ACTA SCIENTIFIC CANCER BIOLOGY (ISSN: 2582-4473)

Volume 4 Issue 3 March 2020

Review Article

RP215 Monoclonal Antibody with O-Glycan Linked Epitope in Cancerous Immunoglobulins and Potential Clinical Applications

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DOI: 10.31080/ASCB.2020.04.0210

Received: January 29, 2020

Published: February 19, 2020

Abstract

RP215 monoclonal antibody was initially generated against OC-3-VGH ovarian cancer cell extract and was shown to recognize a carbohydrate-associated epitope located mainly in heavy chain immunoglobulins expressed on the surface of most human cancer cells. Efforts were made to elucidate the primary structure of this unique epitope through comprehensive biochemical and glycosyl-linkage analysis. Upon 48-hr treatments of cancer cells with glycan analogs or neuraminidase, significant changes were observed regarding the ability of RP215 to induce anti-proliferation of cancer cells. Results of this analysis may suggest the involvement of amino acid residues as well as sugar moiety of immunoglobulin heavy chain in the epitope recognition by RP215. Treatment with tunicamycin to cancer cells in culture has little effect on RP215 immunoactivity, indicating the involvement of O-linked glycan in the RP215-specific epitope recognitions. It was further observed that RP215 binding to cancerous immunoglobulins or CA215 was not affected by anti-human IgG-Fc, but completely inhibited by anti-human IgG-Fab. This observation may suggest the epitope locations in the Fab or variable regions of cancerous immunoglobulin heavy chains. Based on the results of glycosyl-linked analysis, it can be suggested that RP215-specific epitope is associated with CORE-1 structure of O-linked glycans such as Tn or TnS. RP215-specific epitope was identified mainly in cancerous immunoglobulins, but not from those produced by normal B cells. This unique antibody can therefore serve as a useful probe in targeting cancer cells of many tissue origins, including blood tumors. Interactions of RP215 with cancer cells of leukemic origins were demonstrated by comprehensive FACS analysis. The humanized forms of RP215 have been developed as antibody-based anti-cancer drugs or cancer therapy with CAR-T cell or related constructs. In addition, RP215-based immunoassays can be used routinely for monitoring serum levels of CA215 among patients with many types of cancer at different stages, when used singly or in combination with other known cancer biomarkers.

Keywords: CA215 Monoclonal Antibody; RP215 Monoclonal Antibody; O-Glycan Linked Epitope; Cancerous Immunoglobulins; Anti-Cancer Drugs and Therapy

Abbreviations

AFP:α-Fetoprotein; CA125:A Cancer Biomarker; CA15-3:A Cancer Biomarker; CA199: A Cancer Biomarker; CAR-T: Chimeric Antigen Receptor-T Cells Construct; CDC: Complement-Dependent Cytotoxicity Reactions; CEA: Carcinoembryonic Antigen; FACS Analysis: Fluorescence-Activated Cell Sorting; GALNAC: N-Acetyl Galactose; GC-MS: Gas Chromatography-Mass Spectrometry; IL-2: Interleukin-2; IL-7: Inteleukin-7; LC-MS-MS: Liquid Chromatography-Tandem Mass Spectrometry; MALDI-TOF MS: Matrix Adsorption Laser Desorption, Ionization Flight Mass Spectrometry; MHC: Multiple Time of Histocompatibility Locus; MTT Assay: An Assay For Cell Proliferation Based On The Use Of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide; Scfv: Single Chain Fragments In The Variable Regions of Antibody; T: GalNAc-Gal; Tn: GalNAc; Tns: GalNAc-NeuAc; TS: Sialyl-T Antigen

Introduction

RP215 monoclonal antibody reacts specifically with a carbohydrate-associated epitope of immunoglobulin heavy chains expressed on the surface of many human cancer cells, but not found in those from normal B cells [1-4]. In the past decade, numerous biochemical and immunological studies have been performed to elucidate the molecular mechanisms of action of RP215 to cancer cells [5-8]. RP215 was shown to induce apoptosis and/or anti-proliferation to cancer cells *In vitro* [8]. This monoclonal antibody was shown to reduce volumes of implanted tumor in nude mouse animal models [9]. Humanized forms of RP215 were made available and utilized in the constructions of CAR-T cells (chimeric antigen receptor-T cells) for cancer therapy [10]. The efficacy of humanized scFv -RP215 (single chain variable fragments of humanized RP215) has been demonstrated by significant cytotoxic killings of C33A cervical cancer cells in culture [10]. Cytokine releases such as IL-2, IL-7 and Interferon α have been observed following co-incubation of CAR-T cells and cancer cells in culture [10]. Therefore, it was suggested that humanized RP215 can be used to target cancer cells of many tissue origins including those of leukemic cell origins. The functional roles of cancerous immunoglobulins in cancer cells were also investigated by using RP215 as the probe through mutual interactions with human serum proteins [11]. Following affinity isolations of CA215 (mainly cancerous immunoglobulins) and cIgG (cancerous immunoglobulins), they were utilized as affinity ligands to identify those human serum proteins which may interact with CA215 or cIgG [8]. The affinity-isolated human serum proteins were subject to analysis respectively by the MALDI-TOF MS method [8]. It was shown from results of such analysis, quite a few human serum proteins were identified with known properties of anti-cancer or pro-cancer in nature [11]. Therefore, it was hypothesized that immunoglobulins expressed by cancer cells may serve to interact with certain human serum proteins for growth/proliferation (pro-cancer) as well as for protection/survival (anti-cancer) of cancer cells in human circulations [11].

During the last decade, substantial information has been accumulated regarding the functional roles of cancerous immunoglobulins as well as the molecular mechanisms of action of RP215 to cancer cells *In vitro* and *In vivo* [12]. Efforts were also made to elucidate the primary structure of carbohydrate-associated epitope in cancerous immunoglobulins recognized by RP215 [13]. This is an essential step to establish if RP215 is a suitable probe to target cancerous immunoglobulins displayed on cancer cell surface for anti-cancer drug development or for cancer therapy.

Expressions of RP215-specific epitope of many cells of leukemic origins were also demonstrated by FACS analysis. It was further documented that both CA215 and CD19 are co-expressed on several targeted leukemic cells. Data are presented to reveal potential therapeutic application of RP215 in many blood tumors. In this review, structural elucidations of carbohydrate-associated epitope specific to RP215 were presented and summarized to document its significance in cancer immunology and potential clinical applications in cancer therapeutics [14,15].

Results and Discussion

Involvement of O-Glycans in RP215-Specific Epitope in Cancerous Immunoglobulin

Early studies revealed that pre-treatments of cancer cells or CA215 with $NaIO_4$ resulted in a total loss of RP215 binding or immunoactivity [19], indicating possible involvements of carbohydrate moieties in the epitope recognition by RP215 [8,19]. Similarly, it was also observed RP215-specific epitope or CA215 is sensitive to protease treatments [6,16], implying that the amino acid residues of cancerous immunoglobulins may also be part of the epitope recognition [8]. Glycosyl linkage analysis was performed to elucidate the detailed molecular structure of the carbohydrate-associated epitope specific to RP215. Anti-proliferative activities of RP215 to cancer cells were also studied with MTT assays [17,18] following treatments of cancer cells with glycan analogs or neuraminidase in cancer cell culture.

Effects of glycan analogs or neuraminidase on proliferation of cancer cells induced by RP215

RP215 has been shown to affect the proliferation of cancer cells *In vitro* [17,18]. Following 48 hours incubation of RP215 (20 μg/mL) in cancer cells, the anti-proliferative activities of culturing cancer cells were estimated either in the presence or absence of 2 mM GalNAc-Bn (Benzyl-2-acetylamido-2-deoxy-α-D-galactopyranoside) or NeuAc (N-acetyl neuraminic acid). The results of these two studies are presented, respectively in Table 1A with OC-3-VGH ovarian cancer cells as the example [6].

Generally speaking, the anti-proliferative activities by RP215 were reduced significantly upon co-incubation with either glycan

analogs. These results suggested that glycans are involved in the epitope recognition by RP215 (Table 1A). When neuraminidase (at 5 mIU/mL or 10 mIU/mL) was added to culturing cancer cells (OC-3-VGH ovarian cancer cells), anti-proliferative activities of RP215 was also significantly affected as shown in Table 1B. Hydrolysis or removal of neuraminic acid or terminal sialic acid resulted in a reduction of RP215's ability to induce antiproliferation to cancer cells (Table 1A). Compared to the negative control, the antiproliferation activities induced by RP215 upon removal of sialic acid residue by neuraminidase were significantly enhanced either in the case of OC-3-VGH ovarian (Table 1B) or PC-3 prostate cancer cells (Table 1C). The experiments with co-incubation of neuraminidase in culturing cancer cells seemed to suggest that sialic acid residues play important roles in the cell proliferation induced by RP215, as revealed in Table 1B and 1C [17]. Therefore, the enzymatic removal of sialic acid residues from cancerous immunoglobulins can significantly enhance the ability of RP215 to affect cell proliferation on culturing cancer cells. The results also document that both GalNAc and NeuAc are involved in the epitope recognition by RP215 on cancer cells.

Exp NO	Treatment conditions (48 hr incubation in culture medium)	Cell proliferation (% of control)
1	Negative control (none added)	100
2	RP215 (20 μg/ml)	24 ± 2
3	GalNAc α-B_n (2 mM)	80 ± 4
4	NueAc (2 mM)	82 ± 4
5	RP215 (20 μg/ml) and GalNAc α-B_n (2 mM)	43 ± 5
6	RP215 (20 μg/ml) and NueAc (2 mM)	40 ± 2

Table 1A: MTT assays with OC-3-VGH ovarian cancer cells in serum supplemented medium to reveal the involvement of GalNAc and NeuAc moieties in the epitope recognitions of RP215[a].

a. Exp 2 vs. Exp 5, p≤0.05; Exp 2 vs. Exp 6, p≤0.05;

Exp NO	Treatment conditions (48 hr incubation in culture medium)	Cell proliferation (% of control)
7	Negative control	100
8	RP215 (20 μg/ml)	43 ± 1.6
9	Neuraminidase (5 mIU/ml)	91 ± 4
10	Neuraminidase (10 mIU/ml)	76 ± 8
11	RP215 (20 μg/ml) and Neuraminidase (5 mIU/ml)	26 ± 6
12	RP215 (20 μg/ml) and Neuraminidase (10 mIU/ml)	25 ± 7

Table 1B: MTT assays with OC-3-VGH ovarian cancer cells in serum free medium to reveal the effects of neuraminidase and /or RP215 on cell proliferation.

a. Exp 8 vs. Exp 11, p≤0.05; Exp 8 vs. Exp 12, p≤0.05;

Exp NO	Treatment conditions (48 hr incubation in culture medium)	Cell proliferation (% of control)
13	Negative control	100
14	RP215 (20 μg/ml)	27 ± 5
15	Neuraminidase (5 mIU/ml)	105 ± 5
16	Neuraminidase (10 mIU/ml)	110 ± 3
17	RP215 (20 μg/ml) and Neuraminidase (5 mIU/ml)	4.0 ± 0.5
18	RP215 (20 μg/ml) and Neuraminidase (10 mIU/ml)	5.0 ± 0.3

Table 1C: MTT assays with PC-3 prostate cancer cells in serum free medium to indicate the effects of neuraminidase and RP215 on cell proliferation.

a. Exp 14 vs. Exp 17, p≤0.05; Exp 17 vs. Exp 18, p≤0.05;

Table 1: Effect of carbohydrate moieties and neuraminidase on cancer cell proliferation induced by RP215 monoclonal antibody.

Elucidation of primary structure and location of RP215-specific epitope in cancerous immunoglobulins

From the summary of competitive binding and enzyme inhibition studies in Section II of this review, it can be established that RP215-specific epitope in cancer cells are carbohydrate-associated. GalNAc and/or NeuAc might be involved in the recognition of RP215-specific epitope. Efforts have been made to identify and locate RP215-specific epitope in cancerous immunoglobulins or other related minor glycoproteins [20]. Therefore, the cognate antigens designated as CA215 were affinity-purified from cancer cell extract of OC-3-VGH cancer cells and shed medium. The isolated CA215 was subject to molecular analysis by MALDI-TOF MS [8]. It was revealed that the majority of RP215-specific epitope was found to be immunoglobulin heavy chains expressed by cancer cells (~60%), but rarely formed in normal immunoglobulins of B cell origins. The RP215- specific epitope was also found in T cell receptors (~5.7%), MHC proteins (4.9%) and adhesion molecules (8.1%) as well as several other related or unrelated glycoproteins [20,21].

By Western blot assay, it was observed RP215 react predominantly with protein bands having a subunit molecular size of 60 kDa detected either from cancer cell extract or from purified CA215. Therefore, it can be assumed that the majority of glycoproteins recognized by RP215-specific epitope are those of immunoglobulin heavy chains expressed by cancer cells [8].

The exact location of RP215-specific epitope in the heavy chain immunoglobulins or CA215 was determined by RP215-based enzyme immunoassays [6]. The results of such analyses are presented in figure 1 for comparisons. The binding of CA215 purified from shed media of culturing cancer cells was shown to be inhibited by the presence of goat-anti-Human IgG-Fab (1 µg/mL) but not by goat-anti-Human IgG-Fc (Figure 1A). This observation suggests that the glycan-associated epitope recognized by RP215 is located in the Fab or variable regions of heavy chain immunoglobulins, but not in the Fc regions [14,15]. The results of RP215-based immunoassays and inhibition assays of CA215 are presented in Fig 1A for comparisons.

The carbohydrate-associated RP215-specific epitope could either be O-linked or N-linked glycans. Biosynthesis of N-linked glycans is known to be inhibited by tunicamycin in culturing cancer cells. Following 48-hr incubation with tunicamycin (1 µg /mL) and OC-3-VGH ovarian or C-33A cervical cancer cells, the immunoactivity of CA215 extracted from the shed medium of culturing cancer cells was not affected as presented in Figure 1B. This observation leads us to conclude that N-glycan may not be involved in the epitope recognized by RP215.

Figure 1 (A): RP215-based enzyme immunoassays to reveal dose-dependent signal or activity of CA215 (○) expressed in AU/ml) and effects of goat antihuman IgG-Fc (1µg/ml) (●) and goat antihuman IgG-Fab (1µg/ml) (▲) on CA215 signal or activity.

1 (B). Effects of 48 hr tunicamycin treatments (1 µg/ml) on CA215 activity in the supernatant of two cultured cancer cells for OC-3-VGH and C-33A. To each cell line the left column is the negative control (assuming 100% activity) without drug-treatment, and the right column is the relative CA215 activity following the drug treatment (expressed in triplicate)

1 (C). Elucidated and proposed O-glycan structure associated with RP215-specific epitope as well as that of MUC 1 for comparisons (14, 15 with permit).

Glycosyl-linkage analysis of O-glycans recognized by RP215-specific epitope

Studies with inhibition of RP215 binding to cancer cells or CA215 by GalNAc analogs as well as neuraminidase strongly suggest that O-linked glycans are involved. Therefore, glycosyl linkage analysis by GC-MS analysis of purified CA215 and cIgG (cancerous immunoglobulins) was performed and compared. Initially, O-glycan was released by reductive β-elimination followed by prepa-

ration of partially methylated alditol acetates for glycosyl linkage analysis by GC-MS method. Permethylated O-glycans from samples of CA215 and cIgG were analyzed by MALDITOF-MS method to monitor complete reaction and derivatization. Our results of O-glycan analysis revealed that O-glycans derived from CA215 or cIgG consist of tri-saccharides or shorter fragments with terminal N-acetylneuraminic acid (NeuAc) as a major O-glycan component. This study also revealed that identified O-glycans are basically Core-1 structure with 3-linked and/or 3,6-linked GalNAcitol (Tn or Tns). The diagrammatic structure of O-linked glycan structures associated with RP215-epitope are proposed and presented in Figure 1C for comparisons [14,15].

Potential clinical applications of cancerous immunoglobulins with unique RP215-specific epitope

Through years of biological and immunological studies, it was well established that RP215 recognizes O-linked glycan-associated epitope in the heavy chain immunoglobulins expressed by most cancer cells in humans (Section IV). Therefore, RP215 can be used as a probe to study potential roles of cancerous immunoglobulins among human cancer cells. It was well established that apoptosis can be induced by 1 - 10 μg/mL RP215 to all of known human cancer cells studied [17]. Furthermore, complement-dependent cytotoxic toxicity (CDC) reactions were also observed following treatments of RP215 to culturing cancer cells. In addition, by using nude mouse animal model experiments, it was clearly demonstrated that the implanted tumor volumes (implanted ovarian, lung or cervical cancer cell lines) were reduced dose-dependently in response to the injected RP215 [20].

Molecular biological studies were also performed following the incubation of RP215 or goat anti-human IgG with culturing cancer cells for comparisons of changes in gene regulations among those involved. They were highly correlated ($R^2 \geq 0.9 – 0.95$) [22].

The functional roles of cancer immunoglobulins were investigated, especially their interactions with human serum components. Human serum proteins which may interact with RP215 or cancerous immunoglobulins were isolated by two-step immune-affinity chromatography and subject to analysis by LC-MS/MS method [11]. Greater than 80 – 85% human serum proteins detected and identified were found to react with CA215 and/or cancerous immunoglobulins, similarly. These human serum proteins or fragments have been previously detected, studied and classified as "anti-cancer" or "pro-cancer" in nature [11]. These observations led us to hypothesize that cancerous immunoglobulins may serve to interact with "anti-cancer" serum proteins for survival/protection of cancer cells in human circulation. At the same time, some of them may also react with "pro-cancer" serum proteins for growth/proliferation of cancer cells in the human serum environment [11].

Immunodiagnostic applications of RP215-based immunoassays

It was established from many previous biochemical studies that RP215 reacts preferentially with heavy chain immunoglobulins expressed on surface of most human cancer cells [14,15]. O-linked glycans of known structures (Tn and TnS) were involved in the epitope recognition by RP215. RP215-based immunoassays can be used to determine serum CA215 levels among cancer patients for cancer monitoring. Serum levels of CA215 among cancer patients could be monitored to follow their growth and/or proliferation as well as metastatic stages of the tumors [23]. Therefore, RP215-based enzyme immunoassays may be a suitable tool to monitor serum levels of CA215, especially before and after surgical operations or chemo/radio therapy [24]. In addition, monitoring of CA215 levels among cancer patients can also be combined with other cancer known biomarkers which are more or less tissue-specific to certain types of cancer [24] such as CA215, CA15-3, CA19, α-fetoprotein and CEA etc [23]. Combined uses of CA215 assay with those of other biomarkers may result in much higher detection rates or sensitivity in terms of cancer monitoring [23,24]. Therefore, we believe that RP215-based immunoassays should show benefit in suitable monitoring of many types of cancer during therapeutic treatments with anti-cancer drugs and/or cancer therapy.

Immunotherapeutic applications – RP215-based anti-cancer drugs or CAR-T cell constructs

RP215 may be beneficial in targeting mainly cancerous immunoglobulins expressed on cancer cell surface for application in cancer therapy. These cancer cell-expressed immunoglobulins are essential for growth/proliferation as well as protections of cancer cells under our human environments [11]. Upon binding interactions of cancer cells with RP215 or its humanized forms, induced apoptosis and complement-dependent cytotoxicity reactions can be induced to kill cancer cells *In vitro* or *In vivo* [17]. Early proof-of-concept nude mouse experiments also revealed dose-dependent reductions of implanted tumors following injections of RP215

monoclonal antibody to nude mice. Therefore, humanized RP215 may be suitable for clinical applications as anti-cancer drugs [9,20].

Alternatively, cancer therapy can be achieved through constructs of CAR (chimeric antigen receptor)-T cells [10]. Briefly, the scFv gene (single chain antibody variable fragments) of RP215 was inserted into lentiviral vector to form a construct of chimeric antigen receptor (CAR) [10]. T cells isolated from a given patient were transfected with CAR containing the specific RP215 scFv gene. Following *In vitro* expansion and re-transfection of CAR-T cells back to autologous cancer patient, specific cytotoxic cell killing and cytokine releases resulted in destructions of cancer cells. The goal of cancer therapy via the technology of CAR-T cell constructs can therefore be achieved. So far, we have been able to demonstrate that CAR-T cells expressing RP215 scFv gene are capable of cytotoxic killing of C33A cervical cancers *In vitro* [10]. Simultaneously, cytokines such as IL-2, IL-7 and -interferes were released.

Potential therapeutic applications of RP215-CAR-T cell construct for blood tumors

Recently, we have been able to show that many cell lines of leukemic origins co-express both CD19 and CA215, including Nalm-6 (Acute lymphoblastic leukemia), and Burkit lymphoma such as Daudi, NAMALWA and Raji (Table 2). ScFv (single chain fragment) of RP215-CAR-T cell construct can be used to replace CD19-CAR-T cells for effective treatments of the latter for many types of blood tumors [25] with little damage to normal B cells. This assumption was supported by the observations that scFv -hRP215 was shown to bind CD19-positive cell lines by using FACS analysis when FACS analysis was performed with any of including the four leukemic cell lines. Results were presented in Table 2 for comparisons. Among any of these cancer cell lines, luciferase-GFP-conjugated secondary antibody was added to the cells for detection of the primary antibody (scFv -RP215). The controlled group was treated with the second antibody only. In Anti-CD19-scFv -hFC (derived from FMC63) was used to stain Raji cells as the positive control. Through FACS analysis, percentages of the target positive cells were shown to be 61.8%, 57.7%, 14.9%, and 50% for Daudi, Nalm-6, NAMALWA and Raji cells, respectively. Results of FACS analysis are demonstrated in figure 2 for comparisons. Percentages of positive binding to target cells are presented in Table 3. Therefore, in conclusion, CD19-positive leukemic cell lines can be targeted by scFv-hRP215 CAR-T cell constructs. It is worth noting that scFv from hPR215 binds greater than 50% to Dande, Nalm-6, and Raji cells (Table 3).

Figure 2: FACS analysis of four selected leukemic cell lines to reveal percentages fo positive binding with scFv-hRP215 (A,B,C and DL: RP215-scFv) and with Anti-CD19-scFv (E: Anti-CD19-scFv) as the positive control.

Disease	Cell lines	Tissue	Cell type	Age	Gender	Ethnicity
Burkitt's lymphoma	Daudi	Peripheral blood	B lymphoblast	NA	NA	NA
Acute lymphoblastic leukemia (ALL) in relapse	Nalm-6	B cell precursor leukemia	NA	19 yrs	NA	NA
Burkitt's lymphoma	NAMALWA	NA	B lymphocyte	3 yrs	F	NA
Burkitt's lymphoma	RAJI	Lymphoblast	B lymphocyte	11 yrs	M	Black

Table 2: List of four CD19-positive cell lines of leukemic origins and Historical information for FACS Analysis with scFv-hRP215.

Cell line	RP215-scFv (%)	CD19-scFv (%)
Daudi	61.8	NA
Nalm-6	57.70	NA
NAMALWA	14.90	NA
RAJI	50.00	97.70

Table 3: Percentage of the target positive cells: The four CD19+ target cell lines can be targeted by antibody fragments, RP215-scFv.

Conclusions

In this review, based on the results of more than one decade of biochemical and immunological studies, we have been able to establish that RP215 reacts with the epitope associated mainly with O-glycan of defined structures, mainly cancerous immunoglobulins. This unique epitope was found mainly on the variable regions of immunoglobulin heavy chains expressed by most human cancer cells. RP215 can therefore be used to target most of cancer cells for diagnostic and therapeutic applications including those of leukemic origins. For diagnostic applications, RP215-based immunoassay can also be used to monitor serum CA215 (mainly cancerous immunoglobulins) levels among cancer patients, when used singly or in combinations with other known cancer biomarkers [23,24]. Therefore, in conclusion, RP215 or its fragments in humanized forms such as scFv can also be used either as antibody-based anti-cancer drugs or as scFv-RP215-CAR-T cell constructs for cancer therapy of many types of human cancer, following extensive clinical studies.

Acknowledgements

This review article was supported in parts by NRC-IRAP (#821-901) of Canada. Helpful comments by Dr. Joseph Han is acknowledged.

Conflict of Interest

G. Lee is a co-founder of Vancouver Biotech Ltd.

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