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Similar Gene Regulation Patterns for Growth Inhibition of Cancer Cells by RP215 or Anti-Antigen Receptors

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Abstract

RP215 is a monoclonal antibody generated against a carbohydrate-associated epitope of glycoproteins designated as CA215, which consists mainly of immunoglobulin superfamily proteins expressed by cancer cells including antigen receptors such as immunoglobulins and T-cell receptors. Since RP215 was shown to induce apoptosis and inhibit tumor growth in nude mouse models, the effects of RP215 and antibodies against antigen receptors on the gene regulations of cultured OC-3-VGH ovarian and C-33A cervical cancer cells were investigated through semi-quantitative RT-PCR. For both cell lines, RP215 and anti-antigen receptors were found to regulate similarly and consistently a number of genes including NFκB-1, IgG, P21, Cyclin D1, ribosomal P_1 and c-fos with only exceptions for EGFR and ribosomal P_0. Among toll-like receptor genes (TLR-2, -3, -4, -6, -7 and -9), differential levels of gene expressions in different cancer cell lines were observed. RP215 and anti-antigen receptors were found to up-regulate TLR-2 and/or TLR-3, whereas those of TLR-4 and TLR-9 were down regulated for both cancer cells. Based on these preliminary observations, it can be proposed that apoptosis of the two cancer cell lines was induced similarly by RP215 and anti-antigen receptors through consistent regulations of the same groups of genes. The innate immunity of cancer cells can also be affected by any of these antibodies through unidirectional regulations of certain toll-like receptors. Excellent correlations were obtained (R^2=0.90-0.94) in terms of gene regulation patterns affected by any of these binding ligands. Therefore, the anti-cancer therapy of RP215 Mab may be, in part related to the surface bound antigen receptors and/or toll-like receptors in the innate immunity system, all of which may be involved in the growth and survival of cancer cells.

Keywords: RP215 monoclonal antibody; Gene regulations; Cancerous immunoglobulins; Immunoglobulin superfamily proteins; Innate immunity; Toll-like receptors

Abbreviations: CDC: Complement-Dependent Cytotoxicity; C-Fos: Cellular Proto-Oncogene; Cyclin D1: G1/S Phase Regulator Protein; EGFR: Epidermal Growth Factor Receptor; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; IgSF: Immunoglobulin Super Family Proteins; Mab: Monoclonal Antibody; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; NFκB-1: Nuclear Factor Kappa-B P105 Subunit 1; P_0, P_1, P_2: Ribosomal Proteins; P21: Cyclin-Dependent Kinase Inhibitor 1; RT-PCR: Reverse Transcription Polymerase Chain Reaction; TCR: T-Cell Receptor; TLR: Toll-Like Receptor; TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling; KLH: Keyhole Limpet Hemocyanin; LPS: Lipopolysaccharide

Introduction

RP215 is a monoclonal antibody (Mab) generated against an ovarian cancer cell line, OC-3-VGH in 1987 and shown to react with the carbohydrate-associated epitope of glycoproteins known as CA215 expressed by cancer cells [1,2]. Following MALDI-TOF MS analysis of affinity-purified CA215 from shed medium of cultured cancer cells, the molecular identity of CA215 was initially revealed as heavy chains of cancerous immunoglobulins [1,2]. Subsequently, it was further documented that RP215 not only reacts with the epitope of immunoglobulin heavy chains, but also many other immunoglobulin superfamily (IgSF) proteins including antigen receptors, such as immunoglobulins and T-cell receptors, as well as cell adhesion molecules [3,4]. These IgSF proteins of CA215, in particular the antigen receptors, immunoglobulins and T-cell receptors, may have preferential glycosylation sites to generate the epitope which is favorably recognized by RP215 [3,5].

Functional studies by TUNEL apoptosis assay revealed that apoptosis of cultured cancer cells could be induced by RP215 and anti-human IgG [5,6]. It was therefore suggested that cancerous immunoglobulins might be essential for the growth and proliferation of cancer cells in vitro and in vivo [7-12]. However, the mechanisms of action for expressions of cancerous immunoglobulins as well as other IgSF proteins to regulate the growth of cancer cells remain to be explored [3,4]. Therefore, in this study, attempts are made to study changes in regulations of genes involved in cell proliferation, protein synthesis and cell cycle regulations in cancer cells in response to treatment by RP215 and antibodies against human IgG and T-cell receptors. Noticeable changes in gene expressions upon antibody treatment of cancer cells were determined by semi-quantitative reverse transcriptase-PCR (RT-PCR) and correlations made to elucidate mechanisms of action. In addition, toll-like receptors (TLRs) which are also known to be involved in the growth and proliferation of cancer cells through the action of innate immunity were examined [13-16]. Possible roles of RP215 and antibodies against antigen receptors on the innate immunity were investigated through gene regulation studies

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of selected toll-like receptor genes in cultured cancer cells [4,9].

We believe that the roles of these cancerous IgSF proteins with RP215-specific carbohydrate-associated epitope can be better understood through these investigations. Furthermore, the information obtained from this study should be beneficial in determining whether RP215 Mab or its humanized form, hRP215, is suitable for development as an antibody-based anti-cancer drug to target cancer therapeutically in humans.

Materials and Methods

Chemicals and reagents

All the chemicals and reagents used in this study were obtained from Sigma Chemicals Co (St Louis, MD) unless otherwise specified.

Cancer cell lines and cell culture

OC-3-VGH is an ovarian cancer cell line established in 1986 by the Department of OBS/GYN of Veterans General Hospital, Taipei, Taiwan [17]. This cancer cell line is of serous origin and can be cultured or maintained in RPMI 1640 medium containing 10% bovine serum and penicillin-streptomycin. Other human cancer cell lines including DU-145 (prostate), A549 (lung), C-33A (cervix) and MDA-MB-435 (breast) were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and cultured according to instructions provided by the supplier.

Monoclonal and polyclonal antibodies

RP215 Mab was generated against the ovarian cancer cell line, OC-3-VGH as reported previously [17]. From our initial MALDI-TOF MS analysis, RP215 was shown to react with a carbohydrate-associated epitope of a pan cancer marker, CA215, which was shown mainly to be cancer cell-expressed Ig heavy chains [2,18]. This antibody as well as its humanized forms were used for all the biochemical and immunological studies presented in this study including apoptosis, and complement-dependent cytotoxicity assays as well as gene regulation studies.

Rabbit anti-T cell receptor (TCR) β antisera were generated through immunizations of the synthetic oligopeptide selected from the β-subunit of TCR (GeneScript, MD, USA). The amino acid sequence of the oligopeptide is: KEVHSGVSTDPQPLK EQPALN (N50-70). The synthetic peptides were conjugated with hemocyanin from Keyhole Limpet (KLH) according to the published procedures [19]. Two rabbits were immunized separately with 100 μg each of the KLH-conjugated peptides at 10-day interval according to the standard protocols [19]. After the fourth immunization, the rabbits were bled and sera collected separately. The specificity of these antisera was determined by enzyme-linked immunosorbent assay (ELISA) with microwells coated with the synthetic peptide at 1 μg/ml according to the established procedures [19]. The IgG fractions of rabbit antisera were purified by protein affinity chromatography as described previously [17,20]. Monoclonal antibodies against the extra-cellular domains of toll-like receptor 4 (TLR-4) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA) for comparative purposes. These antibodies were dialyzed against phosphate-buffered saline to remove trace of sodium azide prior to any bioassays.

TUNEL assay for assessment of cellular apoptosis

The anti-proliferative or apoptotic effect was investigated with treatments of murine RP215 Mab (mRP215), humanized RP215 Mab (hRP215), goat anti-human IgG, rabbit anti-T cell receptors β and anti-TLR4 on cultured OC-3-VGH cancer cells. An In Situ Cell Death Detection Kit, POD (Roche, Canada), was employed for detection and quantitation of apoptosis at cellular levels. Briefly, OC-3-VGH cancer cells were cultured in RPMI 1640 medium at 37°C in a CO_2 (5%) incubator for 24 h until all cancer cells became attached to the micro-wells. RP215 Mab, goat anti-human IgG, rabbit anti-T cell receptors or anti-TLR4 of known concentrations were added separately for the co-incubation for 24 h and/or 48 h. As the negative control, normal mouse IgG or normal rabbit IgG of the same concentration (10 μg/ml) was used under the same incubation periods. At the end of the incubation, the attached cells were removed from the tissue culture wells. Apoptosis of treated cancer cells was determined quantitatively by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay with the instruction provided by the supplier [21]. Apoptosis assays of other established human cancer cell lines were also performed in a similar manner.

Complement-dependent cytotoxicity assays

Complement-dependent cytotoxicity (CDC) assay was performed according to the standard protocol described previously [3]. Briefly, 1×10^5 cultured OC-3-VGH cancer cells in 1 mL of appropriate culture medium (RPMI 1640 with 10% FCS) were plated in 24-well plates for 2 h before treatment. Murine RP215 Mab (mRP215), humanized RP215 Mab (hRP215), goat anti-human IgG, rabbit anti-T cell receptors β or anti-TLR4 were added separately to give a final concentration of 10 μg/mL and incubated for 15 min at room temperature, respectively. Three microliters of freshly prepared rabbit baby complement (CL3441; Cedarlane labs, Burlington, NC, USA) were added to each well followed by incubation at 37°C for 2 h. After incubation at room temperature, the cells were recovered by centrifugation. Trypan blue (0.4%; SV30084.01; Thermo Scientific, Waltham, MA, USA) was added and mixed gently. The percentages of cells stained with Trypan blue were determined by cell counting under a regular microscope. Normal mouse IgG or normal rabbit IgG of the same concentration was used as the negative control. Incubation with the complement plus the antibody or the complement alone served as the respective negative controls for parallel comparisons in this experiment. Statistical analysis was performed to determine the significance of the assay by the Trypan blue method [22-24].

Effect of antibody treatments on gene expressions of cultured cancer cells

Generally speaking, cancer cells were cultured in culture medium in log phase, antibodies such as RP215, anti-human IgG, anti-T cell receptors β or anti-TLR4 at 10 μg/mL each were added separately to 10^4-10^6 cells / experiment for 24 hr incubation at 37°C in a 5% CO_2 incubator. As a negative control, normal mouse or rabbit IgG at 10 μg/mL were used. Following incubations, the cells were harvested by trypsinization. If not directly used for RNA extraction, the cell pellets were kept at -70-80°C, mRNA was extracted from cultured cancer cells and cDNA prepared as described in the next section.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from OC-3-VGH cell lines (10^5-10^7 cells) by using QIAGEN RNeasy mini kit (Mississauga, ON, Canada) according to the manufacturer's manual. RNase-free DNase set was included to avoid genomic gene interference. Reverse transcription

(RT) of total RNA (0.5-5 μg/20 μl) to cDNA was performed by using oligo $(dT)_{15}$ primers and EasyScript™ First Strand cDNA Synthesis Kit from Applied Biological Materials (Abm) Inc. (Richmond, BC, Canada) following the manufacturer's protocol. Reaction mixtures with RNA template but without reverse transcriptase or with reverse transcriptase but without RNA template were used as the negative controls for cDNA synthesis.

PCR

Semi-quantitative RT-PCR was performed as described previously with a number of genes listed. All primers required for PCR amplification were obtained from Integrated DNA Technologies (San Diego, CA) and listed as follows [9]: IgG: 5'-ACGGCGTGGAGGTGCATAATG-3' (sense) and 5'-CGGGAGGCGTGGTCTTGTAGTT-3' (antisense); T cell receptor α chain constant region: 5'-GTGCTAGACATGAGGTCTATGGAC-3' (sense) and 5'-GGATTCGGAAGGGAATCACTGACAGG-3' (antisense); NFκB-1: 5'-GGATCTGCACTGTAACTGCTGGAC-3' (sense) and 5'-CCTTGTGAAGCTGCCAGTGC-3' (antisense); P_0: 5'-TTGTGTTCACCAAGGAGG-3' (sense) and 5'-GTAGCCAATCTGCAGACAG-3' (antisense); P_1: 5'-CAAGGTGCTCGGTCCTTC-3' (sense) and 5'-GAACATGTTATAAAAGAGG-3' (antisense); P_2: 5'-TCCGCCGCAGACGCCGC-3' (sense) and 5'-TGCAGGGGAGCAGGAATT-3' (antisense); EGFR: 5'-AAGGAAATCCTCGATGAAGCCT-3' (sense) and 5'-TGTCTTTGTGTTCCCGGA CATA-3' (antisense); c-fos: 5'-GAGATTGCCAACCTGCTGAA-3' (sense) and 5'-AGACGAAGGAAGACGTGTAA-3' (antisense); P21: 5'-GCAGACCAGCATGACAGATTT-3' (sense) and 5'-GGATTAGGGCTTCCTCTTGGA-3' (antisense); Cyclin D1: 5'-GTCTCAAAGCTTG CCAGGAG-3' (sense) and 5'-ATATCCCGCACGTCTGTAGG-3' (antisense); TLR-2: 5'-CCTCTCGGTGTCGGAATGTC-3' (sense) and 5'-TCCCGCTCACTGTAAGAAACA-3' (antisense); TLR-3: 5'-CAAACACAAGCATTCGGAATCTG-3' (sense) and 5'-AAGGAATCGTTACCAACCACATT-3' (antisense); TLR-4: 5'-TCAGAGCCTAAGCCACCTCTCTAC-3' (sense) and 5'-TCATAGGGTTCAGGGACAGGTC-3' (antisense); TLR-6: 5'-AGACCTACCGCTGAAAACCAA-3' (sense) and 5'-ACTCACAATAGGATGGCAGGA-3' (antisense); TLR-7: 5'-GACCTCAGCCACAACCAACT-3' (sense) and 5'-CCACCAGACAAACCACACAG-3' (antisense); TLR-9: 5'-ATGTCACCAGCCTTTCCTTG-3' (sense) and 5'-TGAGGGACAGGG ATATGAGG-3' (antisense). A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to check the functional integrity of cDNA and used as internal control by using 5'-GAAATCCCATCACCATCTTCC-3' (sense) and 5'-CCAGGGGTCTTACTCCTTGG-3' (antisense) as primers.

PCR was performed by using 2 × PCR Plus MasterMix kit (Abm, Richmond, BC, Canada) according to the manufacturer's protocols. After denaturing at 94°C for 4 min, 20-35 cycles were performed under the following conditions: denaturing at 94°C for 40 s, annealing at 58°C for 60 s and extension at 72°C for 1 min. A final complete extension was then executed at 72°C for 7 min. At the end, the PCR product was checked by 1.5% agarose gel electrophoresis. The relative signal intensities of different PCR products on the agarose gel were semi-quantitatively analyzed by Image Quant image analysis software [25,26]. The intensity of negative control was adjusted to 100% in each case for comparative purposes.

Statistical analysis

All experiments were performed in triplicate. All the results were presented as mean ± SD (standard deviations). Student t-test was performed to estimate the statistical significance. The pair-wise correlation analysis regarding the effects of three different ligands on gene regulation changes was performed according to the established method [27].

Humanized RP215 monoclonal antibodies

RP215 Mab was originally generated in mouse (mRP215) and converted into humanized form by CDR grafting method through contract work by Lake Pharma Inc. (Belmont, CA). The humanized form of RP215 designated as hRP215 was shown to have affinity and specificity comparable to those of mRP215. Bioequivalence between mRP215 and hRP215 was demonstrated in this study through assays of induced apoptosis and complement-dependent cytotoxicity reactions.

Results

Antibody selections for functional and gene regulation studies

In this study, antibodies of three related ligands to cancer cells were selected for functional studies. Based on previous MALDI-TOF MS analysis, the "sugar" epitope recognized by RP215 can be detected in many CA215 glycoproteins which consist mainly of immunoglobulin superfamily proteins, especially the dominant antigen receptors such as immunoglobulins and T-cell receptors (≥ 50% of the detected CA215 peptides) [4]. Biological effects of RP215 on two different cultured cancer cells from OC-3-VGH (ovary) and C-33A (cervix) cell lines, respectively were compared with respect to those of the two antigen receptors and presented in this study.

Comparative studies of induced apoptosis on cancer cells

Cultured ovarian cancer cell line, OC-3-VGH was employed as a model for studies of induced apoptosis of cultured cancer cells. Several antibodies of known concentrations (1 and 10 μg/ml) were incubated separately with cultured cancer cells for 24-48 hours [3-5]. These antibodies included murine RP215 Mab (mRP215), humanized RP215 Mab (hRP215), goat anti-human IgG (affinity-purified), rabbit anti-T cell receptors β (IgG fraction) and anti-TLR4. The results of this study are presented in (Figures 1A and 1B) [3,5]. Following incubation with different antibodies, it was demonstrated that apoptosis was induced by these added antibodies at concentrations of 1 or 10 μg/ml. The corresponding negative controls with various normal IgG from mouse, human or rabbit showed little or no effect on induced apoptosis under the same experimental conditions for incubation either at 24 or 48 hours [3,4].

Similarly, apoptosis was induced in four other cultured cancer cell lines including DU-145 (prostate), A549 (lung), C-33A (cervix) and MDA-MB-435 (breast) [6,9]. Results of TUNEL apoptosis assay upon treatment of cultured cancer cells with either RP215 or goat anti-human IgG are presented in (Figure 1C) for comparisons. With no exceptions, both RP215 and goat anti-human IgG were shown to induce apoptosis similarly to any of these cancer cells.

Complement-dependent cytotoxicity (CDC) reactions in the presence of antibodies and complement

Previous studies have suggested that antibodies against antigen receptors and RP215 Mab can induce complement-dependent

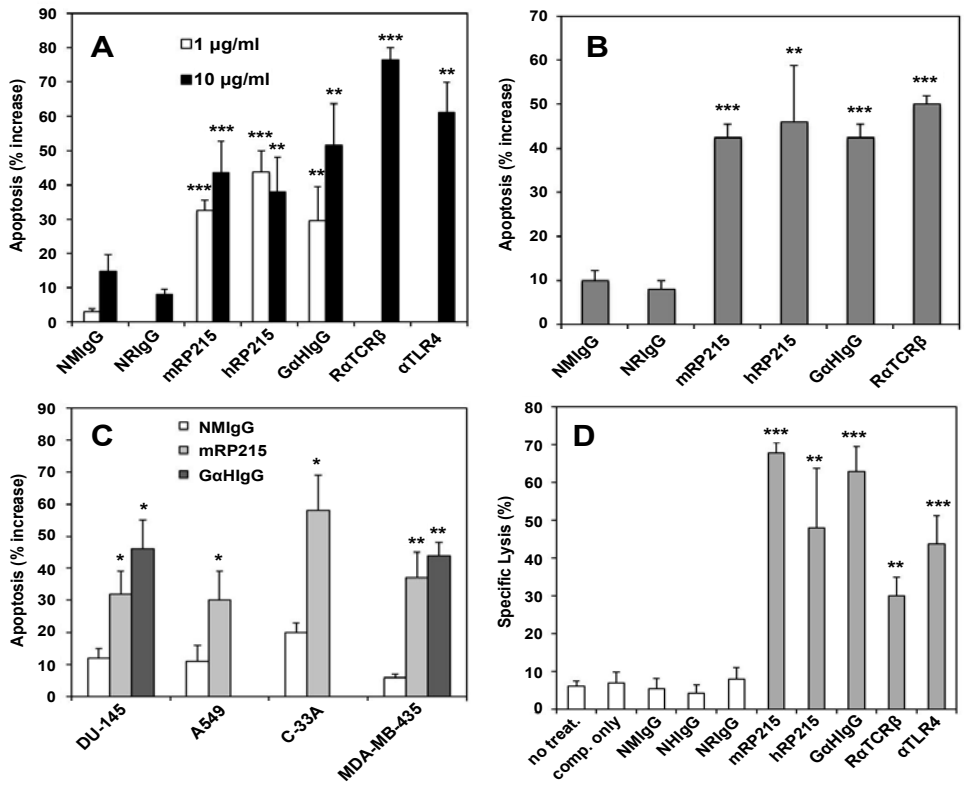

Figure 1: Effects of RP215, anti-human IgG and anti-T cell receptors on induced apoptosis of cancer cells. A) Induced apoptosis on treated cultured OC-3-VGH cancer cells by normal mouse IgG (NMIgG) (Lane 1), normal rabbit IgG (NRIgG) (Lane 2), mRP215 (Lane 3), hRP215 (Lane 4), goat anti-human IgG (GαHIgG) (Lane 5), rabbit anti-T cell receptors β (RαTCRβ) (Lane 6) and anti-TLR4 (αTLR4) (Lane 7) for 24h incubation. □ And ■ represent 1 µg/ml and 10 µg/ml, respectively of the ligands used for the apoptosis assay. **B)** ■ Represents the corresponding data obtained for 48h incubation (10 µg/ml). **C)** Induced apoptosis of several cultured cancer cells including DU-145 (prostate) (Lane 1), A549 (lung) (Lane 2), C-33A (cervix) (Lane 3) and MDA-MB-435 (breast) (Lane 4) [6,9]. □, ■ and ■ represent the treatments with normal mouse IgG (NMIgG), mRP215 and goat anti-human IgG (GαHIgG) (10 µg/ml), respectively. **D)** Complement-dependent cytotoxicity (CDC) reactions in the presence of 10 µg/ml each of mRP215, hRP215 and antibodies against antigen receptors, as well as normal immunoglobulins used as the negative control. Lane 1: no treatment (no treat.); Lane 2: 3 µL freshly prepared rabbit baby complement (comp. only); Lane 3: normal mouse IgG plus complement (NMIgG); Lane 4: normal human IgG plus complement (NHIgG); Lane 5: normal rabbit IgG plus complement (NRIgG); Lane 6: mRP215 plus complement (mRP215); Lane 7: hRP215 plus complement (hRP215); Lane 8: goat anti-human IgG plus complement (GαHIgG); Lane 9: rabbit anti-T cell receptors β plus complement (RαTCRβ); Lane 10: anti-TLR4 plus complement (αTLR4). All data presented are statistically significant at * P<0.05, ** P<0.01 and *** P<0.001.

cytotoxicity (CDC) to cultured cancer cells [3-5]. Following incubations with the antibody and complement, cytotoxicity of cancer cells could be demonstrated in the presence of complement as presented in (Figure 1D). The antibodies used for comparative assay were mRP215, hRP215, anti-human IgG, anti-T cell receptors β and anti-TLR4. No cytotoxicity to cancer cells was observed in the absence of complement. Similarly, normal immunoglobulins gave no complement-dependent cytotoxicity to cancer cells as expected [3,6].

Effects of RP215 and antibodies against antigen receptors on expressions of selected genes

Since apoptosis was induced upon incubation of cultured cancer cells with RP215 and antibodies against antigen receptors [3-5], changes in expressions of genes involved in cell growth regulations were studied and compared. In the case of OC-3-VGH ovarian cancer cells, RP215, anti-human IgG and anti-T cell receptors were found, respectively to up-regulate the genes expressed by NFκB-1, IgG, TCR, P21 and ribosomal P_1, although to different degrees. On the other hand, down regulations of Cyclin D1 and c-fos were consistently observed. In the case of epidermal growth factor receptor (EGFR) and ribosomal P_0, down regulation was observed upon treatment with anti-TCR and RP215, whereas little or up regulation with anti-human IgG was observed instead. It was generally observed that patterns of gene regulations of cultured cancer cells do not vary significantly with the origin of cell lines. The results of such comprehensive analysis are presented as histograms in (Figure 2A, 2B and 2C), respectively with statistical analysis of significance. In addition, under the same conditions, changes in gene regulations upon the treatments of C-33A cervical cancer cells were also investigated following the treatment with RP215 or other two antibodies, respectively. Due to the relatively low expression levels of certain genes such as EGFR in C-33A cervical cancer cells, only selected results are presented in (Figure 2D) for comparisons. The results of semi-quantitative RT-PCR to reveal the relative expressions of various genes selected in this study are detailed in supplement (SI).

Effects of RP215, anti-human IgG and anti-T cell receptors on the regulation of toll-like receptor genes

The effects of antibody treatments on genes of toll-like receptors expressed by cultured cancer cells were also investigated. Two established cancer cell lines were employed in this comprehensive study. By using RT-PCR, mRNA expressed by various toll-like receptor (TLR) genes including TLR-2 to TLR-9 was amplified. The relative

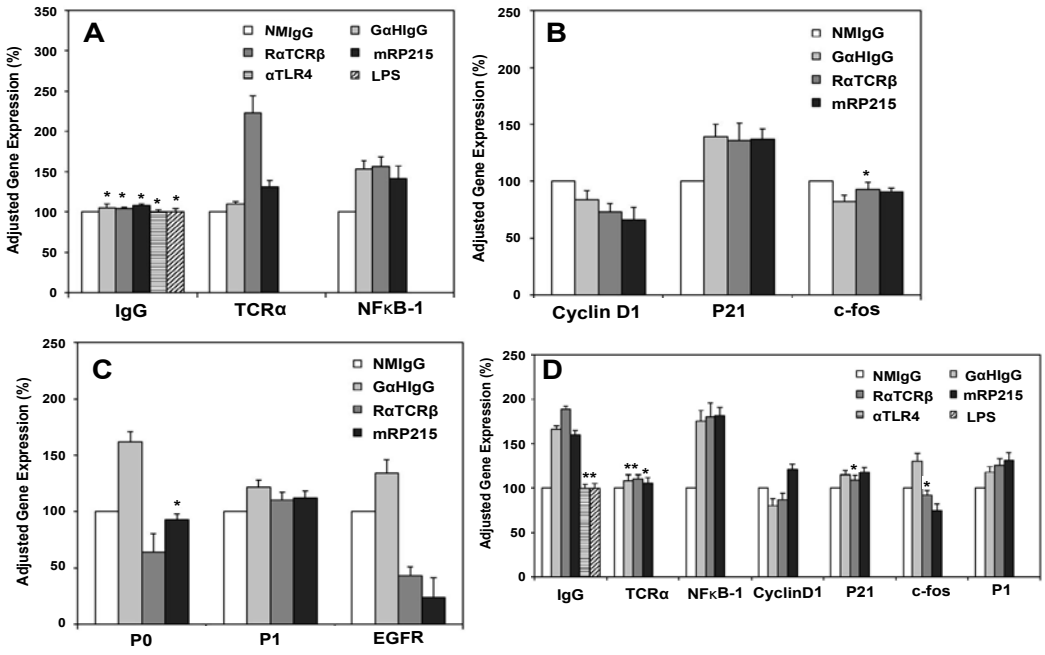

Figure 2: (A-C) Effects of treatments of cultured OC-3-VGH ovarian cancer cells with goat anti-human IgG (GαHIgG) ( ), rabbit anti-T cell receptors β (RαTCRβ) ( ), mRP215 ( ), anti-TLR-4 (αTLR4) ( ) and LPS ( ), respectively on the expressions of genes involved in cell proliferation, protein synthesis, cell cycle regulations as well as the innate immunity; Expressions of 9 genes involved were adjusted with that of GAPDH. The negative control ( ) with normal mouse IgG (NMIgG) was considered 100% in all cases. Antibody concentration of 10 μg/ml was used for all parallel studies of comparisons. List of genes involved: **(A)**: Lane 1: IgG; Lane 2: T-cell receptor α and Lane 3: NFκB-1; **(B)**: Lane 4: Cyclin D1; Lane 5: P21 and Lane 6: c-fos; **(C)**: Lane 7: P0; Lane 8: P1 and Lane 9: EGFR. **(D)** Changes in selected gene regulations upon treatments of cultured C-33A cervical cancer cells for comparison with those of ovarian cancer cells demonstrated in **(A), (B) and (C),** respectively. Lane 1: IgG; Lane 2: TCR; Lane 3: NFκB-1; and Lane 7: P_1. In the case of C-33A cervical cancer cells, the expression of EGFR gene is too low to be quantitated. Effects of anti-TLR-4 and LPS on the gene regulation of IgG are presented in **(A)** and **(D)**, respectively for OC-3-VGH and C-33A cancer cells. All data presented are statistically significant at P<0.01 except those labeled with * which are not statistical different from the negative control.

expression levels of toll-like receptor genes are presented in (Figure 3) for comparisons. Only those of TLR-2 and/or TLR-3, TLR-4 and TLR-9 were clearly detected [28-34] from both cultured OC-3-VGH ovarian and C-33A cervical cancer cells (Figure 3) and pursued for further gene regulation studies. Effects of RP215, goat anti-human IgG and rabbit anti-T cell receptors β on the changes in expression of TLR genes were determined by semi-quantitative RT-PCR. Following respective treatments with RP215, goat anti-human IgG and rabbit anti-T cell receptors β, expression of TLR-3 gene was significantly up-regulated (226%, 143% and 219%, respectively) [28,29,34]. On the other hand, expressions of TLR-4 and TLR-9 genes were significantly down-regulated (50%-80%) [30-33]. Results of the selected TLR gene expression analysis are presented in (Figure 4A) for OC-3-VGH ovarian cancer cells. RP215, goat anti-human IgG and anti-T cell receptors were shown to affect the regulation of TLR genes almost in a consistent manner. The results of semi-quantitative RT-PCR to reveal relative expressions of TLR-3, TLR-4 and TLR-9 genes for OC-3-VGH cancer cells are also shown, respectively in (Figure 1S) of the supplement.

When C-33A cervical cancer cells were employed for such investigations, toll-like receptor genes (TLR-3, TLR-4 and TLR-9) were affected similarly to those of OC-3-VGH ovarian cancer cells although with significant quantitative variations (Figure 4B). Upon treatments with RP215 or anti-antigen receptors, up-regulation of TLR-3 was observed, whereas those of TLR-4 and TLR-9 were down regulated but to a smaller extent as compared to those of OC-3-VGH ovarian cancer cells. In addition, the expression of TLR-2 gene is further up regulated upon the antibody treatments in C-33A cancer cells. The results of semi-quantitative RT-PCR for C-33A cervical cancer cell line were presented in (Figure 2S).

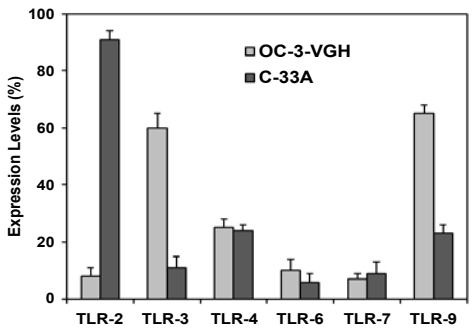

Figure 3: Semi-quantitative RT-PCR to reveal relative expression levels of various toll-like receptor (TLR) genes including TLR-2 to TLR-9 from cultured OC-3-VGH ovarian ( ) and C-33A cervical ( ) cancer cells, respectively. Lane 1: TLR-2; Lane 2: TLR-3; Lane 3: TLR-4; Lane 4: TLR-6; Lane 5: TLR-7; Lane 6: TLR-9. Expression of GAPDH gene served as the internal control. Percent expression levels of various TLR genes with respect to that of GAPDH are presented for comparisons.

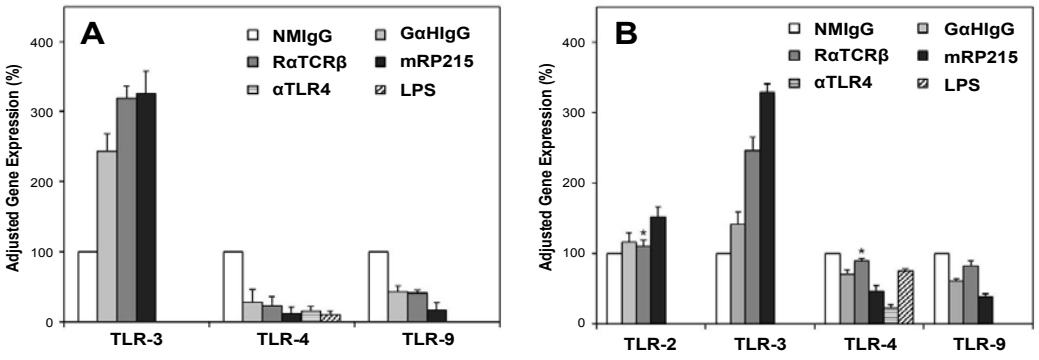

Figure 4: Changes in gene expressions of toll-like receptors (TLR-2, TLR-3, TLR-4 and TLR-9) of cultured OC-3-VGH ovarian cancer cells **(A)** and C-33A cervical cancer cells **(B)** upon treatments with 10 μg/ml each of goat anti-human IgG (GαHIgG) ( ), rabbit anti-T cell receptors β (RαTCRβ) ( ), mRP215 ( ), anti-TLR-4 (αTLR4) ( ) and LPS ( ). The negative control ( ) with normal mouse IgG (NMIgG) was adjusted to 100% in all cases. **(A):** Lane 1: TLR-3; Lane 2: TLR-4 and Lane 3: TLR-9; **(B)**: Lane 1: TLR-2; Lane 2: TLR-3; Lane 3: TLR-4; Lane 4: TLR-9. In the case of OC-3-VGH ovarian cancer cells, the expression of TLR-2 gene is too low to be quantitated. All data presented are statistically significant at P<0.01 except those labeled with * which are not statistically different from the negative control.

Correlation of gene expression levels with antibody treatments in cancer cells

Based on the changes in levels of gene expressions in response to antibody treatments, correlation analysis was performed and presented in (Figures 5A-5C), respectively. Expression levels for most of the genes selected in this study were well correlated among comparisons with different pairs of antibody treatments. Correlation coefficients estimated in the semi-quantitative PCR analysis ranged from 0.90 to 0.94.

Effects of anti-TLR-4 and LPS on the gene regulations of TLR-4 and immunoglobulins

The effects of antibodies against TLR-4 or its ligand, lipopolysaccharide (LPS) on gene expressions of IgG and selected TLR genes were also studied for cultured OC-3-VGH ovarian and C-33A cervical cancer cells by using semi-quantitative RT-PCR (Figure 2A, 4A for OC-3-VGH ovarian cancer cells, and Figures 2D and 4B for C-33A cervical cancer cells). From the histograms presented, it can be shown that anti-TLR-4 treatments of OC-3-VGH and C-33A cancer cells can result in significant down-regulation of TLR-4 gene (Figures 4A and Figure 4B), but not the corresponding IgG expression (Figures 2A and 2D). On the other hand, LPS treatment of OC-3-VGH cancer cells causes more down-regulation of TLR-4 gene expression than that of C-33A cells, but has little effect on the gene expression of cancerous IgG (Figures 2A and 2D).

Discussion

In this study, efforts were made to investigate changes in regulations of a number of genes in response to treatment of cultured OC-3-VGH ovarian as well as C-33A cervical cancer cells with RP215 or antibodies against antigen receptors [3,5]. The results of this study seem to indicate that genes involved in the proliferation and the innate immunity were consistently regulated by both RP215 and anti-antigen receptors.

In view of the nature of carbohydrate-associated epitope recognized by RP215, significantly more CA215 glycoprotein molecules are affected by the binding of this Mab, when compared with those of human immunoglobulins and T-cell receptors expressed by almost all cancer cells [3-5]. Therefore, in terms of the efficacy of cancer therapy, RP215 should be more effective in inducing apoptosis than that of antibodies against antigen receptors. Furthermore, mRP215 and hRP215 do not recognize immunoglobulins or T-cell receptors originated from normal B or T cells, respectively. Therefore, hRP215 Mab can be used uniquely to target various cancerous IgSF proteins on cancer cell surface without affecting normal immune system in humans [3,4].

In fact, the similarity in biological actions between RP215 and antibodies against antigen receptors to induce apoptosis of cultured cancer cells has been clearly established through gene regulation studies [4,35]. With very few exceptions (2 out of 12 genes), changes in expressions of many genes involved in cell growth regulations and innate immunity are consistent among the three different ligands used in this study. RP215 and antibodies against human IgG and T-cell receptors were shown to induce apoptosis and CDC reactions on cancer cells to a similar extent as shown in (Figures 1A-D). Therefore, the common mechanisms of action may be proposed for these three antibody ligands against cancer cells. For example, down regulation of Cyclin D1, c-fos and EGFR might indicate the inhibition of cell growth and proliferation by RP215 as well as antibodies against the other two antigen receptor ligands.

Based on results of semi-quantitative PCR analysis, expression levels of genes selected in this study were well correlated in comprehensive analysis presented in (Figures 5A-C). In response to RP215 treatment of cancer cells, the changes in gene expression levels were well correlated with those by either anti-human IgG ($R^2 = 0.9135$) or anti-T cell receptors ($R^2 = 0.9071$). In addition, the changes in gene expression levels by anti-human IgG and anti-T cell receptors treatments were well correlated ($R^2=0.9433$). This result further supports our contention that the growth of cancer cells is inhibited similarly by RP215 or anti-antigen receptors through more or less the same mechanisms of action.

Toll-like receptors (TLRs) are known to be a family of receptors related to pathogen-associated molecular patterns (PAMP) which have important roles in host-defense from infections [13-16]. Experimental evidence also indicated roles of TLRs in carcinogenesis and growth/

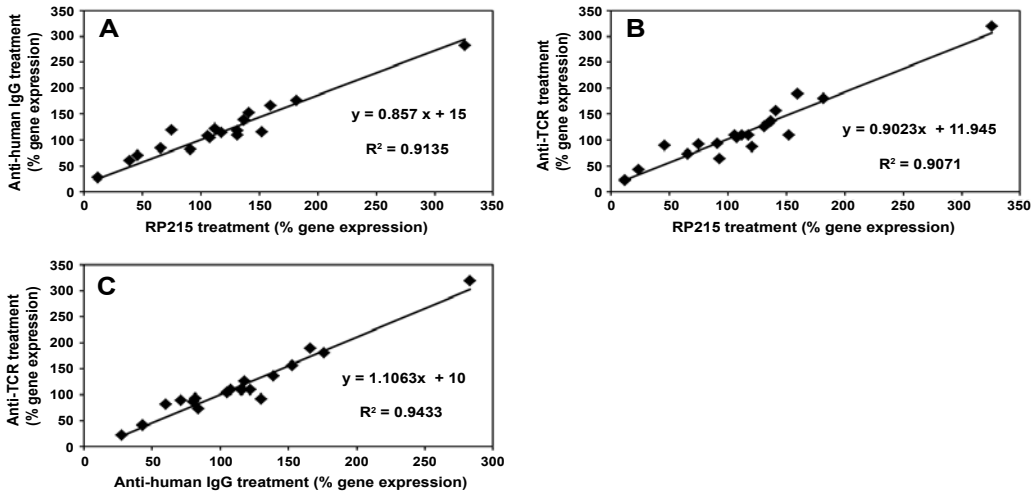

Figure 5: Correlation analysis of changes in gene expression levels with different pairs of antibody treatments. **(A):** RP215 and anti-human IgG treatments; **(B):** RP215 and anti-T cell receptors treatments; **(C):** anti-human IgG and anti-T cell receptors treatments. The correlation coefficients (R^2) are 0.9135, 0.9071 and 0.9433, respectively.

proliferation of cancer cells [28-34]. The innate immunity of cancer cells has been extensively studied and the regulations of TLRs have been implicated in proliferation and survival of cancer cells. Upon treatment with RP215, goat anti-human IgG or anti-T cell receptors, significant changes of expressions in TLR-2 or TLR-3, TLR-4 and TLR-9 genes have been closely associated and consistent with the growth and proliferation of cancer cells reported previously by others [28-34]. Similarly, anti-TLR4 was also shown to induce apoptosis and CDC reactions on cancer cells in (Figure 1) which indicates the surface nature of TLR-4.

Through gene regulation studies, it was demonstrated that expressions of toll-like receptors, especially TLR-3 [28,29,34], TLR-4 [30,36-38] and TLR-9 [31,33,35,39] in cultured OC-3-VGH ovarian cancer cells are strongly influenced by treatments with RP215 or antibodies against antigen receptors. TLR-2 gene expression is also significantly up regulated in C-33A cervical cancer cells. On the contrary, the antibody against TLR-4 or LPS has no significant effects on the expression of cancerous IgG's. This observation seems to suggest that the controls of selected TLR genes by cancer cell-expressed immunoglobulins are unidirectional. Both ovarian and cervical cancer cells revealed the similar effects of antibody treatment on gene regulation of TLR's. The observed patterns of selected TLR gene regulations are so far consistent with the survival, as well as metastasis of various cancer cells reported previously [28-34]. This observation seems to indicate that cancerous immunoglobulins and T-cell receptors are essential or part of components for the innate immune system of cancer cells. Since toll-like receptors are required for the innate immune systems of cancer cells [13-16], the expressions of cancerous immunoglobulins and/or T-cell receptors may in part explain their requirements for the growth and survival of cancer cells [40,11].

It was noticed through these studies that expression of NFκB gene was significantly up regulated by treatments with RP215 or antibodies against antigen receptors in cancer cells. Since NFκB is a transcription factor involved in the activation by various intra- and extra- cellular stimuli, activated NFκB can translocate into nucleus and stimulate the expressions of more than 200 genes involved in many biological functions of various cell types [13-16,41-45]. Similarly, expressions of almost all TLR genes in innate immunity are known to be affected or regulated by activations of NFκB and/or indirectly by interactions with RP215 or antibodies against antigen receptors.

In this study, semi-quantitative RT-PCR was employed to study gene expression pattern changes of cancer cells in response to treatments by RP215 Mab and anti-antigen receptors. Although the results are considered to be preliminary and need further verification by other more advanced methods such as cDNA microarray and miRNA profilings, high degrees of correlations among three related binding ligands are somewhat expected. Our current results would strongly suggest that any of these ligands for the induced apoptosis, or inhibition of growth and proliferation of cancer cells regulates cancer cells similarly and consistently. Furthermore, significant effects of anti-antigen receptors on the gene expressions of different toll-like receptors also imply that the antigen receptors are highly involved in or may be part of the innate immunity system of cancer cells, even though the roles are yet to be defined.

The involvements of key immune components in gene regulations of cancer cells are drawing more attentions with time and will have far reaching implications in cancer immunology [3,4,10-12], not only for the basic understanding of cancer, but also for therapeutic benefits as well. Since the involvement of antigen receptors as part of innate immune system, the present study certainly represents a major advance in our current understanding of cancerous immunoglobulins and T-cell receptors [4].

It was previously established that mechanisms of expressions of cancerous IgG are distinctly different from those of normal immune system, which involves B and T cell interactions, class switching as well as somatic hypermutations [46]. Only limited repertoire (≤100)

of cancerous immunoglobulins in the Fab or V_HDJ_H domains was known to be available for expressions among various cancer cell lines. Therefore, their interactions with the toll-like receptors in the innate immunity as well as their specific recognitions of undefined pathogens or antigens cannot be under-estimated and should deserve further investigations [46,47].

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