Identification, immunoaffinity purification and partial characterization of a human decidua-associated protein*

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Summary. A crude extract of pooled early-pregnancy decidual tissue was enriched for soluble decidual proteins by exhaustive affinity absorption with antibodies to human serum proteins immobilized on Eupergit C. The partly purified extract was used to prepare monoclonal antibodies. A monoclonal antibody was obtained recognizing an antigen present in extract of decidual tissue and not in extract of proliferative endometrium. The monoclonal antibody was used for immunoaffinity purification of the decidua-associated protein. By SDS-PAGE analysis, under reducing conditions it yielded 2 bands at apparent molecular weights of 55 000 and 25 000. Under non-reducing conditions a single protein band at apparent molecular weight of 200 000 was observed. The M_r 200 000 protein was named hDP200 and the M_r 55 000 protein was named hDP55. It is suggested that hDP55 is a subunit of the hDP200. The hDP200 did not react with polyclonal antibodies specific for PP12 and PP14. PP14 has been shown to be immunologically indistinguishable from PEP and α_2 -PEG. Our data therefore suggest that hDP200 is a novel human decidua-associated protein.

Keywords: monoclonal antibody; decidua-associated secretory protein; man

Introduction

Endometrial decidualization is growth and differentiation of a 'new', progestagen-dependent tissue, the decidua, which gradually replaces the functional layer of the endometrium. Pregnancy is not mandatory for growth and differentiation of decidual tissue. It is produced in each normal cycle in the late luteal phase in response to progesterone. Eventually, at birth or menstruation the decidua is shed.

Because decidualization is growth and differentiation of new tissue, the appearance of decidua-specific proteins can be anticipated. Human decidual tissue, obtained at early pregnancy, synthesizes and secretes 7 specific proteins in culture (Kisalus *et al.*, 1987). Several decidua-associated proteins have also been described, e.g. PAPP-A (Bischof *et al.*, 1974), AUP (Sutcliffe *et al.*, 1980), PEP (Joshi, 1983), PP12 (Wahlstrom & Seppala, 1984; Rutanen *et al.*, 1985), EP14, EP15 (Bell *et al.*, 1985a, b), PP14 (Westwood *et al.*, 1988), α_2 -PEG (Bell *et al.*, 1987) and prolactin (Riddick *et al.*, 1978). Immunological identity between the majority of these proteins has been established (Sutcliffe *et al.*, 1982; Julkunen *et al.*, 1986; Bell & Bohn, 1986), but it is not clear whether these represent a single or several decidua-associated proteins.

In this study, we describe the identification, immuno-purification by a monoclonal antibody, and initial characterization of a human decidua-associated protein.

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Materials and Methods

Antisera. Rabbit antisera specific for PP14 (Riittinen *et al.*, 1989) and PP12 (Koistinen *et al.*, 1987) were obtained from Dr M. Seppala.

Decidual tissue extraction. Samples of decidual tissue were acquired from 12 elective pregnancy terminations (6–12 weeks after conception) performed by dilatation and curettage. The decidual tissue was manually cleared of embryonic tissues and large blood clots. After thorough washing in cold phosphate-buffered saline, pH 7.4 (PBS), the samples were blotted, frozen and stored at -20°C .

Thawed tissues were homogenized (Omnimixer) in 3 volumes of cold PBS for 5 min at maximal speed. The homogenate was then centrifuged at $3000 g_{\text{max}}$ for 20 min. The collected supernatants were recentrifuged at $100\,000 g_{\text{max}}$ for 1 h. The supernatants were stored at -20°C until further use. Protein concentrations were measured by the method of Bradford (1976) using the BioRad assay kit (Hercules, CA, USA).

Purification of the crude decidual extract by immunosorption. Eupergit C beads (Rohm Inc., Darmstadt, W. Germany) were washed 3 times with PBS and then with 1.0 M-potassium phosphate buffer, pH 7.5. Blood-related proteins were removed from the crude decidual extract with antibodies to human serum proteins. Goat antiserum to whole human serum (GAHuS) was concentrated by precipitation with ammonium sulphate at 40% saturation. The concentrated GAHuS was dissolved in 1.0 M-potassium phosphate buffer, pH 7.5, 96 mg in 5 ml; 2.5 g washed Eupergit C beads were added. The test tube containing the mixture was agitated by rotation for 16 h at 4°C . Residual oxirane groups were blocked by reacting them with 1.0 M-ethanolamine at pH 9.5, for 4 h at 4°C . The GAHuS–Eupergit C complex was washed with 1.0 M-ammonium acetate buffer, pH 10, for 30 min at 4°C to elute any non-specifically adsorbed protein. The beads were then thoroughly washed with PBS until the pH of the effluent reached 7.0.

An extract of pooled decidual tissue containing about 40 mg protein in 2 ml PBS was gently shaken with 2.5 g Eupergit C–GAHuS conjugate for 16 h at 4°C . The beads were then sedimented by centrifugation ($5000 g_{\text{max}}$) for 5 min, rinsed once with PBS and the rinse was added to the supernatant. The immunological binding activity of the immobilized GAHuS was then regenerated; the immunobound proteins were eluted from the beads with 20 ml 1.0 M-ammonium acetate buffer, pH 10, and several washings of PBS.

The immunoabsorption of the pooled supernatants was repeated 7 times by which time there was no further absorption of protein from the tissue extract. The resulting solution of decidua-associated proteins was concentrated on a PM10 membrane in a Diaflo ultrafiltration cell (10 ml, Amicon).

Preparation of monoclonal antibodies. CB6F1 mice were immunized by 2 injections, 10 days apart, of 10 μg decidua-associated proteins emulsified in an equal volume of Freund's complete adjuvant. After a further 20 days their spleen cells were fused with NSO myeloma cells (Galfre & Milstein, 1981) as previously described (Hadas & Theilen, 1987). A resultant hybridoma line (denoted by us as DEC21) was cloned twice by limiting dilution. Ascites fluid was produced in CB6F1 mice by intraperitoneal inoculation of hybridoma cells 1 day after priming with 0.5 ml Freund's incomplete adjuvant (Mueller *et al.*, 1986). In all subsequent experiments (except as specifically noted) the monoclonal antibody preparation used (mAb) was partly purified from the ascites fluid by 50% ammonium sulphate precipitation.

Preparation of the immunoaffinity column. After conjugation of 1 g washed Eupergit C beads with mAb, the mAb–Eupergit C conjugated beads were packed into a column and washed with 10 volumes of PBS. The column was loaded with 10 ml crude decidual extract, containing about 20 mg total protein/ml PBS, and then washed with PBS at a flow rate of 30 ml/h until the effluent was protein-free. Specifically bound proteins were then eluted from the mAb–Eupergit C with 1.0 M-ammonium acetate buffer, pH 10, and concentrated to 1 ml using a Centrifo CF25 ultrafiltration membrane cone (nominal molecular weight cut-off 25 000; Amicon), and dialysed against PBS.

Polyacrylamide gel electrophoresis. The electrophoresis was performed in the presence of 0.6 M-mercaptoethanol in the samples (the samples were boiled before application onto the gel) and sodium dodecyl sulphate (Laemmli, 1970). Protein bands were stained with Coomassie Brilliant Blue R or silver stain (BioRad).

Western blot analysis. Proteins were transferred from the polyacrylamide gels into nitrocellulose sheets (450 nm nominal pore size) in 20 mM-Tris–HCl buffer, pH 8.8, containing 150 mM-glycine and 20% (v/v) methanol using a TE 22 transfer apparatus (Hoeffer); a current of 1 A was applied for 1 h. The sheets were then cut into strips corresponding with the lanes of the original gel. The strips were stained in a solution of 1 mg Amido black/ml aqueous 5% methanol + 7.5% acetic acid. The strips were destained by washing in the solvent.

For immunostaining, the strips were incubated with the appropriate antibody diluted (1:500) with low-fat milk for 1 h at room temperature and then washed several times with tap water. The strips were then reacted for 1 h at room temperature with goat anti-mouse IgG conjugated with horseradish peroxidase (BioRad) diluted 1:3000 with low-fat milk, and then washed several times with tap water. Immunoreactive bands were visualized by reaction with chloronaphthol (BioRad) reagent (0.5 mg 4-chloro-1-naphthol/ml PBS containing 0.018% hydrogen peroxide). The reaction was terminated by washing the strips in tap water.

ELISA. The wells of microtitre ELISA plates were coated with the mixture of proteins being examined. They were exposed for 16 h to a solution containing 1 μg protein/well in 0.1 ml 0.1 M-carbonate buffer, pH 9.6, at 4°C . After

Fig. 1. Analysis of a crude extract of decidua (a) and an absorbed decidua extract (b) by SDS-PAGE (10% gel). Lane A was stained by Coomassie Brilliant Blue R and Lane B by silver stain (BioRad).

washing several times in tap water, mAb (non-diluted hybridoma culture media or ascites fluids diluted (1:500) with low-fat milk) was added, 0.1 ml/well. After 1 h at 37°C the wells were washed 5 times in tap water. To each well, 0.1 ml goat anti-mouse IgG-horseradish peroxidase complex (BioRad), diluted 1:3000 with low-fat milk, was added and the plates were incubated for 1 h at 37°C. After washing several times in tap water, 0.1 ml *o*-phenylenediamine reagent was added to each well and the colour was allowed to develop. The *o*-phenylenediamine reagent contained 2 mg 1,2-phenylenediamine/ml in 50 mM-sodium citrate buffer, pH 5.0, containing 0.08% hydrogen peroxide. The colour was determined by measuring the increase in absorbance at 492 nm compared to the non-specific absorbance at 405 nm in an EAR400 Elisa Reader (SLT-Labinstruments, Grödig, Austria).

Staining for the presence of sugar residues. Graded quantities of the decidua-associated protein were applied to a nitrocellulose membrane and stained for the presence of sugar residues by using the hydrazine derivative of horseradish peroxidase (Gershoni *et al.*, 1985).

Results

Preparation of decidua-specific soluble proteins

The most obvious sources of contamination of the decidual tissue used in this study were blood and embryonic tissue. At the time of isolation by mechanical separation, an attempt was made to eliminate as much of the fetal tissues as possible. Blood-derived proteins and other antigens cross-reacting immunologically with them could be eliminated by repeated batch absorption of the decidual extract with commercial antibodies to whole human serum conjugated to a Eupergit C carrier. After each absorption, a large decrease in total protein concentration was seen, until, after the 7th absorption, no bands were found in a mercaptoethanol-SDS-PAGE gel stained with Coomassie Brilliant Blue R. Silver staining revealed 7 clearly distinguishable bands (Fig. 1) in SDS-PAGE.

The protein mixture was tested for the presence of albumin by ELISA using a commercial rabbit anti-human albumin antiserum (BioYeda, Rehovot, Israel). None was detected. The absence

Fig. 2.

Fig. 3.

Fig. 2. SDS-PAGE analysis of immunoaffinity-purified hDP200. The hDP200 was purified by a single immunoaffinity extraction from the crude extract of decidua by DEC21 mAb immobilized on Eupergit C (see 'Materials and Methods'). **(a)** Electrophoresis performed under reducing conditions. **(b)** Electrophoresis performed under non-reducing conditions.

Fig. 3. Identification of the antigen recognized by the DEC21 mAb by western blot analysis of immunoaffinity purified decidual extract which was separated by SDS-PAGE under reducing **(a)** and non-reducing **(b)** conditions.

of albumin provided support for the belief that all of the blood proteins had been successfully eliminated. The remaining 7 proteins were tentatively classed as decidua-specific proteins.

Serological purification and analysis of decidua-specific proteins

Mice were inoculated with the mixture of putative human decidua-specific proteins, and hybridomas producing decidua-specific antibodies were cloned. The criterion for selection of the hybridomas was that their antibody reacted to a significantly greater extent with decidual extract than with extracts of proliferative endometrium and not at all with serum from males: 9 mAbs were identified which fulfilled these criteria and one, designated DEC21, was selected for further characterization.

This mAb was partly purified from its ascites fluid by ammonium sulphate fractionation and was coupled to Eupergit C beads. A column of these beads was used to separate the decidua-specific protein. After removal of non-binding proteins, approximately 1 mg immunobound protein(s) was eluted and concentrated. In SDS-PAGE analysis performed under non-reducing conditions, one major protein band was seen with an apparent molecular weight of about 200 000 and two minor protein bands at 165 000 and 125 000. Under reducing conditions a major and a minor protein band with apparent molecular weights of 55 000 and 25 000, respectively, were visible (Fig. 2).

The immunoaffinity purified antigens by mAb DEC21 were also analysed by western blot performed with the same mAb. MAb DEC21 recognized two hDP protein bands with apparent

Fig. 4. Comparative immunological analysis of specificity of mAb DEC21 (1), polyclonal anti-sera specific for PP14 (2) and polyclonal antisera specific for PP12 (3). The antibodies were reacted with western blots prepared from the preparation of decidua-specific soluble proteins.

molecular weights of 55 000 and 25 000 on reduced gels and a major band of 200 000 and several minor protein bands with apparent molecular weights of 165 000, 125 000, 100 000 and 55 000 on non-reduced gels (Fig. 3). The immunological cross-reactivity of the proteins of M_r 55 000 and 200 000 indicated that the latter is probably a tetramer of the former and the minor protein bands are probably degradation products of the M_r 200 000 species or dimers and trimers of the 55 000 subunit and its degradation product. The 200 000 unit was named hDP200 and the 55 000 monomer was named hDP55.

Some characteristics of hDP55 are similar to those of PEP, PP14 and α_2 -PEG. Immunological analysis was used to find out whether hDP55 is different from PP14. Western blots were established from the preparation of decidua-specific soluble proteins and were reacted with mAb DEC21 and with antisera specific for PP14 and PP12. As shown in Fig. 4, the hDP55 and the protein of M_r 25 000 did not react with these antibodies, thus ruling out the possibility of immunological cross-reactivity between hDP200 and PP14 or PP12.

Graded quantities of hDP200 were applied to a nitrocellulose membrane and stained for the presence of sugar residues. Positive results were obtained (data not shown), proving that hDP200 is a glycoprotein.

Discussion

The hDP200 was immunopurified from crude decidual extract by mAb. The hybridoma culture which produced this mAb (designated by us as mAb DEC21) was originally selected on the basis of

its reaction with the major protein remaining in the decidual tissue extract after removal of non-specific proteins by immunosorption. Isolation of the protein from purified decidual tissue extract by immunopurification with this same mAb yielded two bands with apparent molecular weights of 55 000 and 25 000 in SDS-PAGE analysis performed under reducing conditions. Under non-reducing conditions one major band with an apparent molecular weight of 200 000 and two minor bands with apparent molecular weights of 165 000 and 125 000 were observed. All the bands reacted with mAb DEC21 in western blot analysis. In the western blot analysis performed under non-reducing conditions two additional minor bands appeared with apparent molecular weights of 100 000 and 55 000.

The complex pattern that emerges from our results may be interpreted as follows. The basic unit of the system is probably the protein of M_r 200 000 that may give rise, under reducing conditions, to the (monomer) protein of M_r 55 000. The unit of M_r 25 000 is probably a degradation fragment of the M_r 55 000 protein, or its subunit, and the proteins of 165 000, 125 000 and 100 000 may be degradation products of the hDP200 or partly polymerized forms of the M_r 55 000 and 25 000 proteins. The existence of small quantities of the hDP55 in non-reduced gels probably indicates that the hDP200 is highly susceptible to reduction even under conditions which are generally considered as non-reducing when performing SDS-PAGE analysis. However, additional information is needed to prove the validity of any of these suggestions. The protein was named hDP200 because that of M_r 200 000 is the major form observed under non-reducing conditions. The monomer, which is the major form observed after reduction of the hDP200, was named hDP55. Several lines of evidence support the hypothesis that hDP200 is a secretory protein. In immunochemical staining, the protein appears to be localized in the glandular and surface epithelium (data not shown). The protein is detected in uterine luminal flushings and it is a glycoprotein (data not shown). We therefore suggest that the hDP200 is a secretory product of the decidual tissue.

As mentioned in the 'Introduction', several secretory glycoproteins of human decidua, immunohistologically localized to the glandular and surface epithelium (Mazurkiewicz *et al.*, 1981; Waites *et al.*, 1988) and exhibiting at least partial serological identity, have already been described. These include PEP, PP14 and α_2 -PEG. The hDP55, being a glycoprotein of M_r 55 000 secreted from decidual tissue and localized by immunostaining in the glandular and surface epithelium, resembled those proteins. However, hDP55 did not react with antiserum specific for PP14, which has been shown to be immunologically indistinguishable from PEP and α_2 -PEG. Similarly, hDP55 did not react with antibodies specific for PP12. These results suggest, therefore, that the hDP55 is a novel decidua-associated protein.

Three putative major roles for the decidual tissue have been suggested: nutritive support for the developing embryo, defence against aggressive trophoblastic invasiveness and prevention of immune rejection of the embryo. Currently, we can only guess the role of hDP200 in the uterus.

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