Placental Protein 14 Induces Apoptosis in T Cells but Not in Monocytes

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Substantial evidence exists in literature to suggest that placental protein 14 (PP14) (recently renamed glycodelin A), exhibits immunosuppressive properties and is an indispensable macromolecule in the maternal system for the establishment, maintenance, and progression of pregnancy. Though there are several reports substantiating the above, the mechanism of its action at the molecular level has not been elucidated as yet. In this paper we provide data that suggest that amniotic fluid PP14 and recombinant PP14 expressed in Pichia pastoris induce apoptosis in human peripheral blood lymphocytes upon activation, independent of monocytes. That PP14 has a direct apoptotic action on T cells but not on monocytes was also demonstrated by utilizing human cell lines. PP14 was shown to induce apoptosis in the human T cell lines, Jurkat and MOLT-4 cells, but not in the human monocyte cell line, U937.

Placental protein 14 (PP14) or glycodelin A belongs to the family of hydrophobic molecule transporter proteins known as lipocalins. PP14, a 162-amino acid glycosylated protein, is secreted by the late secretory phase endometrium during the menstrual cycle (1) and has been proposed as a biochemical marker of endometrial function in women (2). The gene encoding the protein consists of three putative progesterone/corticosterone response elements (3). Consistent with this is the finding that PP14 concentrations in the endometrial tissue, as well as in circulation, are highest in the late luteal phase and lowest in the periovulatory phase of the menstrual cycle (4–6) in concert with progesterone levels (7). During pregnancy, PP14 concentration rises, peaking at ~10–12 weeks, being high during the first and second trimesters of pregnancy, and declining thereafter (8–10).

PP14 has been shown to inhibit sperm-zona interaction (11) and is associated with endometrial preparation for blastocyst implantation (12, 13). Apart from this, PP14 has been shown to have immunosuppressive properties in that it inhibits both phytotrhagglutinin (14) and anti-CD3 antibody-induced lymphocyte proliferation (15). PP14 also inhibits Natural Killer cell activity (16). That the protein is essential for normal pregnancy progression has been established by the association of low levels of PP14 with habitual abortion (17, 18), unexplained infertility (19), and establishment of pregnancy in women during those cycles in which normal concentrations of PP14 were detected (20). Despite the growing literature on the multifunctional role of PP14, the mechanism of action of this protein has not been delineated at the molecular level. Our studies demonstrate that the immunosuppressive effect of PP14 is the result of induction of apoptosis in T cells. The protein also induced apoptosis in the human T cell lines Jurkat and MOLT-4 but not in the monocyte cell line U937.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Lines—**Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque (Sigma) from freshly drawn blood of normal healthy donors (male and female; age 25–45 years), according to the method described by Boyum (21).

Raji, a human B cell line, and Jurkat and MOLT-4, human T cell lines, were obtained from the Cancer Research Institute, Mumbai, India, and U937, a macrophage cell line, was from Dr. Manjunath, Indian Institute of Science, Bangalore, India. All three cell lines were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and passaged three times a week.

**Purification of Pichia pastoris PP14 (Pic-PP14) and Amniotic Fluid PP14 (AF-PP14)—**Expression of PP14 in P. pastoris (GS115) has been described elsewhere (22). Recombinant P. pastoris (GS115-PP14) supernatant was reverse dialyzed against sucrose and loaded on a preparative Superose-12 fast protein liquid chromatography column (Amersham Pharmacia Biotech), equilibrated against 10 mM phosphate buffer, pH 7.2, containing 0.9% NaCl (PBS). 1-ml fractions were collected, and the fractions corresponding to the molecular mass range of ~45 kDa were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing the recombinant protein (Pic-PP14) were pooled, concentrated, dialyzed extensively against RPMI 1640, and finally filter-sterilized using 0.45 μm SPIN-X tubes (Costar). The purity of PP14 was confirmed by silver staining. As negative control, untransformed P. pastoris (GS115) supernatant was used after concentration and subsequent equilibration against RPMI 1640.

Amniotic fluid was collected from women undergoing elective abortion at 10–12 weeks of gestation. After dialysis against PBS, amniotic fluid was concentrated 10-fold by reverse dialysis against sucrose and fractionated on a Superose-12 fast protein liquid chromatography column. The presence of PP14 in the fractions was detected by Western blotting with two monoclonal antibodies (mAbs), one, namely B1C2, against PP14 (23) and the other, B7B10, against bovine β-lactoglobulin cross-reactive with PP14 (24). The peak fractions of PP14 were pooled (AF-PP14), dialyzed against RPMI, and filter-sterilized. The most abundant protein in the amniotic fluid, namely human serum albumin (HSA) was used as the negative control.

Treatment with peptide-Ν-glycosidase F was carried out according to the method described previously (25). The protein solution at 2 mg/ml in PBS containing 1% SDS and 10 μM N-mercaptoethanol was boiled for 2 min. Nonidet P-40 was then added so that the ratio of Nonidet P-40 to SDS was 7:1 in the final reaction. The enzyme was added (0.5 milli-units/ml), and the mixture was incubated at 37 °C for 48 h. To prepare

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the sample for SDS-polyacrylamide gel electrophoresis, the samples were precipitated with equal volumes of 50% trichloroacetic acid containing 10 mM sodium deoxycholate, boiled with sample buffer and electrophoresed on 12.5% polyacrylamide gel under denaturing conditions. The proteins were transferred onto nitrocellulose membrane and probed with PP14-specific mAb B1C2 (23).

Inhibition of glycosylation of the recombinant PP14 was carried out using tunicamycin. The GS115-PP14 culture was grown in 100 ml of BMGY medium (2% peptone, 1% yeast extract, 1.25% yeast nitrogen base, 2% glycerol, and 16.5 µm M biotin) for 36 h. The cells were collected by centrifugation and resuspended in 2 ml/g cell weight of BMMY medium (BMGY without glycerol supplemented with 0.5% methanol). To one half of the culture, tunicamycin was added at a concentration of 200 µg/ml. The cells were then grown for 120 h at 30 °C with constant agitation at 250 rpm, replenishing the methanol every 24 h. The cells were then centrifuged, and the supernatant was concentrated and electrophoresed on 12.5% acrylamide gel. The separated proteins were visualized by staining with Coomassie Blue.

**Proliferation Assay**—PBMCs at 0.2 ¥ 10^6 cells/200 µl or cell lines at 0.1 ¥ 10^5 cells/100 µl in RPMI 1640 supplemented with 5% FBS were cultured in the presence or absence of PP14 at varying time interval in 96-well plates (NUNC). Phytohemagglutinin (PHA) at 5 µg/ml or OKT3 hybridoma culture supernatant at a dilution of 1:2500 was used for inducing T-lymphocyte proliferation.

At the end of the time interval, as indicated in the figure legends, 1 ¥ 10^4 cpm of 3H-thymidine (methyl T; Bhabha Atomic Research Center, Mumbai, India) was added to the cultures and incubated for 12 h in the case of lymphocytes and 6 h in the case of the cell lines. The cells were harvested on glass fiber paper (Whatman) using a cell harvester (NUNC). The filters were dried, and radioactivity was measured in a liquid scintillation counter. Data presented are representative of at least five to seven different experiments, and each bar represents 3H-thymidine counts of samples in triplicate.

Depletion of monocytes was carried out by incubating the isolated PBMCs for 2 h at 37 °C, in FBS-coated plastic Petri dishes, in RPMI supplemented with 10% FBS. Non-adherent cells were collected for proliferation studies.

**DNA Fragmentation Assay**—DNA fragmentation assay was carried out as described earlier (26) for the cell lines. 1–2 ¥ 10^6 cells at different time intervals after treatment with PP14 or untreated cells were collected by centrifugation and suspended in 100 µl of PBS. 1 ml of ice-cold 70% ethanol was added to cells and kept on ice for 30 min (or –20 °C for 16 h). The cells were centrifuged at 800 ¥ g for 5 min at 4 °C, and ethanol was removed completely, suspended in 40 µl of 0.1 M citrate phosphate buffer, pH 7.8, and kept at room temperature for 30 min with occasional shaking. The cells were then centrifuged at 1000 ¥ g for 5 min at 4 °C, and the supernatant was concentrated. To this, 10 µl of 0.25% Nonidet P-40 and 3 µl of RNase A were added and incubated at 37 °C for 30 min. After addition of 3 µg of proteinase K the incubation was continued for 30 min. 10 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol in 30% glycerol in water) was added to the mixture and electrophoresed on a 0.8% agarose gel at 2 V/cm for 10 h. The gel was then stained with ethidium bromide (5 µg/ml) for 15 min, destained in water for 5 min, and visualized under a UV transilluminator. The genomic DNA was isolated from 1–2 ¥ 10^6 lymphocytes by the procedure described earlier (27). The cells were lysed in 20 µl of 50 mM Tris–HCl buffer, pH 7.0, containing 10 mM EDTA, 0.5% SDS, 500 ng of proteinase K and incubated for 1 h at 50 °C and then for 10 min at 70 °C. To this, 1 µg of RNase A was added and incubated for another hour at 37 °C. The contents of the tube were then loaded on an agarose gel as described above.

**Propidium Iodide (PI) Staining and Fluorescence-activated Cell Sorter (FACS) Analysis**—Cells (1 ¥ 10^6), either untreated or after treatment with PP14, were centrifuged at 300 ¥ g for 5 min at room temperature and resuspended in 100 µl of PBS. 1 ml of ice-cold 70% ethanol was added to the cells and kept on ice for 30 min (or –20 °C for 1 week). The cells were thawed on ice and centrifuged at 800 ¥ g for 5 min at 4 °C. After washing once with PBS, 500 µl of staining solution of propidium iodide at 50 µg/ml of PBS, containing 0.1 mg/ml of RNase A, 1% Triton X-100, and 40 µg/m of sodium citrate, was added to the cells and incubated for 1 h. Analysis was carried out by fluorescence-activated flow cytometer (FACScan; Beckton Dickinson).

**Western Blotting**—Samples were electrophoresed at 30 mA of constant current on a 12.5% polyacrylamide gel. The separated proteins were transferred to nitrocellulose membrane at 125 mA of constant current using a semi-dry transfer apparatus (Bio-Rad) for 1.5 h. After blocking the unbound sites with 0.5% gelatin in PBS, the membranes were incubated with the primary antibody for 1 h and washed 3 times with high salt wash buffer (1 x NaCl in 50 mM phosphate buffer, pH 7.2, containing 0.05% Triton X-100 and 0.2% EDTA) and 3 times with low salt wash buffer (50 mM NaCl in 50 mM phosphate buffer, containing 0.05% Triton X-100 and 0.2% EDTA). Further incubation was carried out with appropriately diluted rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dako) for 1 h. Binding of the antibodies was visualized with the enzyme substrate 3,3’-diaminobenzidine (Sigma) at 1 mg/ml in 100 mM citrate phosphate buffer, pH 5.5, containing 0.03% H2O2 or with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

To measure PP14 concentrations in the amniotic fluid fractions obtained on gel filtration, known concentrations of purified recombinant PP14 were electrophoresed, along with the amniotic fluid fraction, and transferred to nitrocellulose membrane followed by Western blot analysis with the monoclonal antibody B1C2 raised to PP14 (23). Intensities of the chemiluminescence signals as determined by densitometric analysis us-
Fig. 3. Inhibitory effect of PP14 on proliferation of Jurkat (A and B) and U937 (C and D) cells. Cells were cultured for 24 h in the presence or absence of either AF-PP14 (A and C) or Pic-PP14 (B and D). GS115 supernatant and HSA were used at the same protein concentration as the Pic-PP14 and AF-PP14, respectively, which gave maximum inhibition of proliferation. Cultures were pulsed with \(^{3}H\)-thymidine for 6 h after which the cells were harvested on glass fiber filters and cell-associated radioactivity was measured in a liquid scintillation counter. Data presented are representative of at least five different experiments, and each bar represents \(^{3}H\)-thymidine counts of samples in triplicate.

whether peripheral blood monocytes play any role in the observed inhibition of T cell proliferation was investigated by depleting the PBMCs of the monocytic population. Pic-PP14 had the same inhibitory effect on T cells of PBMCs cultured in the absence or presence of monocyes (Fig. 2C).

Effect of Pic-PP14/AF-PP14 on Proliferation of Jurkat, U937, and Raji Cells—With the ultimate aim of delineating the inhibitory effect of PP14 at the molecular level, we chose the human cell lines Jurkat U937 and Raji as representatives of...
human T, monocyte, and B cell lineages, respectively. The cells were cultured in the presence of varying concentrations of PP14, and the incorporation of 3H-thymidine was measured. Data presented in Fig. 2 show that proliferation of Jurkat cells was inhibited by both the proteins in a dose-dependent manner. The concentration of both forms of PP14 required for 50% inhibition of proliferation of Jurkat cells were comparable, 37 nM of the AF-PP14 and 35 nM of the recombinant PP14. No inhibition was seen with either HSA or the P. pastoris (GS115) supernatant. Proliferation of the U937 cells was also inhibited. AF-PP14 at 40 nM and Pic-PP14 at 67 nM brought about 50% reduction in proliferation. However neither of the two protein preparations inhibited the proliferation of Raji cells (data not shown).

To answer the question of whether the inhibitory effect of PP14 is reversible, Jurkat and U937 cells were incubated with PP14 for 3 or 6 h, after which the cells were washed free of the protein, followed by pulsing with 3H-thymidine at the end of 24 h. No difference was found between cells incubated with PP14 only for 3 or 6 h when compared with those that were cultured in the presence of the protein throughout the culture period (data not shown).

**Generation of apoptosis in PBMCs, Jurkat, and MOLT-4**

**PI Staining and FACS—**Cells cultured with either Pic-PP14 or AF-PP14 were stained with PI and subjected to FACS analysis. All treated cells showed DNA loss as indicated by the pre-G2/G1 peak. In the case of lymphocytes, 14% of the cell population was apoptotic after treatment with Pic-PP14 as compared with untreated cells, which showed only 4% apoptosis (see Fig. 4A). The apoptotic population in the Jurkat cell line increased to 69% on culturing with AF-PP14 and to 72% with Pic-PP14 (see Fig. 4B). Untreated MOLT-4 cells had a higher population of apoptotic cells (29%) after a 24-h culture, and on culturing with AF-PP14 and Pic-PP14 this population increased to 95 and 98%, respectively (see Fig. 4B). Neither of the two forms of PP14 induced apoptosis in U937 cells when cultured with PP14 for 24 h (see Fig. 4B). No DNA loss was observed in cells either untreated or treated with HSA/GS115 supernatant (see Fig. 4B).

**DNA Fragmentation—**Though DNA fragmentation was observed in PBMCs, Jurkat, and MOLT-4 cells after culturing with both forms of PP14 (see Fig. 5), DNA ladders were prominent only in the case of the MOLT-4 cells (see Fig. 5, lanes 8 and 9). Importantly, no DNA fragmentation either as ladder or smear was observed in the U937 cells with or without treatment with PP14 (see Fig. 5, lanes 10–12) or the other cells either untreated or treated with control proteins.
AcOr/EtBr Staining—Morphological observations of apoptosis was carried out by dual staining of the cells with AcBr and AcOr after culturing with PP14 for 0, 6, 12, and 18 h. None of the cells exhibited any morphological changes up to 6 h. On culturing with PP14, MOLT-4 cells showed maximum apoptosis at 18 h, and Jurkat cells showed it at 24 h. At 24 h, more than 95% of MOLT-4 cells were seen to be in the late apoptotic stage. Fig. 6 depicts the 18-h time interval for all of the cell lines. As can be observed from this figure, whereas U937 cells did not show any change on treatment with the two proteins, Jurkat, as well as MOLT-4, exhibited chromatin condensation that is very typical of apoptosis.

Caspase 3 Assay—To identify the protease species responsible for PP14-induced apoptosis, the cleavage of the fluorogenic substrate, Ac-DEVD-AMC for caspase-3 protease, was examined by spectrofluorimetry. The cleavage activity for the substrate was marginal in the lysates of cells either untreated or treated with control substances as indicated by low fluorescence emission values (see Fig. 7, panel a). However, there was a significant increase in the fluorescence emission with the lysates of Jurkat and MOLT-4 cells after culturing with AF-PP14 (see Fig. 7, panel b) and Pic-PP14 (see Fig. 7, panel c) at concentrations that inhibited the cell proliferation more than 50%. This activity could be completely inhibited by the specific peptide Ac-DEVD-CHO establishing the specificity of the substrate for the caspase 3 enzyme. The U937 cells showed no increase in caspase 3 activity either in the presence (see Fig. 7, panels b and c) or absence (see Fig. 7, panel a) of PP14.

DISCUSSION

Though the biological role of PP14 has not been elucidated at the molecular level, the protein is known to be associated with several functions, viz. immunosuppression (14–16, 30), transport of a hydrophobic molecule (29), and inhibition of sperm-zona interaction (11). The fetal allograft survives maternal rejection during normal pregnancy, but an allograft at sites other than the uterus is rejected efficiently by the maternal immune system. Therefore, studies on immunological events at the feto-maternal interface have been the major focus for researchers in this area. Experimental observations suggest that endometrial PP14 is involved in the down-regulation of the maternal immune response to foreign fetal antigens. It has been postulated that PP14 action is through immunosuppression, which is essential for protection of the embryo from the immune response of the mother (30). In this context, PP14 has been shown to inhibit the proliferation of peripheral blood lymphocytes induced by the mitogen PHA (14) and by cross-linking of CD3 receptors by specific antibody (15).

We have demonstrated earlier that Pic-PP14 (22) exhibited an inhibition of sperm-ovum interaction as has been reported in case of AF-PP14 (11). To demonstrate whether Pic-PP14 could also exhibit the immunosuppressive activity of the native PP14, we tested both proteins for their effect on lymphocyte proliferation. Pic-PP14 inhibited proliferation of human PBMCs triggered by the OKT3 mAb (as also by PHA) in a dose-dependent manner showing that the recombinant protein is comparable with the AF-PP14 in its activity. Depletion of the monocytes from PBMCs did not abrogate the PP14-mediated inhibitory effect on T cells (Fig. 2C) indicating that PP14 exerts this effect directly on T cells.

The proliferation studies were then extended to human cell lines, namely, Jurkat, which is a T cell line, Raji, a B cell line, and U937, which is of monocyte origin. PP14 inhibited proliferation of Jurkat and U937 cells (Fig. 3) but not Raji cells. Because cell lines are homogeneous populations, these observations confirmed that the inhibitory effect of PP14 is direct on T cells, as well as monocytes. All the above studies were carried out to establish the potential of the recombinant Pic-PP14 comparable with that of the AF-PP14. Further studies were then initiated to look into the molecular events that are triggered by PP14. The first experiment in this direction was to determine whether PP14 action was reversible. The studies provided conclusive evidence that the direct effect on both cell types was irreversible indicating that the signal could be triggered through specific receptors. A cell surface-associated binding protein for PP14 has been reported earlier for the human monocytes (31) but not on T cells.

To explore the antiproliferative effect of PP14 further, PI staining studies were initiated to determine whether PP14 blocks any stage of the cell cycle, leading to a state of anergy. The appearance of the pre G0/G1 cell population in PBMCs after treatment with PP14 indicated that PP14 induces apoptosis. The studies were then extended to Jurkat and U937 and another T cell line, namely MOLT-4 (Fig. 4). Interestingly, whereas PP14 induced apoptosis in both the T cell lines, this effect was not seen in U937, even though the protein inhibited U937 cell proliferation (Fig. 3B). The inhibition of proliferation in U937 cells therefore appears to be induced by a pathway different from that in the T cells in PBMCs, as well as the T cell lines. That AF-PP14, as well as Pic-PP14, had an apoptotic effect on T cells was further confirmed by DNA fragmentation studies (Fig. 5) and EtBr/AcOr staining (Fig. 6). The final confirmation came from our studies on measuring the increase in caspase 3 enzyme levels induced in T cells on culturing with PP14. Caspase 3, a member of the caspase subfamily, is a crucial mediator of apoptosis in many mammalian cells. Our data show that this protease is responsible for PP14-induced apoptosis. That this activity was inhibited by the specific inhibitor Ac-DEVD-CHO (32, 33) established the specificity of the enzyme assay. That the arrest in the proliferation of U937 cells by PP14 is not because of apoptosis was borne out once again by the results of the caspase 3 assay (Fig. 7C).

Lymphocytes appear in the decidua of the first trimester of pregnancy and disappear toward the third trimester (34). Those cells that recognize non-self paternal antigens of the trophoblast would need to be deleted from the local environment, should the embryo survive. Death by apoptosis would be the preferred choice as apoptotic cells are quickly phagocytosed by macrophages via a receptor-mediated process (35) ensuring that there is no release of intracellular contents (containing inflammatory components) in the surrounding tissue, which would prove to be harmful to the developing fetus. PP14 is probably one of the many factors required for destroying activated T cells to abrogate the deleterious maternal response to paternal antigens.

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