Progression and Molecular Biology of the Progesterone-Associated Endometrial Protein: A Constitutive Marker of Human Erythroid Precursors

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Progesterone-associated endometrial protein (PAEP/PP14) is a 28-kD glycoprotein with sequence homology to β-lactoglobulins containing a retinol-binding motif. PAEP/PP14 is present at nanomolar concentrations in human serum. It is produced by secretory and decidualized endometrium in women and by seminal vesicle epithelium in men. We report here that PAEP mRNA is constitutively expressed in normal hematopoietic tissue. Western immunoblotting of bone marrow cells with rabbit antibodies to PAEP gave a band at 28 kD, and immunochemical staining with monoclonal antibodies localized PAEP into the cytoplasm of erythroid precursors representing different stages of the normoblast series. PAEP was not detected in mature red blood cells, platelets, or in cells of the myeloid lineage. Untreated K562 leukemia cells did not contain PAEP, whereas treatment of the cells with tetradecanoylphorbol acetate (TPA) induced strong expression of PAEP mRNA and synthesis of the intact protein that was found both in the cytoplasm of the differentiating cells and in the supernatant of TPA-treated cultures. These findings add a new member to the growing family of genes that are constitutively expressed both in the reproductive tract and in the hematopoietic system.

HUMAN PROGESTERONE-associated endometrial protein (PAEP/PP14) is a 28-kD glycoprotein synthesized by secretory and decidualized endometrium. Studies by in situ hybridization histochemistry have localized the expression of PAEP/PP14 mRNA in the glandular epithelial cells of endometrium and decidua spongiosa. This protein has also been found in the epithelium of seminal vesicles and it is secreted into the seminal plasma. PAEP was previously known as placental protein 14 (PP14), progestagen-dependent endometrial protein (PEP), and pregnancy-associated endometrial protein (PEP), classified as a retinoic acid (RA) binding motif. Human Gene Mapping Committee has suggested the name PAEP for this protein. PAEP/PP14 has significant structural homology to β-lactoglobulins of various species. This class of proteins also contains the retinoic acid (RA) binding motif. The N-terminus of PAEP/PP14 has 22% sequence homology to human retinol binding protein.

The biologic functions of PAEP are still enigmatic. Recent findings indicate that PAEP has immunoregulatory activities. The addition of PAEP/PP14 suppresses proliferation of lymphocytes stimulated in vitro by mitogens by or by allogeneic cells in mixed lymphocyte cultures. Okamoto and co-workers have found that the cytolytic activity of natural killer (NK) cells is inhibited in the presence of purified PAEP/PP14.

In this study, we show that precursors of the erythroid lineage in normal human bone marrow (BM) constitutively express PAEP protein. Human erythroleukemia cell line K562 also expresses PAEP mRNA and protein after induced differentiation in vitro.

MATERIALS AND METHODS

Tissues. Endometrium was collected after informed consent from women undergoing hysterectomy for medical reasons (fibroids). The study was approved by the Ethical Committee of the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. After removal of the representative tissues for routine histopathologic examination the remaining tissues were frozen in liquid nitrogen. Samples from secretory phase endometrium were used for this study.

Hematopoietic cells. Normal BM was obtained from diagnostic aspirates or recovered from pieces of ribs normally resected during thoracic surgery. The mature red blood cells (RBCs) were lysed in 0.84% ammonium chloride and used for the analysis without fractionation. The cell composition of the final sample was determined from May-Grünwald Giemsa-stained cytospin slides. In the samples used here, the proportion of morphologically distinguished erythroid precursors was around 10% of the total cell population. Purified platelets were obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland).

Cell culture and induction of cell differentiation. The hematopoietic cell lines K562, U937, and HL-60 were purchased from the American Type Culture Collection (Rockville, MD). The human megakaryoblastic cell lines Dami and HPRC were kindly provided by Dr Riitta Alitalo (Transplantation Laboratory, University of Helsinki). The cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories, Strathclyde, UK) at 37°C in a humidified atmosphere of 5% CO₂ in air. To induce differentiation, K562, U937, and HL-60 cells were cultured in the presence of 10⁻⁹ mol/L tetradecanoylphorbol acetate (TPA), 10⁻⁶ mol/L RA, or 2 mmol/L sodium butyrate for 3 days.

Immunofluorometric assay. To measure the PAEP production, K562 cells were cultured with or without 8 × 10⁻⁸ mol/L TPA for 24, 48, 72, 96, or 120 hours, and PAEP immunoreactivity was quantitated from the medium by an immunofluorometric assay essentially as described previously using monoclonal antibodies (MoAbs) produced against purified PAEP/PP14. Antibody-containing ascitic fluids were purified using protein G-Trap G; Pharmacia LKB Biotechnology Inc. Piscataway, NJ). MoAbs F23-9G2 were labeled with DELFIA Eu-labelling reagent (Alkpharma, Inc., Madrid). MoAbs F23-9G2 were labeled with DELFIA Eu-labelling reagent (Alkpharma, Inc., Madrid). MoAbs F23-9G2 were labeled with DELFIA Eu-labelling reagent (Alkpharma, Inc., Madrid).

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pregnancy serum containing measured PAEP concentrations) or samples were first incubated overnight at 4°C. After washing, the wells were incubated with labeled MoAbs for 2 hours and further treated as described before. The detection limit of the assay (1.5 μg/L) was defined as the PAEP concentration corresponding to the mean fluorescence signal of 20 replicates of a zero sample plus 2 SD. The measuring range we used was 3.1 to 400 μg/L. The intra-assay variation (n = 10) was 4.4% at the level of 105 μg/L, 8.2% at the level of 21 μg/L and 25% at the level of 5 μg/L. The interassay variations (n = 11) were 4.7%, 15% and 18%, respectively.

Immunostainings. Cytocentrifuged smears of BM cells were immunostained using 1:300 diluted anti-PAEP MoAbs (clone 105 DH1F1) in an indirect peroxidase method and counterstained with hematoxylin to allow morphologic identification of the cells. For double immunofluorescence, smears of BM cells were stained with 1:50 diluted anti-PAEP MoAbs together with 1:100 diluted rabbit anticyclopHENIN A antibodies, followed by affinity purified, fluorescent isothiocyanate (FITC)-conjugated goat antimouse Ig and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antirabbit Ig (Dako A/S, Denmark). Cytocentrifuged smears of K562, U937, and HL-60 cells before and after induced differentiation and the megakaryoblastic cell lines Dami and HPRC were fixed for 20 minutes in 3.5% paraformaldehyde, permeabilized with 0.1% NP-40 in phosphate-buffered saline (PBS) for 10 minutes and stained with anti-PAEP MoAbs, followed by affinity-purified, FITC-conjugated goat antimouse Ig.

Metabolic labeling and immunoprecipitation. K562 cells were incubated for 2 hours in methionine-free Eagle’s minimum essential medium (GIBCO, Paisley, UK) before labeling for 2 hours at 37°C with 0.2 mCi/mL 35S-methionine. The cells were collected, washed twice in PBS and lysed in immunoprecipitation buffer (10 mmol/L TRIS; pH 7.5, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1 U of aprotinin per mL). Three microliters of mouse ascitic fluid containing anti-PAEP MoAbs (clone 105 DH1F1) was added to the lysates, and immunoprecipitations were performed using protein A-Sepharose with and without the addition of 10 μg purified PP14 protein. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. The radioactive proteins were visualized by autoradiography.

Western immunoblotting. Lysates of BM cells were immunoprecipitated with polyclonal rabbit anti-PAEP antibodies. The precipitates were separated under nonreducing conditions on SDS-PAGE, transferred to nitrocellulose membranes and blotted with polyclonal rabbit anti-PAEP antibodies, followed by 125I-labeled swine antirabbit Ig (Amersham International plc, Amersham, UK). The blots were developed by autoradiography. Western blots of lysates of K562 cells, before and after induced differentiation as well as of lysates of platelets, were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

Northern blot analysis. RNA was isolated from whole cells by the guanidinium isothiocyanate extraction method. Poly(A)+RNA was isolated using oligo(dT)-cellulose. Samples (5 μg) were denatured with 6.3% formaldehyde and 50% formamide, electrophoresed, and transferred to nylon membranes. The filters were hybridized with 32P-labeled PP14 cDNA generously provided by Dr Mervi Julkunen (Departments I and II of Obstetrics and Gynecology, University of Helsinki). Labeling was performed by the random oligonucleotide priming technique, using a Maxiprime DNA labeling systems kit (Amersham). For autoradiography, Kodak XAR film (Eastman Kodak, Rochester, NY) was exposed to the filters for 1 and 14 days at -70°C. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to quantify the amount and quality of loaded Poly(A)+RNA.

In situ hybridization. Cytocentrifuged smears of BM cells were fixed in 4% paraformaldehyde and stored at 4°C in PBS until used. 32P-labeled PAEP cRNA probes were generated by digestion of a pGEM-3-blue vector containing PP14 cDNA generously provided by Dr Mervi Julkunen (Departments I and II of Obstetrics and Gynecology, University of Helsinki). Labeling was performed by the random oligonucleotide priming technique, using a Maxiprime DNA labeling systems kit (Amersham). For autoradiography, Kodak XAR film (Eastman Kodak, Rochester, NY) was exposed to the filters for 1 and 14 days at -70°C. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used to quantify the amount and quality of loaded Poly(A)+RNA.

In situ expression. Cytocentrifuged smears of BM cells were fixed in 4% paraformaldehyde and stored at 4°C in PBS until used. 32P-labeled PAEP cRNA probes were generated by digestion of a pGEM-3-blue vector containing PP14 cDNA in sense and antisense orientation with Sal I restriction endonuclease, followed by in vitro transcription with T7 RNA polymerase. A pGEM-3-blue vector was linearized by Xmn I restriction endonuclease to allow synthesis of
Fig 3. PAEP-immunostaining with anti-PAEP MoAbs of BM cells, counterstained with hematoxylin and photographed (A) at 200 × and (B) at 1,000 × magnification using a green filter to visualize the reaction product. C shows Western immunoblot analysis of bone marrow cells. Lane a shows patterns of standard proteins. Lane b shows pattern obtained with rabbit anti-PAEP antibodies of immunoprecipitates obtained from lysates of BM cells using rabbit anti-PAEP antibodies. The position of PAEP is indicated by arrow. Lane C shows control with nonimmune serum. D shows immunofluorescence staining for PAEP with anti-PAEP MoAbs followed by affinity-purified, FITC-conjugated goat-antimouse Ig. E shows immunofluorescence staining with rabbit-antiglycophorin A antibodies followed by affinity-purified TRITC-conjugated goat-antirabbit Ig. F shows the corresponding phase-contrast microscopic presentation.

a 35S-labeled nonsense RNA probe using SP6 RNA polymerase. Nonsense and PAEP sense strands were used for control of nonspecific binding. Pretreatment of slides, prehybridization and hybridization were performed using previously described protocols. Briefly, the cells were pretreated with proteinase K (1 μg/ml), 0.1 mol/L triethanolamine, and 0.25% acetic anhydride in triethanolamine buffer. Prehybridization was performed at room temperature for 2 hours in a buffer composed of 50% formamide, 0.6 mol/L NaCl, 10 mmol/L TRIS-HCl, pH 8.0, 20 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA, 1 × Denhardt’s solution (0.04% each polyvinyl pyrrolidone, bovine serum albumine, and Ficoll [Phar-macia, Uppsala, Sweden]), 10% dextran sulphate, 0.1 mg herring sperm DNA/ml, and 0.5 mg tRNA/ml. Hybridization was performed overnight at 50°C in the same solution as was used for prehybridization with labeled probes at a concentration of 2 × 106 cpm/μL. The slides were washed in 4 × SSC (1 × SSC; 0.15 mol/L NaCl, 0.05 mol/L Na citrate, pH 7.0), 10 mmol/L DTT and in 2 × SSC, 10 mmol/L DTT for 15 minutes at room temperature, 1 × SSC, 10 mmol/L DTT for 30 minutes at 50°C and in 0.1 × SSC for 2 hours at 60°C, then incubated in RNAseA (10 μg/ml) for 30 minutes at 37°C in 0.5 mol/L NaCl, 10 mmol/L TRIS, pH 7.0, 1 mmol/L EDTA. After the final dehydration the slides were immersed in Kodak NTB-2 photographic emulsion (Eastman-Kodak) and exposed at 4°C for 10 days. After developing, the cells were stained with Giemsa.

Complementary DNA synthesis and polymerase chain reaction (PCR) amplification. One to three micrograms of total RNA extracted from BM cells was used for cDNA synthesis performed using the First Strand cDNA synthesis Kit (Phar-macia, Uppsala, Sweden). The reaction was primed with 200 ng of oligo-dT primer or with 60 pmol of PAEP-specific primer. A negative control was included in the first-strand synthesis that contains the above reagents, but no RNA. Cross-contamination of RNA was controlled by reverse transcription reaction that contains RNA and all reagents except reverse transcriptase enzyme. Secretory endometrium and TPA-induced K562 cells were used as positive controls. Amplification of the PAEP cDNA was performed using the following primers: 5'CCAACAACATCTCCCTCATG (nt 165-194, exon 2) forward primer and 5'GTGCAGGTGTGACGAGGAA (nt 720-741, 3' untranslated region) reverse primer. Thirty cycles of PCR were performed on a Techne PHC-2 (Techne Ltd, Cambridge, UK) thermocycler with the following conditions: 95°C, 30 seconds; 55°C, 1 minute 30 seconds; 72°C, 2 minutes. To control the efficiency of reverse transcription reaction, a control amplification of the ubiquitous protein, cyclophilin, was performed. After amplification, the samples were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under ultraviolet light. Southern transfer and hybridization with PP14 cDNA probe were performed to confirm that PAEP cDNA was specifically amplified.

RESULTS

Complementary DNA from BM cells was initially subjected to reverse transcription (RT)-PCR using PAEP-specific primers. A major band of appropriate size (567 bp) was visualized in ethidium bromide stainings corresponding to the size of the positive control (Fig 1). Specificity of the amplified products was established by PAEP/PP14 cDNA probe hybridization under stringent conditions (Fig 1).
In situ hybridization was performed to show PAEP gene expression in BM. Strong hybridization signals were seen in a small population of cells. Control hybridizations with sense and nonsense probes were negative (Fig 2).

After the detection of PAEP mRNA in normal BM, immunocytochemistry was applied to identify the cells containing PAEP protein. When the cytocentrifuged smears of normal BM were stained with anti-PAEP MoAbs by the indirect peroxidase antiperoxidase (PAP) method, PAEP immunoreactivity was found in cells of the erythroid lineage. Early maturation stages of the normoblast series displayed cytoplasmic and membrane staining with the highest intensity seen in the Golgi complex (Fig 3A and B). Double immunofluorescence with anti-PAEP MoAbs and rabbit antiglycophorin A showed that the same nucleated erythroid precursor cells expressed both antigens (Fig 3D through F). Mature RBCs and cells of the myeloid and lymphoid lineages did not stain with anti-PAEP MoAbs, whereas weak reactivity was seen in some megakaryocytes. The antigen was not detectable in platelets as studied by Western immunoblotting (results not shown).

Immunoprecipitation with rabbit anti-PAEP antibodies from detergent lysates of whole BM cells, followed by Western immunoblot analysis, gave a band (Fig 3C) corresponding to the size of PAEP/PP14.5

To further investigate the expression of PAEP in hematopoietic cells, we used human leukemia cell lines with an in vitro differentiation potential. Before induction, the K562 cells did not contain any mature 0.9 kb PAEP/PP14 mRNA detectable by Northern blotting, whereas a faint band was seen at 6 kb. Treatment of K562 cells with TPA for two days induced dramatic accumulation of mature 0.9-kb PAEP mRNA (Fig 4). A hybridization signal at 6 kb was also present in the TPA-treated K562 cells. The higher molecular band may represent unprocessed PAEP/PP14 mRNA.

When cytocentrifuged smears of K562 cells were stained by indirect immunofluorescence with anti-PAEP MoAbs, intense cytoplasmic reactivity was seen in the cells treated with TPA for 3 days. Like in the BM normoblasts, the strongest immunostaining in TPA-treated K562 cells was seen in the Golgi area (Fig 5A). Uninduced K562 cells gave no specific fluorescence above background reactivity (Fig 5B), whereas cells induced with RA, or with sodium butyrate for 3 days, displayed a weak-positive staining (results not shown). Immunostainings of the megakaryoblastic cell lines Dami and HPRC, the promonocytic cell line, U937, and the promyelocytic line HL-60 were negative before and after induced differentiation (results not shown).

Immunoblot analysis with polyclonal rabbit anti-PAEP...
Western immunoblots using rabbit anti-PAEP antibodies of lysates from (A) untreated K562 cells and from cells treated for 3 days with (B) RA, (C) sodium butyrate, and (D) TPA. The position of PAEP is indicated by the arrow.

Fig 6

Antibodies of lysates from TPA-treated K562 cells showed a distinct band that comigrated with PAEP. Cells treated with RA or sodium butyrate only gave barely detectable bands (Fig 6).

K562 cells, either untreated or treated with TPA for 3 days, were metabolically labeled with 35S-methionine for 2 hours and lysed. Immunoprecipitates obtained with anti-PAEP MoAbs were separated on SDS-PAGE and analyzed by autoradiography. A distinct band at 28 kD was seen in the lanes containing precipitates from TPA-treated K562 cells, whereas no specific radiolabeled band was obtained from the lysates of control cells (Fig 7). Precipitation of the PAEP band was largely abolished by the addition of excess of purified decidual PAEP/PP14 to the lysate.

Immunofluorometric measurements showed that PAEP protein accumulates in culture medium of K562 cells grown in the presence of TPA. PAEP became detectable after 48 hours and its concentration increased up to 120 hours of culture (Fig 8). No PAEP was found in the culture media of untreated K562 cells (<3.1 µg/L).

DISCUSSION

There is a growing list of molecules expressed in both the hematopoietic system and the reproductive tract. This includes prolactin, polypeptide growth factors, and tyrosine kinase receptors of the PDGF/CSF-1 receptor family.

Results of the present study show that PAEP, previously believed to be specific for the human reproductive tract, is present in hematopoietic cells. Our immunocytochemical findings indicate that PAEP is expressed in the erythroid lineage. Positive staining was seen exclusively in the cells representing different stages of maturation of the normoblastic series. Attempts to show the presence of PAEP in mature RBCs gave negative results. Because of methodologic limitations of immunocytochemistry and the low frequency of early erythroid precursors in normal BM, it remains to be clarified whether PAEP is expressed already in proerythroblastic cells. This question is now being addressed.

In their preliminary work, Morrow and his coworkers have reported the occurrence of cDNA corresponding to PAEP/PP14 in a cDNA library of the erythroleukemia cell line K562. The present study confirms and extends this observation and lends support to the view that the initiation of PAEP expression may occur at later stages of erythropoiesis. PAEP cannot be detected in normal K562 cells, whereas treatment of the cells for 2 to 3 days with compounds known to induce in vitro differentiation brings about expression of the PAEP message and the protein.

PAEP was found to accumulate in the culture supernatant...
of differentiating K562 cells. The question of whether this is because of release from disintegrating cells or results from active secretion is now under investigation. The serum PAEP/PP14 concentration is higher in women than in men.\textsuperscript{2,24} Although the endometrium and the seminal vesicles are believed to be the main sources, it cannot be excluded that erythropoietic cells may contribute to the circulating PAEP pool.

Details of the regulation of PAEP expression in erythroid cells remains to be elucidated. Interestingly genes for human PAEP/PP14 and of the ABO blood group antigens are mapped to the same chromosomal band 9q34.\textsuperscript{25} Karhi and his coworkers\textsuperscript{26} reported that during erythropoiesis, the expression of blood group A antigen occurs at the stage of basophilic normoblast differentiation. The present findings suggest that the expression of PAEP may coincide with that of the major blood group antigens. However, whereas the structures carrying the ABO blood group substance are abundant in mature RBCs, PAEP is not.

In view of the reported immunosuppressive functions of PAEP, it is of interest to note that K562 cells, which are the standard in vitro target for human NK cells, become virtually resistant to NK cell-mediated lysis during induced differentiation.\textsuperscript{27} In that respect, the finding that PAEP/PP14 inhibits NK cell activity\textsuperscript{28} on K562 cells is of great interest. It remains to be studied whether the induced expression of PAEP contributes to the acquisition of NK resistance.

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