified (4).

It is generally assumed that the BCG-induced antitumor activity is critically dominated by a local nonspecific immunologic reaction reflecting the activity of immunocompetent cells (1, 5). Furthermore, current insights into the mode of action of BCG, ranging from its introduction into the bladder to killing of residual tumor cells, have revealed a complex sequence of processes (1). After adhering to the bladder epithelium and passage through the glycosaminoglycan layer, BCG is internalized and processed by professional antigen-presenting cells and cancer cells. The modified gene expression of these professional cells stimulates the secretion of particular cytokines and presentation of BCG antigens via human leukocyte antigen (HLA) class I and II to $CD8^+$ and $CD4^+$ T cells, respectively. Then, up-regulation of the Th2 response may occur and adversely affect the functioning of the Th1 response, introducing recruitment and maturation of cytotoxic effector cells (2). Thus, various immune systems play important roles in the efficacy of intravesical BCG therapy. Recently, we detected a novel monoclonal antigen, EMR 8-5, for HLA class I and reported that recurrence-free survival of patients with superficial bladder cancer expressing HLA class I was significantly better than that of patients with cancer not expressing HLA class I (6). HLA class I in tumor cells and various immune system

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Table 1. Primary antibodies and conditions

Antibody against	Main cellular expression/ target molecule	Monoclonal or polyclonal antibody	Source	Catalog no./ clone	Dilution
HLA class I	Heavy chains of HLA-A, HLA-B, and HLA-C	Monoclonal	Original	EMR 8-5	1:20
CD4	HLA class II – restricted T cells	Monoclonal	Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom	1F6	1:20
CD8	HLA class I – restricted T cells	Monoclonal	DakoCytomation, Glostrup, Denmark	M7103	1:50
CD20	B cells	Monoclonal	Abcam Ltd., Cambridge, United Kingdom	L26	1:20
CD68	Monocytes, macrophages, etc.	Monoclonal	DakoCytomation	M814	1:100
TIA-1	Active CTLs	Monoclonal	Abcam	ab2712	1:50
S-100	Dendritic cells	Monoclonal	DakoCytomation	Z311	1:400
FOXP3	Regulatory T cells	Monoclonal	Abcam	ab2481	1:40

cells infiltrating to/around the tumor has not been sufficiently evaluated for patients who undergo intravesical BCG immunotherapy, although many prognostic factors, including clinical, pathologic, and molecular ones, have been investigated. In this study, we attempted to determine immunologic prognostic factors of BCG immunotherapy for bladder cancer.

Materials and Methods

Patients and specimens. Surgical specimens were obtained from 30 consecutive patients with bladder cancer who were treated with intravesical BCG. Informed consent was obtained from each patient. They consisted of 16 newly diagnosed patients and 14 previously treated ones. First, those patients underwent transurethral resection of bladder tumors. All endoscopically visible tumors were resected. After 2 to 4 weeks, the patients received weekly intravesical BCG instillations six to eight times as a rule. The Tokyo 172 and Connaught strains were used for 25 and 5 patients, respectively. The BCG (80 mg of Tokyo 172 and 81 mg of Connaught) was instilled into the bladder with 40 mL of normal saline and retained for 2 hours. After completion of the instillations, we obtained surgical specimens from the site where the primary tumor was located to confirm that there was no neoplastic pathology of the mucosa. The median interval from the final BCG instillation to the operation or biopsy was 2.8 months. Additional or maintenance therapy was not done.

All H&E-stained slides were reviewed, and the diagnoses were confirmed using the 2002 version of the tumor-node-metastasis system. All of these specimens showed transitional cell carcinoma. The distribution of their stages and grades was as follows: pTa, $n = 3$; pT1, $n = 9$; pTis, $n = 18$; G1, $n = 1$; G2, $n = 10$; and G3, $n = 19$. The median follow-up period of the patients was 25.4 months.

Immunohistochemical staining. Sections (5 μm) of the formalin-fixed, paraffin-embedded tumors were immunostained after steam heat-induced epitope retrieval using monoclonal or polyclonal antibodies for HLA class I, CD4, CD8, CD20, CD68, TIA-1, S-100, and FOXP3 (Table 1). Subsequent incubations with a secondary biotinylated antibody, avidin-conjugated peroxidase complex, and chromogen were carried out on Ventana NexES (Ventana Medical Systems, Inc., Tucson, AZ). Slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into nonaqueous solution, and coverslipped with mounting medium. Human tonsil sections were used as positive controls for HLA class I, CD4, CD8, CD20, CD68, TIA-1, and FOXP3. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed independently using light microscopy by investigators who were blinded to clinicopathologic data (H.K. and T.T.).

Membrane immunoreactivity levels for HLA class I were categorized as undetectable to +3. A score of zero was defined as undetectable staining. A score of +1 was defined as faint, incomplete membrane staining in >20% of the tumor cells. A score of +2 was defined as moderate to complete staining in cytoplasm but negative membrane staining in the tumor cells. Finally, a score of +3 was defined as complete membrane staining in >80% of the tumor cells. HLA class I

Table 2. Cox regression analysis for recurrence-free survival

Factor	Univariate analysis		Multivariate analysis	
	Risk ratio label (95% confidence interval)	*P*	Risk ratio label (95% confidence interval)	*P*
HLA class I	0.27 (0.08-0.93)	0.0394	0.06 (0.01-0.40)	0.0030
CD4	0.44 (0.03-1.79)	0.3107	0.14 (0.00-2.04)	0.1695
CD8	0.65 (0.26-1.57)	0.3342	0.89 (0.12-6.59)	0.9066
CD20	0.51 (0.22-1.10)	0.0848	0.93 (0.21-3.49)	0.9144
CD68	0.56 (0.16-1.79)	0.3296	0.31 (0.03-2.86)	0.2950
TIA-1	0.18 (0.01-0.93)	0.0393	0.36 (0.02-2.80)	0.3593
S-100	1.15 (0.43-2.83)	0.7631	0.68 (0.06-6.11)	0.7332
FOXP3	0.39 (0.11-1.24)	0.1097	0.19 (0.02-1.41)	0.1061
Pathologic T stage	0.82 (0.36-2.08)	0.6538	0.33 (0.05-2.12)	0.2391
Grade	1.20 (0.41-3.99)	0.7523	2.62 (0.31-21.9)	0.3499

expression was then classified as negative (scores 0, 1, and 2) or positive (score 3). The analysis of other protein-positive cells was semiquantitative as follows: 0 to <10 positive cells/high power field (×400), − (minus); 10 to 50 positive cells/high power field, +; and >50 positive cells/high power field, ++.

Statistical analysis. Recurrence was defined as positive cytology or a tumor that was resected or fulgurated. The time to recurrence was measured from the date of the first BCG treatment to the date of the first positive cystoscopy. Kaplan-Meier survival curves were constructed to show the proportion of patients free of recurrence. The log-rank test was used to analyze prognostic factors related to recurrence. The Cox proportional hazards regression model was used for calculating the hazard ratio and 95% confidence interval, and independent predictors of recurrence were selected in the models. Furthermore, we compared the survival of patients whose posttherapeutic specimens showed increased immune system cells with that of the patients whose posttherapeutic infiltrating cells did not exhibit any increase in CD4, CD8, CD20, CD68, TIA-1, S-100, or FOXP3. If the score of infiltrating cells became higher or the score remained double positive, it was defined as an increase (e.g., the $CD8^{increased}$ group). $P < 0.05$ was considered to indicate statistical significance.

Results

Predictive factors for tumor recurrence. The 5-year recurrence-free survival was 47.1% for all patients. Univariate analysis revealed that HLA class I expression and infiltration of CTL were significant factors influencing recurrence-free survival of patients with superficial bladder cancer (Table 2). Multivariate analysis revealed that HLA class I expression on tumor cells was the only significant and independent factor that affected the recurrence after BCG immunotherapy (Table 2). Patients with HLA class I–positive bladder cancer had longer recurrence-free survival than those with negative expression in the 5-year follow-up (55.7% and 19.1%, respectively; Fig. 1).

Immune system cells infiltrating to/around the tumor after BCG instillations. Statistical analysis (Fig. 2) showed a significant, positive correlation of the occurrence of CD8, CD20, and CD68 cells infiltrating to/around the tumor with survival ($P = 0.0007$, 0.0001, 0.0161, and 0.0039, respectively, log-rank test). Only 3 (15.8%) of the 19 patients in the $CD8^{increased}$ group had recurrence, whereas 9 (81.8%) of the 11 patients in the $CD8^{not\ increased}$ group did. The survival of the $CD8^{increased}$ group was significantly better than that of the $CD8^{not\ increased}$ group ($P = 0.0012$, log-rank test; Fig. 3). Their 5-year recurrence-free survivals were 74.4% and 11.4%, respectively.

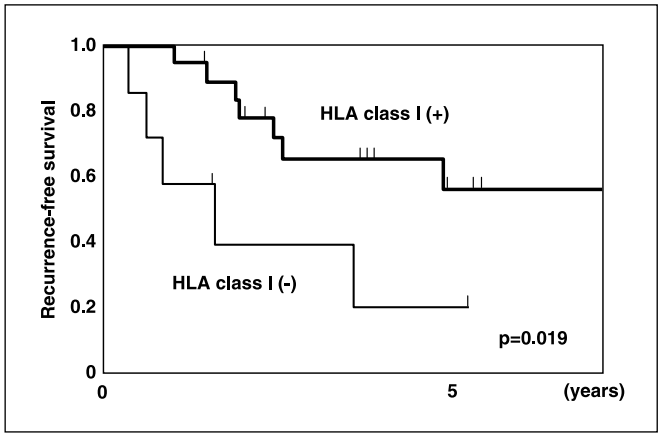

Fig. 1. Recurrence-free survivals for HLA class I–positive ($n = 23$) and HLA class I–negative ($n = 7$) patients who underwent BCG immunotherapy. HLA class I down-regulation is associated with inferior recurrence-free survival. P was determined by log-rank test.

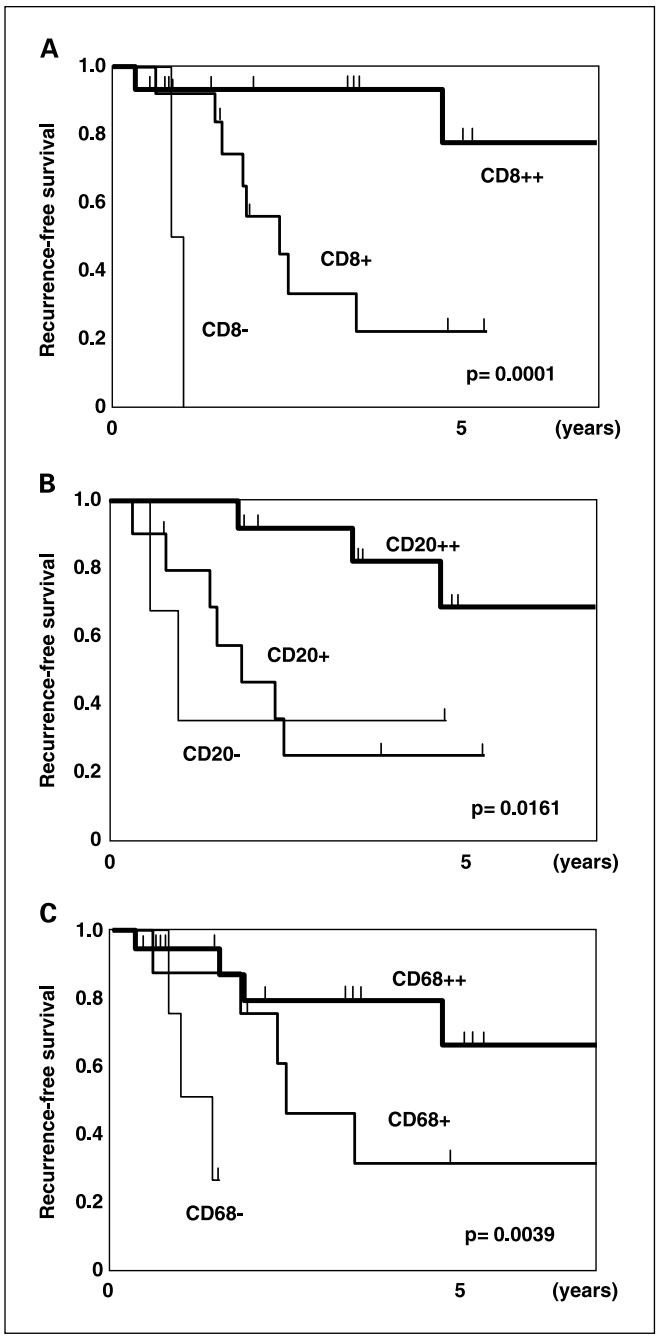

Fig. 2. Association of peritumoral infiltrating $CD8^+$ (*A*), $CD20^+$ (*B*), and $CD68^+$ (*C*) cells with patients' recurrence-free survival. The numbers of patients were as follows: CD8, ++ (15), + (13), and − (2); CD20, ++ (17), + (13), and − (3); and CD68, ++ (18), + (8), and − (4).

Discussion

BCG is considered to be one of the most effective treatments for superficial and *in situ* bladder cancer (1, 2, 7). However, the exact mechanism of its antitumor effect has been not completely clarified. Thus far, the main effector in BCG

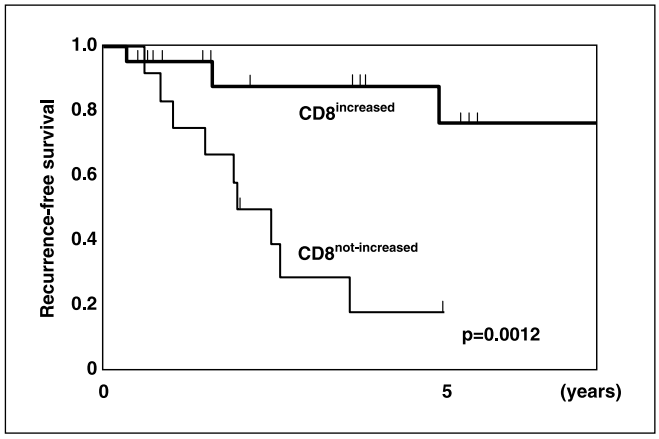

Fig. 3. Recurrence-free survivals for $CD8^{increased}$ (n = 19) and $CD8^{not\ increased}$ (n = 11) patients who underwent BCG immunotherapy. Increased or remaining double-positive $CD8^+$ cell infiltration is associated with favorable survival.

immunotherapy has been thought to be $CD4^+$ T lymphocytes (8, 9). Bevers et al. (1) suggested that BCG is internalized and processed by antigen-presenting cells and tumor cells, and BCG antigens are presented to $CD4^+$ cells, resulting in altered gene expression of these cells that leads to the presentation of BCG antigens and secretion of particular cytokines. We showed that HLA class I expression in cancer cells was the most significant prognostic factor for bladder cancer patients treated with BCG. The patients whose tissue after BCG therapy showed sufficient infiltration of $CD8^+$ T lymphocytes had better recurrence-free survival. This suggested that CTLs were one of main effectors in intravesical BCG immunotherapy. However, we also showed that patients whose posttherapeutic specimens showed much infiltration of $CD20^+$ or $CD68^+$ cells had better recurrence-free survival. Thus, it was suggested that there are various immunologic mechanisms, including a HLA class II–mediated pathway.

Down-regulation of HLA class I in cancer cells is disadvantageous for presentation of a cancer antigen and its peptide to the patient's immune system (10). This is found frequently in breast and prostate carcinomas but less in melanoma and head and neck, lung, kidney, colorectal, and cervical carcinomas (11). Unfortunately, however, there has been no large study of the relationship between HLA class I down-regulation and outcomes of immunotherapy in various cancers because there have been few excellent antibodies for HLA class I that can react with formalin-fixed, paraffin-embedded tissue sections. We developed a monoclonal antibody, EMR 8-5, for heavy chains of HLA-A, HLA-B, and HLA-C, which can react with such sections (12). We previously reported that HLA class I was down-regulated in 33% of bladder cancers (6). The rate is relatively low compared with other malignant tumors. The results of this study indicated that the antigen presentation on HLA class I played an important role in the efficacy of intravesical BCG immunotherapy. Therefore, we suggest that tumor cells with down-regulated HLA class I escape from T-cell recognition and that BCG immunotherapy is not effective for patients with such bladder cancer.

Saint et al. (13) assessed peritumoral infiltrating cells before and after BCG therapy using semiquantitative immunohistochemical analysis and concluded that intravesical BCG instillation recruits specific (CD8, HLA class I) and nonspecific (CD4, antigen-presenting cells, HLA class II) cellular effectors. Mehmut et al. (14) investigated immunofluorescence staining of tumor tissue specimens and reported increases of both $CD4^+$ and $CD8^+$ cells by BCG instillation. Unfortunately, however, the relationship between the immunohistochemical results and recurrence-free survival of the patients was not evaluated in those studies. To the best of our knowledge, this is the first study to report the effect of HLA class I expression in cancer cells on recurrence-free survival of bladder cancer patients.

In conclusion, our results show that down-regulation of HLA class I in cancer cells is a significant risk factor for recurrence in patients with intravesical BCG immunotherapy for bladder cancer. Furthermore, peritumoral infiltration of $CD8^+$ T cells enabled us to identify a patient subgroup ($CD8^{increased}$) that was characterized by a favorable outcome with recurrence-free survival, although a limitation of this study is that it included only a few patients. As the next step, we will evaluate mechanisms of HLA class I down-regulation in bladder cancer and investigate how to up-regulate HLA class I in cancer cells with its down-regulation, which can contribute to improving the prognosis of patients who undergo intravesical BCG immunotherapy for bladder cancer.

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