Cellular origin and induction of pregnancy-associated α_2 -glycoprotein synthesis in pregnant rats*

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Summary. The synthesis of α_2 -PAG was measured and compared in tissues and cells from normal non-pregnant females, and maternal and fetal rats *in vitro* to define the target cells hormonally regulated during pregnancy. Synthesis was measured by [14 C]leucine incorporation into immunochemically isolated α_2 -PAG and confirmed by radioimmunoassay. α_2 -PAG synthesis was demonstrated in maternal peripheral blood leucocytes, placenta, breast, spleen, liver and fetal peripheral blood leucocytes and liver. Maternal and fetal liver were the most active tissue producers and fetal liver synthesized 4 times more α_2 -PAG than did maternal liver. Furthermore, fetal peripheral blood leucocytes synthesized 2 times more α_2 -PAG per cell than did these same maternal cells. A direct comparison of synthesis by cells from pregnant and non-pregnant female rats revealed that (1) maternal peripheral blood leucocytes synthesized 5 times more α_2 -PAG per cell than did normal leucocytes, although these same cells synthesized approximately equal amounts of total cell protein per cell, (2) maternal peritoneal exudate macrophages also synthesized 5 times more α_2 -PAG per cell than did macrophages obtained from normal female rats, and total protein synthesis by these cells also closely paralleled each other, (3) maternal and fetal plastic-adherent peripheral blood monocytes synthesized 22 and 58 times more α_2 -PAG per cell respectively than did normal monocytes, (4) maternal and fetal non-adherent lymphocytes synthesized 8 and 16 times more α_2 -PAG per cell respectively than did normal lymphocytes and (5) fetal monocytes and lymphocytes synthesized 3 and 2 times more α_2 -PAG per cell than did maternal monocytes and lymphocytes.

We conclude that maternal and fetal peripheral blood monocytes and lymphocytes and maternal exudate macrophages are target cells hormonally induced to synthesize increased amounts of α_2 -PAG during pregnancy.

Keywords: pregnancy-associated α_2 glycoprotein; synthesis; induction; rat

Introduction

Elevated concentrations of pregnancy-associated α_2 -glycoprotein (α_2 -PAG) in serum are detectable in pregnant women, non-pregnant women receiving oestrogen-containing steroids and patients with diverse types of disseminated malignancies and a variety of other pathological conditions such as Crohn's disease, Bechet's syndrome and rheumatoid arthritis (Thomson & Horne, 1980; Horne *et al.*, 1982). In pregnant women, serum α_2 -PAG concentrations increase

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rapidly and reach maximum values as high as 1.0 mg/ml during the third trimester of gestation, then usually decrease rapidly to normal low values ($\sim 10 \mu\text{g/ml}$) 6–8 weeks *post partum*. The biological function of α_2 -PAG is unknown, but its temporal appearance during each stage of gestation and subsequent disappearance *post partum* suggest that this protein plays a role during pregnancy. Treatment of non-pregnant women and men with exogenous oestrogen produces markedly elevated serum α_2 -PAG concentrations (Horne *et al.*, 1973; von Schoultz & Stigbrand, 1982) and clearly indicates that this protein is under hormonal control. On the other hand, the precise humoral–endocrine pathways and target cells regulated to produce increased amounts of α_2 -PAG during pregnancy are poorly understood.

Recently, rodent analogues to human α_2 -PAG have been described by several groups of investigators, i.e. rat PAG (Porstmann & Schmechta, 1980), mouse pregnancy-associated PAMP-1 (Hau *et al.*, 1981), rat α_2 -PAG (Sarcione & Dell'Uomo, 1981), mouse α_1 -PAP (Waites & Bell, 1984; Waites *et al.*, 1985) and rat α_1 -PAP (Waites *et al.*, 1986). In the present study, synthesis of α_2 -PAG was measured and compared in normal non-pregnant female, maternal and fetal rat tissues and cells to define the target cells hormonally regulated during pregnancy.

Materials and Methods

Tissue and cells. Rats of the Sprague–Dawley strain, 4–5 months old and weighing 325–400 g were used. Maternal and fetal tissues and cells were obtained at 16–18 days after conception as determined by the presence of a vaginal plug.

Blood samples were obtained from ether-anaesthetized animals by retro-orbital puncture or from the abdominal aorta. Peripheral blood leucocytes were buffed from heparinized whole blood mixed with an equal volume of Plasmagel (Associated Biomedic Systems, Buffalo, NY, USA) and incubated at 37°C for 2 h. Leucocytes were separated from the underlying red blood cell fraction, centrifuged at $800g$ for 5 min and resuspended in distilled water to lyse contaminating red blood cells then adjusted to 0.9% NaCl (w/v) by addition of 3% NaCl (w/v). White blood cells were then washed with 0.9% NaCl, resuspended in RPMI 1640 medium and counted. Cell viability was monitored microscopically by trypan blue exclusion. In some studies, the mononuclear fraction obtained from whole blood by Ficoll gradient centrifugation was further separated into plastic-adherent 'monocytes' and non-adherent 'lymphocytes'. Microscopic examination revealed that $>99\%$ of the adherent cells were morphologically identical to monocytes and $>97\%$ of the non-adherent cells were similar to lymphocytes.

Peritoneal exudate cells (PEC) were obtained from pregnant and non-pregnant female rats 4 days after intraperitoneal injection of 10.0 ml sterile paraffin oil. Exudate cells were removed from the peritoneum by three separate washings with 10.0 ml heparinized RPMI 1640 medium. The washings were pooled, centrifuged, washed twice, resuspended in RPMI 1640 medium, then further separated into plastic-adherent macrophages. Adherent cells were scraped from the plastic with a rubber policeman, resuspended and counted. Cell viability was assessed by trypan blue exclusion. Microscopic examination indicated that $>99\%$ of the adherent cells were morphologically identical to macrophages.

Tissue and cell protein synthesis. Rat tissue slices (300–350 mg) or isolated blood or exudate cells (1×10^6) were incubated in flasks containing 3.0 ml RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), Hepes buffer (5 mM) and 3 μCi L-[U- ^{14}C]leucine (New England Nuclear, Boston, MA, USA) at 37°C with gentle shaking in 5% CO_2 –95% air. Puromycin (Calbiochem, San Diego, CA, USA) was added to some tissue and cell incubations at zero time minus 15 min at a final concentration of 100 $\mu\text{g/ml}$. After incubation, the total contents of each flask were rapidly frozen and thawed 3 times in a solid CO_2 –ethanol bath, homogenized and adjusted to a final concentration of 0.1% Triton X-100 to dissolve cellular membranes and release protein. Samples were then dialysed against frequent changes of 0.9% (w/v) NaCl containing 0.1% unlabelled L-leucine at 4°C to remove free radiolabelled leucine. Dialysed samples were centrifuged and clear supernatants reconstituted to their original 3.0 ml volume by the addition of 0.9% (w/v) NaCl.

Isolation of α_2 -PAG and total cell protein. α_2 -PAG was isolated immunochemically from 500 μl of the dialysed sample by addition of 50 μl pooled serum from pregnant rats to provide sufficient carrier α_2 -PAG for immunoprecipitation, and 200 μl monospecific rabbit antiserum reagent to rat α_2 -PAG prepared as described previously (Sarcione & Dell'Uomo, 1981). The monospecificity of this antiserum reagent was reconfirmed by double immunodiffusion and immunoelectrophoresis. The amount of antiserum used as determined in preliminary titrations to result in precipitation of 98–100% of the total α_2 -PAG present in these samples based on observations that (a) a second addition of antiserum did not result in additional visible immune precipitation and (b) a second addition of serum from pregnant rats followed by anti-rat α_2 -PAG to these samples did not result in the appearance of additional radioactivity in the collected immune precipitates. To reduce non-specific absorption of radioactivity to a minimum, collected immune precipitates were washed three times by resuspension and centrifugation in cold 0.9% (w/v) NaCl.

Total cell protein was isolated by precipitation with trichloroacetic acid (TCA). A 0.5 ml aliquant of dialysed samples was diluted 5-fold with 0.9% (w/v) NaCl, then an equal volume of cold 20% TCA was added. The samples were stored at 4°C for 2 h, centrifuged, and the collected protein precipitates washed twice with cold 10% (v/v) TCA and twice with cold 0.9% (w/v) NaCl.

All immuno- and acid-precipitated protein samples were dissolved in 0.1 N-NaOH and radioactivity in aliquots was measured in a liquid scintillation counter. Radiolabelled leucine incorporation into α_2 -PAG and total cell protein was expressed as d.p.m./100 mg tissue or d.p.m./ 10^6 cells.

Confirmation of α_2 -PAG synthesis by radioimmunodiffusion. Tissues and cells were incubated for 24 h and treated exactly as described above except that 3.0 μ Ci L-[U- 14 C]leucine were added at zero time and at 12 h to increase the amount of leucine radioactivity incorporated. Aliquants of dialysed samples were concentrated 10-fold with a Minicon B-15, then used for double immunodiffusion and autoradiography. Immunodiffusion slides were washed for 24 h in repeated changes of 0.9% (w/v) NaCl and finally in distilled water. The slides were dried slowly at 37°C using paper wicks to prevent distortion of the patterns. Precipitation bands were fixed in 12% TCA for 30 min, then stained for protein with 1% amido black. Stained slides were placed against Kodak non-screen industrial type X-ray film and exposed for up to 2 months to obtain positive radiographs.

Transplacental passage of rat maternal leucocytes to the fetus. Peripheral blood mononuclear leucocytes were obtained from 12.0 ml heparinized whole blood from pregnant near-term rats by Ficoll gradient centrifugation, washed and counted as described earlier. The mononuclear cell fraction contained 82% lymphocytes and 18% monocytes. A total of 7.43×10^6 leucocytes suspended in 10.0 ml RPMI 1640 medium supplemented as described above and containing 100 μ Ci [U- 14 C]leucine were incubated for 19 h at 37°C with gentle shaking in 5% CO₂-95% air, washed three times in phosphate-buffered saline pH 7.4 (Ca²⁺ and Mg²⁺ free) (PBS) and the final cell pellet was resuspended in 1.0 ml PBS. After the radiolabelling procedure 90% of the leucocytes remained viable when assessed by trypan blue exclusion. A total of 1.5×10^6 of these radiolabelled mononuclear cells suspended in 0.2 ml PBS containing 2.3×10^7 d.p.m. were slowly infused intravenously via an exposed femoral vein into each of 4 pregnant near-term rats. Maternal blood samples were obtained from the warmed tail vein at 15-min intervals. The rats were killed at 15-min intervals and fetal blood was obtained by decapitation. Whole blood and maternal and fetal tissues were solubilized in NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, IL, USA) at 50°C for 30 min for blood and overnight for tissues. Samples were decolourized by addition of benzoyl peroxide and incubated for 30 min at room temperature, then counted in a liquid scintillation counter. The number of maternal leucocytes detected in each fetus was calculated from the specific radioactivity (d.p.m./cell) of the radiolabelled maternal cells infused and the total radioactivity measured in fetal whole blood and liver.

Results

Tissue and cellular sites of α_2 -PAG synthesis in pregnant rats

Figure 1 shows a direct comparison of α_2 -PAG synthesis by maternal and fetal tissues and cells from pregnant rats measured by L-[14 C]leucine incorporation into immunochemically isolated α_2 -PAG. Maternal peripheral blood leucocytes placenta, breast, spleen, liver and fetal liver all incorporated radioactivity into α_2 -PAG which was consistently greater than that incorporated into puromycin-inhibited controls. The inhibition produced by puromycin supports the conclusion that incorporation of leucine radioactivity observed in its absence represents de-novo α_2 -PAG synthesis rather than non-specific adsorption of other radiolabelled components to collected immune precipitates. A comparison based on weight of tissue indicated that (1) maternal and fetal liver were the most active tissue producers of α_2 -PAG and (2) fetal liver produced 4 times more α_2 -PAG than did maternal liver. A comparison based on cell number revealed that (1) both maternal and fetal peripheral blood leucocytes actively produced α_2 -PAG and (2) fetal leucocytes synthesized 2 times more α_2 -PAG per cell than did maternal leucocytes.

Induction of cellular α_2 -PAG synthesis in pregnant rats

To investigate whether cellular α_2 -PAG synthesis was stimulated above normal levels during pregnancy, α_2 -PAG and total cell protein synthesis were measured and compared in peripheral blood leucocytes obtained from pregnant and normal adult non-pregnant female rats. After 24 h, maternal leucocytes synthesized 5 times more α_2 -PAG per cell than did normal leucocytes (Fig. 2a). During this same time, however, leucocytes from pregnant and normal animals synthesized approximately equal amounts of total cell protein (Fig. 2b). After 24 h, α_2 -PAG isolated from

Fig. 1. A comparison of incorporation of L-[¹⁴C]leucine into α₂-PAG after incubation of maternal and fetal rat tissues and cells for 24 h without and with added puromycin. Results are the mean of triplicate samples of tissues and cells obtained from 3 pregnant rats.

maternal blood leucocytes contained 3·8% of the total cell protein radioactivity whereas this pregnancy protein isolated from normal peripheral leucocytes contained only 0·8% of the total cell protein radioactivity.

Fig. 2. A comparison of incorporation of L-[¹⁴C]leucine into (a) α₂-PAG and (b) total cell protein during incubation of peripheral blood leucocytes (PBL) obtained from pregnant (●) and non-pregnant normal adult female rats (■) for 24 h without (●, ■) and with (○, □) added puromycin. Each point is the mean of triplicate samples of leucocytes obtained from 3 pregnant and 3 non-pregnant normal adult female rats.

To establish which types of peripheral blood mononuclear leucocytes were selectively stimulated during pregnancy, α_2 -PAG synthesis was measured and compared in plastic-adherent 'monocytes' and non-adherent 'lymphocytes' obtained from normal female, maternal and fetal rats. Figure 3 shows that (1) maternal and fetal 'monocytes' synthesized 22 and 58 times more α_2 -PAG per cell respectively than did normal 'monocytes', (2) maternal and fetal 'lymphocytes' synthesized 8 and 16 times more α_2 -PAG per cell respectively than did normal 'lymphocytes', and (3) fetal monocytes and lymphocytes synthesized 3 and 2 times more α_2 -PAG per cell than did maternal monocytes and lymphocytes, respectively.

Fig. 3. A comparison of incorporation of L-[¹⁴C]leucine into α_2 -PAG after incubation of peripheral blood monocytes and lymphocytes obtained from normal adult non-pregnant female, near-term maternal and fetal rats for 24 h. Results are the mean of triplicate samples of monocytes and lymphocytes obtained from 3 pregnant and 3 non-pregnant normal adult female rats and from 3 pooled whole blood samples obtained from all fetuses found in each pregnant rat.

Fig. 4. A comparison of incorporation of L-[¹⁴C]leucine into (a) α_2 -PAG and (b) total cell protein during incubation of peritoneal exudate macrophages (PEM) obtained from pregnant (●) and non-pregnant normal adult female rats (■) for 24 h without (●, □) and with (○, □) added puromycin. Each point is the mean of triplicate samples of peritoneal exudate macrophages from 3 pregnant and 3 non-pregnant normal adult female rats.

Fig. 5. Stained double immunodiffusion patterns (a) and their respective autoradiographs (b) obtained after incubation of maternal and fetal rat tissues and cells with L-[^{14}C]leucine for 24 h *in vitro*. L = Maternal liver; BT = breast; SPL = spleen; UT = uterus; PL = placenta; FL = fetal liver; PBL = fetal peripheral blood leucocytes; PEC = maternal peritoneal exudate macrophages. Immunoprecipitation bands were developed using monospecific rabbit anti-rat α_2 -PAG.

To investigate the role of tissue macrophages, α_2 -PAG and total cell protein synthesis were measured and compared in peritoneal exudate macrophages obtained from pregnant and normal adult non-pregnant female rats. After 24 h, maternal exudate macrophages also synthesized 5 times more α_2 -PAG per cell than did macrophages obtained from normal females (Fig. 4a). In this study, total cell protein synthesis by maternal and normal macrophages paralleled each other during 24 h, and maternal macrophages actually produced less total protein per cell than did normal macrophages (Fig. 4b). After 24 h, α_2 -PAG isolated from maternal and normal rats contained 5.3 and 1.4% of the total cell protein radioactivity respectively.

Confirmation of α_2 -PAG synthesis by double immunodiffusion and radioautography

Double immunodiffusion of radiolabelled tissue and cell extracts, containing added carrier rat pregnancy sera, against rabbit anti-rat α_2 -PAG resulted in a single continuous immunoprecipitation line (Fig. 5a) and produced a positive superimposable autoradiograph (Fig. 5b) for maternal liver, breast tissue, spleen, uterus, placenta and fetal liver, and also for maternal peripheral blood leucocytes and peritoneal exudate macrophages. Fetal peripheral blood leucocytes also generated a positive autoradiograph (not shown). These results confirm the monospecificity of the rabbit

Fig. 6. Distribution of L-[^{14}C]leucine radioactivity in maternal and fetal whole blood and selected tissues after intravenous administration of maternal peripheral blood mononuclear leucocytes previously radiolabelled by incubation with L-[^{14}C]leucine for 19 h at 37°C. Each point is the mean of triplicate samples of tissues and cells from 1 pregnant rat at each time interval.

antiserum to rat α_2 -PAG used throughout these studies, and demonstrate and confirm α_2 -PAG synthesis by these maternal and fetal tissues and cells by a method independent of the immunochemical precipitation procedure.

Transplacental passage of rat maternal leucocytes to fetus

Since some maternal rat (Beer *et al.*, 1972) and human (Field & Caspary, 1971; Schroder, 1974) lymphocytes have been reported to enter the fetal circulation, the magnitude of this possible transplacental passage of rat maternal leucocytes to the fetus was evaluated under the same experimental conditions as used to measure maternal and fetal α_2 -PAG synthesis. After intravenous infusion of [^{14}C]leucine maternal peripheral blood mononuclear leucocytes into pregnant near-term rats, 85% of the total pre-labelled maternal leucocyte labelled with radioactivity rapidly disappeared from the maternal circulation during 1 h, and a large proportion (44%) of these radiolabelled leucocytes accumulated in maternal lung, liver and spleen (Fig. 6). During this same time, however, only 0.003% of this total administered radioactivity appeared in the fetal circulation which did not increase over time, and only 0.007% of this radioactivity accumulated in fetal liver. Calculations based on the specific radioactivity of the maternal peripheral blood leucocytes infused (d.p.m./cell) indicated that 46 and 107 radiolabelled maternal peripheral blood mononuclear cells accumulated in total whole blood and liver per fetus after 1 h. During this same time, little or no radioactivity was detected in maternal or fetal serum, which indicates that little or no leucocyte lysis and release of radioactivity occurred.

Discussion

This study demonstrated de-novo α_2 -PAG synthesis by all pregnant rat tissues and cells studied which included maternal peripheral blood leucocytes, placenta, breast, uterus, spleen, liver and fetal peripheral blood leucocytes and liver. Specific types of cells shown to synthesize α_2 -PAG were maternal and fetal peripheral blood monocytes and lymphocytes and maternal peritoneal exudate macrophages. It is possible that other tissues and cells not examined in this study also synthesize α_2 -PAG. Since maternal macrophages produced α_2 -PAG and spleen, liver and placenta are multiple cellular organs containing large amounts of reticuloendothelial tissue, it is likely that at least part of the α_2 -PAG synthesis observed in these maternal organ tissues was due to spleen, liver and placental cells *per se* rather than due exclusively to peripheral blood monocytes and lymphocytes which are known to infiltrate these organs in large numbers. The immunohistological localization of analogous mouse and rat proteins within the liver and placenta *per se* (Chemnitz *et al.*, 1982; Waites *et al.*, 1986) supports the supposition that the infiltrating cell population was not solely responsible for the α_2 -PAG synthesis measured in these tissues. The evidence that α_2 -PAG was synthesized by rat maternal peripheral blood leucocytes and liver confirms and extends reported synthesis of this protein by human maternal peripheral blood leucocytes and liver (Stimson *et al.*, 1979) and strengthens the conclusion that rat α_2 -PAG is analogous to its human counterpart. Since peripheral blood monocytes and lymphocytes would also be found in breast and uterine tissues, the types of cells responsible for α_2 -PAG synthesis observed in these organ tissues remain ambiguous. On the other hand, evidence that MCF-7 and BT-20 human breast cancer cells synthesize and release α_2 -PAG (Sarcione *et al.*, 1983) suggests that maternal breast cells *per se* may also have the capacity to produce this pregnancy protein.

The conclusion that fetal blood leucocytes, monocytes, lymphocytes and liver *per se* synthesized α_2 -PAG, and that synthesis observed in these cells and tissue was not due to translocated maternal peripheral blood leucocytes was based on observations that: (1) fetal liver synthesized 5 times more α_2 -PAG per 100 mg tissue than did maternal liver, (2) fetal peripheral blood leucocytes, monocytes and lymphocytes synthesized 3, 5 and 4 times more α_2 -PAG per cell respectively than did these same maternal cells, and (3) after infusion of radiolabelled maternal leucocytes into pregnant near-term rats, relatively few radiolabelled cells were found in fetal whole blood or liver during 1 h when most of these cells were rapidly cleared from the maternal circulation. The conclusion that fetal cells *per se* produce α_2 -PAG and evidence that human breast cancer cells also synthesize this pregnancy protein indicates that α_2 -PAG should be classified as an 'oncofetal' protein analogous to alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA).

The evidence that maternal and fetal rat peripheral blood leucocytes, monocytes, lymphocytes and maternal macrophages synthesized 5–58 times more α_2 -PAG per cell and 4–5 times more as a percentage of total cell protein per cell, without appreciable change in total cell protein synthesis when compared to these same cells obtained from normal female animals, indicates that (1) α_2 -PAG synthesis was selectively induced in these maternal and fetal target cells during pregnancy and (2) the results obtained were not a reflection of increased total cellular protein synthesis or possible differences in intracellular unlabelled leucine pool sizes within these cells. Furthermore, the observations that fetal peripheral blood leucocytes, monocytes, lymphocytes and liver consistently synthesized 2–3 times more α_2 -PAG per cell or per mg tissue than did these same maternal cells and liver indicates that fetal cells were more sensitive to stimulatory induction of α_2 -PAG synthesis than were maternal cells. We conclude that maternal and fetal peripheral blood monocytes and lymphocytes and maternal exudate macrophages are target cells hormonally induced to synthesize increased amounts of α_2 -PAG during pregnancy.

The precise humoral–endocrine pathways and mechanisms directly responsible for regulating induction of cellular α_2 -PAG synthesis remain to be elucidated. Although treatment of non-pregnant women, men and rats with exogenous oestrogen is known to produce markedly elevated serum α_2 -PAG concentrations, a direct regulatory role for endogenous oestrogen is unlikely

because serum α_2 -PAG concentrations are (1) decreasing during the last stages of pregnancy when oestrogen values continue to increase (Stimson, 1975), (2) are lower in twin pregnancies associated with higher oestrogen values (Westergaard *et al.*, 1982) and (3) are higher in post-menopausal women than in premenopausal women (Damber, 1976). It has been suggested that indirect effects of oestrogen are mediated via the pituitary and that mouse and rat serum concentrations of α_2 -PAG analogues are regulated by growth hormone secretion (Fröhlander *et al.*, 1987; Eriksson *et al.*, 1988).

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