

***In vitro* induction of anterior gradient-2-specific cytotoxic T lymphocytes by dendritic cells transduced with recombinant adenoviruses as a potential therapy for colorectal cancer**

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Abbreviations: Ad, adenovirus; AGR2, anterior gradient-2; CEA, carcinoembryonic antigen; CRC, colorectal cancer; DC, dendritic cell; MOI, multiplicity of infection; TAA, tumor-associated antigen

Abstract

Anterior gradient-2 (AGR2) promotes tumor growth, cell migration, and cellular transformation, and is one of the specific mRNA markers for circulating tumor cells in patients with gastrointestinal cancer. We investigated the feasibility of AGR2 as a potent antigen for tumor immunotherapy against colorectal cancer (CRC) cells using dendritic cells (DCs) transduced with a recombinant adenovirus harboring the AGR2 gene (AdAGR2). DCs transduced with a recombinant adenovirus encoding the AGR2 gene (AdAGR2/DCs) were characterized. These genetically-modified DCs expressed AGR2 mRNA as well as AGR2 protein at a multiplicity of infection of 1,000 without any significant alterations in DC viability and cytokine secretion (IL-10 and IL-12p70) compared with unmodified DCs as a control. In addition, AdAGR2 transduction did not im-

pair DC maturation, but enhanced expression of HLA-DR, CD80, and CD86. AdAGR2/DCs augmented the number of IFN- γ -secreting T-cells and elicited potent AGR2-specific cytotoxic T lymphocytes capable of lysing AGR2-expressing CRC cell lines. These results suggest that AGR2 act as a potentially important antigen for immunotherapy against CRC in clinical applications.

Keywords: AGR2 protein, human; antigens, neoplasm; colorectal neoplasms; dendritic cells; immunotherapy, active

Introduction

Cancer immunotherapy based on dendritic cell (DC) vaccination is an exciting prospect for the treatment of cancer (Banchereau and Palucka, 2005). A central tenet of cancer immunotherapy is the generation of an antigen-specific cytotoxic T lymphocyte (CTL) response (Melief *et al.*, 2002). The CTL response is dependent on suitable tumor-associated antigens (TAAs). In turn, TAAs can induce T lymphocytes that recognize tumors, but not normal tissues (Parmiani *et al.*, 2007). TAAs should be expressed in a significant proportion of tumor cells and are obligatory for the survival of tumor cells. DCs pulsed with various TAAs have been shown to be effective in producing specific anti-tumor effects *in vitro* and *in vivo* (Nakamura *et al.*, 2005).

One of the most interesting tumor antigens in colorectal cancer (CRC) is the carcinoembryonic antigen (CEA) because CEA is extensively expressed in the vast majority of patients with CRC. However, the induction of immune responses against antigens that are expressed not only by tumor cells but also by benign tissues, such as CEA, is more difficult because rapid induction of tolerance against the so-called "self antigens" occurs (Thompson *et al.*, 1991). Human tumors express a number of specific antigens that can be recognized by T cells, thus the discovery of a variety of novel TAAs, especially those shared among patients, is needed to improve the efficacy of DC-based cancer immunotherapy in patients with CRC.

Different strategies have been attempted to load DCs with tumor antigens, including irradiated tumor cells, tumor lysates, tumor RNA, antigen peptides, or antigen-gene transfer *via* non-viral or viral vectors (Mayordomo *et al.*, 1995; Ashley *et al.*, 1997; Nestle *et al.*, 1998). The most commonly used method, loading with peptides or proteins, is not a very efficient method because the half-life of complexes of MHC molecules and peptides is very short. Prolonged presentation due to stable antigen expression by a genetic modification of DCs appears to be a more promising method. Recently, several studies have reported that TAA gene-modified DCs can provide extended antigen presentation and induce antigen-specific CTLs more efficiently than peptide pulsing (Oh *et al.*, 2006). In previous studies, DCs have been shown to be amenable to adenovirus (Ad) vector infection *in vitro* (Zhong *et al.*, 1999). Moreover, an Ad-modified DC vaccine, produced using the Ad to transfer the TAA *ex vivo*, successfully induced T cell immunity against target tumor cells expressing relevant antigen *in vitro* and *in vivo* in experimental tumor models (Song *et al.*, 1997).

Anterior gradient-2 (AGR2) is a secreted protein, which was initially described in *Xenopus laevis*, and identified in a screen for differentially-expressed genes in neural development (Aberger *et al.*, 1998). In humans, AGR2 was first identified in studies which focused on differentially-expressed genes in estrogen receptor-positive breast cancers. Subsequent studies showed elevated AGR2 expression in adenocarcinomas of the breast (Liu *et al.*, 2005), esophagus (Hao *et al.*, 2006), pancreas (Ramachandran *et al.*, 2008), and prostate (Zhang *et al.*, 2007). Recently, it has been reported that AGR2 promotes tumor growth, cell migration, and cellular transformation and is one of the specific mRNA markers for circulating tumor cells in cancer patients with gastrointestinal cancer (Valladares-Ayerbes *et al.*, 2008). The fact that AGR2 overexpression may provide a survival benefit for tumor cells and the enhanced expression is almost completely restricted to malignant tissues makes AGR2 an interesting target for the development of immunotherapeutic strategies.

In the present study the efficiency of Ad encoding AGR2 transduction in monocyte-derived DCs was evaluated. We showed that AdAGR2 is capable of transducing human DCs, the transduction was not compromised, and the transduced DCs were able to elicit AGR2-specific CTL responses against AGR2-expressing CRC cells. Taken together, the results of the current study have shown that AGR2 is a potentially useful antigenic target for cancer immunotherapy using DCs

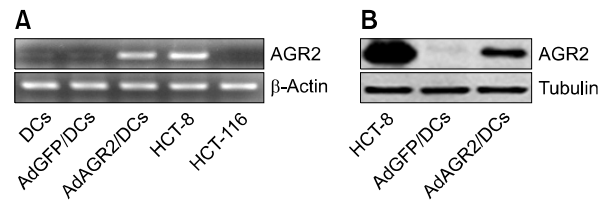


Figure 1. Ad-mediated gene transfer and expression in DCs *in vitro*. AGR2 gene was cloned and monocyte-derived DCs were infected with AdAGR2 at a multiplicity of infection (MOI) of 1,000. Seventy-two hours later, the expression of AGR2 was analyzed by RT-PCR (A) and WB (B). HCT-8 and HCT-116 cells were used as AGR2-positive and -negative controls, respectively.

against CRC.

Results

Adenoviral transduction of DCs *in vitro*

In preliminary experiments (submitted), most CRC cell lines were shown to express AGR2 protein based on WB and flow cytometric analyses (AGR2⁺ cell lines: KM12C, HCT-8, DLD1, DK01, and HT-29; AGR2⁻ cell lines: HCT-116 and SW620).

To produce recombinant adenovirus encoding AGR2 (AdAGR2), the AGR2 gene was cloned into BglIII/XhoI sites of an Ad transfer vector pShuttle-CMV. Recombinant AdAGR2 stocks were generated using a helper virus-free packaging system. The infectious titer of AdAGR2 was 2×10^{11} viral particles per ml. The transduction efficiency of AdAGR2 in DCs at a multiplicity of infection (MOI) of 1,000 was determined by RT-PCR and WB. DCs expressed AGR2 mRNA (Figure 1A) and protein (Figure 1B) efficiently. HCT-8 and HCT-116 CRC cell lines were used as positive and negative controls, respectively.

Characteristics of AdAGR2-transduced DCs

An MOI of 1,000 accomplished transgene expression in $47.1 \pm 6.5\%$ of the DCs (Figure 2A). To evaluate the effect of AdAGR2 transduction on DCs, viability, phenotypic expression, and cytokine secretions were analyzed. AdAGR2 transduction did not show any detrimental effect on the viability of DCs, as estimated by intravital staining with trypan blue (Figure 2B). As shown in Figure 2C, AdGFP- and AdAGR2-transduced DCs exhibited a strongly increased expression of CD80, CD86, and HLA-DR compared to imDCs. However, there was no significant difference between AdGFP- and AdAGR2-transduced DCs. In addition, the production of IL-12p70, IL-10, and IL-6 from DCs was not

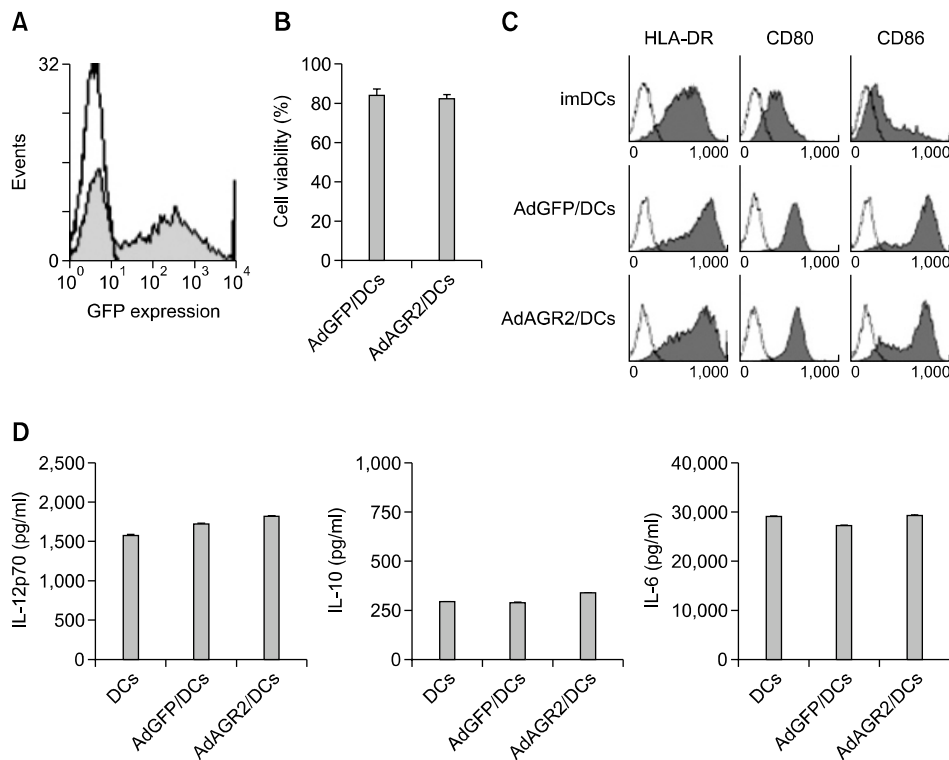


Figure 2. Ad transduction efficiency in DCs and characterization of AdAGR2-transduced DCs. Monocyte-derived DCs were transduced with AdGFP on day 5, then 2 days after maturation cells were analyzed for the expression of GFP by flow cytometry. The results showed $47.13 \pm 6.48\%$ cells were GFP-positive at a MOI of 1,000 (A). Data represent the mean \pm SD of 5 independent experiments. Cell viability after transduction in DCs was measured by 0.4% trypan blue exclusion (B). FACS was performed to analyze the cell surface markers of DCs and the expression of CD80, CD86, and HLA-DR molecules was enhanced by AdAGR2 transduction (C). Isotype control in each group is indicated in white. Three experiments with similar results were performed. Cytokine secretion of IL-12p70, IL-10, and IL-6 after transduction was measured by ELISA (D).

altered by AdAGR2-transduction compared to untreated and AdGFP-transduced DCs (Figure 2D).

Induction of AGR2-specific CTL responses by AdAGR2-transduced DCs

Autologous CD8⁺ T cells were sensitized for CTL induction with AdAGR2-transduced or untreated DCs. The effector T cells were compared to the ability to recognize AGR2-positive CRC cell lines, such as DLD1, HT-29, HCT-8, and DK01, by an IFN- γ ELISPOT assay at an E:T ratio of 20:1 (Figure 3). CTLs generated by AdAGR2-transduced DCs recognized the AGR2-positive CRC cell lines and the responses were specifically inhibited by MHC class I blockade. HCT-8 and HT-29 cell lines were shown to have more sensitive targeting to AGR2-specific CTLs, whereas no significant IFN- γ secretion occurred in the control group using AdGFP-transduced DCs.

Characterization of AGR2-specific CTLs induced by AdAGR2-transduced DCs

The functional capability of the CTL response to AdAGR2-transduced DCs was tested by determining whether or not the CTLs could specifically lyse target cells using the JAM and 4-h standard

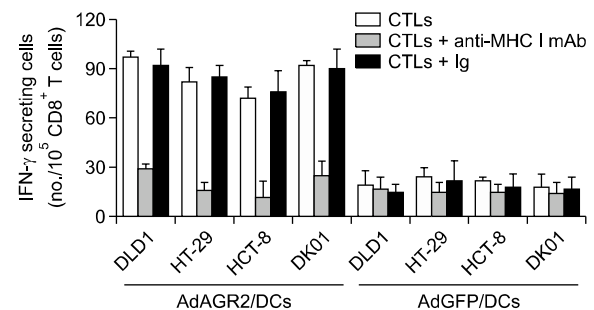


Figure 3. AGR2-specific CTL response by transduced DCs. ELISPOT assay showing the number of IFN- γ -secreting cells per 10^5 CD8⁺ T cells induced by AdGFP- and AdAGR2-transduced DCs, respectively, at an E:T ratio of 20:1. Several AGR2-expressing CRC cell lines were used as targets. MHC class I restriction of the CTLs was analyzed using a MHC class I specific monoclonal Ab (clone W6/32). Nonspecific response was determined by irrelevant Ig blockade. Representative results of 4 independent experiments are shown.

⁵¹Cr-release assays. In the JAM assay (Figure 4A), the CTLs sensitized by AdAGR2-transduced DCs had high lytic activity against the AGR2-positive HCT-8 CRC cell line at an E:T ratio of 40:1. The response was specifically inhibited by MHC class I mAbs. No significant killing activity was observed against the K562 cell line, excluding the possibility that NK cells contributed to the cytotoxicity. We next examined the cytotoxic activity of these CTLs

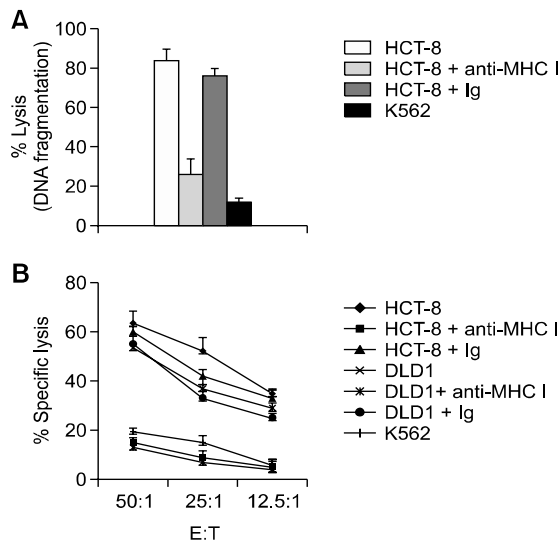


Figure 4. Cytotoxicity of AGR2-specific CTLs. CTLs were generated by autologous CD8⁺ T cells from healthy donors stimulated with AdAGR2-transduced DCs. Lytic activity of CTLs generated by AdAGR2-transduced DCs was analyzed by DNA fragmentation at an E:T ratio of 20:1 (A) and 4-h standard ⁵¹Cr-release assay at different E:T ratios (50:1, 25:1, and 12.5:1) (B) against AGR2-positive HCT-8 and DLD1 target cells. MHC class I restriction of the CTLs was analyzed using a MHC class I-specific mAb. Nonspecific response was determined by irrelevant Ig blockade. The NK-sensitive K562 cell line was used to exclude the possibility that NK cells contributed to the cytotoxicity. Results of 4 independent experiments are shown.

against AGR2-positive CRC cell lines, including HCT-8 and DLD1, using a standard 4-h ⁵¹Cr-release assay. As shown in Figure 4B, CTLs induced by AdAGR2-transduced DCs effectively lysed AGR2-positive CRC cell lines, such as HCT-8 and DLD1, in a dose-dependent manner according to the E:T ratio. MHC class I restriction of the CTLs was detected using a MHC class I-specific mAb. No killing was observed against the K562 cell line. The results demonstrated that CTLs generated by autologous DCs transduced with AdAGR2 showed strong cytotoxic activity against AGR2-positive CRC cell lines *in vitro*. These findings suggest that an AdAGR2/DCs vaccine may serve as a feasible approach for the treatment of patients with AGR2-associated CRC.

Discussion

Identification of appropriate target TAAs and effective delivery into DCs with stable expression are major limitations for DC-based immunotherapy. Recently, several studies have revealed that TAA gene-modified DCs can provide extended antigen presentation and induce antigen-specific CTLs

more efficiently than peptide pulsing (Nakamura *et al.*, 2005). Previous studies have shown that self-antigens overexpressed in malignant tissue can provide a basis for a tumor-specific immune response. The majority of TAAs recognized thus far by tumor-infiltrating T cells in patients are non-mutated proteins (Rosenberg, 1997). It is therefore of great interest to establish whether or not these self-antigens can be used to stimulate a tumor-specific T cell response.

Several studies have shown elevated AGR2 expression in adenocarcinomas of the breast, esophagus, pancreas, and prostate (Liu *et al.*, 2005; Hao *et al.*, 2006; Zhang *et al.*, 2007; Ramachandran *et al.*, 2008). The aberrant expression of AGR2 in CRC remains unknown. Recently, it has been reported that AGR2 promotes tumor growth, cell migration, and cellular transformation and is one of the specific mRNA markers for circulating tumor cells in cancer patients with gastrointestinal cancer (Valladares-Ayerbes *et al.*, 2008). Here, for the first time, we report that AGR2 protein was expressed at a high level in CRC cells. The fact that AGR2 overexpression may provide a survival benefit for tumor cells and the enhanced expression is almost completely restricted to malignant tissues, thus making AGR2 an interesting target for the development of immunotherapeutic strategies. Also, recombinant adenovirus encoding AGR2 was efficiently transduced in DCs without alteration of functional DC capacity, elicited AGR2-specific CTL generation, and lysed AGR2-positive CRC target cells.

Gene transfer is a powerful means to manipulate the properties of DCs for therapeutic advantage to augment the immune response and is currently under extensive evaluation in pre-clinical and clinical trials. Most investigations have focused on transferring genes encoding activating cytokines and tumor antigens to DCs (Gong *et al.*, 1997). While a variety of vectors, including adenovirus, adeno-associated virus, retrovirus, and lentivirus, are capable of producing gene transfer to DCs (Aicher *et al.*, 1997), adenoviral vectors hold several distinct advantages. First, adenovirus is one of the most efficient vehicles for DC gene therapy (Di Nicola *et al.*, 1998). Moreover, in addition to achieving a high degree of transgene expression (Benihoud *et al.*, 1999), the adenoviral vector itself, without the addition of a therapeutic transgene, matures and activates DCs by enhancing the expression of MHC and co-stimulatory molecules in human (Rea *et al.*, 1999) and murine (Hirschowitz *et al.*, 2000) models. In addition, it has been reported that adenoviral gene transduction to DCs produce IL-12 in significant amounts and

increased CCR7 expression on DCs, leading to enhanced migratory capacity of DCs to draining LNs in which DCs can stimulate T cells (Hirschowitz *et al.*, 2000; Nakamura *et al.*, 2005).

In the present study we revealed that recombinant adenovirus efficiently transduced in DCs and GFP expression in DCs was successfully accomplished at a MOI of 1,000 without significant cell death. Hirschowitz *et al.* reported that adenovirus infection increases DC surface expression of MHC class II proteins and B7 co-stimulatory molecules. Consistent with previous reports (Benihoud *et al.*, 1999; Rea *et al.*, 1999; Hirschowitz *et al.*, 2000), AdAGR2 transduction in DCs enhanced DC activation and maturation by showing higher expression of CD80, CD86, and HLA-DR than untreated DCs. However, cytokine production, such as IL-12p70, IL-6, and IL-10 from transduced DCs, was not altered compared to the untreated DCs in the current study.

In conclusion, cancer immunotherapy using DCs transduced with the entire gene encoding AGR2 elicited efficient immune responses against CRC, as shown by AGR2-specific CTL generation. Thus, this study is the first to provide strong and direct evidence to support the application of AGR2-based immunotherapy in CRC. In addition, the identification of T-cell epitopes from AGR2 as a TAA protein in CRC is available. Further studies to enhance the *in vivo* anti-tumor effect of AGR2 as a tumor antigen against CRC will be necessary before clinical application.

Methods

Target cell lines

The following human CRC cell lines were used as target cells: DK01; DLD1; HCT-8 (all HLA-A*0201⁺, A*2402⁺); HCT-116 (HLA-A*0101⁺ and A*0201⁺); and HT-29 (HLA-A*24+). All cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS; all from Gibco-BRL, Grand Island, NY). K562 cells were obtained from the Korea Cell Line Bank (KCLB; Seoul, Korea) and used as natural killer (NK) cell-sensitive targets.

Construction and production of recombinant Ad encoding AGR2

The recombinant Ad encoding AGR2 (AdAGR2) was constructed using the AdEasyTM vector system (Qbiogene Inc., Carlsbad, CA), according to the manufacturer's instructions. Briefly, the full length of AGR2 cDNA was amplified by a polymerase chain reaction (PCR) using AGR2 sense primer 5'-atggagaaattccagtgtca-3' containing the *Bgl* II site and AGR2 antisense primer 5'-ttacaattcagctt-

cagcaa-3' containing the *Xho* I site. The obtained PCR products were cloned in Ad transfer vector (pShuttle-CMV; AES1021) at the *Bam*HI and *Xho* I enzymes in multiple cloning sites (MCS), and sequenced to identify possible *Taq* polymerase errors. Subsequently, the AGR2 expression cassette was inserted into viral DNA vector (pAdEasy-1; AES1010). After packaging the recombinant adenoviral DNA into infectious Ad by transfecting HEK 293A cells, the adenovirus containing AGR2 gene was amplified and purified from cell lysates by banding twice in CsCl density gradients, as described previously (Eloit *et al.*, 1990). Viral products were desalted and stored at -80°C in phosphate-buffered saline (PBS) containing 10% glycerol (v/v). A similar protocol was used to generate recombinant Ad encoding green fluorescent protein (GFP; AdGFP). The titer of the viral stock was determined using the tissue culture infectious dose (TCID₅₀) method.

Generation of DCs

Peripheral blood was collected from healthy donors with HLA-A0201⁺ after obtaining informed consent and approval by the institution ethical committee of the Chonnam National University Hwasun Hospital. The monocytes were isolated by two-step density gradient centrifugation followed by plastic adherence, as previously described (Muthuswamy *et al.*, 2008). Briefly, the adherent monocytes (purity > 95 %) were cultured for 6 days at 5×10^5 cells per well in 24-well plates (BD Biosciences-Labware, San Jose, CA) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 1% PS in the presence of recombinant human granulocyte macrophage-colony-stimulating factor (rhGM-CSF, 50 ng/ml; LG Biochemical, Daejeon, Korea) and interleukin-4 (rhIL-4, 20 ng/ml; PEPROTECH, Rocky Hill, NJ). For the generation of AGR2-specific CTL lines, the immature DCs were cultured for an additional 2 days using α DC1-polarizing cocktails composed of IL-1 β (25 ng/ml; PEPROTECH), TNF- α (50 ng/ml; PEPROTECH), IFN- α (3,000 units/ml, Intron-A-IFN- α -2b; Schering-Plough International, Kenilworth, NJ), IFN- γ (1,000 units/ml; Strathmann Biotech GmbH, Hannover, Germany), and poly-I:C (20 μ g/ml; Sigma-Aldrich, St. Louis, MO) for final maturation.

Ad transduction in DCs

To determine transduction efficiency, 1×10^6 immature DCs were suspended in IMDM with 10% FBS and exposed to AdGFP at a multiplicity of infection (MOI) of 1,000 for 2 h at 37°C. After washing twice, the transduced DCs were re-suspended in fresh cytokine-supplemented medium and cultured for an additional 2 days using a maturation cytokine cocktail. Subsequently, the expressions of GFP and DC phenotypes were assayed by a FACSAria cell sorter (Becton Dickinson, San Jose, CA) and the data were analyzed with FACSDivaTM (Becton Dickinson) and WinMDITM version 2.9.

Immunophenotyping of DCs

For phenotype analysis, AdGFP- and AdAGR2-infected DCs were stained with phycoerythrin (PE)-conjugated

CD1a, CD11c, CD38, CD54, CD80, and CD86 (AdGFP/DCs and AdAGR2/DCs; Becton Dickinson) and fluorescein isothiocyanate (FITC)-conjugated CD14, CD40, CD83, HLA-DR, and CCR7 for unmodified and AdAGR2/DCs (Becton Dickinson).

AGR2 expression

AGR2 surface expression of CRC cell lines was determined by serial incubation with primary rabbit anti-AGR2 monoclonal antibody [Ab] (ab76473; Abcam, Cambridge, UK) and secondary AffiniPure goat anti-rabbit IgG(H+L) conjugated with FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For Western blot (WB) analysis, proteins in the cell extracts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto a nitrocellulose (NC) membrane. The NC membrane was incubated with 5% non-fat milk in PBS, then with rabbit anti-human AGR2 polyclonal Ab (ab43043; Abcam) for 2 h at room temperature (RT). After washing, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (ab6721; Abcam) for 1 h at RT. Immuno-reactive bands were detected using the WEST-one™ Western Blot Detection System (iNtRON BIOTECHNOLOGY, Daejeon, Korea) and were analyzed with a LAS-3000 imaging system (Fujifilm Corporation, Tokyo, Japan).

RT-PCR

Expression of AGR2 mRNA in DCs or AdAGR2 transduced-DCs and CRC tumor cell lines was assayed by RT-PCR amplification using a cellular mRNA control. The mRNA from the cells was extracted according to the manufacturer's instructions. Total RNA was isolated from tumor cells with a RNeasy Mini kit (QIAGEN, Valencia, CA). First-strand cDNA synthesis was performed using oligo (dT) 18 primers. The following PCR primers were used: AGR2 forward, 5'-ATTGGCAGAGCAGTTTGTCC-3' and reverse, 5'-GAGCTGTATCTGCAGGTTTCGT-3'; and β -actin forward, 5'-CCCTCCATCGTCCACCGCAAATGCTTC-3' and reverse, 5'-CGACTGCTGTACCTTCACCGTTCCAG-3'. Each of the primer sets was confirmed by running samples on agarose gels. β -actin transcript levels were used to normalize the amount of cDNA in each sample.

ELISA

ELISA for IL-12p70, IL-10, and IL-6 were used to measure the secreted cytokine. Supernatants of DCs were collected on day 8, and the amounts of secreted cytokines in the supernatants were quantified using commercially available ELISA kits (BD Biosciences, San Diego, CA).

Generation of AGR2-specific CTLs

AGR2-specific CTLs were generated *in vitro* by stimulation of autologous CD8⁺ T cells from healthy donors with autologous DCs transduced with AdAGR2. CD8⁺ T cells stimulated with autologous DCs without transduction was used as a control. Briefly, CD8⁺ T cells (purity > 90%) were positively isolated using a MACS system (Miltenyi Biotec Inc., Auburn, CA) from the lymphocyte fraction after Percoll

isolation. CD8⁺ T cells (1×10^6) were sensitized by AdAGR2/DCs ($1-2 \times 10^5$ cells) in 2 ml of medium (RPMI-1640: AIM-V = 1:1) containing 10% FBS and 1% PS. On day 3, 5 ng/ml of rhIL-2 and 10 ng/ml of rhIL-7 (both from PEPROTECH) were added in the medium and the medium was replenished with cytokines every 3 days. On day 10, the CTL lines were re-stimulated with the same DCs. On day 17, the number of AGR2-specific CTLs was analyzed by an IFN- γ ELISPOT kit (BD Biosciences) and the cytolytic activity of CTLs was evaluated by DNA fragmentation and standard 4-h ⁵¹Cr release assays.

Enzyme-linked immunosorbent spot (ELISPOT) assay

Detailed methods of the ELISPOT assay have been described previously (Yi *et al.*, 1993). AGR2-positive CRC cell lines (DLD1, HT-29, HCT-8, and DK01) were used as target cells with or without anti-MHC class I mAb (30 μ g/ml) for the antigen-specific IFN- γ ELISPOT assay. Nonspecific response was determined by irrelevant Ig blockade. The number of IFN- γ spots was enumerated by an automatic CTL immunospot® analyzer (Cellular Technology, LTD., Shaker Heights, OH). All samples were run in triplicate. Data are expressed as the mean number of IFN- γ -secreting cells \pm SD per 10^5 CD8⁺ T cells.

DNA fragmentation assay (JAM assay)

The JAM assay was performed as described previously (Matzinger, 1991). Briefly, target cells (HCT-8) were labeled with [³H] thymidine for 4 h at a final concentration of 5 μ Ci/ml at 10^6 cells, washed, and plated into wells that contain CTLs (E:T ratio of 20:1). After co-culture for 8 h, cells were harvested onto glass fiber filter mats using a TOMTEC plate harvester (TOMTEC, Inc., Hamden, CT). Data are expressed as a percentage of DNA fragmentation based on the percentage decrease in counts per minute (cpm) as compared with untreated controls using the following formula: (% DNA fragmentation = [control cpm - sample cpm/control cpm] \times 100; control and sample mean labeled target cells alone and labeled target cells plus CTLs, respectively).

⁵¹Cr-release cytotoxicity assay

The standard 4-h ⁵¹Cr-release assay was performed to measure cytolytic activity of the T cells with target cells, including AGR2-positive CRC cell lines (HCT-8 and DLD1). Target cells were incubated with 100 μ Ci of ⁵¹Cr-sodium chromate for 1 h, washed extensively, seeded (1×10^4 cells/well) into 96-well U-bottomed plates in T-cell medium, and co-cultured for 4 h with various numbers of T cells (E:T ratios of 50:1, 25:1, and 12.5:1). All assays were performed in triplicates. Results are expressed as the mean percentage of ⁵¹Cr-release calculated as follows: [(experimental release-spontaneous release) / (maximum release-spontaneous release)] \times 100%. Maximum release was determined after adding 100 μ l of 2% Triton X-100 solution to the targets and spontaneous release, which was always < 20% after incubating targets without effectors.

Nonspecific response was determined by irrelevant Ig blockade. To determine whether or not the cytolytic activity was restricted by MHC class I molecules, 30 µg/ml of mAb against HLA-ABC (W6/32) were added to the cultures at the start of the assay.

Statistical analysis

All statistical analyses were performed with SPSS 13.0 for Windows. The Mann-Whitney *U* test was performed to analyze the statistical significance of non-parametric differences between the groups. *P* < 0.05 was considered statistically significant.

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