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Original Articles

Reproductive Biology Section

Serum levels of the progesterone induced blocking factor do not precipitously rise in women with gynecologic cancer in contrast to women exposed to progesterone

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Summary

Purpose: To determine if an immunomodulatory protein (progesterone induced blocking factor [PIBF]) that is progesterone induced and found in higher concentration during pregnancy is similarly found with increased levels in women with gynecologic cancers. *Materials and Methods:* A newly developed enzyme linked immunoabsorbent assay (ELISA) assay was used to measure PIBF in the sera of six women with various gynecologic cancers and compare them to five controls (three with benign tumors and two having gynecologic procedures for non-tumors. *Results:* The PIBF levels in women with gynecologic cancer did not rise precipitously as historical controls of women or men exposed to progesterone. The two highest PIBF levels of the 11 subjects were in women with gynecologic cancer. *Conclusions:* The data suggest that if PIBF helps cancer cells to evade immune surveillance, it probably operates through an intracytoplasmic presence. If an increase in sera PIBF could have been detected in women with gynecologic cancer, then this ELISA test could have been used to detect tumor recurrence. Future studies may concentrate on evaluating intracytoplasmic PIBF to possibly help determine which tumors may respond to progesterone antagonist receptors.

Key words: Progesterone induced blocking factor; PIBF; Gynecologic cancer; Pregnancy.

Introduction

One of the functions of progesterone during the luteal phase and throughout pregnancy is to help suppress immune rejection of the fetal semi-allograft. Immune rejection of the fetus occurs mostly through the cellular immune system and the two most important cytolytic cells that need to be suppressed are natural killer (NK) and cytolytic T cells.

Early studies suggested that NK cell activity was predominantly inhibited during pregnancy by a 34 kDa protein which acted at least partially by stabilizing perforin granules in NK cells [1, 2]. This 34 kDa protein seems to be expressed by gamma/delta T cells [3, 4]. Since the use of the progesterone receptor modulator mifepristone was able to abrogate the immune suppression by these gamma/delta T cell, this suggested progesterone was needed to react with a progesterone receptor on these gamma/delta T cells to activate them [5-7]. The term coined for this 34 kDa immunosuppressive protein was the progesterone induced blocking factor (PIBF) [1,8].

The early studies did not have a pure 34 kDa protein to establish more sensitive assays, e.g., the enzyme linked immunoabsorbent assay (ELISA) and thus most studies used a less sensitive immunocytochemistry technique [9-11]. Using this less sensitive immunocytochemistry technique, early studies suggested that the actual pregnancy state was responsible for the increased sensitivity of the pregnancy lymphocytes to react to progesterone to secrete PIBF since some studies suggested that there was a need to increase the progesterone concentration 100 fold to obtain the same suppressive effect on NK cell activity by non-pregnant *vs.* pregnant lymphocytes [7, 9]. This led to the concept that the allogeneic stimulus of the fetus may cause an increase in progesterone receptors in gamma/delta T cells. This was supported by the demonstration that the allogenic stimulus of lymphocyte immunotherapy not only increased progesterone receptor expression in pregnancy lymphocytes, but it also increased PIBF secretion [12, 13].

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For a malignant tumor to proliferate it must borrow mechanisms already existent to allow cell growth while at the same time suppress mechanisms that signal cells to stop growing to accomplish this. The oncogenes will cause certain proteins on the tumor cell to be present which will not be normally present on the cell and thus make the tumor cells somewhat immunogenic and thus prone to immune rejection by the host.

The continued proliferation of the tumor cells thus requires escape from immune surveillance. There are at least six different mechanisms by which tumor cells may evade immune rejection: 1) downregulation of major histocompatibility complex class I expression, 2) lack of co-stimulatory molecules that are needed to activate T cells, 3) immunoselection of tumor cells with weak immunogenicity, 4) failure of the host to respond to tumor antigens, i.e., tolerance because of neonatal antigen exposure, 5) induction of suppressor T cells, and 6) suppression of immune response by tumor secreted or directed production of certain cytokines, prostaglandins, soluble antigenic material, and even hormones [14].

Immune methods to suppress tumor growth would not require inhibiting all six of the aforementioned methods of escape (there could be more then six) but theoretically any one of these mechanisms. A model for potential tumor immunotherapy was proposed suggesting tumors may be able to direct gamma/delta T cells in the tumor microenvironment to secrete PIBF and thus inhibit NK cell cytolytic activity and allow cancer cells to proliferate. The model suggested a mechanism for cancer cells to secrete P and thus interact with P receptors in gamma/delta T cells in the tumor microenvironment [15]. The hypothesis suggested that if this mechanism was operative, blocking P action by treating with P receptor antagonists, e.g., mifepristone could inhibit tumor cell growth [15]. Though possibly not as high as seen with pregnancy some of the PIBF made by the gamma/delta T cells in the tumor microenvironment may spill over and cause a perceptible rise in serum PIBF.

Thus this hypothesis suggested that possibly by secreting hCG some tumor cells can secrete enough progesterone to interact with the progesterone receptors and the gamma/delta T cells and consequently make PIBF [15, 16]. The PIBF, in turn, could inhibit NK cells in the tumor microenvironment from attacking the tumor cells despite foreign oncofetal antigens [15].

Support for the possible role of PIBF in helping cancer cells to evade immune surveillance was provided by the demonstration that all 29 human leukemia cell lines evaluated were found to produce a considerable amount of mRNA for PIBF [17]. Furthermore four of ten leukemia cell lines tested by the less sensitive immunocytochemistry technique for PIBF was found to express the PIBF protein. Interestingly following the addition of mifepristone to the media PIBF protein expression was down-regulated [17].

The hope of detecting a relationship with PIBF and cancer cells is that the information could possibly be used in the treatment or the prevention of cancer. The hypothesis is that progesterone plays a continual role in the production of PIBF with cancer cells similar to the pregnancy state. However it is possible that PIBF could be made or directed by cancer cells through another mechanism that does not require progesterone. However, if progesterone is involved there are already drugs on the market, i.e., progesterone receptor antagonists, that could inhibit PIBF production and thus remove theoretical suppression of NK cell immune rejection.

Indeed treatment of a variety of murine and human cancers not known to be associated with progesterone receptors with mifepristone resulted in considerable palliation from their cancers [18-22]. These data lent support for the hypothesis that some cancers may escape immune surveillance from NK cells through the stimulation of increased production of the immunomodulatory protein PIBF [23]. The inhibition of the production of the 34 kDA PIBF protein found in the circulation of pregnant women could be inhibited from being produced by gamma/delta T cells in the tumor microenvironment as originally hypothesized [15]. However, another possibility exists as to the location as to where the main inhibitor of PIBF takes place and that is in the tumor cell itself rather than the microenvironment.

Using a Western blot analysis more information accrued about the nature and origin of PIBF in 2003 and 2004 [24, 25]. The parent compound actually resides in the nucleus at a centrosomal position [24]. Interestingly the PIBF protein seems to be unique showing no amino acid sequence homology with any known protein [25]. The full length protein consists of 757 amino acid residues and is encoded by PIBF1 CDNA [25]. The 48 kDa N terminal part of PIBF is biologically active [25].

The parent and dominant 90 kDa form of PIBF has been found to be present in most rapidly growing cells especially cancer cells as evidenced by Western blot analysis using PIBF specific antibodies [24]. There has been identification of the exon 1-5+17-18 transcript encoding for a 35 kDa protein [24]. The deletion observed in this transcript preserves the open reading frame for the full length PIBF protein [24]. Translation of the transcript results in a 35 kDa isoform of PIBF containing the N terminal 222 and C terminal 75 amino acids [24].

The PIBF gene has been identified on chromosome 13 in the vicinity of BRCA1 and BRCA2 mutations [24]. RNA expression analysis has shown that centrosomal PIBF is overly expressed in rapidly proliferating cells irrespective of whether they have been shown to be positive or not for progesterone receptors [24].

Immunofluorescence microassay demonstrated a 35 kDa form of PIBF localized to the cytoplasm of tumor cells [24]. Since this split isoform of the parent compound has a similar size to the circulating immunomodulatory protein in

the serum of pregnant women, it may be that PIBF may confer immunoprotection to the tumor cell itself (or for that matter fetal cells or trophoblast cells) by this intracytoplasmic position and may not need circulating PIBF to suppress NK cell attack. Since both the fetal placental unit and cancer cells are rapidly growing, thus the original hypothesized mechanism of suppressing NK cell activity in the tumor microenvironment by secretion of PIBF by gamma/delta T cells may not be the main operative mechanism of immune suppression. It is possible that the intracytoplasmic presence could also confer a degree of immune protection, but possibly because the fetal semi-allograft is more immunogenic than cancer cells, perhaps extra PIBF secreted externally and concentrated at the maternal-fetal interface is needed to allow the fetus to grow.

These new data suggest that the tumor cells themselves may actually produce the PIBF. Thus instead of inhibiting NK cells in the tumor microenvironment the intracytoplasmic location itself may directly confer immune protection to the tumor cell by suppression of NK cell immunosurveillance.

The PIBF protein has been purified and synthesized by recombinant DNA technology [26]. A purified protein is required to develop a monoclonal antibody and this was achieved [26]. The protein must be soluble which has been established [27].

With these new medical advances, the less sensitive immunocytochemistry technique using a polyclonal antibody to PIBF has been replaced with a much more sensitive ELISA test [28]. There is now found to be a marked difference in women whose PIBF levels are obtained in the follicular phase *vs.* three days after embryo transfer [28].

The objective of the present study was to obtain blood samples of patients with gynecological malignances prior to surgery to determine if an increased level of PIBF using a non-commercial ELISA assay for PIBF could be detected in women with a variety of gynecologic malignances.

Materials and Methods

Subjects

Serum was obtained from women about to have surgery for gynecologic problems including malignant and benign disorders. The samples would then be measured for PIBF using a new non-commercial ELISA for PIBF and for serum progesterone. Women with serum progesterone level greater than two ng/ml were eliminated.

Methodology of PIBF assay

A non-commercial ELISA was used to measure PIBF in serum. Serum specimens were stored at -20°C. Fifty microliters of recombinant PIBF standard was added to each pre-coated goat anti-rabbit antibody well in duplicate. The concentrations of the PIBF standard were S0 – 0, S1 – 3.2, S2 – 11.2, S3 – 40, S4 – 160, S5 - 802 ng/ml. The patient's serum was then added to each well. Next 50 microliters of horse radish peroxidase conjugated PIBF antigen was added to each well except the zero standard. Next anti-PIBF IgG antibody was added to each well. The microtiter plate was then incubated in the dark for one hour at 37°C. After one hour, the wells were washed with PBS and decanted three times. Next 50 microliters of Substrate A (carbamide peroxide) and 50 microliters of Substrate B (tetramethyl-benzidine) were added. The microliter trays were then incubated in the dark at 37°C for 15 minutes. Next 50 microliters of stop solution was added whose main component is H_2S04. The plates were read within ten minutes using a microplate reader at 450 nm. The results were calculated using a four-parameter logistic curve fit.

Results

The PIBF levels (ng/ml) from lowest to highest in women with various gynecologic cancers (in all cases serum progesterone ≤ two ng/ml) were 10.06 (64-year-old woman with endometrioid type of adenocarcinoma of uterus), 17.35 (63-year-old woman with clear cell adenocarcinoma of ovary), 32.59 (72-year-old woman with papillary serous adenocarcinoma of uterus), 35.62 (66-year-old woman with primary peritoneal papillary serum cystadenocarcinoma), 54.7 (77-year-old woman with mucinous adenocarcinoma of the gastrointestinal tract), and 57.17 (68-year-old woman with a recurrent adult granulosa cell tumor). The average serum PIBF was 34.6 ng/ml.

There were three women with benign gynecologic tumors and the serum PIBF was 14.76 (45-year-old woman with leiomyomata), 15.7 (58-year-old woman with mucinous cystadenoma of the ovary), and 36.64 (57-year-old with adenomyosis and leiomyomata). Their average serum PIBF was 22.5 ng/ml).

There were two women with no tumors having gynecologic surgery and their serum PIBF levels (ng/ml) were 9.56 (21-year-old) and 35.27 (48-year-old with cervical dysplasia) with an average of 22.4 ng/ml.

Discussion

Exposure to progesterone even in males will cause the serum PIBF levels to exceed 100 ng/ml and frequently > 800 ng/ml using the new ELISA assay [28-30]. Though it is true that of the 11 women tested (six with cancer and five without) the highest levels (> 50ng/ml) were seen in the two women with cancer, clearly the levels do not come close to what is seen with exposure to progesterone [28-30].

Possibly a larger series may show evidence that there is a significantly higher level of PIBF seen in some women with cancer. However, if PIBF is effective in suppressing an immune response against the cancer cells, it seems to be more likely through a different mechanism than pregnancy where the serum PIBF levels are very high.

There is the possibility that mifepristone improves immunosurveillance through some other mechanism than PIBF. There is evidence that progesterone interacting in a non-genomic manner with progesterone receptor membrane 1 may suppress in an epigenetically manner T cell rejection of the fetal semi-allograft. Possibly this could apply to cancer cells [31].

Most of the data supporting the hypothesis suggesting that PIBF may be a way in which tumor cells, similar to the fetus, escapes immune surveillance, is based on the fact that most tumors in mice and humans will be suppressed by mifepristone whether the tumor is known to have progesterone receptors or not [18-22]. It is beyond the discussion of this manuscript to discuss the various ways that the progesterone receptor may be involved in factors needed for tumor growth but the possibility exist that progesterone receptor antagonist benefit has nothing to do with the immune system. For an excellent review of the way progesterone receptors may help in tumor growth, at least in tumors, e.g., breast cancer known to possess progesterone receptors, has been summarized by Daniel *et al.* [32].

The possibility exists that gynecologic cancers, as opposed to the others responding to mifepristone, just do not express PIBF and possibly these gynecologic tumors would not have responded to mifepristone therapy. There was only one gynecologic cancer, a leiomyosarcoma, in the study of mifepristone on providing palliative benefit to people with advanced cancer [22]. The woman with the leiomyosarcoma dramatically responded to mifepristone but her serum level of PIBF was not measured [22]. None of the gynecologic cancers in the present study were leiomyosarcoma.

One case does show that some cancers may dramatically respond to mifepristone even if no increase in serum PIBF is determined. A woman without elevated sera PIBF levels had either the acute phase of leukemia progression from her chronic lymphocytic leukemia or primary lung cancer. Death was considered imminent. All her lung lesions disappeared after six weeks of mifepristone. She remains well with good energy two years after therapy just continuing on 200 mg mifepristone daily [33]. This could suggest that the main benefit of mifepristone may be to inhibit the conversion in the tumor cell cytoplasm of the 90 kDa parent form of PIBF to the intracytoplasm 34-36 kDa isoform. It should be recalled that 29 of 29 human leukemia cell lines were found to have an enormous amount of mRNA devoted to the manufacturing of PIBF and yet no increase in serum PIBF was detected in this woman who responded so well to mifepristone [17].

A study is in progress to see if breast cancer that is progesterone receptor positive may show higher serum levels of PIBF than women with progesterone receptor negative breast cancer. If significantly higher levels of PIBF are found in the sera of tumors that are positive for progesterone receptors, perhaps these tumors may respond the best to progesterone receptor antagonists. Perhaps the high sera levels help protect the cancer from immune surveillance.

If more extensive testing of various cancers confirms a lack of significant elevation of serum PIBF levels, this test may not prove worthwhile to use as a method to determine who should be treated with progesterone receptor antagonists. If people with certain tumors can demonstrate slightly higher levels of PIBF than the sera of other patients, with other types of cancer, measurement of baseline levels before therapy, could be potentially useful as a marker for disease recurrence.

Thus this small pilot study does not support the importance of PIBF as a means of cancer cells escaping immune surveillance because in contrast to the pregnancy state no significant rise in serum PIBF was detected in the sera of women with gynecologic cancer. However, in view of the marked improvement seen in certain cancers treated by mifepristone, and the known increase in intracytoplasmic PIBF in all rapidly growing cells, attention should be placed on measuring intracytoplasmic PIBF in the tumor specimens directly. Possibly some tumors will have higher concentration than others and these may be the ones that best respond to progesterone receptor antagonists. Such a demonstration could at least generate interest in the oncologic group to consider progesterone receptor antagonists as a treatment even for cancers not known to be associated with progesterone receptors.

Once the PIBF assay is better refined, perhaps after studying a larger series of patients with cancer some discriminatory level may be detected, that could suggest that a malignancy is possibly present (even if not nearly as high as seen in people exposed to progesterone). It should be recalled that only two women in this study had PIBF levels > 50 ng/ml and both of these women had cancer. If mifepristone suppressed these levels perhaps these women would be found to have good palliation from therapy *vs.* those that failed to lower serum PIBF levels. Perhaps monitoring PIBF in women responding to progesterone receptor antagonists could alert the treating physician if a rise approaching baseline is occurring to influence the treating physician to either raise the dosage of the progesterone receptor antagonist or change to another progesterone receptor antagonist or add another type of chemotherapy or monoclonal antibody therapy.

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