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Studies of a tumor-associated antigen, COX-1, recognized by a monoclonal antibody

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Summary. Monoclonal antibodies against an ovarian tumor cell line, OC-3-VGH, were generated using modified hybridoma technology. Among the seven that were selected for their high specificity and affinity to ovarian cancer cells and low cross-reactivity to most normal human tissues, RP 215 was shown to react specifically with a tumor-associated antigen, COX-1, from certain ovarian/cervical cancer cell lines. By Western blot assay, COX-1 was shown to have a subunit molecular mass of about 60 kDa and exist as an aggregate in the native state. COX-1 could also be detected in the shed medium of certain cultured tumor cells. A solid-phase sandwich enzyme-immunoassay procedure was designed for quantitative determinations of COX-1 in the shed medium or in patients' sera using RP 215 for both well-coating and the signal detection. Highly purified COX-1 was obtained from the shed medium of cultured OC-3-VGH tumor cells mainly by hydroxyapatite and immunoaffinity chromatography with RP 215 as the affinity ligand. At neutral pH, purified COX-1 also exists as an aggregate and is relatively stable at temperatures below 50° C. Its immunoactivity was found to decrease with time in the presence of trypsin. However, the immunoactivity of COX-1 was not affected upon incubation with carbohydrate-digestive enzymes or concanavalin A and only partially inactivated in the presence of $NaIO_4$ or iodoacetamide. Treatments of COX-1 with dithiothreitol and guanidine thiocyanate resulted in a complete loss of activity. Furthermore, rabbit antisera raised against purified COX-1 exhibited similar immunospecificity to that of RP 215. The results of this study suggest that COX-1 is a glycoprotein consisting of a 60 kDa subunit, which is recognized by RP 215 through its peptide determinant. Preliminary retrospective clinical studies were performed to assess the utility of a COX-1 enzyme immunoassay kit for detection and monitoring of patients with ovarian and cervical cancers.

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Introduction

Ovarian cancers are among the most lethal gynecological cancers. About 47% of the mortality caused by various gynecological malignancies is derived from ovarian cancers [15]. However, if they can be detected at early stages and receive proper clinical treatment, the 5-year survival rate can be as high as 76% [44].

Although human chorionic gonadotropin and α-fetoprotein could be useful markers for the detection of ovarian cancers of germ cell origin, no reliable biochemical markers have been identified for early diagnosis of epithelial ovarian tumors. Carcinoembryonic antigen was once shown to be a promising marker; however, the positive identification rate of ovarian tumors remains low [1, 16, 27, 44]. The use of heterologous polyclonal antisera such as OCA, OCAA and NB/70K against certain types of ovarian tumor extracts or tumor cells has been reported [4, 8, 11, 22, 29–32]. The antisera raised have then been used to develop radioimmunoassay or immunohistological assays for the detection of tumor-associated antigens in the sera or tissues of cancer patients during early diagnosis and monitoring [4, 22–29]. However, the results of this approach have not been satisfactory owing to the lack of tumor specificity.

With hybridoma technology, numerous monoclonal antibodies have been generated against different ovarian tumors by a number of investigators. The most notable and established ones are those reactive to CA 125, CA 19-9, HMFG-2 and NB/70K [1, 5–11, 18, 24, 27, 30, 36–39, 42, 45–47, 48]. The monoclonal antibody specific to CA 125 was used to detect related tumor-associated antigens among 83.1% of patients with nonmucinous ovarian carcinomas [24]. However, CA 125 has also been found in

normal adult tissues that are derived from coelomic epithelium and other types of tumors [6, 37, 51]. Moreover, CA 125 could not be used to predict the absence of microscopic disease in patients with complete remission [2, 3]. In addition, the diagnostic positive rate was only 18% for patients in the early stages of cancer, when CA 125 was used [49, 50]. Similarly, the cases predicted by CA 19-9, HMFG-2 and NB/70K remained low for the same types of patients [7, 27, 49].

Since none of the available tumor markers is ideal for the management of ovarian cancer patients, identification of additional complementary markers would be highly desirable for future clinical utility. In this communication, we would like to report our preliminary studies of a new tumor-associated antigen, COX-1, which is recognized by a monoclonal antibody generated against an established ovarian tumor cell line.

Materials and methods

Chemicals. The following chemicals were purchased from Sigma Chemical Company, St. Louis, Mo.: Dimethylsulfoxide, lipopolysaccharide, methylcellulose, bovine serum albumin, complete and incomplete Freund's adjuvant, pristane, 3,3′,5,5′-tetramethylbenzidine, trypsin, neuraminidase, β-galactosidase, β-*N*-acetylglucosaminidase, fucosidase, concanavalin A (ConA), ConA-Sepharose, sodium periodate and dithiothreitol. Cell culture media and supplements including IMDM, RPMI-1640, penicillin/streptomycin (100×) and glutamine were from Gibco, Burlington, Ontario, Canada. Tissue-culture plates and selection media, including hypoxanthine/aminopterin/thymidine) and hypoxanthine/thymidine, were from Flow Laboratories, Mississauga, Ontario, Canada. Fluorescein-isothiocyanate-labeled goat anti-(mouse IgG+M+A) was from Cappel-Worthington, Malvern, Pa. All the analytical-grade reagents required for sodium dodecylsulfate (SDS)/acrylamide gel electrophoresis and Western blot assay were from Bio-Rad Laboratories, Richmond, Calif. Polyethylene glycol (PEG, M_r = 1500) was from British Drug House Chemical Ltd. The iodine-125 radioisotope was purchased from Amersham, Oakville, Ontario, Canada.

Ovarian cancer cell lines and production of monoclonal antibodies. An ovarian cancer cell line of serous origin, OC-3-VGH, was established by the Department of Obstetrics and Gynecology, Veterans General Hospital, Taipei, Taiwan, China [12]. Cultured tumor cells derived from this cell line were used as immunogens. BALB/c mice were used for immunization, cell fusions and production of monoclonal antibodies.

Briefly, for immunization, about 1×10^6 cell of OC-3-VGH in 100 µl PBS (phosphate-buffered saline containing 15 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.14 M NaCl and 2 mM KCl, pH 7.0) were emulsified with an equal volume of complete Freund's adjuvant. The mixture was injected subcutaneously into each mouse. Two more injections were given 2 and 4 weeks after the primary immunization, except that incomplete Freund's adjuvant was used. The immunized mice were injected through the tail vein with 1×10^6 tumor cells in 200 µl PBS 1 week after the third immunization. Spleen cells of immunized mice were fused with NS-1 cells in the presence of 50% PEG 3 days after the booster injections. Hybrid cells were cultured initially in a semi-solid medium containing methylcellulose as previously described [14]. The hybrid colonies were removed from the semi-solid medium 7–10 days after the cell fusion and cultured in RPMI-1640 medium containing 10% fetal calf serum in 96-well microtiter plates. Cell culture supernatant was screened for the presence of antibodies against OC-3-VGH cells by both enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent assay as described previously [26, 35]. The hybrid cell lines, which secrete antibodies of high affinity and specificity, were subcultured until they reached a cell density of 1×10^6/ml. The hybrid cells were frozen and stored in liquid nitrogen tanks [35]. Mass production of selected monoclonal antibodies was achieved by inducing ascites fluid from BALB/c mice primed with pristane according to the published procedures [13, 35].

For comparative purposes, several established human epithelial tumor cell lines including CaOV-3, C-33A, JEG-3, ME-180, SiHa and AN3 CA were obtained from American Type Culture Collection (Rockville, Md.). Among these cell lines, C-33A, Me-180 and SiHa are those of cervical carcinoma. AN3 CA and JEG-3 are those of endometrial adenocarcinoma and choriocarcinoma respectively. CaOV-3 is a tumor cell line of ovarian origin.

Western blot assay. Molecular mass analysis of tumor-associated antigens that react with monoclonal antibodies was performed by using the Western blot assay. Details of this assay procedure involving the application of the SDS gel/protein blot radioimmunobinding method have been described previously [34]. The minimum (or subunit) molecular mass of those antigens reactive to monoclonal antibodies was determined following the assay using ^{125}I-labeled goat anti-(mouse IgG+M) as the second antibodies and autoradiography using Kodak AR-2 film. Iodination of goat anti-(mouse IgG+M) was performed according to the published procedure [19].

Immunohistochemical study of tissue sections. Formalin-fixed, paraffin-embedded normal human tissue sections were used to determine the specificity or cross-reactivity of the generated monoclonal antibodies to various tissues including those of brain, liver, heart, kidney, ovary, testis, cervix and muscle. These tissues were obtained at the time of surgery or at autopsy. Paraffin tissue sections were deparaffinized in xylene, then sequentially dipped in 95%, 75% and 50% ethanol, and finally washed with PBS. Cryostat tissue sections were also prepared, dipped in 95% ethanol and then washed with PBS. The avidin-biotin-peroxidase complex immunohistochemical staining method was performed according to the previously reported procedure [21, 25].

Sandwich enzyme immunoassay. One of the monoclonal antibodies, RP 215, was shown to recognize multimeric epitopes of the complementary tumor-associated antigen, COX-1 in its native form. Therefore, this antibody was used to determine COX-1 by means of a solid-phase sandwich enzyme immunoassay. Briefly, RP 215 was first purified from ascites fluid and coated on microwells at a final concentration of 5 µg/ml in 50 mM TRIS/HCl, pH 8.0 [13]. At the same time RP 215 was labeled with horseradish peroxidase and served as the detecting antibody for the sandwich assay [13].

The enzyme immunoassay was initiated by first adding 100 µl specimens to each microwell. Following incubation for 18 h at 4°C, the well contents were removed by suction. The wells were washed once with distilled water. A sample of 100 µl horseradish-peroxidase-labeled RP 215 of known dilution was then added to each well. This was followed by incubation at 37°C for 3 h. The microwells were then washed three times with PBS/Tween and then three times with distilled water. The microwell-bound peroxidase activity was determined in a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (0.3 mg/ml) and 0.02% H_2O_2 in 0.1 M citrate/phosphate, pH 5.0. Following 15 min incubation, the color reaction was stopped by adding 1 M H_2SO_4. The color intensity in each well at 450 nm was determined by a Molecular Device microplate reader. The amount of COX-1 in the sonicated cultured OC-3-VGH tumor cell extract at a protein concentration of 1 mg/ml was arbitrarily designated as 100 AU/ml for this enzyme immunoassay.

Preparation of the immunoaffinity column. Purified RP 215 (5 mg/ml in 0.1 M $NaHCO_3$, pH 8.3) was coupled to Affi-Gel-10 (from Bio-Rad Laboratories) with a ligand density of 10–20 mg antibody/ml affinity gel. Following coupling, the affinity gel was incubated with 1 M ethanolamine at pH 9.5 to block all the uncoupled sites and stored at 4°C in the PBS until use.

Purification of COX-1 from the shed culture medium. COX-1 was purified from shed culture medium by ammonium sulfate fractionation, hydroxyapatite column and immunoaffinity chromatography using RP 215 as the affinity ligand. Briefly, COX-1 in shed medium was first

precipitated by ammonium sulfate of 35% saturation. Following centrifugation, the pellet was redissolved in a minimal amount of PBS and dialyzed overnight against PBS. Following centrifugation to remove the denatured proteins, the clear supernatant was then passed through a hydroxyapatite column, which was equilibrated with 0.1 M sodium phosphate buffer, pH 7. The unbound proteins were washed out from the column with the initial buffer and the bound proteins were then eluted with 0.2 M phosphate buffer, pH 7. After assaying by enzyme immunoassay, the fractions containing the highest activity were pooled and further purified by a RP 215 immunoaffinity column. After the sample was loaded, the column was washed extensively with 300 ml PBS at a flow rate of 50 ml/h, followed by a continuous wash with an additional 300 ml PBS containing 1 M NaCl to eliminate all the possible non-specifically bound proteins. Finally, COX-1 was eluted with 50 mM glycine/HCl, pH 2.2. Fractions of 1 ml were collected and neutralized immediately with 1 M phosphate buffer, pH 7. During the course of antigen purification, COX-1 immunoactivity in the shed medium and purified preparation was determined by the sandwich enzyme immunoassay described above. The purity of the COX-1 preparation was examined by SDS gel electrophoresis (10% acrylamide gel).

Determination of the dissociation constant between COX-1 and RP 215. Samples of 100 µl each of $I^{125}I$-labeled COX-1 and RP 215 of different dilutions were incubated at room temperature for 1 h. A 1-ml aliquot of PEG/second antibody solution [containing 3.6% polyethylene glycol 4000, 50 µl rabbit anti-(mouse IgG) in 60 mM potassium phosphate, pH 7.2, 150 mM NaCl, 0.1% NaN_3 and 0.25% bovine serum albumin] was then added to each tube and this was followed by an additional 20 min incubation. Following centrifugation at 3000 rpm for 20 min, the supernatant was removed and the pellet counted for radioactivity. The residual radioactivity of COX-1 in each tube was plotted as a function of RP 215 concentration. The association constant between RP 215 and COX-1 was then calculated [20]. Purified COX-1 was labeled with ^{125}I by the chloramine T method with a specific activity of 0.1 mCi/ µg [19].

Thermal stability of COX-1 immunoactivity. The stability of COX-1 aggregates was determined by its immunoactivity using the described sandwich enzyme immunoassay (EIA). The COX-1 preparation was incubated at different temperatures (50, 60, 80, and 1003° C) for various time intervals. At the end of each incubation, aliquots were drawn and cooled at 0° C in the ice. The residual COX-1 immunoactivity was then determined using EIA.

Chemical treatments. The effect of carbohydrate modification on the immunoactivity of COX-1 was studied by the procedure of periodate oxidation [43]. Highly purified COX-1 was mixed with various concentrations of sodium periodate in acetate buffer (50 mM, pH 4.5) to a final concentration of 0–100 mM. The mixtures were incubated at 4° C for 2 h in the dark. Following extensive dialysis in PBS, the residual COX-1 immunoactivity was determined. Reduction was accomplished with dithiothreitol (10 mM in 50 mM TRIS buffer, pH 8) for 2 h at room temperature either in the presence or absence of 4 M guanidine thiocynate. Alkylation of COX-1 was carried out by mixing the sample with iodoacetamide to a final concentration of 20 mM. The mixture was allowed to stand at room temperature for 2 h. After the treatment, all samples were immediately dialyzed overnight at 4° C against PBS. The remaining immunoactivity was assayed by EIA and compared with that of the control.

The effects of urea and different detergents on COX-1 immunoactivity were also determined. The samples of COX-1 were treated with 6 M urea or different types of detergent in 1% solution for 2 h at 4° C. The remaining activity of COX-1 was then measured by EIA.

Effect of trypsin and other hydrolytic enzymes or chemicals on COX-1 immunoactivity. In order to determine whether trypsin could affect COX-1 immunoactivity, the concentrated shed medium was incubated with trypsin at a protein concentration ratio of 50 : 1 for various time intervals at 37° C. At the end of each incubation, the trypsin activity was neutralized with a 3 times molar excess of soybean trypsin inhibitor. The residual COX-1 immunoactivity was determined by the described EIA.

In a separate antigen control, COX-1 was co-incubated with trypsin and soybean trypsin inhibitor, the remaining immunoactivity of COX-1 was then determined at different time intervals. Exoglycosidase digestions were performed in acetate buffer (0.1 M, pH 4.5) except that α-1-fucosidase digestion was performed in phosphate buffer (0.1 M, pH 7). The reaction mixtures were incubated at room temperature for 24 h and the residual activity was assayed by EIA as described above.

Determination of the molecular maas of COX-1 in the native state. The molecular maas distributions of COX-1 in purified form or from the supernatant of sonicated OC-3-VGH tumor cells and shed medium of cultured tumor cells were determined by using a Sepharose 4B or Sephacryl S-300 gel filtration column (size 1×30 cm). Myoglobin (13 kDa), ovalbumin (45 kDa), immunoglobulin G (160 kDa), thryoglobulin (675 kDa) and blue dextran ($>2 \times 10^3$ kDa) were used as molecular mass standards. Fractions of 0.5 ml were collected and assayed for the amount of COX-1 by EIA as described.

Preliminary clinical evaluations. Preliminary clinical studies of COX-1 antigen were conducted in the Department of Obstetrics and Gynecology, Veterans General Hospital, Taipei, Taiwan, China. The control specimens came from women age 25–51 who were disease-free at the time the samples were drawn. None of the control developed cancer in 2 years. Frozen serum specimens from those patients who had been diagnosed with ovarian or cervical carcinoma or benign ovarian tumors were used for retrospective studies. The FIGO staging system of ovarian/cervical cancers was used in the designation and classification of patients' specimens [17]. Ovarian benign tumors were diagnosed on the basis of standard pathological and histological criteria [40]. The serum levels of COX-1 antigens from all patients were determined by a double-blind assay using the described COX-1 EIA kit.

A statistical analysis using the χ^2 method was used to assess the relative clinical significance of the determined COX-1 values derived from defined patients' specimens.

Results

Generation and characterization of monoclonal antibodies

Following typical immunizations and cell fusion experiments, about 3000 hybrid clones were initially recovered. About 30 were finally selected and shown to secrete antibodies reactive to the surface of OC-3-VGH cells using ELISA and indirect immunofluorescent assay procedures [26, 35]. To examine the tissue specificity of selected monoclonal antibodies, avidin-biotin-peroxidase staining of normal human tissue sections and quantitative tissue adsorption experiments were performed [21, 25]. Among these antibodies, RP 215 was shown to have the highest specificity to OC-3-VGH cells and did not significantly cross-react with any of the normal human tissues. RC 26 was reactive to both OC-3-VGH cells and normal ovarian tissue. On the other hand, RC 8, RC 30, RC 32, RC 33 and RC 35 revealed slight cross-reactivity with some of the normal human tissues including ovary and/or testis. While all of the selected monoclonal antibodies react with OC-3-VGH tumor cells, none was found to cross-react with cells from C33-A and AN3 CA tumor cell lines. RP 215 was also shown to cross-react with tumor cells from CaOV-3 (ovarian origin) ME-1800 (cervical origin) and CA-33 (cervical origin) cell lines, as demonstrated by indirect immunofluorescent assay.

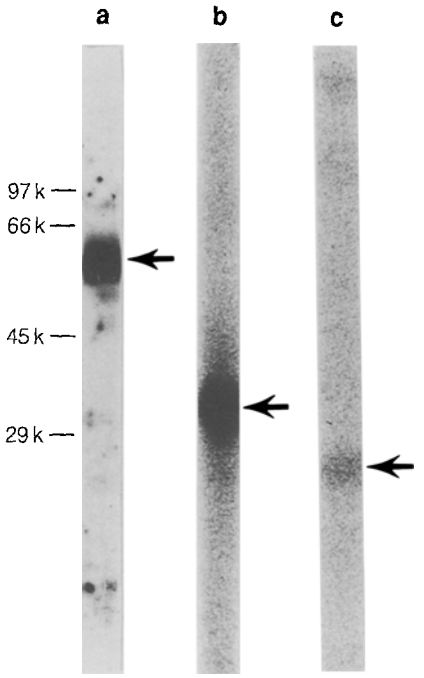

Fig. 1. Western blot assay to reveal the molecular mass of OC-3-VGH tumor-associated antigens (on sodium dodecyl sulfate, SDS, gels) that react specifically with three different monoclonal antibodies. From *left* to *right*, lanes *a*, *b* and *c* represent the blot with RP 215, RC 35 and RC 30 monoclonal antibodies respectively. The molecular mass markers are indicated on the left of the strip by bars (*k* = kDa). A sample of 1×10^6 cpm ^{125}I-labeled goat anti-(mouse IgG+M) was used for initial incubation. The autoradiogram was developed after 24 h of exposure

The immunoglobulin subclass of RP 215 was determined to be IgG1, whereas the others were either IgG1 or IgG2a [41].

The minimum molecular masses of tumor-associated antigens reactive to the selected monoclonal antibodies were determined by Western blot assay [34]. The results of this assay using selected antibodies are presented in Fig. 1 RP 215, RC 35 and RC 30 were shown to recognize tumor antigens with molecular masses of 60, 32 and 20 kDa respectively.

Preliminary characterization of tumor-associated antigen recognized by RP 215

In view of the relatively high specificity of RP 215 to tumor cells of cervical and ovarian origins, its cognate tumor-associated antigen was designated as COX-1. Using a solid-phase sandwich enzyme immunoassay, COX-1 could be detected in the shed medium of cultured OC-3-VGH tumor cells as well as in the tumor cell extract. When analyzed by Sephacryl S-300 gel filtration chromatography, COX-1 was shown to exist as undefined aggregates with molecular masses ranging from 1×10^2 to 1.5×10^3 kDa. The Western blot assay was performed with the high-molecular-mass form of COX-1 recovered from the void volume of the gel filtration column. It was shown that the minimum molecular mass of COX-1 in the aggregates was identical to that in the initial cell extract (60 kDa). Furthermore, when the extract of tumor cells was rechromatographed in the presence of 6 M urea on a Sephacryl S-300 column, only a single form of COX-1 with a molecular mass of 60 kDa was detected.

Table 1. Purification of COX-1 from shed medium of cultured OC-3-VGH tumor cells

Steps	Protein concentration (mg/ml)	Activity[a] (AU/ml)	Specific activity (AU/mg protein)	Purification (fold)
Shed medium[b]	2.38	2.52	1.06	1
Ammonium sulfate (35%)	26.16	1100	42.04	40
Hydroxyapatite	0.315	132	352	333
RP215 affinity chromatography	0.016	22.86	1365	1292

[a] Enzyme immunoassay
[b] 100 ml shed culture medium was employed for this purification

Purification of COX-1

The tumor-associated antigen, COX-1, was purified by ammonium sulfate fractionation, hydroxyapatite column and finally by immunoaffinity chromatography using RP 215 as the affinity ligand. As determined by sandwich enzyme immunoassay, an approximately 1300-fold purification was achieved (Table 1). Under denaturing conditions, the molecular mass of purified COX-1 was found to be about 63 ± 3 kDa as determined by SDS/polyacryamide gel electrophoresis. The isoelectric point of COX-1 was determined by isoelectrofocussing gel electrophoresis. After electrophoresis, the gel was sliced and incubated with PBS overnight at 4° C. The activity of different fractions was assayed and the pI of the major native COX-1 was determined to be 5.05. This finding was consistent with the two-dimensional gel electrophoresis (not shown), when denatured COX-1 was analyzed. At neutral pH, the molecular mass of native COX-1 was shown to be equal to or greater than 2×10^3 kDa as determined by gel filtration on a Sepahrose 4B column. The main COX-1 activity was eluted in the void column (unpublished data). This observation suggests that purified COX-1 may also exist in the form of aggregates in the native condition.

Sensitivity of COX-1 immunoactivity to digestive enzymes and to physical and chemical treatments

Under the assay conditions described, COX-1 immunoactivity was shown to be sensitive to the proteolytic digestion by trypsin at 37° C. When analyzed by the sandwich EIA method, it was observed that less than 1% of COX-1 immunoactivity could be recovered after 60 min incubation with the concentration of trypsin added. In a separate antigen control, when COX-1 was co-incubated with the

Table 2. Effects of chemical treatments or enzymes on COX-1 immunoactivity

Chemical or enzyme treatment	COX-1 activity remaining following treatments (%)
Control	100
1. Reduction:	
Dithiothreitol (10 mM) + guanidine thiocyanate (4 M)	0
Dithiothreitol (10 mM)	0
Guanidine thiocyanate (4 M)	0–10
2. Alkylation:	
Iodoacetamide (20 mM)	72
3. Detergents and chaotropic agents	
Non-ionic detergents (1%)	
Triton X-100	9
Tween-20	9
NP-40	10
Anionic detergents (1%)	
SDS	35
Deoxycholate	56
CHAPS	33
Urea (6 M)	30
4. Periodate oxidation ($NaIO_4$)	
1 mM	68
10 mM	53
100 mM	35
5. Exoglycosidase treatment	
β-galatosidase	97
β-*N*-Acetylglucosaminidase	98
Neuraminidase	98
α-1-Fucosidase	95

same amount of trypsin in the presence of soybean trypsin inhibitor, COX-1 immunoactivity was not affected. COX-1 immunoactivity is relatively stable in a PBS solution at pH 7.2 at temperatures below 50° C. At incubation temperatures higher than 60° C, COX-1 immunoactivity was observed to decrease significantly with incubation time. The immunoactivity of COX-1 was completely diminished after incubation at 100° C for 10 min.

A number of chemical treatments that denature most proteins were employed to study the molecular nature of COX-1 isolated from the shed medium (summarized in Table 2). The results indicate that COX-1 immunoactivity was completely destroyed by the addition of 20 mM dithiothreitol and 4 M guanidine thiocyanate, or 20 mM dithiothreitol alone. However, the COX-1 activity was reduced by 50%–70%, in the presence of 6 M urea or 20 mM iodoacetamide. COX-1 activity was found to be more sensitive to the treatment of non-ionic detergents than to ionic detergents. It was shown that less than 10% of activity remained after the treatment of 1% of various types of non-ionic detergents including Triton X-100, Tween-20 and NP-40. In contrast to non-ionic detergents, after treatment with 1% ionic detergents, such as SDS, deoxycholate and CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid}, the immunoactivity of COX-1 decreased to about 33%–60%.

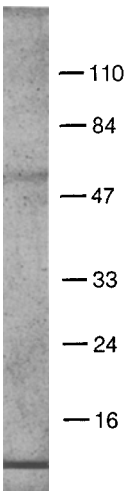

Fig. 2. SDS/acrylamide gel (10% acrylamide) of purified COX-1 (10 μg) to reveal the purity and its molecular mass. The molecular mass markers (kDa) are indicated on the right of the gel strip by *bars*

COX-1 was found to be relatively stable at neutral pH and to become inactivated at pH 3.5 and 10.3, when its immunoactivity was determined by EIA after incubations at various pH levels.

To determine whether COX-1 is a glycoprotein, highly purified COX-1 was subjected to ConA-Sepharose adsorption. After ConA-Sepharose adsorption, the COX-1 immunoactivity remaining in the supernatant was significantly lowered as compared to that of the controls. The bound COX-1 activity can be recovered by washing the gel with 0.5 M D-mannose (unpublished observations).

Modification of carbohydrates of COX-1 by periodate oxidation was found to be effective in lowering its immunoactivity. Although a dose-dependent inhibition was observed, the COX-1 immunoactivity was not completely destroyed by the highest concentration of $NaIO_4$ (100 mM) used. Various exoglycosidase treatments on the COX-1 antigen were performed (Table 2). The results indicate that no significant loss of COX-1 immunoactivity was observed when assayed by EIA. The immunoactivity of COX-1 was not affected by the addition of ConA to the antigen preparation.

Characterization of rabbit anti-COX-1 and RP 215

Purified COX-1 was used as an immunogen for the immunization of rabbits. The titers of rabbit antisera were determined by ELISA using purified COX-1-coated microwells and compared with those of RP 215 monoclonal antibody. The results of this immunoassay (presented in Fig. 3), indicated that after successive immunizations, the titer of rabbit antisera was determined to be between 1:400 and 1:800 serum dilution. A comparable assay result was obtained with purified RP 215 monoclonal antibody. By Western blot assay, the raised rabbit antisera were shown to react with a protein band of 60 kDa molecular mass, which is identical to that recognized by RP 215. Using the radioimmunobinding assay with I^{125}-labeled COX-1 as the tracer, the association constant between RP 215 and COX-1 was determined to be $5 \times 10^8 M^{-1}$.

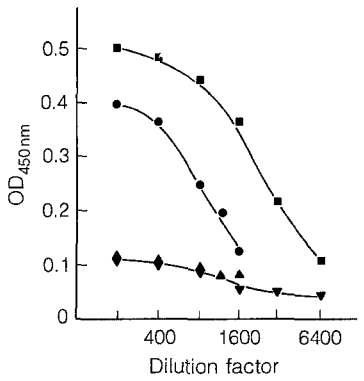

Fig. 3. The titers of rabbit antisera against purified COX-1 and RP 215 monoclonal antibody as determined by ELISA using microwells coated with purified COX-1. ▲, ▼, The preimmune rabbit serum and anti-β-hCG monoclonal antibody (initial concentration 1 mg/ml), respectively, as the negative control. ●, ■, The rabbit anti-COX-1 antiserum and RP 215 monoclonal antibody (initial concentration, 1 mg/ml) respectively. OD_{450}, absorbance at 450 nm

Preliminary clinical evaluations of COX-1 antigen

Using the COX-1 enzyme immunoassay kit, preliminary clinical studies regarding the serum levels of COX-1 antigen were determined retrospectively to assess its clinical utility. As summarized in Table 3, patients with ovarian or cervical carcinoma had significantly higher serum levels of COX-1 than those of the normal control and those with benign tumors. In the case of patients with cervical carcinoma, the serum COX-1 levels appeared to increase with the disease stages. If the mean + 2 SD for the normal control was used as a clinical cut-off level, as many as 80% of the ovarian cancer patients showed elevated COX-1 levels. For patients with cervical carcinoma, as many as 64%–80% showed significantly elevated levels of serum COX-1 antigen. On the other hand, only 5% of patients with benign diseases showed elevated levels.

Discussion

In this study, the effort was made to generate monoclonal antibodies that are highly specific to tumor cells and have minimal cross-reactivity with the normal human tissues. Among the numerous antibodies produced, only RP 215 fits the above criteria. The tumor-associated antigen recognized by this antibody, COX-1, was subsequently identified, purified and characterized biochemically and immunologically. At the same time, a solid-phase sandwich enzyme immunoassay was designed based on the use of RP 215 alone to determine the immunoactivity of this antigen in the shed medium of cultured tumor cells and in the patients' sera.

The results of our study indicated that COX-1 has a subunit molecular mass of 60 kDa and exists as undefined aggregates in the native state. COX-1 was purified from the shed culture medium of ovarian cancer cells (OC-3-VGH) to homogeneity by a combination of ammonium sulfate fractionation, hydroxyapatite chromatography and immunoaffinity chromatography. A 1300-fold purification of COX-1 has been achieved (Table 1). When analyzed with the reducing SDS gel electrophoresis, the subunit molecular mass of COX-1 was determined to be 60 kDa. However, both the crude COX-1 preparation and the purified COX-1 were shown to appear as aggregates as demonstrated by Sephacryl S-300 and Sepharose 4B gel filtration chromatography.

The immunoactivity of COX-1 was very susceptible to trypsin digestion. The immunoactivity was totally lost after prolonged incubation with this protease. This would suggest that the monoclonal antibody RP 215 may react with peptide epitopes of tumor-associated antigen, COX-1. On the other hand, COX-1 immunoactivity is relatively stable upon incubation with several carbohydrate-digestive enzymes tested. These included neuraminidase, β-galactosidase and fucosidase. Furthermore, no significant loss of immunoactivity was observed with the addition of ConA to the COX-1 preparation. In fact, at the lowest periodate concentrations (0.1 mM) that destroyed other tumor antigen activity [43], COX-1 activity was not affected. The loss of activity at higher concentrations of periodate is probably due to non-specific oxidation of the carbohydrate moiety of this antigen, as well as to some amino acid residues. These results indicate that the carbohydrate moiety of COX-1 may not be essential for the binding between RP 215 and this antigen. However, the activity of COX-1 could be reduced if the conformation of the antigen molecule is modified after periodate treatment.

Unlike CA 125 isolated from an ovarian cancer cell line OVCA 433 [5–7], COX-1 activity is very sensitive to the treatments of guanidine thiocyanate or non-ionic deter-

Table 3. Preliminary retrospective clinical evaluations of COX-1 among patients with ovarian or cervical cancers, benign tumors or normal subjects[a]

Parameter	Normal control	Ovarian benign tumors	Ovarian carcinoma	Cervical carcinoma stages		
				I	II	III
Cases	130	19	20	25	20	5
Mean (AU/ml)	29.4	31.4	82.0	72.0	87.2	88.8
SD	19.4	13.1	34.3	25.9	37.0	35.3
Positive (%) (>mean+2 SD)		5	80	64	80	80

[a] Normal control vs ovarian carcinoma $P < 0.001$; vs ovarian tumors (non-significant). Ovarian carcinoma vs ovarian tumors $P < 0.001$. Normal control vs cervical carcinoma I, $P < 0.001$; II, $P < 0.001$; III, $P < 0.001$. Cervical carcinoma I vs II, $P > 0.1$; II vs III $P > 0.2$

gents. The activity loss after such treatments is most likely due to the breakdown of antigen aggregation to a less complex form or due to the denaturation of the antigen. It was observed that COX-1 activity could no longer be detected by EIA when it has been reduced by the addition of dithiothreitol. After alkylation, COX-1 was found to be only partially active as compared to the control. This indicates that RP 215 may recognize a disulfide-dependent configuration in the antigen.

Rabbit antisera raised against purified COX-1 were shown to exhibit similar immunospecificity to that of RP 215 monoclonal antibody (Fig. 3). The polyclonal antisera may prove to be useful for immunoassay and immunohistological studies of this tumor-associated antigen.

Judging from this preliminary study, it can be concluded that COX-1 is a relatively stable tumor marker and may have potential clinical applications for the monitoring of patients with ovarian or cervical cancers.

In preliminary clinical studies, serum levels of COX-1 antigen among patients with cervical or ovarian cancers were determined by the COX-1 sandwich EIA procedure that we designed. As clearly demonstrated in Table 3, serum COX-1 antigen levels were significantly elevated among these groups of cancer patients as compared to those of the normal control or those with benign diseases. As many as 64%–80% of cancer patients revealed clinically significant COX-1 levels in sera, as compared to those of the normal control or those with benign diseases. Further basic and clinical studies are required before we can understand more about the molecular nature of this newly identified tumor-associated antigen and why it is highly associated with these two types of gynecological cancers. At the same time, extensive clinical evaluations of COX-1 in several medical centers are currently in progress to obtain more precise information about this cancer marker.

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