Expression of Costimulatory Molecules on Human Retinoblastoma Cells Y-79: Functional Expression of CD40 and B7H1

Yoshihiko Usui, Youko Okunuki, Takaaki Hattori, Masaru Takeuchi, Takeshi Kezuka, Hiroshi Goto, and Masahiko Usui

Purpose. To examine the expression of various costimulatory molecules on the human retinoblastoma cell line Y-79 and assess the functional roles of selected costimulatory molecules.

Methods. Y-79 cells were incubated in the presence or absence of IFN-γ, with or without irradiation (100 Gy). Expression of major histocompatibility complex (MHC) class I molecules, MHC class II, CD80, CD86, CD40, CD70, B7H1, B7DC, B7H2, OX40L, and 4-1BBL on Y-79 cells was measured by reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometric analysis. The functional role of CD40-mediated interactions in modifying immune responses to Y-79 was assessed in vitro by using recombinant human CD40 ligand (rhCD40L). The costimulatory effect of B7H1-expressing IFN-γ–treated Y-79 cells on proliferation of purified T cells was studied in Y-79/T-cell coculture experiments with a blocking anti-B7H1 monoclonal antibody (mAb).

Results. CD40 and B7H2 were consistently detected on Y-79 cells by RT-PCR and flow cytometry. Cell surface expression of CD40 was upregulated on stimulation by IFN-γ alone, radiation alone, and IFN-γ combined with radiation. B7H1 expression was induced by IFN-γ stimulation and increased further when irradiated Y-79 cells were stimulated by IFN-γ. Treatment of Y-79 cells with rhCD40L enhanced cell surface expression of MHC class I and intercellular adhesion molecule (ICAM)-1 and also stimulated monocyte chemotactic protein (MCP)-1 production. Proliferative response of purified $CD3^+$ T cells costimulated with IFN-γ–stimulated Y-79 was significantly enhanced by the addition of anti-B7H1 mAb.

Conclusions. These results suggest that CD40 expressed on Y-79 plays an important role in augmenting antitumor immunity. In contrast, the expression of B7H1 on IFN-γ–treated Y-79 cells contributes to the suppression of T cells. The dual effects of CD40 and B7H1 on Y-79 cells may contribute to positive or negative regulation of antitumor immune responses in human retinoblastoma. (*Invest Ophthalmol Vis Sci.* 2006;47:4607–4613) DOI:10.1167/iovs.06-0181

Retinoblastoma is the most frequently occurring malignant eye tumor in childhood.[1] Current treatments include enucleation, external beam radiotherapy, cryotherapy, photocoagulation, and chemotherapy.[1] Although these methods achieve over 95% survival, there remains a need for better treatment alternatives to improve visual results and to avoid enucleation in hereditary retinoblastoma.[2–4] Therefore, the studies of new therapeutic strategies including immunotherapy are needed. One attractive concept already applied to other tumors is to modulate the immune system in a way that enables it to recognize and kill tumor cells.[5] Mononuclear cell infiltration is observed in primary retinoblastoma and has been suggested to play an important role in host defense.[6] Barez et al.[7] have shown that major histocompatibility (MHC) class I plays a critical role in antigen presentation, and upregulation of this molecule by IFN-γ may increase the susceptibility of tumor cells to cytotoxic T cells. Moreover, several studies indicate the possibility that the expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and nerve-cell adhesion molecule (NCAM) on human Y-79 cells plays a role in the pathogenesis of human retinoblastoma and that these molecules are also involved in host susceptibility.[6,8,9]

Effective protective immunity against tumor depends on the concordant activity of cytotoxic T cells,[10] and costimulation of T cells on a tumor has been shown to be essential for eliciting cell-mediated antitumor immunity.[11] Optimal T-cell recognition is based primarily on the presentation of an antigen compatible with MHC class I molecules, whereas secondary costimulatory molecules such as the B7 family and the tumor necrosis factor (TNF) receptor superfamily are necessary to initiate maximum stimulation.[11] In recent years, several studies have indicated that tumor cells are able to deliver antigen-specific signals to T lymphocytes[12] and that T-cell activation is the result of a balance between positive and negative signals. CD28, ICOS, 4-1BB, and CD40 are major positive costimulatory receptors, and interaction with the respective ligands on tumor cells is essential for T-cell activation[13–17] In contrast, CTLA-4 and B7H1 expressed on tumor cells inhibit T-cell activation.[18,19] However, the costimulatory molecule expression patterns differ among tumor cells. Tumor-reactive cytotoxic T lymphocytes isolated from the peripheral blood of cancer patients exhibit limited anti-tumor response.[20] In contrast, persistence and progressive growth of tumors indicate that the immune system is not generally competent to control the malignancy. Most tumor cells express little or no costimulatory molecules,[21] but enforced expression of some molecules (e.g., CD80, B7H2, OX40L, and 4-1BBL) by gene transfer has resulted in enhanced antitumor responses and tumor rejection in different animal models.[22–24] The results of these studies have provided the groundwork for various clinical trials of gene therapy in cancer patients,[25,26] and most clinical trials use irradiated tumor cells as vaccine for safety reasons. Although irradiation is known to increase the inherent immunogenicity of some tumor cells,[27,28] the effects of irradiation on the expression of MHC molecules and costimulatory molecules in human retinoblastoma cells have not been reported. Therefore, we sought to determine the in vitro expression patterns of MHC and costimulatory molecules in the human retinoblas-

From the Department of Ophthalmology, Tokyo Medical University, Tokyo, Japan.

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Corresponding author: Masahiko Usui, Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo 160-0023, Japan, usuyoshi@aol.com.

toma cell line Y-79 under various conditions and clarified their functional roles in the immune system.

Materials and Methods

Cells and Culture Conditions

A human retinoblastoma cell line, Y-79, originally characterized by Reid et al.,[29] was obtained from ATCC (HTB-18; Rockville, MD). The cell line was maintained as a suspension culture at a concentration of 2.5×10^5/mL in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM glutamine, 0.1 mg/mL penicillin, and streptomycin, in 5% CO_2/95% air at 37°C. The cells were incubated with medium alone or in the presence of IFN-γ (500 U/mL), and/or irradiated with an X-irradiator (Gammacell 40 Exactor; MDS Nordion International, Inc., Ottawa, Ontario, Canada).

Monoclonal Antibodies and Reagents

FITC-conjugated anti-HLA-DR, -DP, and -DQ (TÜ39, IgG2a) mAbs; PE-conjugated anti-human HLA-ABC (G46-2.6, IgG1), HLA-DR (G46-6, IgG2a), HLA-DQ (TÜ169, IgG2a), CD80 (L307.4, IgG1), CD86 (IT2.2, IgG2b), 4-1BBL (C65-485, IgG1), B7-H2 (2D3/B7-H2, IgG2b), ICAM-1 (HA58, IgG1), NCAM (NCAM16.2, IgG2b), LFA-2 (RPA-2,10, IgG1), CD95 (DX2, IgG1), CD95L (NOK-1, IgG1), CD14 (M5E2, IgG2a), CD16 (3G8, IgG1), and CD20 (2H7, IgG2b) mAbs; mouse IgG isotype control; and recombinant human IFN-γ were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-CD40 (5C3, IgG1); PE-conjugated and purified blocking anti-human B7H1 (MIH1, IgG1), PE-conjugated anti-human PD-1 (J116, IgG1), and B7-DC (MIH18, IgG1) mAbs; and agonistic anti-CD3 mAb (OKT3, IgG2a) were purchased from eBiosciences (San Diego, CA). PE-conjugated anti-OX40L (ANC10G1, IgG1) and recombinant human CD40L were purchased from Alexis Biochemicals (Carlsbad, CA).

Flow Cytometric Analysis

For the flow cytometry analysis of costimulatory expression on the human retinoblastoma cell line, cells were washed once in PBS and 10^6 cells were incubated with FITC- or PE-labeled mAbs. For OX40L staining, cells were incubated with the anti-OX40L monoclonal mAb, washed, and then incubated with PE-conjugated goat anti-mouse mAb. After washing with PBS twice, the stained cells (live-gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ). Data were processed using the accompanying software (CellQuest; BD Biosciences), and expressed as mean fluorescence intensity (MFI).

RNA Extraction, cDNA Synthesis, and RT-PCR

Total RNA was extracted using the an RNA isolation system (Isogen; Wako, Osaka, Japan) from untreated, IFN-γ–treated, irradiated, or IFN-γ–treated and irradiated Y-79 cells, according to the manufacturer's protocol. Complementary DNA was synthesized at 42°C for 2 hours in the presence of reverse transcriptase and random primers (SuperScript II RNaseH$^-$; Invitrogen Corp. Carlsbad, CA) in 20-μL reaction volumes. RT-PCR was conducted in 50 μL of reaction mixture using 0.05 μg (1 μL) of cDNA as template. The PCR mixture consisted of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM of each dNTP, and 2.5 units of *Taq* DNA polymerase (TaKaRa, Shiga, Japan). Sequences of sense and antisense primers and expected product sizes were as follows: for HLA-DRβ (473 bp): 5′-CTCCAGCATGGTGTGTCTGA, 3′-GGAGGTTGTGGTGCTGCAGG; for CD80 (773 bp): 5′-GGTCTTTCTCACTTCTGTTC, 3′-CTTTCCCTTCTCAATCTCTC; for CD86 (278 bp): 5′-ACAGCAGAAGCAGCCAAAAT, 3′-CTTGTCCGGCCCATATACTT; for B7-H1 (446 bp): 5′-ACGCATTTACTGTCACGGTTCC, 3′-GACTTCGGCCTTGGGGTAGC; for B7-DC (492 bp): 5′-AAAGAGCCACTTTGCTGGAG, 3′-TGAAAGCAATGATGCAGGAG; for B7-H2 (566 bp): 5′-CCGAGCCCTGATGTCACC, 3′-CCGCCACGACCACAAGCA; for CD40 (612 bp): 5′-CTGGGGCTGCTTGCTGAC, 3′-TCCTGGGGTTCCTGCTTG; for OX40L (177 bp): 5′-TCAACATTAGCCTTCATTACC, 3′-GAATCAGTTCTCCGCCATTCA; for 4-1BBL (194 bp): 5′-ACAAAGAGGACACGAAGGAG, 3′-GGAGGAGGCGGGTGGCAGGT; and for β-actin (540 bp): 5′-GTGGGGCGCCCCAGGCACCA, 3′-CTCCTTAATGTCACGCACGATTTC. The thermal cycle consisted of denaturation at 94°C for 1 minute; annealing at 51°C (OX40L), 52°C (CD80, CD86), 53°C (B7-DC, CD40), 54°C (B7-H1), 55°C (HLA-DRβ), 58°C (B7-H2, β-actin), or 61°C (4-1BBL) for 1 minute; and extension at 72°C for 1 minute. Each cycle of amplification was repeated 35 times in a PCR thermal cycler (model 480; TaKaRa). Five μL of each PCR product was electrophoresed on 1.3% or 2% agarose gel containing ethidium bromide in Tris-borate EDTA buffer and photographed.

Y-79 Stimulation via CD40

Human retinoblastoma cell line Y-79 was cultured for 48 hours in medium, with or without 500 U/mL IFN-γ unless stated otherwise, and then washed twice with PBS. They were then stimulated for 24 hours with rhCD40L or isotyped control Ig or medium alone.

Chemokine Production

Cell culture supernatants were assessed for the human chemokines monocyte chemotactic protein (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES), and IL-8 by using cytometric bead array (CBA) kits and CBA software supplied by BD PharMingen. Briefly, particles (polystyrene beads; Bangs Laboratories, Indianapolis, IN) were dyed in five different fluorescence intensities. The proprietary dye has an emission wavelength of approximately 650 nm (FL-3). Each type of particles is coupled to an antibody (BD PharMingen) against MCP-1, MIP-1α, MIP-1β, RANTES, or IL-8 via covalent linkage based on thiol-maleimide chemistry and represents a discrete population unique in FL-3 intensity. The Ab-particles serve as capture for a given cytokine in the immunoassay panel and can be detected simultaneously in a mixture. The captured cytokines are detected by a direct immunoassay using five different antibodies coupled to phycoerythrin (PE), which emits at approximately 585 nm (FL-2). Two-color flow cytometric analysis was performed.

T-Cell Proliferation Assay

Heparinized blood samples were obtained from healthy individuals, who provided informed consent in accordance with the Declaration of Helsinki, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. $CD3^+$ T cells were purified with microbeads on auto-MACS columns with a pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purified $CD3^+$ T-cell populations contained >97% $CD3^+$ T cells and <1% $CD14^+$, $CD16^+$, and $CD20^+$ cells, as determined by flow cytometry. Purified $CD3^+$ T cells (3×10^5/well) were cocultured with the irradiated (30 Gy) and IFN-γ-stimulated (500 U/mL for 48 hours) or non-IFN-γ–stimulated Y-79 cells in the presence or absence of agonistic anti-CD3 mAb. For mAb blocking assay, purified mAb against B7H1 or control mouse IgG was added at a final concentration of 10 μg/mL at the start of the assay. The cultures were incubated for 96 hours at 37°C in 5% CO_2 in air, pulsed with [^{3}H] thymidine (1.0 μCi/10 μL/well) during the last 18 hours of incubation, and then were harvested onto glass filters with an automated cell harvester. Radioactivity was assessed by liquid scintillation spectrometry (Tomtec, Orange, CT), and the amount was expressed as counts per minute.

Statistical Analysis and Reproducibility

Experiments were repeated at least twice and usually three times. Statistical analyses were performed by independent *t*-test. $P < 0.05$ was considered significant. Significance is denoted by an asterisk in the figures.

RESULTS

Expression of Costimulatory Molecules, with or without IFN-γ, with Irradiation, or with IFN-γ after Irradiation

The expression of costimulatory molecule mRNA by the human retinoblastoma cell line Y-79 was examined by RT-PCR under various conditions. To find the dose of recombinant IFN-γ used in the assay, Y-79 cells were cultured in the presence of titrated amounts of IFN-γ and MHC class I expression was analyzed by flow cytometry. More than 500 U/mL of IFN-γ did not induce an obvious expression of MHC class I, consistent with a previous report.[7] IFN-γ at 500 U/mL was therefore used in subsequent experiments for assessing the expression of costimulatory molecules in Y-79 cells.

CD40 and B7H2 mRNA were found to be expressed constitutively by Y-79 (Fig. 1). CD40 mRNA expression increased on stimulation by IFN-γ (500 U/mL) alone, irradiation (100 Gy) alone, or IFN-γ (500 U/mL) after irradiation (100 Gy). MHC class II, DR, B7H1, and OX40L mRNA expression was induced after IFN-γ stimulation. Y-79 stimulated with IFN-γ or irradiation alone expressed very low or undetectable levels of CD80, and 4-1BBL. CD86 and B7DC expression was undetectable under all conditions. These results indicated that MHC class II, CD40, OX40L, B7H1, and B7H2 mRNA were either expressed constitutively or could be induced in the human retinoblastoma cell line Y-79.

The surface expression of various costimulatory molecules on the Y-79 cell line was analyzed by flow cytometry (Fig. 2). The expression of MHC class I (A, B, C) molecules increased after IFN-γ stimulation, which agreed with previous data on the Y-79 cell line.[7] Consistent with the mRNA expression results, the CD40 molecule was expressed constitutively on the surface of Y-79 cells, and was enhanced after stimulation with IFN-γ alone, irradiation alone, or IFN-γ after irradiation. In contrast, B7H2 expression did not increase after IFN-γ stimulation. Moreover, only B7H1 expression was enhanced significantly after IFN-γ stimulation, and this result was consistent with the mRNA expression data. Y-79 did not express surface molecules of MHC class II (DR, DP, DQ), CD80, CD86, CD70, 4-1BBL, and B7DC under any condition.

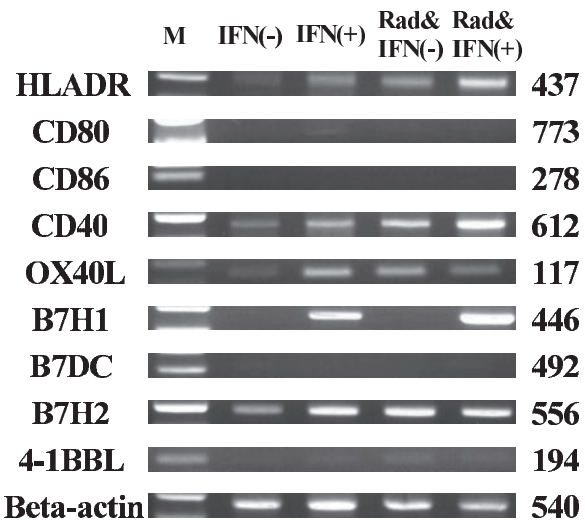

FIGURE 1. MHC class II (DR) molecules and costimulatory molecule mRNA expression in the human retinoblastoma cell line Y-79, as assessed by RT-PCR. RNA was isolated, reverse transcribed, and used for PCR analysis. This figure represents the analysis of reverse transcription-PCR–amplified transcripts of various costimulatory molecules and β-actin from nonstimulated, IFN-γ (500 U/mL)-stimulated, irradiated (100 Gy), or irradiated (100 Gy) and IFN-γ (500 U/mL)-stimulated Y-79 cells. The *first lane* on the *left* shows the amplification product of the β-actin housekeeping gene as control. On the *right*, the expected molecular weights of the amplified bands are shown.

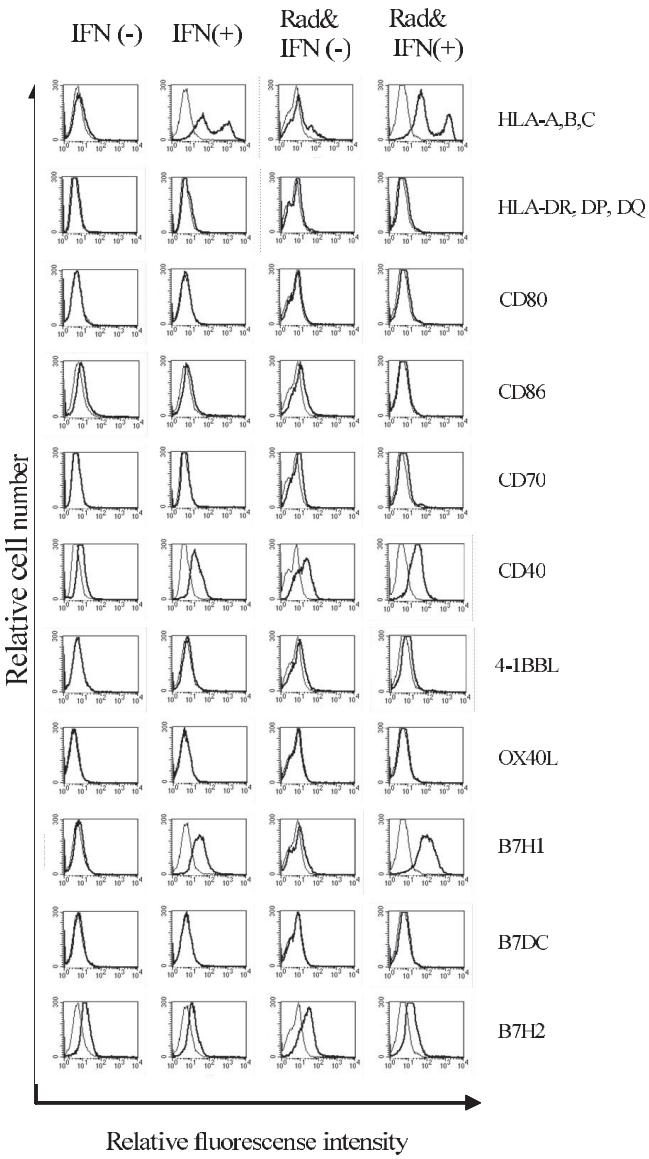

FIGURE 2. MHC molecule and costimulatory molecule expression in the human retinoblastoma cell line Y-79, as assessed by flow cytometry. Y-79 cells were grown to subconfluence and not stimulated or stimulated with IFN-γ (500 U/mL) for 48 hours, irradiated (100 Gy), or irradiated (100 Gy) and then stimulated with IFN-γ (500 U/mL) for 48 hours. The cells were then harvested for immunofluorescence staining and flow cytometry. The experiment was repeated five times, and a representative set of data is shown. *Black traces*: staining with the indicated mAb; *gray traces*: staining with an isotype-matched antibody as the negative control.

Effect of CD40 Ligation on MHC Class I and ICAM-1 Surface Molecules

Interaction between CD40 and CD40L activates antigen-presenting cells (APCs), leading to the upregulation of molecules involved in T-cell adhesion, antigen presentation, and activation. Examples of such molecules are ICAM-1, MHC class I and II molecules, and costimulatory molecules of the B7 family.[30–32] In the analysis of cell surface antigen expression by

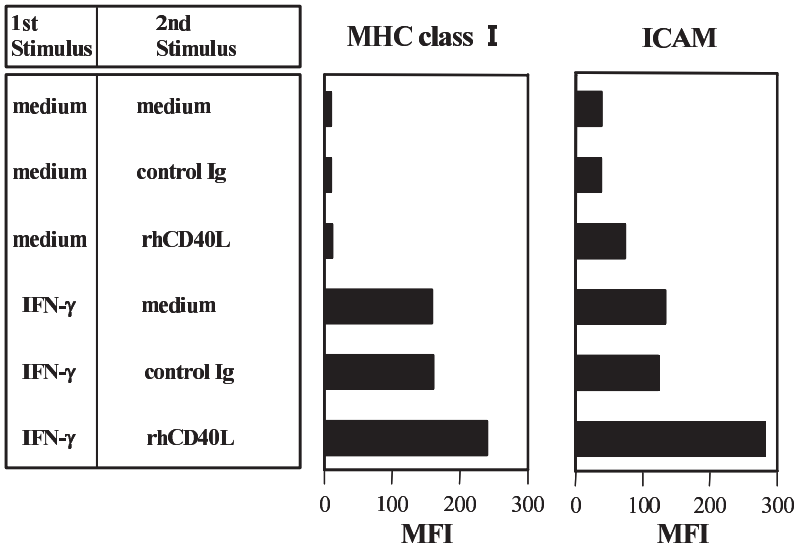

FIGURE 3. CD40 triggering upregulated expression of MHC class I and ICAM-1 on the human retinoblastoma cell line Y-79. Y-79 cells, with or without IFN-γ priming (48 hours, 500 U/mL), were stimulated with rhCD40L, control Ig (1 μg/mL), or medium alone for 24 hours. Y-79 cells were then analyzed by flow cytometry for MHC class I and ICAM-1 surface expression. The data are mean fluorescence intensities of surface expression corrected for background staining of an appropriate control mAb. The experiment was repeated three times, and a representative set of data is shown.

flow cytometry (described earlier), CD40 was found to be constitutively expressed on the cell surface of Y-79. To determine whether the CD40 expressed on Y-79 cells is functionally active, we investigated the effects of CD40 ligation on the expression of ICAM-1, NCAM (CD56), LFA-2 (CD2), MHC classes I and II, CD80, CD86, CD95, and CD95L on the surface of Y-79 cells. The cells were either untreated or primed with IFN-γ (500 U/mL, 48 hours) and then incubated for 24 hours with rhCD40L, isotype control Ig, or medium alone. Y-79 constitutively expressed a low level of surface ICAM-1 molecules, as reported previously.[8,9] Y-79 cells stimulated with rhCD40L without IFN-γ priming showed only weak upregulation of MHC class I molecules (Fig. 3). In contrast, after IFN-γ priming, stimulation of CD40 by rhCD40L induced significant upregulation of MHC class I molecules compared with Y-79 primed with IFN-γ alone or incubated with isotyped control Ig. Likewise, Y-79 stimulated with rhCD40L with or without IFN-γ priming, showed twofold upregulation of ICAM-1 molecules compared with Y-79 primed with or without IFN-γ alone, respectively, or incubated with isotyped control Ig. CD40 stimulation by rhCD40L had no effect on the surface expression of NCAM, LFA-2, MHC class II molecules, CD80, CD86, CD95, and CD95L (data not shown).

CD40 Mediation of In Vitro MCP-1 Production in IFN-γ–Treated Cells

CD40 ligation is known to induce the secretion of chemokines such as IL-8 and MCP-1 by a human malignant melanoma cell line and human cervical carcinoma cell line.[14,33] This study analyzed the effects of CD40 ligation on chemokine secretion (MCP-1, MIP-1α, MIP-1β, RANTES, and IL-8) by the human retinoblastoma cell line Y-79. Y-79 cells were either untreated or primed with IFN-γ (500 U/mL, 48 hours) and then incubated for 24 hours with rhCD40L, isotype control Ig, or medium alone. Y-79 cells primed with IFN-γ (500 U/mL) 24 hours before CD40 stimulation resulted in significant release of MCP-1 into the culture supernatant (Fig. 4). However, other chemokines including MIP-1α, MIP-1β, RANTES, and IL-8 were not detected in this culture condition. This confirms that rhCD40L-induced MCP-1 production from IFN-γ–treated Y-79 cells is mediated through the CD40/CD40L receptor interaction.

Effect of Blocking B7H1 on IFN-γ–Treated Y-79–Mediated T-Cell Proliferation

As described earlier, B7H1 expression was detected on the cell surface of IFN-γ–treated and irradiated Y-79. Recent studies have demonstrated that B7H1 (also termed PDL1) and B7DC (also termed PDL2) can bind to PD-1, a member of the immunoglobulin (Ig) superfamily involved in lymphocyte proliferation and activation, and that PD-1 functions as an important negative regulator of the immune system.[34–37] To determine whether B7H1 expressed on IFN-γ–treated Y-79 cells was functionally inhibited in T-cell proliferation, human $CD3^+$ T cell responses to agonistic anti-CD3 mAb (OKT3) were examined in the presence of Y-79 and antibodies blocking B7H1. We used this approach to test the hypothesis that B7H1 expressed on Y-79 suppresses the proliferative response of $CD3^+$ T cells.

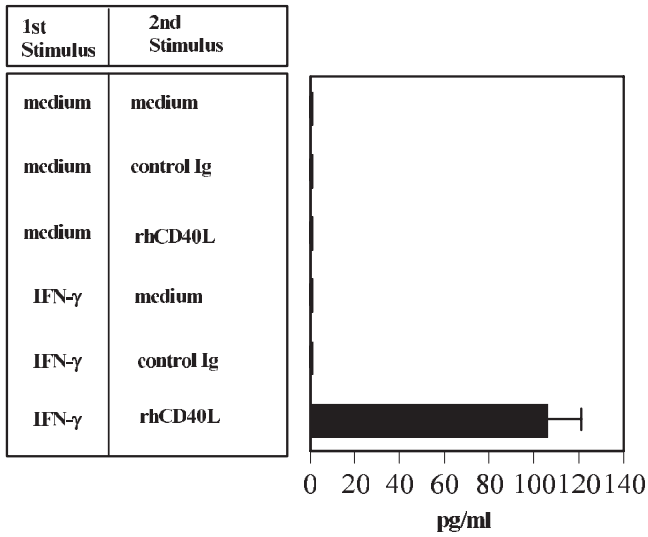

FIGURE 4. Induction of MCP-1 by rhCD40L in the human retinoblastoma cell line Y-79. Y-79 cells, with or without IFN-γ priming (24 hours, 500 U/mL), were stimulated with rhCD40L, control mAb (10 μg), or medium alone for 24 hours. After 24 hours, supernatants were harvested and analyzed for MCP-1 by using cytometric bead array immunoassay. The data are mean values of triplicate determinations. The experiment was repeated three times, and a representative set is shown.

Highly purified $CD3^+$ T cells were used to exclude the contribution of professional APCs such as B cells, dendritic cells, and monocytes. The preparation was verified by flow cytometry to contain >98% of $CD3^+$ T cells and <1% $CD14^+$ cells. $CD3^+$ T cells were cocultured with irradiated (30 Gy) and IFN-γ–treated or non-IFN-γ–treated Y-79 cells in the presence of agonistic anti-CD3 mAb. As shown in Figure 5, the proliferative response of $CD3^+$ T cells cultured with agonistic anti-CD3 mAb and IFN-γ–treated Y-79 cells was significantly enhanced by the antagonistic anti-B7H1 mAb. These results suggested that B7H1 expressed on IFN-γ–treated Y-79 cells is functionally involved in the suppression of the proliferative response of $CD3^+$ cells through costimulation. The proliferative response of $CD3^+$ T cells was not altered by PDL2 blockade (data not shown).

Discussion

In this study, the expression of nine costimulatory molecules (CD80, CD86, CD40, CD70, B7H1, B7DC, B7H2, OX40L, and 4-1BBL) was investigated in the Y-79 cell line. The rationales for the study were as follows: (1) Costimulatory molecules are poorly expressed on the surface of tumor cells.[21] (2) Transfer of some costimulatory-molecule–coding genes into tumor cells leads to tumor rejection in vivo[38,39]; (3) No information is available on the expression and function of costimulatory molecules in human retinoblastoma. In the present study, CD40 and B7H2 proteins were expressed constitutively, and B7H1 protein was enhanced significantly by IFN-γ in Y-79 cells. Irradiated Y-79 cells displayed enhanced expression of MHC class I molecules as well as CD40 and B7H2 proteins. To the best of our knowledge, this is the first report to show that IFN-γ induces CD40 and B7H1, whereas irradiation induces MHC class I molecules and enhances CD40 and B7H2 protein expressions in Y-79 cells. Nevertheless, human retinoblastoma may be a poor immunogenic tumor because of the absence of or low expression of other positive costimulatory molecules (CD80, CD86, CD70, 4-1BBL, and OX40L) under various conditions.

The most important finding of the study is the constitutive expression of CD40 mRNA and protein in Y-79 cells. Furthermore, we demonstrated that CD40 expressed on human Y-79 cells is functional because CD40L–CD40 interaction acts synergistically with IFN-γ to stimulate the cells to release the chemokine MCP-1, which attracts not only monocytes but also CD4- and CD8-positive memory T cells.[40] These results suggest that IFN-γ produced by infiltrating activated T cells may sensitize human retinoblastoma cells to produce MCP-1 on contact with CD40L in the body. Further studies are needed to verify whether these phenomena occur in vivo. We also demonstrated that cross-linking of CD40 with its ligand on human Y-79 cells upregulates surface expression of MHC class I molecule and ICAM-1, which parallels observations made in immune cells such as APCs.[30,31] However, in contrast to immune cells, CD40-mediated upregulation of MHC class II, CD80, or CD86 was not detected. It has been reported that the level of surface MHC class I and ICAM-1 expression on tumor cells is crucial for their recognition by specific cytotoxic T cells.[41,42] Our findings that CD40 triggering upregulates MHC class I and ICAM-1 expression on human retinoblastoma may thus explain their enhanced susceptibility to specific cytotoxic T-cell–mediated killing. In human retinoblastoma showing no intense inflammatory reaction, therapeutic application of IFN-γ may be considered a strong sensitizer for endogenous defense mechanisms via CD40L–CD40-mediated responses. Moreover, a trimeric recombinant form of soluble CD40L, recently tested in phase 1 studies on patients with lymphoma or solid tumor, showed limited toxicity, indicating the potential feasibility of this therapeutic approach in patients with retinoblastoma.[43]

Many recent studies have indicated that B7H1 and B7DC are involved in negative regulation of immune responses through PD-1 expressed on activated T cells, B cells, and myeloid cells,[34,36,37] which is considered a candidate strategy by which tumor cells evade host immune surveillance. The present study is the first to demonstrate that anti-B7H1 mAb significantly enhances the proliferation of $CD3^+$ T cells costimulated with IFN-γ–stimulated Y-79. The regulatory mechanisms of B7H1 expression on tumor cells are not known. Inflammatory mediators are implicated based on findings of upregulated B7H1 expression on the surface of several tumor cell lines after exposure to IFN-γ.[19,44] T cells or natural killer cells that infiltrate tumor tissue secrete many cytokines including IFN-γ Therefore, one possible scenario is that the lymphocytes that initially infiltrate the retinoblastoma secrete IFN-γ, which upregulates B7H1 expression on the tumor cells. Consequently, the upregulated B7H1 on tumor cells may transfer negative

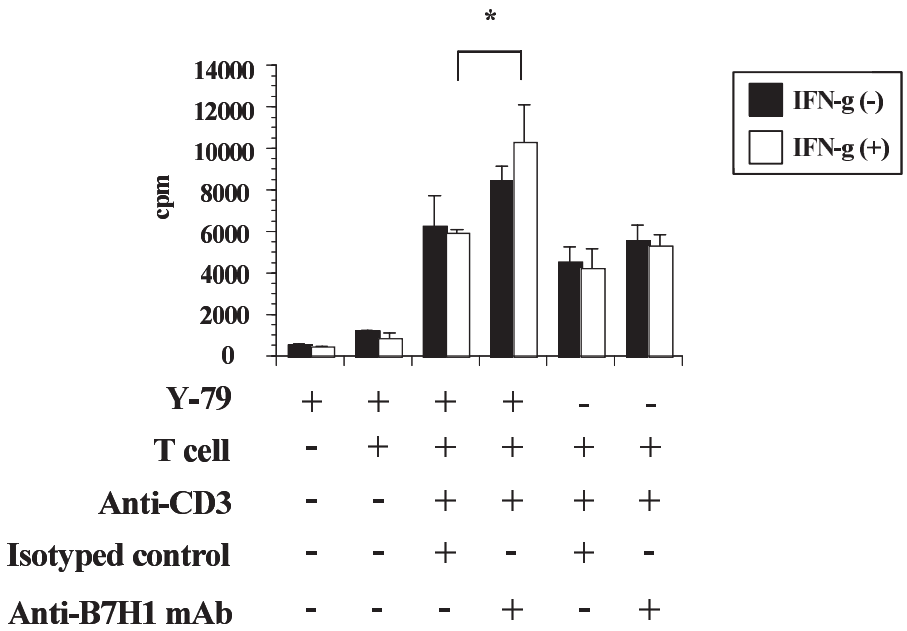

Figure 5. $CD3^+$ T cell proliferative responses costimulated with IFN-γ–treated Y-79 cells. Purified $CD3^+$ T cells (5×10^6) were cocultured with or without irradiated (30 Gy) and IFN-γ–stimulated or nonstimulated Y-79 cells for 96 hours. Blocking mAb against B7H1 was added at the start of the assay at a final concentration of 10 μg/mL. Cultures were pulsed during the final 8 hours of incubation, and incorporation of [^{3}H]thymidine was measured. Data are the mean ± SD of results in three experiments from blood sample donors. $^*P < 0.05$ compared with control IgG-treated IFN-γ-stimulated Y-79 cells.

signal to T cells via PD-1. The present findings suggest that the expression of B7H1 on Y-79 cells may contribute to negative regulation of immune responses against tumor-infiltrating lymphocytes in human retinoblastoma. Furthermore, negative response of T cells by the tumor cells through B7H1 may be expected to reduce T-cell cytolytic activity. However, a limitation of this study is that although we demonstrated negative regulation by T-cell proliferation, this phenomenon was not confirmed by direct killing ability, because the human T cells purified from PMBC did not induce sufficient cytolytic activity against Y-79 cells. Recent studies have reported that B7H1 blockade improves antitumor immunity and represents one approach for cancer immunotherapy.[44–46] The blockade of B7H1 in human retinoblastoma could be another strategy to pursue future immunotherapy in human retinoblastoma.

In conclusion, we have demonstrated the expression of several costimulatory molecules on retinoblastoma cells under various conditions. These data could explain the enhanced immunogenicity of human retinoblastoma cells after inflammation and/or irradiation, and could lead to new immunotherapy protocols. Moreover, CD40 and B7H1 expressed on the tumor cells are functionally active. These results suggest that the expression of CD40 and B7H1 on Y-79 cells and the interactions of these molecules with their receptors expressed on infiltrated immune cells may contribute to the regulation of immune responses in the eye or at metastatic sites. Recently, regulation of costimulatory molecules has attracted attention as a possible strategy for exploring novel immunotherapy for tumors, and gene transfer of ocular melanoma cells with the CD80 vector results in the stimulation of tumor-specific T cells.[47] The present data may have important implications for human retinoblastoma immunotherapy in the future.

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References

1. Ferris FL. 3rd, Chew EY. A new era for the treatment of retinoblastoma. *Arch Ophthalmol*. 1996;114:1412.
2. O'Brien JM. Alternative treatment in retinoblastoma. *Ophthalmology*. 1998;105:571–572.
3. Chantada G, Fandino A, Davila MT, et al. Results of a prospective study for the treatment of retinoblastoma. *Cancer*. 2004;100:834–842.
4. Shields CL, Meadows AT, Leahey AM, Shields JA. Continuing challenges in the management of retinoblastoma with chemotherapy. *Retina*. 2004;24:849–862.
5. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother*. 2005;54:307–314.
6. Madigan MC, Penfold PL. Human retinoblastoma: a morphological study of apoptotic, leukocytic, and vascular elements. *Ultrastruct Pathol*. 1997;21:95–107.
7. Barez S, Boumpas DT, Percopo CM, Anastassiou ED, Hooks JJ, Detrick B. Modulation of major histocompatibility complex class 1 genes in human retinoblastoma cells by interferons. *Invest Ophthalmol Vis Sci*. 1993;34:2613–2621.
8. Madigan MC, Penfold PL, King NJ, Billson FA, Conway RM. Immunoglobulin superfamily expression in primary retinoblastoma and retinoblastoma cell lines. *Oncol Res*. 2002;13:103–111.
9. Conway RM, Madigan MC, King NJ, Billson FA, Penfold PL. Human retinoblastoma: in vitro differentiation and immunoglobulin superfamily antigen modulation by retinoic acid. *Cancer Immunol Immunother*. 1997;44:189–196.
10. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today*. 1996;17:138–146.
11. Chambers CA. The expanding world of co-stimulation: the two-signal model revisited. *Trends Immunol*. 2001;22:217–223.
12. Urban JL, Schreiber H. Tumor antigens. *Annu Rev Immunol*. 1992;10:617–644.
13. Pirozzi G, Lombari V, Zanzi D, et al. CD40 expressed on human melanoma cells mediates T cell co-stimulation and tumor cell growth. *Int Immunol*. 2000;12:787–795.
14. von Leoprechting A, van der Bruggen P, Pahl HL, Aruffo A, Simon JC. Stimulation of CD40 on immunogenic human malignant melanomas augments their cytotoxic T lymphocyte-mediated lysis and induces apoptosis. *Cancer Res*. 1999;59:1287–1294.
15. Salih HR, Kosowski SG, Haluska VF, et al. Constitutive expression of functional 4–1BB (CD137) ligand on carcinoma cells. *J Immunol*. 2000;165:2903–2910.
16. Schreiner B, Wischhusen J, Mitsdoerffer M, et al. Expression of the B7-related molecule ICOSL by human glioma cells in vitro and in vivo. *Glia*. 2003;44:296–301.
17. Guinan EC, Gribben JG, Boussiotis VA, Freeman GJ, Nadler LM. Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. *Blood*. 1994;84:3261–3282.
18. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol*. 2001; 19:565–594.
19. Wintterle S, Schreiner B, Mitsdoerffer M, et al. Expression of the B7-related molecule B7–H1 by glioma cells: a potential mechanism of immune paralysis. *Cancer Res*. 2003;63:7462–7467.
20. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. *Annu Rev Immunol*. 1994;12:337–365.
21. Hurwitz AA, Kwon ED, van Elsas A. Costimulatory wars: the tumor menace. *Curr Opin Immunol*. 2000;12:589–596.
22. Wu TC, Huang AY, Jaffee EM, Levitsky HI, Pardoll DM. A reassessment of the role of B7–1 expression in tumor rejection. *J Exp Med*. 1995;182:1415–1421.
23. Weinberg AD, Rivera MM, Prell R, et al. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J Immunol*. 2000; 164:2160–2169.
24. Liu X, Bai XF, Wen J, et al. B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes in vivo. *J Exp Med*. 2001;194:1339–1348.
25. Tepper RI, Mule JJ. Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum Gene Ther*. 1994;5:153–164.
26. Yoshizawa H, Kagamu H, Gejyo F. Cancer immunogene therapy. *Arch Immunol Ther Exp* (*Warsz*). 2001;49:337–343.
27. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA*. 1993;90: 3539–3543.
28. Friedman EJ. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des*. 2002; 8:1765–1780.
29. Reid TW, Albert DM, Rabson AS, et al. Characteristics of an established cell line of retinoblastoma. *J Natl Cancer Inst*. 1974;53:347–360.
30. Stout RD, Suttles J. The many roles of CD40 in cell-mediated inflammatory responses. *Immunol Today*. 1996;17:487–492.
31. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol*. 1998;16:111–135.
32. Alexandroff AB, Jackson AM, Paterson T, et al. Role for CD40-CD40 ligand interactions in the immune response to solid tumours. *Mol Immunol*. 2000;37:515–526.
33. Altenburg A, Baldus SE, Smola H, Pfister H, Hess S. CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. *J Immunol*. 1999;162:4140–4147.
34. Carter L, Fouser LA, Jussif J, et al. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *Eur J Immunol*. 2002;32:634–643.
35. Curiel TJ, Wei S, Dong H, et al. Blockade of B7–H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med*. 2003;9:562–567.

36. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000; 192:1027–1034.
37. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*. 2001;2:261–268.
38. Katsanis E, Xu Z, Bausero MA, et al. B7–1 expression decreases tumorigenicity and induces partial systemic immunity to murine neuroblastoma deficient in major histocompatibility complex and costimulatory molecules. *Cancer Gene Ther*. 1995;2:39–46.
39. Enomoto A, Kato K, Yagita H, Okumura K. Adoptive transfer of cytotoxic T lymphocytes induced by CD86-transfected tumor cells suppresses multi-organ metastases of C1300 neuroblastoma in mice. *Cancer Immunol Immunother*. 1997;44:204–210.
40. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci USA*. 1994;91:3652–3656.
41. Katsanis E, Bausero MA, Xu H, et al. Transfection of the mouse ICAM-1 gene into murine neuroblastoma enhances susceptibility to lysis, reduces in vivo tumorigenicity and decreases ICAM-2-dependent killing. *Cancer Immunol Immunother*. 1994;38:135–141.
42. Rivoltini L, Barracchini KC, Viggiano V, et al. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res*. 1995;55:3149–3157.
43. Vonderheide RH, Dutcher JP, Anderson JE, et al. Phase I study of recombinant human CD40 ligand in cancer patients. *J Clin Oncol*. 2001;19:3280–3287.
44. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7–H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793–800.
45. Strome SE, Dong H, Tamura H, et al. B7–H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res*. 2003;63:6501–6505.
46. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, 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*. 2002;99:12293–12297.
47. Uno T, Chen PW, Murray TG, Podack ER, Ksander BR. Gene transfer of the CD80 costimulatory molecule into ocular melanoma cells using a novel episomal vector. *Invest Ophthalmol Vis Sci*. 1997;38:2531–2539.