Simultaneous Expression of Allogenic Class II MHC and B7.1 (CD80) Molecules in A20 B-Lymphoma Cell Line Enhances Tumor Immunogenicity

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We expressed the allogenic class II MHC antigen and B7.1 (CD80) co-stimulatory molecule in A20 B-lymphoma cells in order to test their efficacy as immuno-stimulating adjuvant agents in inducing tumor-specific immunity. The transduction of the allogenic I-Aβ and I-β chain genes into A20 cell resulted in a surface expression of the allogenic class II MHC molecules. The expression of the allogenic class II MHC antigen (I-Aβ) in A20 cells enhanced the proliferation of T cells in a mixed lymphocyte tumor culture and in vitro cytotoxic T lymphocyte (CTL) generation against parental cells. The B7.1 gene, which is known to be a potent co-stimulatory molecule, was also transduced and expressed in A20 cells, either alone or in combination with I-Aβ. The B7.1 transduction alone leads to a similar in vitro immune enhancing effect as I-Aβ. When both the I-Aβ and B7.1 genes were transduced, the in vitro immunostimulating capacity was further enhanced. Finally, we also tested the A20 cells that were transduced with I-Aβ and/or B7.1 for their efficacy as preventive tumor vaccines in vivo. The results indicate that the A20 cells that express both the I-Aβ and B7.1 have more potent vaccinating potential, compared to the cells that express only one of the molecules.

Keywords: Allogenic Class II MHC; B7.1; Tumor Vaccine.

Introduction

The recent development of gene transfer technology allowed the efficient transduction of various genes with immune stimulating capability (Bueler and Mulligan, 1996; Mulligan, 1993). The development of more efficient genetically engineered tumor vaccines is now one of the major goals of many biomedical industrial companies and research centers. So far, virtually all cytokine and co-stimulatory molecule genes have been tested in various settings as immuno-stimulating genes in manipulating tumor immunity (Dranoff et al., 1993; Hara et al., 1995; Mulligan, 1993). Several of these genes are currently in clinical trial (Fenton et al., 1995; Gleich et al., 1998; Rini et al., 1999).

As for co-stimulatory molecules, B7.1 (CD80) is one of the most effective molecules in stimulating tumor immunity (Gimmi et al., 1993; Wang et al., 1996). Although still largely unknown, the immuno-stimulating activity that is acquired by B7.1-expressing tumor cells may be partly attributable to the newly acquired co-stimulating activity of the genetically engineered tumor cells (Iezzi et al., 1996; Ostrand-Rosenberg et al., 1998). However, there is a report suggesting that host antigen presenting cells may play crucial roles in eliciting effective antitumor immunity, even after vaccinating the animal with the B7.1-transduced tumor cells (Marti et al., 1997). In

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule-1; IRES, internal ribosome entry site; MLTC, mixed lymphocyte tumor culture; MMC, mitomycin C; puromycin.
this case, the direct stimulation of host T cells by engineered tumor cells was evident only after a secondary vaccination. However, the precise way in which the engineered tumor cells interacts with the host immune system may vary, depending on the host strains and/or the tissue origins of tumor cells used.

The allogenic MHC molecules have been used as adjuvants in stimulating tumor immunities in several tumor models. The HLA-B7 was the first MHC molecule tested in clinical trial (Fenton et al., 1995; Gleich et al., 1998; Rini et al., 1999; Rubin et al., 1997). A class I MHC molecule, such as HLA-B7, will induce CD8+ T cell-dependent allo-response, resulting in typical tissue rejection phenomena in vivo when introduced into the tumor cells. However, the absence of co-stimulatory molecules in most tumor cells will make it highly unlikely that this strategy will be a generally effective therapeutic modality in different settings of tumor immunotherapy. In this regard, a recent report of enhanced tumor immunity in animals vaccinated with tumor cells that were transduced with both the allogenic class I MHC and B7.1 genes is a significant improvement over previous studies by providing a co-stimulatory signal along with the allogenic MHC (Bellone et al., 1994; Iezzi et al., 1996; Wang et al., 1996). In addition to the class I MHC, the syngenic class II MHC was tested in a vaccine study to enhance tumor immunity (Baskar et al., 1995; Ostrand-Rosenberg et al., 1998). In this strategy, the syngenic class II MHC molecules, engineered to be expressed on the surface of tumor cells, are expected to stimulate the CD4+ T cell-dependent anti-tumor response by presenting tumor antigens in association with class II MHC molecules. We hypothesized that we may be able to enhance tumor immunity further by expressing co-stimulatory molecules along with allogenic class II MHC in a single tumor cell, thus providing a strong helper function that is mediated by alloreactive CD4+ T cells instead of classical Ag-specific CD4+ T cells. We expect that the T cells that respond to the allogenic class II MHC will provide stronger help to the nearby cytotoxic T lymphocytes (CTLs) that recognize the tumor antigens. To test this idea, we expressed allogenic class II MHC and B7.1 molecules on A20 B lymphoma cells, and tested the immunogenicity of the cells that expressed transgenes in both the in vitro and in vivo model systems.

Our results show that the A20 B-lymphoma cells that expressed both the allogenic class II MHC and B7.1 co-stimulatory molecules elicited stronger anti-tumor immunity than the cells that expressed either molecule alone, both in vitro and in vivo. The results of this study show that the simultaneously expressing allogenic class II MHC and co-stimulating molecules may be a new and effective way of genetically engineering tumor vaccine.

Materials and Methods

Mice and cell lines Balb/c and C57BL/6 mice (6–8 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME) and maintained in an animal care facility at Hanyang University. The A20, a B-lymphoma cell line from Balb/c mouse, was purchased from the American Type Culture Collection (ATCC, Rockville, MD). MCA205, a methylcholanthrene-induced fibrosarcoma cell line established from C57BL/6 mice, was obtained from D. S. Heo (Cancer research Center, Seoul National University, Korea). These cell lines were maintained in DMEM (Gibco-BRL, USA) that was supplemented with 10% heat-inactivated (56°C, 30 min) FBS (Gibco-BRL, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml). The retrovirus packaging cell line, PA317, was purchased from ATCC. The PA317 cell lines were maintained in DMEM with 10% FBS (Gibco-BRL) with 10% FBS that was supplemented with penicillin (100 U/ml)/streptomycin (100 µg/ml). The NIH3T3 cell line was purchased from ATCC, and maintained in DMEM with 10% bovine serum (Gibco-BRL) that was supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml).

Retroviral vector construction The structure of the MFG retroviral vector was described previously (Kim et al., 1999). Standard molecular cloning techniques were used to construct the MFG.B7.puro, and MFG.I-Aβ plasmids. The cloning of B7.1 in the MFG retroviral vector was described previously (Kim et al., 1997, 1999). For MFG.I-Aβ, the α and β chain genes of I-Aβ were linked through the EMCV internal ribosome entry site (IRES) sequence (Gimmi et al., 1993; Oh et al., 2001). The detailed strategy of cloning is available upon request.

Antibodies and flow cytometry FITC-conjugated anti-B7.1 (1G10, Pharmingen, USA), anti-H-2Kβ.D6 (Cedarlane, Canada), FITC-conjugated anti-Rat κ and λ light chains (Sigma, USA), FITC-conjugated anti-H-2Kβ (Pharmingen), anti-I-Aβ (Clone 25-9-3, Parbingen), FITC-conjugated anti-mouse IgM (Pharmingen), FITC-conjugated anti-mouse CD4 (AK1.5, Pharmingen), PE (Phycerothrine)-conjugated anti-mouse CD8 (53-6.7, Pharmingen) and FITC-conjugated anti-Thy-1 (G7, Pharmingen) Abs were purchased and used at concentrations suggested by the manufacturers. Data were collected on the FACSort (Becton Dickinson, San Jose, CA) flow cytometer, and analyzed with the Cell Quest software (Becton Dickinson).

Transduction of tumor cell lines To make retrovirus producing cell lines, retroviral vector plasmids were introduced into the PA317 packaging cell line by the calcium phosphate precipitation method (Riviere et al., 1995). For MFG.B7.puro, the puromycin resistant cells were selected in a selective medium that contained 2 µg/ml of puromycin (Sigma, USA). For MFG.I-Aβ, the I-Aβ-positive PA317 cells were stained with an antibody and the positive cells were sorted through FACSort. After two rounds of sorting, the cells were subjected to a limiting dilution, and the clones with the highest titers were further expanded.
The viral supernatants were harvested from the overnight cultures of the 75% confluent cell layers of the retrovirus producing cell lines. The viral infection was performed by incubating the target cells with the viral supernatant for 4 h in the presence of 8 µg/ml polybrene. Forty-eight hours after the infection, the cells were either placed in a selective medium that contained 2 µg/ml puromycin, or sorted after staining with anti-I-A^b^ antibodies. The cells were further characterized for the expression levels of the transgenes and used as stimulator cells in MLTC, or as tumor cell vaccines.

**T cell preparation** Splenic T cells were prepared from the whole spleens of the native mice. The spleens were dissected from the mice and gently mashed with the blunt side of the sterile syringe. The cells were incubated in pre-warmed nylon wool column for 2 h to remove adherent cells. The non-adherent cells were collected from the column after rinsing the column with 20 ml of a pre-warmed culture medium. The cells that were eluted from the columns were further enriched for T cells by treating the eluted cells with the culture supernatant of HB35 hybridoma (28-16-8S, ATCC), which secretes anti-I-A^b^ specific antibodies and the rabbit complement (Accurate Chemical & Scientific Corporation, USA). The live cells were separated from the dead cells over a single step Ficoll-Hipaque (Sigma, USA) gradient. The purity of the collected T cells reached more than 95%, as determined by the surface staining with FITC-conjugated anti-Thy-1 Abs. The absence of contaminating APCs in the T cell preparation was confirmed by the lack of a Con A response of the purified T cells in a three-day culture.

**Measurement of T cell proliferation in mixed lymphocyte tumor cultures (MLTCs)** The stimulator cells for MLTCs were prepared by treating the tumor cell lines with 100 µg/ml Mitomycin C (MMC; Sigma, USA) for 30 min at 37°C in the dark. After an extensive wash with the culture medium, MLTCs were set up in flat-bottomed 96-well culture plates (Nunc, Denmark). MMC-treated tumor cells (2 × 10^5 cells/well) and nylon wool-purified T cells (3 × 10^5 cells/well) were co-cultivated for indicated periods of time in a total volume of 0.2 ml. In some experiments, IL-2 (25 U/ml, Endogen Inc., USA) was included during the culture. In all of these cultures, half of the medium was replaced with fresh medium on day 3. At the end of the cultures, 1 µCi of [3H]-thymidine was added to each well, and the [3H]-thymidine incorporation was measured in a scintillation counter after 16 h. For the MLTC with MCA205 as a stimulator, the cells were plated first in 96-well culture plates on the previous day, and the MMC treatment and washing was performed while the cells were adherent to the culture plates.

**Generation of CTLs and CTL assay** The CTL activity was measured in a standard [51Cr] (Amersham Corp., Arlington Height, IL)-release assay (Kim et al., 1999). The target cells were labeled with [51Cr]Na_2CrO_4 (100 µCi/10^6 cells) for 1 h at 37°C. After labeling, the cells were washed three times, and 5 × 10^5 cells were plated onto the U-bottom 96-well culture plates. The effector T cells were added at the indicated effector to target (E/T) ratios, and the plates were incubated for 4 h at 37°C. After incubation, the plates were centrifuged at 200 × g for 10 min, and 0.1 ml of the supernatant was removed from each well, and the radioactivity-released were measured in a gamma counter. The percent of the specific release of [51Cr] was calculated by the equation, [(E-S)/(M-S)] × 100, where E is the average experimental release, S is the average spontaneous release, and M is the average maximum release. The spontaneous [51Cr] release is the radioactivity that is released by the labeled target cells alone. The maximum [51Cr] release is the radioactivity that is released after treating the labeled target cells with 1% Triton X-100. For blocking experiments, the target cells were pretreated with anti-H-2K^d^ (mouse IgG2a, Cedarlane Laboratories, Canada) antibody, and used as target cells.

**Tumorigenicity test and vaccination study** For the vaccination study, the A20 cells that were modified to express I-A^b^ and/or B7.1 were treated with MMC, and injected subcutaneously into Balb/c mice (2 × 10^6 cells/mouse). The mice were immunized two more times at two-week intervals. Three days after the final immunization, 5 × 10^5 parental A20 cells were challenged, and the tumor-free mice were counted after 28 d. Mice with tumors of less than 5 mm in diameter were considered tumor-free. For the tumorigenicity test, different A20 cells (2 × 10^6 cells/mouse) were injected s.c., and tumor-free mice were counted after 28 d.

**Results**

**Retroviral vectors and expression of I-A^b^ and B7.1 in A20 cells** The expression of I-A^b^ and B7.1 in A20 cells after retroviral transduction was confirmed by fluorescence staining (Fig. 1). As illustrated in **Materials and Methods**, the cells that expressed B7.1 were positively selected by puromycin selection. The cells that expressed I-A^b^ were sorted after fluorescence staining. For the cells that expressed both I-A^b^ and B7.1, the I-A^b^ expressing cells were sorted after transducing the B7.1 expressing cells with the retroviral vectors that encoded the I-A^b^ α and β chain genes. After a single round of retroviral infection, around 25% of the A20 cells expressed I-A^b^ (data not shown).

**Effect of I-A^b^ and/or B7.1 expression on syngenic T cell proliferation in MLTC** To evaluate the effect of expressing allogenic I-A^b^ and/or B7.1 on the allo-reactive T cell stimulating capacity, we measured the proliferation of syngenic Balb/c T cells in the presence of A20 cells that expressed the I-A^b^ and/or B7.1 genes. Since the A20 cells originated from Balb/c mouse (I-A^b^), the I-A^b^ molecule will stimulate allo-reactive Balb/c T cells that are syngenic to parental A20 cells. The A20 cells that expressed
Fig. 1. The structure of the retroviral vectors and expression of I-A\(^b\) and B7.1 in A20 cells after retroviral transduction. A. I-A\(^b\) \(\alpha\) and \(\beta\) chain genes were simultaneously expressed by inserting the IRES sequence between the two coding sequences, the B7.1 and puromycin-resistance genes were expressed from a single recombinant retroviral vector by the same strategy. B. The A20 cells that expressed both I-A\(^b\) and B7.1 were stained and analyzed in FACS. The B7.1-positive cells were enriched by puromycin selection of the transduced cells. The peaks on the left are the staining results of parental A20 cells. The B7.1-transduced cells were secondarily infected with retroviral vectors that expressed I-A\(^b\) \(\alpha\) and \(\beta\) chain genes, and the I-A\(^b\)-positive cells were sorted in FACSort after staining with the FITC-conjugated anti-I-A\(^b\) monoclonal antibody.

Fig. 2. T cell proliferation in MLTCs with A20 cells that expressed I-A\(^b\) and/or B7.1 as stimulators. T cells that were isolated from naive Balb/c mice were mixed with MMC-treated A20 cells that expressed I-A\(^b\) and/or B7.1. T cell proliferation was measured by \([^{3}H]\)-thymidine incorporation after three days of culture. Bars represent the standard error.
Fig. 3. CTL generation from syngenic T cells against parental A20 cells in MLTCs with A20 cells that expressed I-A\(^b\) and/or B7.1 as stimulators. The T cells that were isolated from MLTCs were mixed with \(^{51}\text{Cr}\)-labeled parental A20 or MCA205 cells, and the radioactivity that was released into supernatants was plotted against different effectors to target the ratios used. A. T cells isolated at day 3. B. T cells isolated at day 5. C. The CTL activities against the A20 cells can be blocked by an anti-class I MHC antibody. The anti-K\(^d\)D\(^d\) specific antibody blocks CTL activities against A20 cells. However, the same antibody treatment shows no effect on the CTL activity against MCA205 cells that have H-2\(^b\) haplotype. This demonstrates the specificity of the antibody blocking.

cells expressing I-A\(^b\) and/or B7.1 genes. The A20 cells expressing both the I-A\(^b\) and B7.1 induced more potent anti-tumor immunity against the parental A20 cells, compared to the cells that expressed I-A\(^b\) or B7.1 alone (Table 2). This result indicates that, at least in A20 cells, the allogenic class II MHC antigens (I-A\(^b\)) and co-stimulatory molecules (B7.1) collaborate in inducing anti-tumor imm

Table 1. Tumorigenicity of the A20 cells expressing I-A\(^b\) and/or B7.1 in syngenic Balb/c mice.

<table>
<thead>
<tr>
<th>Cells used for tumor formation(^1)</th>
<th>No. of mice with tumor/total No. of mice (^2)</th>
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<tbody>
<tr>
<td>A20</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>A20. I-A(^b)</td>
<td>7/15 (67%)</td>
</tr>
<tr>
<td>A20.B7.1</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>A20.I-A(^b).B7.1</td>
<td>0/10 (0%)</td>
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\(^1\) Two \(\times 10^6\) live cells were subcutaneously injected into Balb/c mice.  
\(^2\) Mice with tumors of less than 5 mm in diameter on day 28 were counted as tumor-free.

Table 2. Frequencies of tumor formation in mice vaccinated with modified A20 cells.

<table>
<thead>
<tr>
<th>Cells used for vaccination(^1)</th>
<th>No. of mice with tumor/total No. of mice (^2)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>A20.I-A(^b).B7.1</td>
<td>0/10 (0%)</td>
</tr>
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</table>

\(^1\) Mice were immunized three times (at two-week intervals) with \(2 \times 10^7\) A20 cells expressing I-A\(^b\) and/or B7.1. Three days after final immunization, \(5 \times 10^5\) live parental A20 cells were challenged s.c. for tumor formation.  
\(^2\) Mice with tumors of less than 5 mm in diameter at day 28 were considered as tumor-free.

Discussion

Currently, many different strategies are being developed to synthesize more potent tumor vaccines. One of the major breakthroughs is the genetic engineering of the tumor cells in order to express proteins that can enhance anti-tumor immunity (Bueler and Mulligan, 1996; Dranoff et al., 1993; Martin et al., 1999; Mulligan, 1993; Pulaski and Ostrand-Rosenberg, 1998). Recently, several reports demonstrated the importance of the CD4\(^+\) T cell dependent tumor Ag(s) in the systemic anti-tumor immune response (Ladanyi et al., 2000; Pulaski and Ostrand-Rosenberg, 1998). For example, tumor cells that were engineered to express the syngenic class II MHC antigens and co-stimulatory molecules were demonstrated to induce a strong anti-tumor immune response when injected into an animal as a tumor vaccine (Baskar et al., 1995; Ladanyi et al., 2000). Furthermore, when the invariant (Ii) chain expression was suppressed (Qiu et al., 1999) or absent (Armstrong et al., 1997), the immunogenicity of the tumor cells was further enhanced. These results demonstrate that the CD4\(^+\) T cell-dependent anti-tumor im-
mune response is important in maintaining and/or eliciting the tumor-specific immune response. In addition, several antigenic peptides that are responsible for establishing the CD4+ T cell-dependent anti-tumor immune response were characterized. All of these reports indicate that the helper function that is provided by the tumor-specific CD4+ T cells are important in the anti-tumor immune response.

Based on these findings from previous reports, we reasoned that the tumor cells that can stimulate strong CD4+ T cell-dependent allo-reaction will be equally or more effective in inducing the helper function that is required in generating tumor-specific CTLs. The only previous attempts to elicit allo-reactive T cell help was performed by Trefzer et al. (2000). There the hybrid cells between tumor cells and cells that expressed allogenic class II Ags were used as vaccines. However, the hybrid cells in this attempt express both class I and II MHC Ags and the molecules that are responsible for the enhanced efficacy of the hybrid cell vaccine were not thoroughly characterized. In another attempt, Hock et al. (1996) reported that neuroblastoma cells that are engineered to express either xenogenic or allogenic class II MHC were superior to the ones that expressed the syngenic class II Ag in inducing anti-tumor immunity both in vitro and in vivo. In this report, we described an approach that can vaccinate the potential of tumor cells by simultaneously expressing the allogenic class II MHC and co-stimulatory molecules genes in tumor cells. The A20 cells were engineered to express the I-A<sup>b</sup> α and β chain genes by transducing recombinant retroviral vector, where the two genes were linked through the IRES sequence. The A20 cells that expressed I-A<sup>b</sup> alone stimulated the allo-reactive T cells, when mixed with syngenic Balb/c T cells. This result indicates that A20 cells, even without B7.1, provide a sufficient co-stimulatory signal to the allo-reactive T cells. The candidates for these are either the B7.2 or ICAM-1 molecules that are strongly expressed in A20 cells. We also transduced the B7.1 gene into the A20 cells. The B7.1 is one of the most potent co-stimulatory molecules for T cell activation (Bellone et al., 1994; Iezzi et al., 1996; Townsend et al., 1994). The A20 cells that were engineered to express the B7.1 stimulated the proliferation of syngenic T cells and CTL generation. The results indicate that B7.1, when over-expressed, can induce the proliferation of syngenic T cells. Although we do not know the exact molecules that the T cells are recognizing, we frequently observed T cell aggregation around the A20 cells that expressed B7.1. The A20 cells that simultaneously expressed I-A<sup>b</sup> and B7.1 showed a stronger in vitro stimulatory potential both in terms of T cell proliferation and CTL generation. The T cells that were activated in this system include both the CD4+ and CD8+ subpopulations, based upon the analysis of surface phenotype of the cells that were transformed into blasts (data not shown).

These results indicate that the stimulation of allo-reactive T cells by allogenic class II MHC molecules, when combined with the co-stimulation by B7.1, provide strong stimulatory signals for T cell proliferation and CTL generation. The CTL activity that was generated in MLTCs were class I MHC-restricted, according to the Ab-blocking experiment that was performed in Fig. 3C. Therefore, the CTLs that were obtained in MLTCs are probably self-reactive to the Ag(s) that was expressed in A20 cells, and presented through the class I MHC-restricted pathway.

To determine whether the in vitro immune-enhancing effect that was observed is relevant to the in vivo vaccinating potential, we vaccinated Balb/c mice with A20 cells that expressed I-A<sup>b</sup> and/or B7.1. The result showed that the A20 cells that expressed both I-A<sup>b</sup> and B7.1 were superior in inducing anti-tumor immunity, when compared to the cells that expressed either molecule alone. Since the A20 is a relatively immunogenic tumor cell line, we cannot conclude that the strategy we employed can be applied to other tumor models. In fact, we previously showed that tumor cell lines of various origins have different capacities in stimulating T cell proliferation in vitro after B7.1 transduction (Kim et al., 1999).

In this report, we showed that tumor cells that simultaneously expressed allogenic class II MHC and B7.1 genes are very effective in inducing the tumor-specific immune response both in vitro and in vivo. We expect that the strategy we demonstrated in this report will be useful in synthesizing more efficacious tumor vaccines in the future.

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