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Potential Roles of Cancerous Immunoglobulins in the Immunology of Cancer Cells

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Abstract

Expressions of immunoglobulins among various cancer cells have been known for decades. However, their potential roles and mechanisms of action are not fully understood and need further investigations. A monoclonal antibody designated as RP215 was found to react mainly with the carbohydrate-associated epitope of antigen receptors including immunoglobulins and T cell receptors on the surface of cancer cells, but not in normal immune cells. Therefore, RP215 was used as a probe to replace antibodies against cancerous immunoglobulins to study their roles in the immunology of cancer cells, through extensive biochemical and immunological studies. Both antigen ligands were found to have high correlations in terms of regulations of a number of genes involved in growth/proliferation of cancer cells (e.g. NFκB-1, IgG, P21, Cyclin D1, ribosomal P_1 and c-fos) as well as toll-like receptors. These observations are consistent with the roles of cancerous immunoglobulins in growth/proliferation of cancer cells. Attempts were made to employ cancerous immunoglobulins isolated as CA215 from RP215 immunoaffinity column to detect any specific antigen or autoantibodies in pooled human serum samples. We believe that these anti-CA215 components may be present in human circulation for immune surveillance. From these studies, we believe that both normal and cancer immune systems may co-exist in our body and operate independently and simultaneously, for respective immune surveillance and protection. The balance of these two immune factors in our human body environment may be relevant to the outcome of cancer immunotherapy in humans.

Keywords: RP215; CA215; Cancerous Immunoglobulins; Gene regulations; Immune protection

Abbreviations

ALP: Alkaline Phosphatase; CDC: Completement-Dependent Cytotoxicity; c-fos: Cellular proto-oncogene; CIgG: Cancer cells expressed Immunoglobulin G; Cyclin D1: G1/S phase regulator protein; EIA: Enzyme Immunoassay; ELISA: Enzyme-linked immunosorbent assay; FCS: Fetal Calf Serum; IgG: Immunoglobulin G; IgM: Immunoglobulin M; MALDI-TOF MS: Matrix Adsorption Laser Desorption Ionization-Time of Flight Mass Spectrometry; NFκB: Nucle Factor of kappa-B P105 subunit 1; P_0, P_1, P_2: Ribosomal proteins for protein synthesis; P21: Cyclin-dependent kinase inhibitor 1; POD: Peroxidase; RT-PCR: Reverse Transcription Polymerase Chain Reaction; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; TLR: Toll-Like Receptor; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

RP215 is one of the three thousand monoclonal antibodies generated against OC-3-VGH ovarian cancer cell extract in 1987 and later shown to react mainly with a carbohydrate-associated epitope of cancerous immunoglobulins located on the surface of almost all cancer cells in humans, but not found in normal immune cells [1,2].

Basically, the respective effects of RP215 and anti-human IgG on cancer cells can be used to evaluate the biosimilarity through known functional assays such as induced apoptosis and complement-dependent cytotoxicity [3,4]. Expressions of genes involved in the growth/proliferation [5-8] as well as the innate immunity of cancer cells can also be correlated between RP215 and antibodies against human IgG or cancerous IgG [9]. Results of such comparative analysis should enable us to judge if RP215 is a suitable probe to study the roles of cancerous immunology in cancer immunity [4,10].

With RP215 as a unique probe to elucidate roles of cancerous immunoglobulins in cancer cells, one can then ask if cancer immune system is operated independently of our normal immune system for cancer immune protection [10]. Furthermore, it remains to be demonstrated if any serum antigen (or autoantibodies) can be detected in our body fluid and shown to interact specifically with cancerous immunoglobulins for possible mechanisms of immune protection or surveillance. Through such a comparative analysis, we may be able to reach a better understanding of molecular interactions of cancerous immunoglobulins within our normal immune system. The results of this study may lead to designs of a more successful strategy in human cancer immunotherapy [4,11,12]. Therefore, in this mini-review, experimental evidences through biochemical and immunological studies are presented to identify the possible roles of cancerous immunoglobulins in the immunity of cancer cells by using RP215 as the unique probe [9,11,12].

Materials and Methods

Chemicals

All the chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise mentioned.

Cell lines

OC-3-VGH is an ovarian cancer cell line established in 1986 by Department OBS/GYN of Veterans General Hospital, Taipei, Taiwan. This cell line is of serous origin and can be cultured and maintained in RPMI 1640 medium containing 10% bovine calf serum and penicillin-streptomycin [1]. Other human cancer cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and cultured according to supplier's instructions.

Isolation of CA215 and cancerous Immunoglobulins from shed media of cultured cancer lines

To perform a large scale purification of CA215 and/or cancerous IgG, shed media from cultured cancer cells (OC-3-VGH ovarian cancer cell) were used for purification by affinity chromatography with RP215 as the ligand. Basically, following affinity adsorption and extensive buffer wash, CA215 was eluted from the affinity column with 5 mM citric acid, and then neutralized with 0.2M K_2HPO_4, dialyzed and concentrated. Alternatively, the elution of bound CA215 was made with 3M urea followed by dialysis against PBS and concentrated [13,14].

Cancerous IgG was purified by affinity chromatography with goat anti-human IgG as the ligand with procedure similar to those of CA215 [13].

Affinity isolation of serum antigen from pooled human serum samples

To identify if there are any specific antigen in human sera which may react with CA215 or cancerous immunoglobulins, affinity chromatographies were performed with either purified CA215 or cancerous immunoglobulins as the ligand. By using the same affinity isolation procedure, 2-5 ml of pooled human serum samples was loaded to 5 ml affinity column with either CA215 or cancerous IgG as the separate affinity ligand. The adsorbed proteins were then eluted with 5 mM citric acid followed by collecting fractions, neutralization and concentration. The eluted serum proteins were designated as CA215 (S+), CIgG (S+), respectively, depending on the type of affinity columns used for purification. They were then coated separately on microwells for EIA binding assay to be described later in Results section.

TUNEL assay for induced apoptosis and assay for complement-dependent cytotoxicity reactions

TUNEL apoptosis assay

Relative efficacy was compared between RP215 and anti-human IgG for induced apoptosis of cultured cancer cells [3,9,15,16]. In Situ Cell Death Detection kit, POD (Roche Canada) was employed for detection and quantitation of apoptosis to the cultured cancer cells [3,15,16]. Briefly, OC-3-VGH ovarian cancer cells were cultured in RPMI 1640 medium at 37°C in a CO_2 incubator for 24 hrs until all cells were attached to tissue culture plates. RP215 and goat anti-human IgG were added separately at a final concentration of 1 μg/ml or 10 μg/ml for co-incubation of additional 24 hrs. As the negative control, normal mouse IgG or normal goat IgG of the same concentration was used. At the end of the incubation, the cells were removed from tissue culture wells by trypsinization. Apoptosis of treated cancer cells were quantitatively determined by terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay with supplier's instructions.

Complement-dependent cytotoxicity assay

Complement-dependent cytotoxicity (CDC) assay was performed as reported previously [14]. Briefly, 1×10^5/ml cultured cancer cells in RPMI 1640 containing 10% FCS were added in 24 well tissue culture plate for 2 hrs prior to any treatment. RP215 and goat anti-human IgG at a final concentration of 10 μg/ml were added to separate wells for 15 min. incubation. 3 μl of freshly prepared rabbit baby complement (CL3441; Cedarlane labs Burlington, NC, USA) was then added to each well followed by incubation at 37°C for 2 hrs. At the end of incubation the cells were recovered by centrifugation. Trypan blue (0.4% in PBS; 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 the regular microscope. Normal mouse IgG of the same concentration was used as the negative control. Statistical analysis was performed to determine the significance of the CDC assay [9,17-19].

Effects of RP215 or anti-human IgG treatments on gene expressions of cultured OC-3-VGH ovarian cancer cells

OC-3-VGH ovarian cancer cells were cultured and maintained in exponential phase, RP215 or goat anti-human IgG was added (final concentration 10 μg/ml) separately to 10^4-10^6 cells/experiment in tissue culture wells for 24 hrs incubation at 37°C in a 5% CO_2 incubator. Normal mouse IgG at 10 μg/ml was added as the negative control [9].

Following incubations, the cells were harvested by trypsinization. mRNA were extracted from each cultured and reagent-treated cancer cells and cDNA prepared as described in previous studies [9].

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 RP215 and anti-human IgG on gene regulation changes were performed according to the established method [9,17-19].
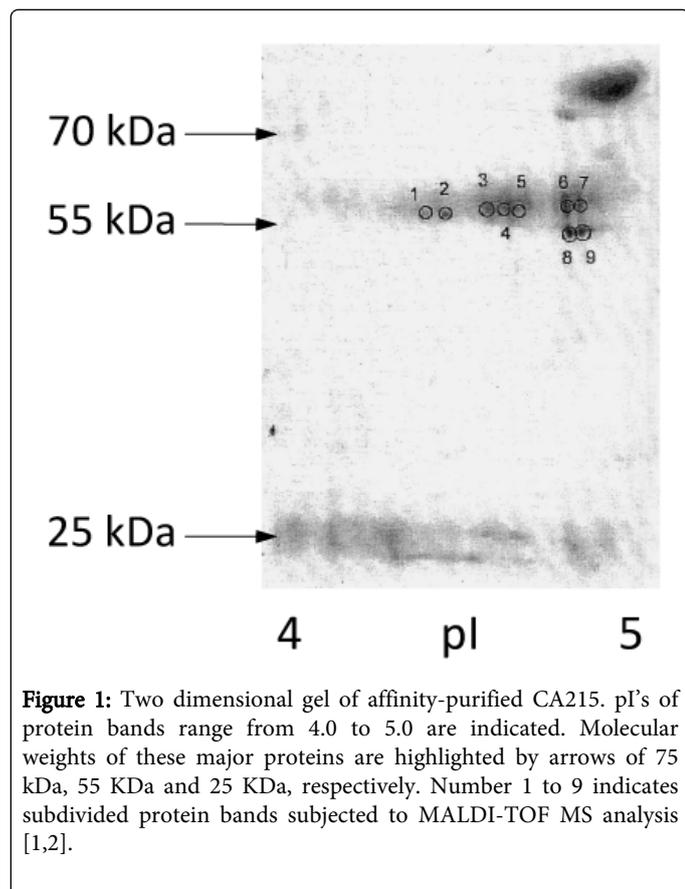
Semi quantitative RT-PCR was performed with a number of genes listed as follows: [9] IgG, NFκB-1, P_0, P_1, P_2, c-fos, P21, Cyclin D1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-7 and TLR-9. Primers required for PCR amplification were reported previously and obtained from Intergrated DNA Technologies Inc. (San Diego, CA, USA) [9].

Results

Purification and protein analysis of affinity-purified CA215, cancerous IgG and serum antigen

By using RP215 as the affinity ligand, the corresponding antigen designated as CA215 can be affinity-purified from shed media of

cultured cancer cells (OC-3VGH ovarian and C-33A cervical cancer cells) [2,13]. The affinity-purified CA215 was concentrated and analyzed by two-dimensional gel as shown in Figure 1. It can be demonstrated that two major groups of broad protein spots were detected with molecular weights ranging from 50-55 kDa and 70-75 kDa, respectively, and pI's ranging from 4.0 to 5.0. By MALDI-TOF MS analysis, the molecular identity of the tryptic peptides of CA215 has been previously determined and found to consist mainly of immunoglobulins heavy chains [2] as well as others related to immunoglobulin super family proteins [14].

Figure 1: Two dimensional gel of affinity-purified CA215. pI's of protein bands range from 4.0 to 5.0 are indicated. Molecular weights of these major proteins are highlighted by arrows of 75 kDa, 55 KDa and 25 KDa, respectively. Number 1 to 9 indicates subdivided protein bands subjected to MALDI-TOF MS analysis [1,2].

Figure 2: A. SDS-PAGE (10% acrylamide) of purified human IgG (lane 1), CA215 (lane 2) and CIgG (lane 3). The molecular weights of protein bands are indicated by arrows as 70 kDa, 55 kDa or 25 kDa, respectively, for immunoglobulin heavy chain (IgM and IgG) and light chains, respectively. **B.** Western blot assay of CA215 to indicate protein bands probed with RP215 followed by ALP-labeled goat anti-mouse IgG (lane 1), or with ALP-labeled anti-human IgM (lane 2) and ALP-labeled anti-human IgG (lane 3), to indicate the position of human IgM and IgG heavy chains, respectively (described in text).

Cancerous IgG (CIgG) was also affinity-purified by anti-human IgG affinity column by a similar procedure to that of CA215. Following purification and concentration, purified CIgG was subject to analysis by SDS-PAGE shown in Figure 2A. It can be demonstrated that three major protein bands with molecular weights ranging from 25, 55 and 70 kDa, respectively were observed in purified CA215. For purified CIgG, two major protein bands corresponding to heavy and light chains (55 & 25 kDa) of human IgG were observed [13,20].

Affinity-purified CA215 was subject to analysis by Western blot assay. When RP215 was used as the probe, two major broad protein bands were detected with molecular size of 50-55 kDa and 70-75 kDa, respectively. When goat anti-human IgG and goat anti-human IgM were used as the separate probes, these two protein bands corresponding to heavy chain of human IgG and IgM were detected at 55 and 75 kDa, respectively. Results of such analysis are presented in Figure 2B.
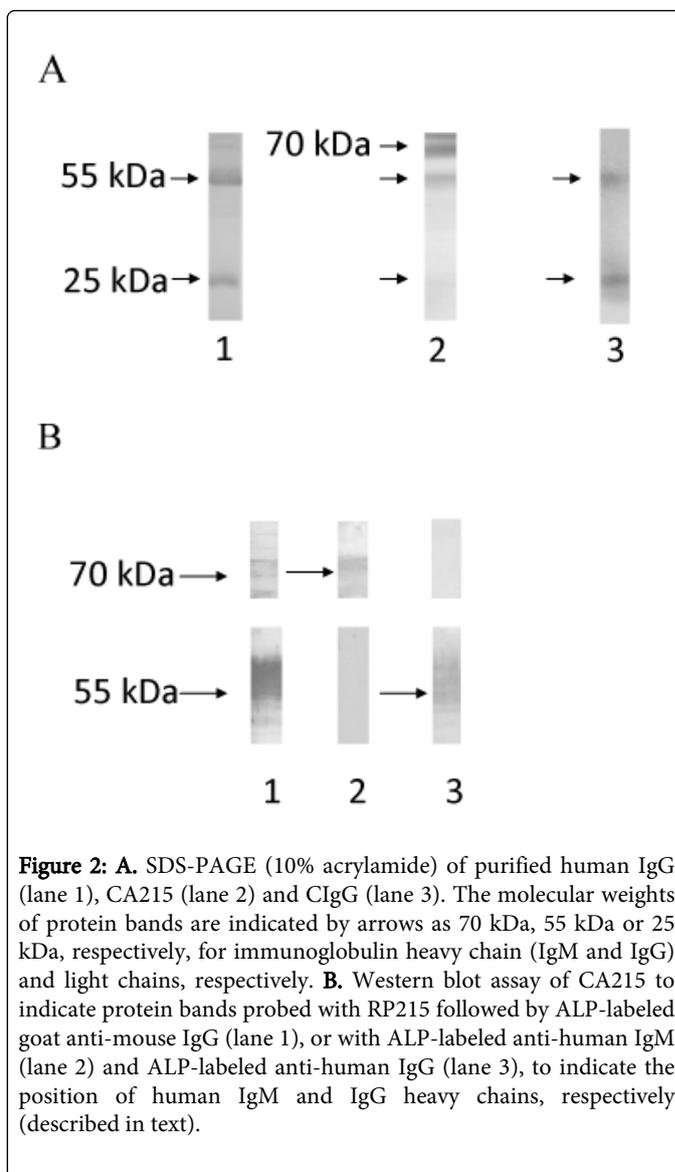
With either CA215 or CIgG as the affinity ligand, specific antigen in pooled human serum specimens can be isolated with respective affinity columns. Similarly, antigen elution can be made with 5 mM citric acid. Following concentration, the eluted proteins designated as CA215 (S+) and CIgG (S+), were collected for further analysis.

Affinity purified CA215 (S+) and CIgG (S+) were characterized by their respective specificity to CA215 or CIgG. Briefly, purified CA215 (S+) and CIgG (S+) were coated separately on microwells for immunobinding ELISA. Biotinylated CA215 was used as the primary probe to study the relative binding between biotinylated CA215 and serum antigen-coated microwells. Biotinylated CA215 of different dilutions (1:50 – 1:200 dilution at 100μg/ml) was added to coated microwells for 3 hr incubation at 37°C. Following wash to remove the unbound biotinylated CA215, alkaline phosphatase (ALP)-labeled avidin (1:500 dilution at 1mg/ml) was added to each well for additional one hour incubation at 37°C. After incubation and wash, the relative binding signals were determined spectrophotometrically at

405 nm by EIA reader following addition of ALP substrate. Microwells coated with unrelated proteins serve as the negative control for the same binding experiments. Dose-dependent binding signals with biotinylated CA215 or with biotinylated CIgG were determined following subtractions of noise from the negative control. They are presented in Table 1 for comparative purposes. Affinity-purified serum antigen was further purified from the same affinity column. The resulting fractions were tested for specific binding with EIA and also shown in Table 1 for comparisons.

Biotinylated Antibodies Ligands used Dilution factor	Serum Antigen-coated Wells		
	CA215 (S+) I[b] first purification	CIG (S+)[b] first purification	CA215 (S+) II[c] second purification
	Δ OD$_{405nm}$ (relative reading)		
CA215			
1:50	1.107 ± 0.261[b]	1.347 ± 0.180	0.312 ± 0.030[d]
1:100	1.175 ± 0.157	1.345 ± 0.092	0.238 ± 0.060
1:200	0.704 ± 0.161	0.917 ± 0.151	0.095 ± 0.005
CIgG			
1:50	1.098 ± 0.172	1.204 ± 0.121	
1:100	0.611 ± 0.048	0.742 ± 0.060	
1:200	0.317 ± 0.061	0.416 ± 0.122	

Table 1: Enzyme immunobinding assay to reveal specific binding between well-coated purified serum antigen, CA215(S+) or CIgG(S+) and CA215 or CIgG[a].

[a]Biotinylated CA215 or biotinylated CIgG of known dilution (100 µg/ml) was added to serum antigen coated microwells for 3hr incubation at 37°C. ALP-labeled Avidin was added for additional 60 min incubation, followed by color substrate development at 405nm (See text). Unrelated proteins coated wells serve as the negative control under the same conditions. The data are presented as duplicates following the subtraction of corresponding negative control which serves as the blank. Relative signals using unrelated non-binding proteins coated wells as the negative control.

[b]Microwells coated with eluted serum antigen from the first purification by affinity column.

[c]Microwells coated with eluted serum antigen [CA215(S+) I] re-purified by the same affinity column [CA215(S+) II].

[d]Non-adsorbed serum antigen after re-purification was coated on microwells and was used as the corresponding negative control to reveal the positive binding between CA215 and re-purified serum antigen.

Enzyme immunoassays (EIA) and Western blot assay were also employed to determine the immunospecificity of the affinity-purified proteins such as CA215 and CIgG. A sandwich enzyme immunoassay was performed by using RP215 for both capturing and signal detection. Affinity-purified CA215, human IgG and CIgG were employed separately to determine relative immunoactivity with RP215-based sandwich enzyme immunoassay. It was found that under the same protein concentrations, CA215 has substantially higher

activity when compared to that of CIgG. In contrast, no binding signal was observed with human IgG. Results of such as enzyme immunoassay are presented in Figure 3.
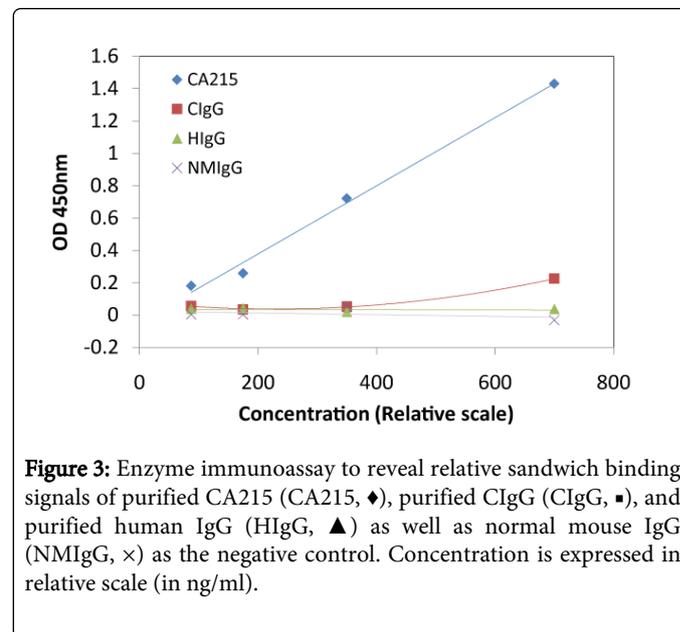

Figure 3: Enzyme immunoassay to reveal relative sandwich binding signals of purified CA215 (CA215, ♦), purified CIgG (CIgG, ▪), and purified human IgG (HIgG, ▲) as well as normal mouse IgG (NMIgG, ×) as the negative control. Concentration is expressed in relative scale (in ng/ml).

Comparative functional studies with induced apoptosis and complement-dependent cytotoxicity reactions

As reported in several previous studies [3,12,14,15], RP215 and anti-human IgG were found to induce apoptosis of cultured cancer cells *in vitro* at antibody concentrations of 1µg/ml to 10µg/ml, respectively [4]. As presented in Figure 4A, both RP215 and anti-human IgG were shown to induce apoptosis of cultured cancer cells after 24 or 48 hours incubation. As a negative control, normal mouse IgG revealed little or no effect on the induced apoptosis of cultured cancer cells [4,9]. Results of this comparative analysis at antibody concentrations of 1 µg/ml or 10 µg/ml are statistically significant.

Complement-dependent cytotoxicity (CDC) reactions in the presence of 10 µg/ml RP215 or goat anti-human IgG were also studied in the presence or absence of complement [3]. As illustrated in Figure 4B, little or no CDC-induced cell lysis was observed in the presence of complement or normal antibody alone. CDC reactions were observed when RP215 or goat anti-human IgG of 10 µg/ml were incubated with cultured cancer cells in the presence of complement. The results of CDC reactions are statistically significant.

Effects of RP215 and anti-human IgG on gene expression of cancer cells

Effects of RP215 and anti-human IgG on expression of selected genes of cultured cancer cells were studied and compared [9]. In the case of OC-3-VGH ovarian cancer cells, both RP215 and anti-human IgG were found, respectively to up-regulate the genes expressed by NFκB-1, IgG, P21, and ribosomal P1. By comparison, down regulation of Cyclin D1 and c-fos was observed. It was generally observed that patterns of gene regulations of cultured cancer cells affected by these two antibody ligands are similar to each other, when studied with either OC-3-VGH ovarian or C-33A cervical cancer cell line [9].
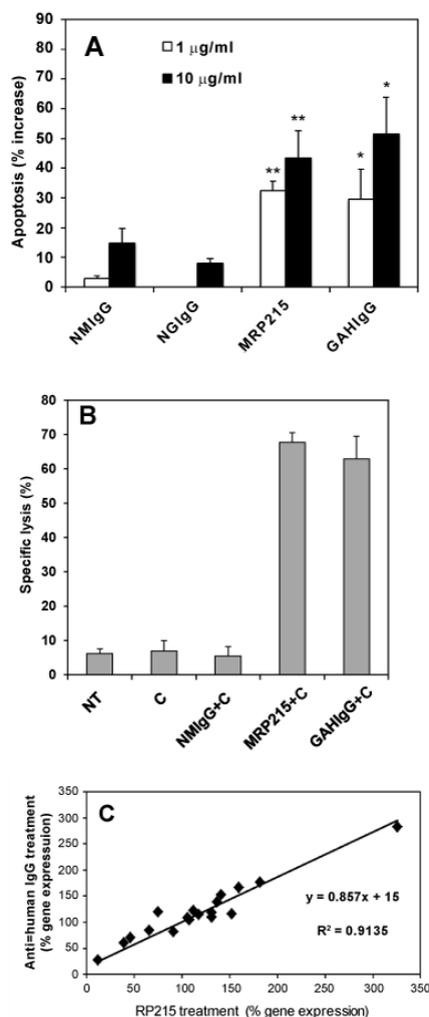
Figure 4: A. Effects of treatment of different immunoglobulins on induced apoptosis of cultured OC-3-VGH cancer cells, including normal mouse IgG (NMIgG), normal goat IgG (NGIgG), mouse RP215 (MRP215), goat anti-human IgG (GAHIgG) Open and solid columns represent antibody concentrations of 1 μg/ml and 10 μg/ml, respectively. All data presented are statistically significant at * P<0.01 and ** P<0.001. B. Complement-dependent cytotoxicity (CDC) reaction in the presence or absence of complement and/or antibodies containing 10 μg/ml of mRP215 or normal immunoglobulins for the assays of cancer cells. Lane 1: no treatment (NT); Lane 2: freshly prepared rabbit baby complement (C) only (3 μl in each experiment); Lane 3: normal mouse IgG plus complement (NMIgG+C); Lane 4: mouse RP215 plus complement (MRP215+C); Lane 5: goat anti-human IgG plus complement (GAHIgG+C). C. Correlation analysis of RP215 and anti-human IgG on gene regulation changes to cancer cells in culture (OC-3-VGH and C-33A cancer cell lines). Each point (♦) represents for one gene used in the semi-quantitative RT-PCR experiment, including: IgG, NFκB-1, P_0, P_1, P_2, c-fos, P21, cyclin D1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-7 and TLR-9. A correlation coefficient of $R^2 = 0.9135$ was obtained statistically. Details of these studies were presented previously in ref [9] (obtained with permission).

Effects of RP215 and anti-human IgG on gene expressions of toll-like receptors in OC-3-VGH ovarian cancer cells were also investigated [9]. Following respective treatments with RP215 and anti-human IgG, TLR-3 gene was up-regulated significantly (2 or 3 fold increase), whereas those of TLR-4 and TLR-9 were down regulated by as much as 2 to 5 fold [9].

The correlation analysis of gene regulations was also performed with RP215 and anti-human IgG. It was generally observed that the expression levels for most of genes selected in this study were well correlated following respective treatments with either RP215 or anti-human IgG on cancer cells [9]. The correlation coefficient in this comparative semi-quantitative RT-PCR analysis was estimated to be 0.91 for between RP215 and anti-human IgG. Results of such correlation analysis by adopting the individual effects with a group of one dozen genes are diagramatically presented in Figure 4C.

Discussion

The main objective of this study is to investigate roles of cancerous immunoglobulins in immunology of cancer cells through several biochemical and immunological studies, including affinity-purification of relevant antigen and antibodies, characterization as well as gene regulation analyses. With RP215 as the unique probe, it has become possible to advance our understanding about the potential roles of immunoglobulins expressed by cancer cells [21].

Initially, with RP215 as the ligand, it has been possible to isolate CA215 from shed media of cultured cancer cell extract from cell lines derived from many different tissue origins (Figure 1) [22]. Similarly, cancerous IgG (CIgG) can also be purified with goat anti-human IgG as the affinity ligand and characterized with respect to its affinity to RP215 which recognizes mainly the heavy chains of cancerous IgG (Figure 3) [2].

Attempts were made to identify the possible serum antigen (or autoantibodies) which might show specific affinity to cancerous IgG. Purified CA215 or CIgG were used as the affinity ligands to isolate serum antigen or autoantibodies and further characterized with enzyme immunobinding assay [23,24]. By using ELISA with purified serum antigen coated on microwells, we have been able to demonstrate specific and significant dose-dependent binding between CA215 or CIgG with purified serum antigen or autoantibodies, when compared to those of the negative control. Judging from the results of enzyme immunobinding assay, it is possible to demonstrate significant binding between CA215 or CIgG and purified serum antigen. The positive binding between CA215 and re-purified serum antigen was also demonstrated following the same binding ELISA as indicated in Table 1. Therefore, the specific binding of re-purified serum antigen to CA215 or CIgG can be documented. However, the re-purified serum antigen was obtained in small quantity with contamination of major serum proteins and difficult to perform molecular characterization at this moment by other means such as Western blot assay and MALDI-TOF MS analysis [1,2]. In view of the fact that highly purified serum antigen was found to cross-react with anti-human IgG or IgM, it was therefore suggested that autoantibodies may be present in human circulation against cancerous immunoglobulins and/or CA215 through the expression of RP215-specific immunodominant epitope [15]. (unpublish observations) Further biochemical and immunological studies of serum antigen or autoantibodies are in progress and would be required to clarify its molecular nature and origins as well as possible biological roles in cancer.

In this study, several functional studies were performed to demonstrate biosimilarity between RP215 and anti-human IgG [3]. Both were shown to induce apoptosis of cultured cancer cells indicating that cancerous IgG is essential for growth/proliferation of cancer cells [3,4,9,12, 14-16]. Both exhibit complement-dependent cytotoxicity reactions to cancer cells indicating the surface nature of cancerous immunoglobulins in cancer cells [3,14-16].

Previous studies with RP215 and antibodies against antigen receptors strongly suggested that cancerous immunoglobulins play significant roles in innate immunity of cancer cells, as demonstrated in previous studies [9]. Both receptor ligands, RP215 and anti-human IgG were shown to have strong effects on the regulation of toll-like receptor genes in cancer cells. For example, the up-regulation of TLR-3 gene and down-regulations of TLR-4 and TLR-9 were consistently demonstrated by respective treatments with RP215 and anti-human IgG [9]. This observation was not totally unexpected, since expressions of cancerous immunoglobulins as well as toll-like receptors were highly regulated by NFκB-1, which is a key transcription factor for both innate and adapted immune system in our human body, including cancer cells [9,25-28]. Based on these studies, it is therefore concluded that expressions of cancerous immunoglobulins may be closely related with the innate immune system of cancer cells by maintaining survival of cancer cells through the functional expression of toll-like receptors.

In addition, cancerous immunoglobulins are expressed with mechanisms which are very different from those of B cells [4,24,29-31]. For example, the antigen receptors are expressed by B and T lymphocytes in normal immune system [8], while both can be co-expressed by the same cancer cell clones [32-34]. Expressions of immunoglobulins in B cells require a series of activations, differentiation, somatic hypermutation as well as class switching [35-37]. Most of these mechanisms in normal B lymphocytes do not exist in cancer cells [38,39]. In addition, expressions of cancerous toll-like receptors are strongly influenced or regulated by cancerous immunoglobulins [9]. Furthermore, cancerous immunoglobulins are known to carry very different O-linked and N-linked glycosylation patterns from those of normal human IgG. [10,13]. These observations led us to hypothesize that there are two separate immune systems in our human bodies. One is our normal immune system for the immune surveillance to fight against foreign pathogens or cancer, including the presence of autoantibodies, or specific binding antigen [4]. Cancer cells, on the other hand, may have their own independent immune system which serves for immune protection of cancer cells. These two systems can operate independently through different mechanisms and objectives within the same body environment. Cancer immunotherapy will work effectively only when the strategy to suppress cancer immune system can be designed.

Acknowledgement

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