Characterizations of A Tumor-Associated Antigen COX-1

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Obstetrics and Gynecology)

(Reproductive and Developmental Science Program)

We accept this thesis as conforming to

the required Standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1991

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Abstract

By using modified hybridoma technology, monoclonal antibodies against an ovarian tumor cell line, OC-3-VGH, were generated in Dr. Lee's laboratory. Among these antibodies, RP 215 was shown to react specifically with a tumor-associated antigen, COX-1. On SDS gel, COX-1 has a molecular weight of 60 KD and exists as an aggregate in the natural state. A highly purified COX-1 was obtained mainly by immunoaffinity chromatography, with RP 215 as the affinity ligand, from the shed medium of cultured tumor cells. A solid phase enzyme immunoassay was established using RP 215 as the capturing and the detecting antibody with a sensitivity of 1 AU/ml. This immunoassay kit could be used to determine the levels of COX-1 in the culture medium and in the sera of cancer patients.

COX-1 was characterized under a variety of experimental conditions. At temperatures higher than 50°C or in the presence of trypsin at 37°C, COX-1 immunoactivity was found to decrease with incubation time. However, COX-1 was not affected by incubation with carbohydrate-digestive enzymes including neuraminidase, Beta-galactosidase and fucosidase or carbohydrate modifying agents such as NaIO₄. Concanavalin A had no effect on the immunoactivity of COX-1 to RP 215. Furthermore, rabbit antisera against COX-1 were raised, and these polyclonal antisera were shown to exhibit
similar immunoactivity to that of RP 215 monoclonal antibody.

Using the established sandwich enzyme immunoassay, serum levels of COX-1 among patients with ovarian or cervical cancers were determined retrospectively through interlaboratory evaluations and collaborations. Compared to those of normal individuals and benign tumors, serum levels of COX-1 were significantly elevated and can be correlated to the progression of the disease among cancer patients. Preliminary data indicated the COX-1 can complement other established tumor markers such as CA 125 for the purpose of monitoring patients with ovarian or cervical cancers.
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V. List of Abbreviations

AFP  Alphafetoprotein
ATCC  American Type Culture Collection Inc.
AU  Arbitrary Unit
BSA  Bovine Serum Albumin
CEA  Carcinoembryonic Antigen
Con A  Concanavalin A
CPM  Count per minute
DEAE  Dethylaminoethyl
EDTA  Ethylenediaminetetraacetic Acid
ELISA  Enzyme-linked Immunosorbent assay
EIA  Enzyme Immunoassay
HCG  Human Chorionic Gonadotropin
HPL  Human Placental Lactogen
HRP  Horse Raddish Peroxidase
IEF  Isoelectronic Focusing
IgG+M  Immunoglobulin G & M
KD  Kilodalton
LB  Luria-Bertani
MAb  Monoclonal antibody
O.D.  Optical Density
PAGE  Polyacrylamide Gel Electrophoresis
PBS  Phosphate Buffered Solution
PEG  Polyethylene Glycol
PI  Isoelectric Point
RIA  Radioimmunoassay
<table>
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<td>RPMI</td>
<td>Russell Park Memorial Institute</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Solution</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS + 0.05% Tween-20</td>
</tr>
<tr>
<td>Tris</td>
<td>Trihydroxymethylaminomethane</td>
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<td>ul</td>
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Acknowledgements

I wish to extend my thanks to all members regarding my thesis work. First of all, I appreciate my supervisor, Dr. Gregory Lee, who gave me this opportunity to learn basic research technique in our labs.

Secondly, I would like to thank Dr. Basal Ho Yuen (Grace Hospital) and Dr. Diane Miller (VGH) for helping me collect blood samples for my clinical evaluation.

Also, I appreciate all of my committee members: Dr. Rajamahendran (Chairman of committee, Dept. of Animal Science), Dr. J. T. Emerman (Dept. of Anatomy, UBC), Dr. Y. S. Moon (Dept. of Obstetrics & Gynecology), and Dr. Dun Rurak (Dept. of Obstetrics & Gynecology and Research Center Shaughnessy Hospital) who organized this committee and gave me valuable suggestions.

Furthermore, I would like to thank Dr. Angus Tsang for his advice in thesis writing and organization, Lebby Balakshin for secretarial assistance, Jennifer Yao for correction, and all my co-workers in our lab during the two years.

Finally, I would like to thank my family - my father who supported my research and unfortunately passed away last year; my wife for moral and spiritual support and my son, Kevin, for his computer work.
Introduction
A. General review:

Cancers are tissues composed of cells that have become independent to some degree of the normal regulation of growth of the host cell. Cancer cells are characterized by the tendency to change their morphologies, biochemical properties and the properties of chromosomal structures. These abnormal cells can proliferate and produce more and more abnormal cells which subsequently spread to different parts of the body, eventually leading to death if untreated.

The incidence of cancer has increased progressively during the last decades, and it has been claimed that if the same trend continues, by the end of this century there will be more than fifty thousand cancer deaths each year in the United States alone. To date, our understanding of the regulation of cell differentiation and development is still very limited and the control mechanism for this complex process has not been elucidated. Although much work has been done in an attempt to identify the regulatory mechanism of the development of cancers, so far there have been very few advances. It is obvious that if cancer patients can be diagnosed at an earlier stage and prompt medical treatment is provided, the survival rate of cancer patients could be greatly improved. Over the last twenty years, development in the area of biological research including the discovery of techniques to produce monoclonal antibodies (Kohler and
Milstein, 1975; Minna et al, 1981.) have opened up a new avenue for cancer research. With the aid of this technology, antibodies with a predefined antigen specificity can thus be obtained. The hybrid clone that produces the antibody can be amplified to provide a renewable source of the highly monospecific reagent which can be exploited for use in different applications in cancer disease. These applications include diagnosis, imaging and treatment of cancers (McIntire 1985).

B. Cancer markers:

The association of biological markers with cancers has been recognized for many decades (Jones, 1848; Zondek, 1930; and Gutman et al, 1936). In certain types of cancers including those of ovarian origins, tumor cells possess the ability to synthesize specific protein molecules that are expressed at the cell surface. These molecules are sometimes shed into the serum, and can be detected by means of specific and sensitive immunoassay. The first cancer marker "Bence-Jones protein" was identified over four decades ago (Bence-Jones, 1847; Edelman & Poulik, 1961; Porter, 1963) from patients who suffered from a tumor of plasma cell-multiple myeloma. Since then, a number of tumor markers including monoclonal immunoglobulins, hormones, secreted serum proteins, specific enzymes and isoenzymes, cell surface components, glycoproteins and carbohydrates, etc., have been identified by various laboratories. The
applications of most of these components are presently being investigated. The clinical usefulness of some of them such as CEA, AFP and HCG has already been well recognized (Sell, 1990) and are already commercially available (Sell, 1990).

It has been noticed that the expression of a given cancer marker is related to the tumor's tissue origin (Sell, 1990). For instance, tumors arising from closely related embryological tissues, such as gastrointestinal cancers, may all produce alpha-fetoprotein (AFP), a substance normally produced by fetal liver and yolk sac. On the other hand, evidence obtained indicates that the concentration of a tumor marker in a patient's body fluid might reflect a dynamic balance that represents a combination of tumor activity and marker turn-over (Accola et al, 1980). In this aspect, these markers are considered to be a useful aid for the assessment of tumor burden. In addition, it is well recognized that certain tumor markers can provide an accurate and constant estimate of the effectiveness of treatment, and serve as a monitor during active tumor therapy.

Most recently, many attempts have been focused on the utilization of tumor markers in targeting antibody-bound cytotoxic agents to preferentially control or destroy malignant cells (Sell, 1990; Nadji, 1990). For cancer therapy, a number of toxins have been used for chemotherapy of cancers and proven to be effective in killing cancer cells. However, the drawback of using these toxins is that
they also exert a toxic effect on proliferating normal cells. Therefore, the potential of using monoclonal antibodies to specifically deliver anti-tumor drugs, toxins, or radionuclides to cancer has received extensive investigation (Sell, 1990; Nadji, 1990) and shed light on the treatment of cancers. Monoclonal antibodies directed against well-defined tumor associated antigens have made it possible to evaluate their suitability as carriers for direct delivery of toxic agents to tumor targets. They could also assist in the development of a treatment which reduces toxic effects on normal cells and facilitates damage on more selective targets.

C. Ovarian cancers:

Ovarian cancer is the fourth most frequent cause of cancer death in women. Over the past 30 years, the mortality of the patients who suffer from ovarian cancer has increased considerably (Green et al 1984). Although ovarian cancer occurs less frequently than some other kinds of gynecological carcinoma, such as cervical or endometrial cancers, they are responsible for more deaths (Hart 1981; Young, et al, 1985). Clinical data indicates that the five year survival rate of ovarian cancer is usually relatively low (20-47%). The high lethal rate of ovarian cancer is mainly due to the the fact that the tumor is often severely advanced by the time of clinical diagnosis, and the tumor cells are likely to have spread throughout the peritoneal cavity. Further decreases in the chance of survival are
thought to be attributed to the fact that most ovarian cancers respond poorly to chemotherapy and radiotherapy (Morrow, 1981; Richardson, 1985b).

It has been reported that among the common female genital cancers, sixty percent of vulvar cancers are diagnosed at stage I & II. At these stages, the cure rates of these cancers are about 80-90%. For vaginal and cervical cancers, more than 50% of the patients can be diagnosed at either stage I or stage II with 5 year survival rates of 85% and 60%, respectively. About 90% of endometrial cancers are detected at stage I & II with a cure rate of 90% for stage I and 57% for stage II. In contrast, less than 50% of ovarian cancers can be diagnosed at stage I or stage II. In fact, approximately 65% of patients with ovarian cancers are already inoperable when first diagnosed (McGown 1978). It has been considered that the failure of diagnosis of ovarian cancer at a curable stage is probably because (i) the ovaries are relatively inaccessible for accurate evaluation (ii) ovarian carcinoma, like other visceral malignancies, do not display early symptoms and (iii) screening procedures, such as specific immunologic or biochemical markers in the blood or urine, have yet to be developed (Lingeman, 1983). On the other hand, it is also believed that ovarian cancer usually spreads throughout the peritoneal cavity and forms multiple metastatic nodules which are often undetected by noninvasive techniques. As a result, in the majority of
cases, initial surgery shows that the tumors have spread beyond the ovaries (Morrow, 1981).

There is a wide range in the malignant potential of ovarian tumors. The complicated histogenesis of ovarian tumors can be understood on the basis of the individual elements making up this organ. The epithelial cells, growing germ cells and supporting mesenchymal cells of the ovary all possess the potential to become malignant cells. A unified classification of cancers has been adopted by the World Health Organization (Scully, 1975) and the International Federation of Gynecology and Obstetrics (FIGO, Table I). According to this classification, neoplasms thought to be derived from the ovarian surface epithelium are classified as "common epithelial tumours" of the ovary which includes serous, mucinous, endometriods, clear cells and Brenners tumors, solid adenocarcinoma, carcinomas and mixed mullerian tumors (Fenoglio, 1980; Scully, 1977; and Serov, 1973). These tumors are notorious for their wide variation in the degree of histologic differentiation that can be found in different areas of the same neoplasm (Hart, 1981). The other two major types of ovarian cancers are those cell tumors developed in the germ cells (lipoid cells tumors) and those developed in the granulosa and stroma cells (sex cord - stromal tumors). In humans, the most common ovarian cancers (over 80%) are found to be derived from epithelial cells (Servo, 1973; Young et al, 1985).
Table 1  FIGO Staging of Ovarian Cancer*

<table>
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<td>Stage I</td>
<td>Growth limited to the ovaries</td>
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| Stage Ia  | Growth limited to one ovary; no ascites  
1. No tumor on the external surface; capsule intact  
2. Tumor present on the external surface and/or capsule ruptured                      |
| Stage Ib  | Growth limited to both ovaries; no ascites  
1. No tumor on the external surface; capsule intact  
2. Tumor present on the external surface and/or capsule ruptured                      |
| Stage Ic  | Tumor either stage Ia or stage Ib, but with ascites present or positive peritoneal washings                                                                               |
| Stage II  | Growth involving one or both ovaries with pelvic extension                                                                                                         |
| Stage IIa | Extension and/or metastases to the uterus and/or tubes                                                                                                              |
| Stage IIb | Extension to other pelvic tissues                                                                                                                                   |
| Stage IIc | Tumor either stage IIa or stage IIb but with ascites present or positive peritoneal washings                                                                               |
| Stage III | Growth involving one or both ovaries with intraperitoneal metastases outside the pelvis and/or positive retroperitoneal nodes; tumor limited to the true pelvis with histologically proved malignant extension to small bowel or omentum |
| Stage IV  | Growth involving one or both ovaries with distant metastases; If pleural effusion present, positive cytology required to assign a case to stage IV, parenchymal liver metastases equals stage IV |

**Special category**  Unexplored cases thought to be ovarian carcinoma

*Obtained from "Cancer Diagnosis in vitro using monoclonal antibodies" edited by Herbert Kupchik (p143)
Ovarian cancers can be found at all ages from infancy to old age. However, nonepithelial neoplasms account for most of the neoplasms in younger patients, while adenocarcinomas predominate in women of 25 years or older in most developed countries (Lingeman, 1983). The incidence of epithelial tumor rises rapidly after age 40 until a plateau occurs at age 80 (Hart, 1981). In general, it appears that ovarian cancer increases with "ovulatory age" - the total time in a woman's life during which her ovarian cycle is not suppressed by pregnancy, lactation or the use of oral contraceptives (Casagrande et al, 1979). Also it has been observed that frequent ovulation could increase the frequency of cyst formation which is thought to be associated with many incidences of ovarian cancers (Hart 1981).

Despite the prevalence of ovarian cancers, knowledge concerning the pathogenesis and etiology of ovarian cancer remains unresolved. General epidemiologic studies suggest that disordered endocrine function may contribute to the development of ovarian cancer (Joly & Lilienfield, 1974; Berval et al, 1978; Lingeman, 1974). Although the relationship between ovarian cancer and the administration of synthetic estrogen has not been clearly defined, it has been suggested that there may be an increased risk in patients receiving stilbestrol for menopausal symptoms (Hoover et al, 1977). On the other hand, considerable
evidence collected implies that some environmental agents such as polycyclic aromatic hydrocarbons, industrial pollutants, smoking (Mattison & Thorgeirsson, 1978), asbestos (Newhous, et al, 1972), talc (Cramer, et al 1982) and antirust oil (Jarvholm, 1981) may be carcinogenic. Familial and genetic factors have also been reported to be causes of ovarian cancers. In addition, geographical factors have been taken into account since migrants from low incidence countries to high incidence countries usually develop a similar incidence rate as the new host country within one or two generations (Liggeman, 1983).

Epidemiologic studies also suggest that cancer of the breast and ovary might share some common etiologic factors. For instance, women with ovarian cancer could have a 3 to 4 fold increase in the frequency of subsequent breast cancer, and women with breast cancer have a much higher (2 times) chance of developing ovarian cancer (Young et al, 1985).
D. Classification of Ovarian Cancer Markers

It is evident that ovarian tumor cells possess the ability to synthesize specific protein molecules that are expressed at the cell surface and sometimes shed into the serum (Sell, 1990; Nadji, 1990). If a sensitive assay of a tumor marker could be developed for the detection of ovarian cancers at an earlier stage, the 5-year survival rate of patients could be substantially improved. During the past years many efforts have been made to identify a reliable serum marker for ovarian cancers. Although some of them have been commercially available, none is totally satisfactory. Recently, with the application of advanced hybridoma technology, monoclonal antibodies (MAbs) with high specificity can be established to react with a distinct antigenic determinant or epitope present on particular antigen molecules, thus providing a more specific and sensitive tool for cancer detection (Accola et al, 1980; Knauf & Urbach, 1980; Bast et al, 1981; Bhattacharyya et al, 1982; Kabawat et al, 1983; Tagliabue et al, 1985; Tsuji et al, 1985; Miotti et al, 1985; Malnguene, 1986; Kawahara et al, 1986; Fleuron et al, 1987; Thor et al, 1987).
Potential tumor markers for ovarian cancer so far identified can generally be classified into three broad categories based on their nature (table 2). These are (1) oncofetal antigens; (2) carcinoplacental proteins; and (3) tumor associated antigens.

(1) Oncofetal antigens:

The application of cancer markers as a more generally applicable tool began with the discovery of AFP and carcinoembryonic antigen (CEA). (Gold & Freedman, 1965; Hoskins et al, 1987). Carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) are examples of oncofetal antigens which are produced only by normal embryonic tissues during fetal life. When a mature cell undergoes neoplastic transformation, the production of these proteins become reactivated as a result of the loss of regulator gene function. CEA is a large family of related cell surface glycoproteins that have a domain structure similar to that of immunoglobulins (Thompson, 1988). CEA was first identified by Gold and Freedman (1965) as an antigen specific for adenocarcinomas of the digestive tract. Its chemical structure is very complicated, and the CEA family consists of about 10 genes. So far, up to 36 different glycoproteins have already been identified. Chemically, CEA is a complex glycoprotein with numerous antigenic sites and a molecular weight ranging from 180,000 - 200,000 daltons. The ratio of protein to carbohydrate varies from 1:1 to 1:5 in
Table 2

Tumor markers with clinical usefulness in gynecologic cancers*

I. Oncofetal antigens
   A. Carcinoembryonic antigen (CEA)
   B. α-Fetoprotein (AFP)

II. Carcinoplacental antigens
   A. Human chorionic gonadotropin (HCG)
   B. Human placental lactogen (HPL)
   C. Regan isoenzyme (RI)

III. Tumor-associated antigens
   A. Ovarian cystadenocarcinoma antigen (OCAA)
   B. Ovarian cancer antigen (OCA)
   C. NB/70K
   D. CA 19-9
   E. CA-125

*Obtained from "Tumor markers and tumor associated antigens" by Bismal C. Ghosh, Luna Gosh (1987)

In ovarian carcinomas, it has been reported that more than 46% of patients have elevated CEA levels in their plasma (Van Nagell et al, 1981). Measurable levels of CEA have been reported in 50% of patients with stage III epithelial ovarian cancer. It has been found that the frequency of elevated CEA levels progressively increases with advancing stage and bulk of tumor.

Although CEA is detectable in all the major cell types of epithelial ovarian cancers, it can also be observed in mucinous ovarian tumors (Heald et al, 1978; Charpin et al, 1982). Besides ovarian cancers, CEA is also found in a large variety of cancers of epithelial origin (McIntire, 1985). Furthermore, the finding of elevated CEA in nonmalignant disease conditions and even in smokers (Hansen et al, 1974) has largely been a problem in applying this marker to screen large populations in an attempt to detect subclinical ovarian cancer (Van Nagell et al, 1976; Hansen et al, 1974; Smith & Oil, 1984). The elevated level of CEA can therefore only be considered as a suggestive parameter
in cancer diagnosis (Go, 1976; Zamcheck, 1981; Staab, 1984 and Terry et al, 1974). Currently, the major usefulness of CEA is limited to the follow-up of ovarian cancer patients whose plasma levels are elevated prior to therapy.

Alpha-fetoprotein (AFP) is another oncofetal protein first described by Abelev et al (Abelev, 1963). It is originally produced during fetal development by the liver, yolk sac, and gastrointestinal epithelium. AFP is also a secretory protein synthesized by tumor cells (Tatarinov, 1964 and McIntire, 1975). This property makes AFP an ideal tumor marker since it can be detected in the circulation of patients. It is a single chain glycoprotein with a molecular weight of 69,000 daltons. The carbohydrate content of AFP is about 4% of the molecular weight (Ruosslahti, et al, 1979). AFP exhibits microheterogeneity from different sources because of variation in sialic acid content (Ruoslahti et al, 1979). Generally, a value of 20 to 25 ng/ml or greater of AFP in serum is considered elevated (Donaldson et al, 1980).

Similar to that of CEA, elevations of serum AFP in ovarian cancer patients have also been reported (Esterhay et al, 1973; Talerman et al, 1974; Ghosh P.157 41, 42). Serum AFP is elevated in most cases of endodermal sinus tumor of the ovary in which a sample is taken prior to surgical excision of the cancer (Kurman et al, 1976; Gallion et al, 1979). It is reported that serum AFP levels reflect
clinical disease status in more than 75% of cases of endodermal sinus tumor (Wilkinson et al, 1973; Horney et al, 1975). Therefore, the serial measurement of AFP is considered to be very helpful in postsurgical evaluation of therapy for patients with endodermal sinus tumors of the ovary (Goldstein & Piro, 1972). Patients with ovarian teratocarcinomas also show elevated serum AFP, but it is unclear if these teratomas contain endodermal sinus tumor elements (Esterhay et al, 1973, Talerman et al, 1974). However, in 53 patients with epithelial carcinoma of the ovary, all had AFP levels lower than the cut off range (25 ng/ml), except one patient with a tumor of uncertain origin (Seppala et al, 1975). In addition, AFP elevation in the serum of the patients with benign conditions, such as acute viral hepatitis, liver cirrhosis, obstructive jaundice, and hepatic trauma, is also observed. In this regard, AFP is not an entirely reliable indicator of ovarian malignancy (Abelev, 1971). Nonetheless, from the clinical data obtained, AFP is still accepted as being useful in diagnosing endodermal sinus tumors in women with a rapidly enlarging solid ovarian mass.

(2) Carcinoplacental proteins:

Carcinoplacental proteins are protein moieties, e.g. human chorionic gonadotropin (HCG), normally produced by placental tissues during pregnancy. These proteins are also found in certain rapidly growing neoplastic cells (Van
Nagell et al, 1981; 1982). HCG is a well known hormonal glycoprotein composed of two subunits, the alpha and beta chains, and a molecular weight of approximately 39,000 daltons. HCG or its beta-subunit is a valuable marker for ovarian germ cell tumors that contain either embryonal cell carcinomas or choriocarcinoma (Kurman et al, 1976). Although HCG can, to a certain extent, reflect the status of ovarian germ cell tumors, it is considered to be of little value in patients with ovarian cancers of epithelial origin (van Nagell et al, 1981).

Human placental lactogen (HPL) is found normally in the human placenta and is detected in a high percentage of patients with gestational trophoblastic disease. Although HPL is observed occasionally in the serum of patients with epithelial and germ cell ovarian cancers, its usefulness in patients with gynecologic malignancy is very limited (Stuhlmill & Seigler, 1987).

Regan isoenzyme (RI) is an alkaline phosphatase found in normal placenta (Stolbach et al, 1969; Rosen, 1975) with a molecular weight of 120,000 daltons. It is detected in a number of gynecologic cancers, and 47% of patients with ovarian carcinoma show elevated levels of RI (Kellen et al, 1976). However, the effectiveness of RI in cancer diagnosis is limited as elevated serum levels of RI are also found in patients with diseases such as hepatitis, colitis, familial...
polyposis, and even in smokers (van Nagell et al, 1976; Smith & Oi, 1984).

(3) Tumor Associated antigens:

It is known that in many types of cancer tumor cells possess the ability to synthesize specific proteins which can be used to distinguish the neoplastic cells from normal cells (Sell, 1990; Nadji, 1990). Ovarian cancer cells also express or secrete unique glycoproteins that can potentially be used for immunodiagnosis and immunotherapy (Kenemans et al, 1985; Kenemans et al, 1988). It is evident that all benign and malignant epithelial tumors of the ovary including serous, mucinous, endometrioid, clear cell and transitional types express two or more proteins similar to keratin. However, by judging the profile of this particular antigen, ovarian epithelial neoplasm cannot be differentiated from epithelial neoplasms of other organs (Dabbs & Geisinger, 1988).

In addition to keratin-like antigens, epithelial tumors of the ovary usually contain other epithelial markers, namely, epithelial membrane antigen (EMA) and human milk fat globulin (HMFG) membranous antigens. Similar to that of keratin, these two antigens are not unique to ovarian cancer tissues (Nadji, 1990) and can also be found in other epithelial neoplasms as well as in malignant mesotheliomas.
Ovarian cystadenocarcinoma antigen (OCAA) is a glycoprotein chemically different from CEA and AFP and unique to neoplastic ovarian tissues (Bhattacharya & Barlow, 1975). Sixty percent of early stage ovarian cancer patients is reported to have elevated serum OCAA levels, and up to 80% of patients with advanced disease exhibits high levels of OCAA (Bhattacharya & Barlow, 1979). Serum concentration of OCAA in ovarian cancer patients correlates with the tumor volume and the status of the disease. In patients with tumor completely resected, the serum level returns to normal shortly after operation. However, this antigen is not specific enough to be used as a screening test for ovarian cancer, since 35% of patients with cancers not of ovarian origin, such as breast and colonial cancers also exhibit a positive reaction to antisera against OCAA (Bhattacharya & Chatterjee, 1980).

OCA (Ovarian cancer antigen) is another tumor associated glycoprotein. First described by Knauf and Urbach (Knauf & Urbach, 1977; 1981), Sixty percent of patients with ovarian cancer are observed to have elevated OCA levels in their plasma. However, more than 10% of patients with benign gynecologic disease or who are pregnant are also detected to be positive (Knauf & Urbach, 1980). Due to the lack of specificity of OCA, it is excluded as an efficient screening tool.
NB/70K is an ovarian tumor-associated antigen identified from ovarian cancer patients by radioimmunoassay (Knauf & Urbach, 1981). It is reported that NB/70K is a glycoprotein with a M.W. of 70,000 daltons and is present in most epithelial ovarian adenocarcinomas but not in normal tissues of the ovaries (Brizzari et al, 1983). The positive predictive rate for ovarian cancers using polyclonal antisera or monoclonal antisera against this antigen is not promising (Ken et al, 1981; Knauf & Urbach, 1981).

A number of tumor-associated antigens for ovarian cancers are also reported by various groups of researchers (Barlow & Bhattacharya, 1983; Cordon-Cardo et al, 1985; Mariani-Costantini, et al, 1985), including CA 19-9 (Finkler et al, 1988), TA-4 (Kato & Torigoe, 1977) TAG-72 (Thor et al, 1986), OV632 (Kaelma et al, 1988), MH 94 C1 (Nouwen et al, 1987) and MF 116 (Nouwen et al, 1987). However, most of these markers are not ovary-specific, and can be detected in other types of tumors and even in normal tissues.

Recently, two monoclonal antibodies(MAb) namely OC 125 which reacts with antigen CA 125 and OV-TL3 which reacts with antigen OA 3, are considered to be the most promising markers with respect to clinical applicability in gynecological oncology (Kenemans, 1990). OV-TL 3 MAb was established by fusion of murine myeloma cells with spleen cells of mice immunized with ovarian endometriod cancer cells (Bast et al, 1981). OV-TL 3 seems to be highly
specific for ovarian epithelial tumors, and useful for the
differential diagnosis, especially of colorectal cancer
(Henzen-Logmans et al, 1988). This may be due to the fact
that OA 3 antigen exhibits a high expression exclusively in
ovarian cancers. However, OA3 antigen is membrane bound and
not shed into the blood serum. Therefore, although it has a
high specificity to ovarian cancer, the feasibility of using
OV-TL3 antibody for serum immunoassay becomes particularly
limited.

CA 125 is an antigen associated with ovarian carcinoma
cells (Davis, et al, 1986). A MAb OC 125, raised by
immunizing mice with an epithelial ovarian cancer cell line
(OVCA-33) isolated from the ascites fluid of a cancer
patient, can identify this antigen CA 125 is reported to be
a glycoprotein with a molecular weight greater than 500,000
daltons (Bolts & D'Inclci, 1978). The MAb OC 125 is found
to recognize the CA 125 antigenic determinant present on the
glycoprotein complex (Bast, et al, 1981). The nature of the
epitope recognized is not known but it could be a
carbohydrate moiety of the protein molecules.

CA 125 is detected in fetal mullerian epithelium,
peritoneum, pleura, pericardium and amnion (Kabawat et al,
1983). Immuno-histological staining of frozen tumor
specimens indicates that 77% of the nonmucinous ovarian
tumors including serous, endometriods and clear cell types
are positive (Castaldo, et al, 1981). The antigen is not
detected in normal ovarian tissues and only 1% of apparently healthy person have elevated antigen levels. Changes in serum CA 125 levels are found to correlate with disease progression or regression in more than 90% of cases studied.

Although CA 125 appears to have a high sensitivity for ovarian cancers, elevated levels are also detected in some benign diseases and in other epithelial cancers (Breast and lung cancers) (Endo et al, 1988). Furthermore, most Krukenberg's tumors express detectable CA 125 (Fukazawa et al, 1988). About 25% and 5% of patients with nongynecologic malignancies and benign disease, respectively, are reported to have elevated antigen levels. The efficacy and reliability of CA 125 in the early diagnosis and monitoring of patients with ovarian carcinoma is still under active investigation.

E. Objective of the present work:

Over the past few decades, much research effort has been directed toward searching for a specific tumor marker for ovarian cancers. Although a number of tumor markers have been identified and studied, so far, none of them appears to be satisfactory. In this regard, intensive research is, therefore, essential for the identification of a new tumor-associated antigen which potentially can be used for the development of a diagnostic aid for the detection of ovarian cancer.
With the application of hybridoma technology, it is possible to establish monoclonal antibodies (MAb) with high specificity to react with a distinct antigenic epitope present on a particular antigen molecule, thus providing a more specific tool for cancer detection (Accola, et al, 1980; Kawahara, et al, 1986). A monoclonal antibody, RP 215, has recently been developed in our laboratory, which is highly specific to a tumor-associated antigen (COX-1) identified from an ovarian cancer cell line (OC-3-VGH) (Chao, et al., 1983). In the present work, efforts are directed toward the isolation, purification and characterization of the antigen COX-1 from the shed culture medium of ovarian cancer cells, OC-3-VGH. In addition, attempts are made to determine the biochemical nature of the determinant recognized by the MAb RP 215 and to develop an immunoassay for this antigen. The usefulness of this assay kit is being evaluated by various groups of collaborators and their preliminary clinical data will be presented and discussed in the thesis.
VIII. Materials and Methods

A. Materials:

A) Chemicals:

Tissue culture media and supplements including RPMI 1640, IMDM, glutamine and penicillin-streptomicin (100X) were from Gibco, Burlington, Ontario, Canada; 2,6,10,14-tetramethyl-pentadecane (pristane), dimethylsulfoxide (DMSO), methylcellulose, bovine serum albumin (BSA), polyethylene glycol (PEG), complete and incomplete Freund's adjuvant were purchased from Sigma Chemical Company, St Louis, MO; fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG+A+M was from Cappel, Malvern, PA; Reagent for sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA; Iodine-125 radioisotope was from Amersham, Oakville, Ontario, Canada.

B) Animal and cell lines:

Inbred male mice of BALB/C strain (6-10 weeks) were from Charles River, Canada; the ovarian cancer cells line, OC-3-VGH, was of serous origin and was established by the Department of Obstetrics and Gynaecology, Veterans General Hospital, Taipei, Taiwan (Chao et al, 1983).

Several other cell lines including ME-180 (human epidermoid cervical cancer), Shiha (human cervical squamous carcinoma), JEG (human choriocarcinoma), C33A (human cervical carcinoma) and AN3A (human endometrial carcinoma)
were obtained from American Type Culture Collection Inc. (ATCC, Rockville, Maryland).

B. Production and characterization of monoclonal antibody (RP 215)

A) Production of monoclonal antibodies

Monoclonal antibodies could be produced either from culture medium of antibody-secreting hybrid cells which were the result of previous cell fusion screened in Dr. Lee's lab, or from ascites fluid collected from BALB/C mice primed with pristane according to the published procedures (Freund, et al, 1982). Briefly, pristane (0.5 ml) was injected 5 days in advance to the intraperitoneal cavity of the BALB/c mice followed by intraperitoneal injection of $1 \times 10^5$ to $1 \times 10^6$ antibody-secreting hybrid cells in serum free medium. The ascites fluid was drawn after one week and stored at $-20^\circ$C.

B) Immunohistochemical characterization of monoclonal antibody, RP-215

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 brain, liver, heart, kidney, spleen, ovary, testis, cervix, and muscle. These tissues were obtained at the time of surgery or at autopsy. Paraffin
tissue sections were deparaffinized in xylene, dipped in 95%, 75% and 50% ethanol, and finally washed with PBS. Cryostat tissue sections were also prepared and dipped in 95% ethanol followed by wash with PBS. The immunofluorescent method was performed according to procedures to be described later (p27).

Several cell lines, including ME-180, Shiha, Jeg, C33A and AN3A, were examined for their respective cross reactivities to the RP 215 monoclonal antibody. For immunofluorescent study, 50 ul of cultured cells with a concentration of $1 \times 10^6$/ml from each cell line were dried on the slides. The slides were then blocked with blocking solution containing 0.5% BSA in PBS and fixed with methanol. Twenty ul of RP 215 from the culture supernatant was added, followed by one hour incubation. After washing three times with PBS-BSA, 20 ul of goat-anti-mouse IgA+M-FITC was added. For ELISA, the cell extract from each cell line was adjusted with PBS to 0.05 absorbance/ml at 280 nm. Details of the assay procedures will be described later (p26).

C. Purification of RP 215 by DEAE column:

Fifty ml of ascites fluid containing RP 215 monoclonal antibody was first precipitated with 50% saturated ammonium sulfate. The suspension was centrifuged at 10,000 rpm for ten minutes. The pellet was redissolved in 20 ml of distilled water. The protein solution was then precipitated
again with 40% ammonium sulfate and centrifuged at 10,000 rpm for 10 minutes. The pellet was redissolved in about 10 ml of 10 mM Tris buffer, pH 8.0 and dialyzed overnight against the same buffer at 4°C. The precipitated protein was removed by centrifugation. The dialized solution was loaded (1 mg protein/ml gel) onto a DEAE column (size 2.5 x 30 cm) which had been equilibrated with 10 mM Tris buffer pH 8. After loading, the column was washed with the same buffer until absorbance at 280 nm decreased to blank level. The solution was then eluted with a linear gradient of 0 to 0.3 M NaCl in 10 mM Tris HCl, pH 8.0. The fractions containing the highest absorbance at 280 nm were collected. The purity of RP 215 was analyzed using SDS-PAGE with 10% acrylamide gels(see p31).

D. Immunological methods:

A) Enzyme-linked immunosorbent assay (ELISA)

The specific binding of monoclonal antibody RP 215 to OC-3-VGH cells was also determined by ELISA (Lee C.Y.G. et al, 1984). Briefly, cells from OC-3-VGH cell line were cultured in each of 96 wells in microtiter plates until a monolayer was formed. Alternatively, OC-3-VGH cells could be harvested from cell cultures and dried on microtiter wells at a final concentration of 1 x 10^6/ml. After removal of culture supernatant, the cell-coated wells were blocked by incubation with PBS-BSA (0.5% BSA in PBS) for one hour prior
to use by ELISA. After one hour of incubation with the test antibody in culture supernatant, wells were washed three times with PBS-BSA. Goat anti-mouse IgG+M+A conjugated with horse radish peroxidase was added to each well for an additional hour incubation at room temperature. Following three washes with PBS-BSA, 3,3',5,5'- tetramethyl benzidine (0.3 mg/ml) and 0.02% H₂O₂ in 0.1 M citrate-phosphate buffer, pH 5.0 were added to initiate the enzymatic reaction at room temperature for 15 minutes. The color reaction was stopped by adding 1 M H₂SO₄. The color intensity in each well was determined spectrophotometrically at 450 nm by a CLS microplate reader (Cambridge, UK). Normal mouse serum in RPMI 1640 medium served as the negative control in all assays, while mouse antisera against OC-3-VGH cells served as the positive control.

B) Indirect immunofluorescent assay

Indirect immunofluorescent assay was used to determine the binding of antibodies to the COX-1 cell surface antigen and to determine antibody titers. This assay was performed using methanol-fixed tumor cells according to the reported procedure (Bast at el, 1984). Due to the fact that methanol-fixed cells or paraffin embedded tissue sections do not consistently preserve their surface antigens which react with monoclonal antibodies, an indirect immunofluorescent assay using a live cell suspension was also performed. Briefly, 50 ul of cell culture supernatant containing
monoclonal antibodies was mixed with a suspension of cultured OC-3-VGH cells (1 x 10^5/ml) in either microfuge tubes or microtiter wells. The mixture was incubated at room temperature for 3 hours. Following three washes with PBS-BSA and centrifugation, FITC-labeled goat anti-mouse IgG+M+A was added to the cell suspension and the mixture was incubated for an additional two hours. Following three washes with PBS-BSA and centrifugation, the final superatant was discarded. Finally, 10 ul of 80% glycerol in PBS was added to the cell pellet and transferred to slides for observation under a fluorescence microscope.

C) Sandwich enzyme immunoassay

One of the monoclonal antibodies, RP 215, was shown to recognize repeated epitopes of the complementary tumor-associated antigen Cox-1. Therefore, this antibody was used to determine Cox-1 levels in the sera of ovarian cancer patients and those of normal healthy subjects by means of sandwich enzyme assay. Briefly, RP 215 was purified from ascites fluid by ammonium sulfate fractionations and DEAE ion exchange chromatography. Purified monoclonal antibody was coated on microwells at a concentration of 5 - 10 ug/ml in 50 mM Tris-HCl, pH 8.0. At the same time, RP 215, labelled with HRP (horse radish peroxidase), at a concentration of 1/400 with conjugate buffer (1% BSA, 0.2% PEG, 0.1% Thimerisol in 0.15M Tris pH 8.0) was added to the wells, and this served as the second antibody tracer in the sandwich
enzyme immunoassay. The immunoassay was initiated by incubating 100 ul of human serum and RP 215 labeled HRP (1:400) in antibody-coated microwells (Duplicate) for three hours at room temperature. After incubation, the content of the microwells was removed by suction. The wells, as in previous procedures, were washed three times with PBS-tween 0.02% and twice with distilled water. PBS containing 5% BSA served as negative control. Concentrate shed medium from OC-3-VGH precipitated with ammonium sulfate served as standard for the assay of Cox-1.

D) Tissue adsorption experiment

Normal human tissues such as liver, kidney, testis, muscle, heart and brain were homogenized. The concentration was adjusted to 10 mg/ml with 1% BSA in PBS. Fifty ul of tissue homogenate sample was added to microtiter wells coated with OC-3-VGH cells. One hundred ul of RP 215-HRP (horse radish peroxidase) of different dilutions was combined with 0.2 M Tris HCl, pH 8, 50% newborn serum or calf serum, 0.1 % thimersol and phenol and added to each well. For the control, 50 ul of 1% BSA in PBS or COX-1 partially purified from shed medium of OC-3-VGH cell were used as negative and positive control. The activity was determined by EIA as described.
E) Dot blot assay

The dot blot assay was modified from the method described by Towbin (Towbin et al., 1979). This method would give rapid detection of antigen in various samples and was especially useful for testing column eluents during antigen purification.

Briefly, 5 ul of sample was dotted onto nitrocellulose filter paper and allowed to dry completely. The nitrocellulose filter was blocked with 5 % BSA in PBS for 1 hour, then incubated with iodinated monoclonal antibody RP 215 for another two and a half. After washing three times with TBST, the nitrocellulose filter was counted on a gamma counter.

E. Purification of COX-1

A) Preparation of Immunoaffinity column:

Monoclonal antibodies were coupled to affini-Gel-10 (BioRad Co.) according to established protocols. Eight ml of RP 215 (1.3mg/ml in 0.1M NaHCO₃, pH 8.3) purified by DEAE column was mixed with 5 ml affini-Gel-10 and shook for two hours at room temperature. The column was washed with PBS and 1M Tris HCl, pH 8.0, (2 volumns each) and then by 2M NaCl containing 6N urea. Finally, the column was washed with 2 volumes of 50 mM glycine HCl pH 2.2 prior to equilibration with PBS for antigen purification.
B) Purification of COX-1 from the shed culture medium:

COX-1 was purified from shed culture medium by ammonium sulfate fractionation and then by immunoaffinity chromatography using RP 215 as the affinity ligand. Briefly, COX-1 in shed medium was first precipitated in the presence of 35% ammonium sulfate. Following centrifugation, the pellet was redissolved in minimal amount of PBS and dialyzed overnight against the same buffer. Following centrifugation to remove the denatured protein, the clear supernatant was then passed through a 10 ml RP 215-immunoaffinity column. The affinity column was then washed extensively with 300 ml of PBS at a flow-rate of 50 ml/hr. This was followed by a continued wash with an additional 300 ml of PBS containing 1M NaCl to eliminate all possible nonspecifically bound proteins. Finally, COX-1 was eluted with 50 mM glycine HCl, pH 2.2., and fractions of 1 ml were collected and neutralized immediately with 1M Tris, HCl, pH 8.0. During the course of antigen purification, COX-1 immunoactivity in the shed medium and purified preparation was determined by sandwich enzyme immunoassay. The purity of COX-1 preparation was examined by SDS gel electrophoresis, two-dimensional gel electrophoresis (O'Farrel et al, 1977) and by Western blot assay (Lee et al 1982).

C) SDS-PAGE (SDS polyacrylamide Gel Electrophoresis)

The purity of the fractions containing the highest OD
at 280 nm and immunoactivity was studied by 10% SDS-PAGE. Samples mixed with an equal amount of sample buffer (15 ul) were heated for five minutes at 100°C to denature the protein and then applied to the gel. The initial voltage for electrophoresis was set at 100 V. When the sample protein reached the top of the running gel, the voltage was set to 200 V. The electrophoresis was stopped when the dye reached 1 cm from the bottom of the running gel. After electrophoresis, the gel was removed carefully for protein visualization with either silver or coomassie staining.

D) Western blot assay

This method was used to identify the specific protein bands separated by SDS-PAGE in the presence of antibodies. Details of this method are described in the instruction manual supplied by Bio-Rad (Towbin et al, 1979). The purified antigen was also analyzed by Western blot assay. After electrophoresis, the gel and the nitrocellulose filter paper were rinsed with transfer buffer. The whole sandwich unit was placed in the electrophoretic transfer apparatus with the gel facing the cathode. The buffer tank was filled with enough transfer buffer to cover the gel. After 2 hours of electrophoretic transfer at a constant voltage of 50 V, the nitrocellulose paper was removed and placed in blocking buffer for one hour.

COX-1 which reacted with monoclonal antibodies or Rabbit polyclonal antibodies could be analyzed by either
direct method or indirect method. By direct method, RP 215 conjugated HRP (Horse Radish Peroxidase) in 1:400 dilution was added after the nitrocellulose paper had been blocked at 4°C overnight. By indirect method, COX-1 reacted with rabbit antisera (1:100) for two hours. After washing with TBST, goat anti-rabbit IgG conjugated with HRP was added for another two hours. After washing with TBST, the protein pattern was stained by adding enzyme substrates.

F. Characterization of COX-1 antigen:

A) Thermal Stability of COX-1 Immunoactivity

The thermal stability of COX-1 aggregates was determined by its immunoactivity using the established sandwich enzyme immunoassay. Briefly, the tumor cell extract or shed medium was incubated at 50 °C or higher temperatures for various time intervals. At the end of each incubation, aliquots were drawn and cooled at 0°C in ice. The residual Cox-1 immunoactivity was then determined using the established EIA procedure.

B) 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 concentration of 1 mg/ml for various time intervals at 37°C. At the end of each
incubation, trypsin activity was neutralized with two fold excess of soybean trypsin inhibitor (1 mg/ml). The residual COX-1 immunoactivity was determined by the established sandwich enzyme immunoassay. Similarly, carbohydrate-digestive enzymes or carbohydrate-binding proteins, namely neuraminidase, beta-galactosidase, fucosidase and Concanavalin A, N-glycosidase and O-glycosidase, were incubated with COX-1 solutions to determine whether COX-1 immunoactivity would be affected.

The carbohydrate modifying agent, NAIO₄ (final concentration 0.1M) was added to COX-1 solution (in PBS) and incubated for 1 hour at room temperature. At the end of incubation, COX-1 solution was dialyzed extensively against PBS at 4°C. The remaining COX-1 immunoactivity was determined by the established EIA and compared to that of the control in which no NAIO₄ was added so to determine its effects.

C) Determination of molecular weight of COX-1 in the native state

The molecular weight of COX-1 in the native state was determined by Sephacryl S-300 gel filtration chromatography. Myoglobin (Mr. 13,000), ovalbumin (45,000), immunoglobulin G (160,000) and thyroglobulin (675,000) were used as molecular weight standards. By using Sephacryl S-300 gel filtration chromatography, the molecular weight distributions of
purified COX-1, tumor cell extract and concentrated shed medium were determined. Briefly, swollen Sephacryl S-300 in a 22 ml column (size 1 x 30 cm) was equilibrated with PBS containing 0.1% Thimersol. One ml of purified COX-1, OC-3-VGH cells extract or concentrated shed medium (10x) was loaded on to the column. Fractions of 0.5 ml were collected. Immunoactivity for COX-1 in each fraction was determined by the established sandwich enzyme immunoassay.

G. Production of polyclonal antisera against purified COX-1

Antisera against purified COX-1 were raised in New Zealand white female rabbits according to typical immunization protocols (Lee et al, 1984). Briefly, 50 ug of purified COX-1 in 0.5 ml of PBS was emulsified with an equal volume of complete Freund’s adjuvant. The mixture was injected into the back of the rabbit subcutaneously in 10 different sites. Two weeks after the primary immunization, the rabbits were immunized twice with the same amount of immunogen except that incomplete Freund’s adjuvant was used. One week after the third immunization, blood was drawn from the retroauricular vein of the rabbit and the titer was checked by EIA or radioimmunobinding assay using I 125 -labeled COX-1 as the tracer (Lee et al 1986).
IX. Results

RP 215 was one of 3000 monoclonal antibodies generated against OC-3-VGH ovarian cancer cell line. Initially, this antibody was selected based on its positive binding to OC-3-VGH ovarian cancer cells coated on microtiter wells as judged from the results of ELISA.

The RP 215-secreting hybrid cell line was further established by the ability of this antibody to react with live or methanol-fixed OC-3-VGH cells by indirect immunofluorescent assay (Figure 1-2).

A. Purification and Characterization of monoclonal antibody, RP 215

The tissue-specificity of RP 215 was generally determined by tissue adsorption experiments and an immunohistochemical study of human tissue sections to be described as follows:

(A) Tissue adsorption experiments (Figure 1-1)

To examine the tissue-specificity of selected monoclonal antibodies, a quantitative tissue adsorption experiment was performed. The results of this experiment shown in Figure 1-1 revealed that normal human tissues do not interfere with the binding between HRP-labeled RP 215 and the OC-3-VGH ovarian cancer cells coated on microwells.
Figure 1-1

Tissue adsorption experiment to demonstrate the lack of inhibition of horseradish peroxidase-labeled RP 215 (HRP-RP 215) binding to OC-3-VGH tumor cells coated on microwells by the normal tissue homogenates, where (●), (○), (▲), (△), (□) and (■) denote those of kidney, liver, muscle, testis, heart and brain, respectively. (▽) denotes soluble extract of OC-3-VGH tumor cells (protein concentration of 10 mg/ml), (▼) denotes the nonspecific binding between HRP-RP 215 and microwells coated with unrelated proteins, and (X) denotes the BSA (10 mg/ml).
Figure 1-2

Indirect immunofluorescent staining of OC-3-VGH ovarian cancer cells by RP 215 monoclonal antibody observed under fluorescence microscope: (A) under UV light; and (B) under visible light.
(B) Immunohistochemical study:

Normal human tissue slides of vaginal wall, uterine cervix, fallopian tube, ovary, heart, colon and some benign and malignant tumors of different tissue were deparaffinized and fixed in methanol for 5 min and blocked with PBS BSA. Immunohistochemical study was performed by the immunofluorescent procedure. RP 215 was shown to have the highest specificity to OC-3-VGH cells (Figure 1-2) and did not cross react with other normal human tissues or other benign and malignant tumors (Table 3).

Several cell lines including ME 180, AN3A, Shiha, C33A and Jeg were also used to determine their respective cross reactivities to RP 215 by ELISA and indirect immunofluorescent method. It was observed that RP 215 can recognize antigens only from ME 180 and OC-3-VGH cells (Figure 1-2) but not any other established cell lines used in this study. The results are presented in Table 4.

(C) Purification of RP 215 monoclonal antibody from ascitis fluid

From 50 miniliters of ascites fluid, about 180 mg of monoclonal antibodies could be purified through the DEAE cellulose column.
Table 3. Immunofluorescent assay of paraffin tissue sections from clinically defined benign and malignant tumors

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Clinical diagnosis</th>
<th>Indirect* Immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>muscle</td>
<td>fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>tongue</td>
<td>mucocele</td>
<td>-</td>
</tr>
<tr>
<td>hypopharynx</td>
<td>adenoid cystica Ca</td>
<td>-</td>
</tr>
<tr>
<td>salivary gland</td>
<td>chronic sialoadenitis</td>
<td>-</td>
</tr>
<tr>
<td>intestine</td>
<td>adenocarcinoma</td>
<td>-</td>
</tr>
<tr>
<td>fallopian tube</td>
<td>ectopic pregnancy</td>
<td>+</td>
</tr>
<tr>
<td>ovary</td>
<td>simple cyst</td>
<td>-</td>
</tr>
<tr>
<td>anus</td>
<td>hemorrhoid</td>
<td>-</td>
</tr>
<tr>
<td>bursa</td>
<td>baker cyst</td>
<td>-</td>
</tr>
<tr>
<td>skin</td>
<td>fibrosis</td>
<td>-</td>
</tr>
<tr>
<td>breast</td>
<td>fibroadenoma</td>
<td>-</td>
</tr>
<tr>
<td>spleen</td>
<td>spleen rupture</td>
<td>-</td>
</tr>
</tbody>
</table>

*OC-3-VGH cells fixed on slides were used as the positive control
Table 4
Cross reactivities of RP 215 with the established tumor cell lines by ELISA and immunofluorescent assay

<table>
<thead>
<tr>
<th>cell line</th>
<th>IIF Assay</th>
<th>ELISA with shed medium</th>
<th>ELISA with cell extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC-3-VGH</td>
<td>+</td>
<td>1.069</td>
<td>0.439</td>
</tr>
<tr>
<td>ME 180</td>
<td>+</td>
<td>0.726</td>
<td>0.349</td>
</tr>
<tr>
<td>Shiha</td>
<td>-</td>
<td>0.190</td>
<td>0.104</td>
</tr>
<tr>
<td>Jeg</td>
<td>-</td>
<td>0.190</td>
<td>0.128</td>
</tr>
<tr>
<td>C33A</td>
<td>-</td>
<td>0.173</td>
<td>0.102</td>
</tr>
<tr>
<td>AN3CA</td>
<td>-</td>
<td>0.170</td>
<td>0.151</td>
</tr>
</tbody>
</table>

Tumor cell lines obtained from American type culture collection Inc..

OC-3-VGH - ovarian cancer cells (serous type)
Me-180 - human epidermoid cervical cancer (ATCC HTB 33)
Shiha - human cervical squamous carcinoma (ATCC HTB 35)
JEG - human choriocarcinoma (ATCC HTB 36)
C33A - human cervical carcinoma (ATCC HTB 31)
AN3CA - human endometrial Ca (ATCC HTB 111)
IIF - indirect immunofluorescence
ELISA - enzyme-linked immunosorbent assay
B. Purification of COX-1:

The tumor-associated antigen, COX-1 was purified mainly from cultured shed medium by using RP 215 as the ligand in the immunoaffinity chromatography. The molecular weight of purified COX-1 was shown to be 60 KD on SDS Gels (Fig 2) and confirmed by Western blot assay. In the neutral state, purified COX-1 existed as an undefined aggregate of 60 KD basic subunit with molecular weight ranging from $1 \times 10^5$ to $1.5 \times 10^6$ daltons. This can be shown by the elution profiles from Sephacryl S-300 gel filtration chromatography as shown in Figure 5. By radioimmuno dot assay the specific activity of purified COX-1 could reach up to 15,000 times that of the starting material (Table 5). The purified COX-1 could be used for iodination, standard antigen preparations or for immunization in rabbit to raise polyclonal antibodies.

C. Characterization of COX-1

A) Thermal stability of COX-1 immunoactivity

As shown in Figure 3, COX-1 immunoactivity was relatively stable in a PBS solution of neutral pH at temperatures below 50°C. At incubation temperatures higher than 50°C, COX-1 immunoactivity was observed to decrease significantly with incubation time. The immunoactivity of COX-1 completely diminished after incubation at 100°C for 10 minutes.
Table 5

Purification of COX-1 from culture shed medium of cultured OC-3-VGH cells

<table>
<thead>
<tr>
<th>Purification</th>
<th>protein concentration (ug/ml)</th>
<th>specific activity</th>
<th>fold of protein purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>shed medium</td>
<td>2780</td>
<td>2.3</td>
<td>1</td>
</tr>
<tr>
<td>ammonium sulfate ppt.</td>
<td>6712</td>
<td>26.3</td>
<td>11</td>
</tr>
<tr>
<td>Immunoaffinity</td>
<td>24)* 11.25</td>
<td>8853</td>
<td>3849</td>
</tr>
<tr>
<td>Chromatography</td>
<td>25)* 10</td>
<td>34720</td>
<td>15056</td>
</tr>
<tr>
<td></td>
<td>26)* 15</td>
<td>12186</td>
<td>5298</td>
</tr>
</tbody>
</table>

*Fraction number of immunoaffinity chromatography profile

**Determined by dot blot immunoassay described in the text
Figure 2

Results of SDS gel electrophoresis and the corresponding Western blot assay to reveal respectively the purity and immunospecificity of COX-1 purified by immunoaffinity chromatography. Lane B is SDS gel of purified COX-1 (5 ug/well, fraction #25 of Table 5); lane A is COX-1 revealed by Western blot assay; lane C is the molecular weight standards of the corresponding SDS gel with size of molecular weight markers indicated by arrows on the right.
Figure 3. Effect of temperature and incubation time on the immunoactivity of COX-1. Samples of COX-1 were incubated at different temperatures for different time intervals, where (●), (■), (▲) and (▲) denote 50°C, 60°C, 80°C and 100°C, respectively. The residual activity of COX-1 was determined by the solid-phase sandwich enzyme immunoassay described in the text.
B) Sensitivity of COX-1 immunoactivity to digestive enzymes, Con A and NaIO₄ and trypsin:

Under the assay conditions described, COX-1 immunoactivity was shown to be sensitive to proteolytic digestion by trypsin at 37°C (Figure 4) when analyzed by sandwich EIA method. It was observed that only 0.7% of COX-1 immunoactivity could be recovered after incubation with trypsin for 60 minutes. However, COX-1 immunoactivity was not affected significantly by incubation with concanavalin A (con A), NaIO₄ and the carbohydrate-digestive enzymes including neuraminidase, beta-galactosidase and fucosidase, N-glycosidase and O-glycosidase.

C) Molecular weight distribution

Using Sephacryl S-300 gel filtration chromatography, the molecular weight distribution of purified COX-1 purified, tumor cell extract and concentrated shed medium was determined. The results of this analysis are presented in Figure 5.
Figure 4. Effect of trypsin digestion on COX-1 Immunoactivity. COX-1 antigen was incubated with trypsin at 37° C for the various time intervals as described in the procedures. The residual activity of COX-1 was determined by the solid-phase sandwich enzyme immunoassay, described in the text.
Figure 5

Sephacryl S-300 gel filtration chromatography to reveal the molecular size distributions of COX-1 in different antigen preparations. Relative immunoactivities of COX-1 were determined by the established solid-phase sandwich immunoassay described in the text. Where (△), (□), and (○) represent those of COX-1 antigens prepared from crude OC-3-VGH extract, purified from shed medium, and concentrated shed medium derived from cultured OC-3-VGH cells, respectively. A gel filtration column (size 1x30cm) equilibrated with PBS-BSA containing 0.1% thimersol was used for this analysis. Fractions of 0.5 ml/tube were collected and assayed for immunoactivity. The void volume and column volume of this column appeared in fractions #19 and #44, respectively, as judged from the calibration with the known molecular weight standards and blue dextran (molecular weight 2x10^6 dalton) described in the text.
As shown in the figure, COX-1 in three different preparations existed in the form of undefined aggregates with molecular weight ranging from $1 \times 10^5$ to greater than $1.5 \times 10^6$ daltons. However, Western blot assay revealed that all of the aggregates were derived from a basic unit of 60 KD.

D. Assay of COX-1 by solid phase Sandwich enzyme immunoassay

In view of the fact that RP 215 recognized repeated epitopes of its complementary antigen, COX-1, a sandwich enzyme immunoassay using this antibody was designed for quantitative determination of COX-1 in different antigen preparations or in human sera. The standard curve of this enzyme immunoassay is presented in Figure 6. The sensitivity of this enzyme immunoassay was determined to be 1 AU/ml. The intra and inter-assay variation is between 5-15%.

E. Characterization of rabbit antisera against COX-1

COX-1 protein spot was removed from two-dimensional gels of the affinity purified COX-1 and used as an immunogen for the immunization of rabbits. The titers of rabbit antisera were determined by radioimmunobinding assay and by enzyme-linked immunosorbent assay using OC-3-VGH cells coated on microwells. The results of this immunoassay indicated that after successive immunizations, the titer of
Figure 6

A standard curve of the solid-phase sandwich enzyme immunoassay for the quantitative determinations of COX-1 immunoactivity from OC-3-VGH cell extract. The immunoactivity of COX-1 was expressed in AU/ml as defined. Details of this assay procedure were described in the text.
rabbit antisera was determined to be 1:400 serum dilution (as shown in Figure 7). By western blot assay, the rabbit antisera were shown to react with a protein band of 60 KD molecular weight which was identical to that of RP 215 monoclonal antibody (Figure 2).
Figure 7

Determination of titer of rabbit anti-COX-1 by ELISA method as described in the text, where (●) and (▲) indicate rabbit antiserum (after the third immunization) and normal rabbit serum, respectively.
X. Discussion

During the last decade, numerous investigations have been performed in the search for "ideal" tumor markers which allow differentiation between the normal and neoplastic conditions. This tumor marker should be highly specific to the tumor cells and at the same time correlate with the prognosis and stage of the malignancy. Despite the continuing development of new therapeutic modalities, the most important factor influencing the prognosis of female genital cancer is early diagnosis.

Early diagnosis of ovarian cancers is difficult, mainly because of the lack of reliable and specific tumor markers (Introna, M at al, 1985). Currently, available cancer treatments such as radiotherapy and chemotherapy are often hampered by their apparent lack of tumor specificity and low therapeutics/toxicity ratios.

Therefore, the development of such a marker for ovarian cancer is essential as there appears to be little chance of early diagnosis without such a diagnostic aid. The rationale and importance of developing reliable tumor markers for female genital cancer, however, are not limited to early diagnosis of cancer. Such markers would enable gynecologic oncologists to follow-up patients after treatment and also to detect the recurrence at an early stage so that an additional or a new treatment modality
could be initiated. Development of a reactive antibody conjugated with a tracer substance to attack a cancer antigen could greatly enhance the accurate identification of tumor cells that have spread beyond the original site of the cancers. Ultimately, chemotherapeutic or radiotherapeutic agents could be conjugated to these antibodies, enabling the physician to achieve selective therapeutic eradication of cancer cells while sparing normal tissues.

As ovarian epithelial cancers usually produce no symptoms prior to generalized intraabdominal dissemination of the disease and since there are no diagnostic screening tests that allow early diagnosis, it could be useful if a serum test for tumor marker could be utilized for early diagnosis. Unfortunately, currently available tumor markers for ovarian cancer are neither sensitive nor specific enough to enable early diagnosis. Development of the new marker in the future would be beneficial for the clinician to diagnose cancers earlier and get better prognosis.

With the development of hybridoma technology of monoclonal antibodies, RP 215 was generated and shown to be highly specific to single epitopes of tumor-associated antigen COX-1.

Monoclonal antibodies secreted by hybrid clones were initially screened simultaneously by ELISA and indirect immunofluorescent assay for their respective specificity to
cultured OC-3-VGH cells. The tissue specificity of the generated antibodies was examined by the quantitative tissue adsorption experiment and immunohistochemical study. Among these monoclonal antibodies, RP 215 demonstrated the highest degree of specificity to OC-3-VGH tumor cells and showed no cross reactivity to normal human tissues including liver, testis, kidney, heart, muscle, and brain homogenate (Figure 1-1) and to tissue sections of vaginal wall, cervix, fallopian tube, ovary, colon and small intestine, as resulted by the immunofluorescent method. In addition, RP 215 cross-reacted with some of the human gynecological cancer cell lines employed in this study, including that of ME-180 cells which are of cervical epithelial origin (Table 4). Therefore it may be reasonable to suggest that COX-1 may be a tumor-associated antigen derived from cancers of either ovarian or cervical origins. COX-1 cannot be detected in the female serum during the proliferative phase of the menstrual cycle which was assayed from 10 patients’ samples collected by Dr. Basal Ho Yuan in Grace Hospital, Vancouver.

The tumor-associated antigen, COX-1 was purified to homogeneity by using an immunoaffinity chromatographic procedure from the cultured shed medium or from the cultured tumor cell extract. COX-1 appeared to exist in the state of aggregates in the shed medium or in tumor cell extract. However, after being purified from affinity column by a low
pH buffer in 50 mM glycine - HCl, pH 2.2, following neutralization, COX-1 continues to exist as an undefined aggregate in PBS. This was clearly demonstrated by a sepharyl S-300 gel filtration chromatography. Following immunoaffinity purification, COX-1 retained immunoactivity to RP 215 when analyzed by dot blot immunoassay or Western blot assay.

The aggregate form of COX-1 was unstable at temperatures above 50°C and sensitive to trypsin (Figure 4) and other proteases. However, treatment of COX-1 with NaIO₄ and several carbohydrate-digestive enzymes including neuraminidase, beta-galactosidase and fucosidase did not affect the immunoactivity of COX-1. This clearly suggested that the carbohydrate moiety of COX-1 may not be essential for the binding between RP 215 and this antigen.

Rabbit antisera raised against purified COX-1 (50 ug) were shown to exhibit similar immunospecificity to that of RP 215 monoclonal antibody. The polyclonal antisera might prove to be useful for immunoassay and immunohistological studies of this tumor-associated antigen.

A solid-phase sandwich enzyme immunoassay using RP 215 monoclonal antibody would enable us to detect the native form of COX-1 either in cultured shed medium of tumor cells or in sera of patients with ovarian or cervical cancer. This enzyme immunoassay kit might prove to be beneficial for
the diagnosis and monitoring of patients with ovarian or cervical cancers.

In order to explore the potential utility of COX-1 enzyme immunoassay kit for the diagnosis and monitoring of patients with cervical and ovarian cancers, multi-center collaborative studies are being conducted. Preliminary clinical evaluation data were available from the following sources:

(1) Department of Obstetrics and Gynecology Veterans General Hospital, Taipei, Taiwan, China

(2) Department of Obstetrics and Gynecology Veterans General Hospital, Taichung, Taiwan, China

(3) Hu-Bei Medical University, Wu Han, Hu-Bei, China

Typical results are presented in Table 6, Table 7, and Table 8. Due to the lack of absolute COX-1 antigen absolute standards, the serum levels of COX-1 among normal individuals varied from the study sources. However, the general conclusions in regard to the performance of this immunoassay kit were quite similar. As shown in Table 6, which reports data from VGH, Taipei, patients with ovarian cancer had higher levels of COX-1 than those with benign tumor or pelvic mass. Serum COX-1 levels among those patients with cervical carcinoma seemed to be correlated with their respective disease stages. As many as 60-80% of patients with cervical or ovarian cancers had
Table 6

Preliminary Clinical Evaluation of COX-1 among Patients with Ovarian, Cervical Cancers, Benign Tumors or Normal Health Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Ovarian Disease</th>
<th>Cervical Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OC*</td>
<td>OT*</td>
</tr>
<tr>
<td>Cases</td>
<td>130</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Mean</td>
<td>29.4</td>
<td>82.0</td>
<td>31.4</td>
</tr>
<tr>
<td>S.D.</td>
<td>19.4</td>
<td>34.3</td>
<td>13.1</td>
</tr>
<tr>
<td>% Positive***</td>
<td>80</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

Normal control vs. OC p<0.001 OC vs. OT p<0.001 OT non-significant OC vs. PM p<0.01 PM p>0.05 OT vs. PM p>0.05

vs. Cervical Carcinoma

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>p&lt;0.001</td>
<td>I vs. II</td>
<td>p&gt;0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>p&lt;0.001</td>
<td>I vs. III</td>
<td>p&gt;0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* OC: Ovarian Cancer, AU/ml
OT: Ovarian Tumor
PM: Pelvic Mass

** data provided by Dr. H. T. Ng, Dept. of Obstetrics & Gynecology, Veterans General Hospital, Taipei, Taiwan.

*** positive: ≥ mean + 2SD (95% Confidence)
Table 7
Serum levels of COX-1 of normal cases and patients with ovarian carcinoma, cervical carcinoma, pelvic benign tumors and other tissue-originated tumors

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Mean Values (Units/ml in serum)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>10</td>
<td>2.1</td>
<td>1.51</td>
</tr>
<tr>
<td>Ovarian Carcinoma</td>
<td>18</td>
<td>43.8</td>
<td>7.90</td>
</tr>
<tr>
<td>Cervical Carcinoma</td>
<td>20</td>
<td>37.5</td>
<td>6.20</td>
</tr>
<tr>
<td>Pelvic benign tumors</td>
<td>4</td>
<td>21.3</td>
<td>5.36</td>
</tr>
<tr>
<td>Other Tissue-originated Carcinoma</td>
<td>10</td>
<td>8.08</td>
<td>5.33</td>
</tr>
</tbody>
</table>

Table 8
Comparison of serum COX-1 levels both in pre-operative and post-operative cases for patients with ovarian carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Pre-operative</th>
<th>Post-operative (0 days)</th>
<th>7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>18</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Mean Values (units/ml in serum)</td>
<td>43.8</td>
<td>47.3</td>
<td>4.6</td>
</tr>
<tr>
<td>S.D.</td>
<td>7.9</td>
<td>6.88</td>
<td>2.49</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

* Clinical data provided by Dr. E. Huang, Dept. of Obstetrics & Gynecology, Hu-Bei Medical University, Hu-Bei, China.
serum COX-1 levels significantly higher than those of the normal individuals (> mean + 2 S.D.). Similarly, the study from Hu-Bei Medical University seemed to suggest that serum COX-1 levels of those patients with cervical or ovarian cancer were higher than those with other cancers or benign tumors (Tables 7,8). Furthermore, following surgical removal of ovarian tumors, serum COX-1 levels appeared to decrease significantly 7 days after operation. Therefore, serum COX-1 levels appeared to respond to the tumor burden of those cancer patients.

Similar to other established tumor markers (CA 125, CA 19-9, NB/70K etc.), the majority of patients with ovarian cancers showed significantly elevated levels of COX-1 when compared with those of the normal control and those with endometriosis and pelvic benign tumor. In contrast to CA 125 and CA 19-9 which were not suitable tumor markers to monitor patients with cervical carcinoma, COX-1 could also serve as a sensitive marker for this type of cancer. On the other hand, those patients with cervical carcinoma could be monitored by a tumor marker derived from squamous cell carcinoma, SCC-antigen (Keto H. et al, 1984; Maruo, T. et al, 1985). COX-1 seemed to have a comparable degree of sensitivity to that of SCC-antigen in terms of the monitoring of patients with cervical carcinoma as indicated in Table 6.
Although serum COX-1 levels are less well correlated with the stage progression, the significantly elevated COX-1 levels could be readily detected among those patients even with stage I of ovarian or cervical carcinoma. The serum levels of COX-1 were also well correlated with conditions of clinical treatments for those patients with ovarian carcinoma. This is clearly demonstrated in Table 8. Generally speaking, there was a significant decrease in serum COX-1 levels for those patients who had prior surgical removal of tumors seven days in advance and those who underwent radio- or chemotherapy.

From the results of this preliminary evaluation, it is logical to assume that serum COX-1 levels in 60-80% of patients may be closely associated with the tumor size or disease progression and are in parallel response to clinical treatments. If this is the case, COX-1 may be a useful tumor marker for monitoring disease progression in patients with ovarian or cervical carcinoma. It can also be used in combination with other tumor markers such as CA 125 or SCC-antigen for monitoring patients with cervical or ovarian cancers.
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