An Update on the Identity of Early Pregnancy Factor and Its Role in Early Pregnancy

INTRODUCTION

With the benefit of hindsight, the major reason for the 20-year gestation period between the initial discovery of EPF (1,2) and its isolation and definitive characterization (3) can be seen to be the nature of the dose–response of the bioassay for EPF, the rosette inhibition test. This has been discussed in some detail previously (3–5). In essence, it is failure to appreciate three elements of the assay which has resulted in conflicting claims about the nature of EPF, as well as the apparently irreconcilable outcomes from a variety of attempts at purification of the active factor (see Ref. 6 for analysis of these different outcomes).

The three essential points are

(1) the dose–response curve is bell-shaped, with high as well as low concentrations giving negative results;
(2) substances are present in various biological samples which, while themselves inactive in the bioassay, can modify the response by shifting the entire dose-response curve by several orders of magnitude; and
(3) the bioassay is sensitive to the molecular level.

Points 1 and 2 in combination mean that when complex biological mixtures are fractionated, the most active fractions can be easily overlooked. The result is the selection of fractions containing either protein with very low activity, which is thus at best of peripheral interest, or predominantly inactive protein and small amounts of EPF. It is only by testing samples over a very wide range of dilutions that the bioassay can be applied semiquantitatively and the most active fractions selected at each fractionation step.

Point 3 means that only trace amounts of EPF need be present for an apparently pure preparation of an inactive protein to be mistakenly identified as "EPF."

ISOLATION AND CHARACTERIZATION OF EPF

These insights into the bioassay were gained from attempts in our laboratory to isolate the EPF-active agent from a wide variety of biological source materials (although point 3 was not fully appreciated until complete purification was achieved). Even with this knowledge, the final breakthrough did not occur until our studies on the biology of EPF (reviewed in Ref. 6) identified human blood platelets as a relatively rich source material.

To ensure that the platelet-derived material was truly representative of EPF, we fractionated, in parallel, all major classes of source materials with activity in the rosette inhibition test, including the archetypal source, pregnancy serum (3,7). The identical biochemical and immunologic behavior displayed by the active agents from all materials indicated that we were dealing with a single substance, or closely related family of substances, acting in diverse biological situations. However the only sources providing sufficient product to study at the protein, as opposed to the activity, level were regenerating rat liver (7) and human platelets (3), yielding, respectively, 5 μg per 40 g of tissue and 45 μg per 300 U (platelets from approximately 150 Liters of blood), with a purification factor of approximately 10^5-fold.

Structural studies were conducted on the more abundant platelet-derived material. Analysis by SDS-PAGE and mass spectrometry established the high degree of homogeneity of the preparation. After proteolytic cleavage, the amino acid sequence of approximately 70% of the molecule was determined. With a single exception, this was identical to the sequence of rat chaperonin 10 [cpn10 (8)] and the determined mass was consistent with this being the only difference. We have since isolated a cDNA for human EPF/cpn10 (9) confirming a single residue difference between the rat and the human molecules.

These results were surprising in light of known properties of chaperonins. cpn10 and its functional

associate, cpn60, are heat shock proteins which function within eukaryote mitochondria and plastids as molecular chaperones (10,11). The *Escherichia coli* homologues are known as groES and groEL, respectively (12). Nevertheless, further studies (3) with platelet-derived EPF, rat mitochondrial cpn10, and groEL established that the structural similarities between EPF and cpn10 extend to functional identity. groES, however, exhibited no activity in the rosette inhibition test. Thus it appears that the EPF activity of cpn10 is a property only of the eukaryotic molecule. The studies also established unequivocally that a cpn10-like molecule is the only source of EPF activity in pregnancy serum. Furthermore, both polyclonal and monoclonal anti-EPF antibodies, used as neutralizing agents in previous studies from this laboratory (13–17), were shown specifically to recognize cpn10 (7).

The final assurance that we had indeed isolated EPF came with the preparation and testing of recombinant protein, as well as antibodies to synthetic peptides corresponding to different parts of the EPF/cpn10 amino acid sequence (18,19; Somodevilla-Torres *et al.*, submitted). Both the recombinant protein and a chemically synthesized form of the molecule prepared very recently (20) are fully active in the rosette inhibition test and this activity is neutralized by the anti-EPF antibodies used in our previous biological studies. Conversely, the EPF activity of pregnancy serum is completely neutralized by affinity-purified, monospecific, polyclonal antibodies to short synthetic peptides, corresponding to residues 1–11 (anti-N) and 34–44 (anti-I) of rat and human EPF/cpn10.

ROLE OF EPF IN PREGNANCY

In vivo and in vitro studies, with the first-generation anti-EPF antibodies discussed above, established that EPF is essential for normal embryonic development and successful establishment of pregnancy. During the pre- and periimplantation stage, EPF is important both for first cleavage (15) and at implantation (17). In the former case, the effect on the embryo appears to be indirect (? paracrine), but by the stage of blastocyst development, EPF acts in an autocrine fashion. Mouse embryos cultured in the presence of anti-EPF antibodies displayed normal blastocyst development, but trophoblast outgrowth was completely inhibited in almost half of the embryos, compared with those cultured with control antibody (17).

The second-generation anti-EPF/cpn10-derived peptide antibodies discussed above have been used in similar passive immunization studies. Pregnant mice were treated, separately, with anti-N and anti-I on days 1 and 2 of pregnancy and the effect was determined on day 7 (18,19). As shown in our previous studies, this treatment disrupts pregnancy. The number of implantation sites per mouse was significantly reduced compared with the number expected from the corpora lutea count. The effect was embryo related because the corpora lutea count did not differ in animals treated with anti-EPF/cpn10 versus control antibodies.

Now that we have prepared recombinant and synthetic EPF, we have begun to examine the direct effect of EPF on the embryo. Collaborative studies with Drs. Hill and Brenneman at the NICHD, NIH, have established that EPF appears to be an endogenous growth regulator of the early postimplantation embryo (Lee *et al.*, in preparation). Treatment of whole cultured E9.5 mouse embryos with exogenous EPF stimulates growth; this effect is overcome by simultaneous addition of anti-EPF antibody (anti-N), while anti-EPF alone has a growth inhibitory effect.

Results for pre- and periimplantation-stage embryos are still very preliminary. Using a Texas red-labeled synthetic EPF preparation, we have demonstrated a specific binding pattern consistent with localization of exogenous ligand to blastocyst cell membranes (Kaye *et al.*, unpublished). Furthermore, immunohistochemical examination of mouse blastocysts with anti-EPF/cpn10 antibodies reveals extremely dense staining in the trophectoderm but little in the inner cell mass, a pattern consistent with protein secretion. Parallel immunoblots of blastocyst lysate show that the antibody recognition pattern is specific because only a single band of the correct size is evident (20).

FOR THE FUTURE

On the question of conflicting claims about the nature of EPF, our laboratory has established, unequivocally, the identity of the serum factor responsible for activity in the rosette inhibition test. In this respect, we are aware of only one outstanding issue. Thioredoxin, itself inactive in the rosette inhibition test and therefore not EPF, appears to be

a modifier of the response of EPF in this assay (22). This has been established by a series of in vitro studies, the interpretation of at least some of which might be disputed. Nevertheless, setting aside such minutiae, these studies raise the possibility that thioredoxin could be a natural regulator of EPF. Now that pure EPF protein is available, this proposition can be tested.

As for the biological role of EPF, we are continuing the types of investigations briefly sketched above. We have established an essential role for EPF in situations involving very rapid cell growth (embryonic, neoplastic and normal regenerative) and have confirmed that EPF is immunosuppressive, something first surmised from its action in the rosette inhibition test. In addition, we are investigating the genetic and regulatory mechanisms leading to the differential localization and activity of EPF and cpn10. We have discovered that EPF and cpn10 are members of a large, evolutionarily conserved gene family, which, in the human genome, is localized on nine different chromosomal arms (9). A number of transcripts are present in mammalian cells and sequencing has established that several genes are involved (unpublished).

We have identified the developing mouse as an appropriate model in which to characterize expression and regulation of the EPF gene. This in turn will provide us with the necessary tools to subject the gene to the ultimate functional test in gene knockout and overexpression models in vivo.

ACKNOWLEDGMENTS

Our laboratory gratefully acknowledges financial support received at various stages of these studies from the National Health and Medical Council of Australia, CSL Ltd., Queensland Cancer Fund, University of Queensland Mayne Bequest Fund, Lalor Foundation, Carter Wallace Inc., Utah Foundation, and Clive and Vera Ramaciotti Foundations.

REFERENCES

1. Morton H, Hegh V, Clunie GJA: Immunosuppression detected in pregnant mice by rosette inhibition test. Nature 1974;249:459–460
2. Morton H, Hegh V, Clunie GJA: Studies of the rosette inhibition test in pregnant mice: evidence of immunosuppression? Proc R Soc B 1976;193:413–419
3. Cavanagh AC, Morton H: Early pregnancy factor: purification to homogeneity from human platelets and identification as chaperonin 10. Eur J Biochem 1994;222: 551–560
4. Cavanagh AC, Morton H, Athanasas-Platsis S, Quinn KA, Rolfe BE: Identification of a putative inhibitor of early pregnancy factor in mice. J Reprod Fert 1991;91:239–248
5. Cavanagh AC, Rolfe BE, Athanasas-Platsis S, Quinn KA, Morton: Relationship between early pregnancy factor, mouse embryo conditioned medium and platelet activating factor. J Reprod Fert 1991;93:355–365
6. Morton H, Rolfe BE, Cavanagh AC: Early pregnancy factor. Semin Reprod Endocrinol 1992;10:72–82
7. Quinn KA, Cavanagh AC, Hillyard NC, McKay DA, Morton H: Early pregnancy factor (EPF) in liver regeneration after partial hepatectomy in rats: its relationship to chaperonin 10. Hepatology 1994;20:1294–1302
8. Hartmann DJ, Hoogenraad NJ, Condron R, Hoj PB: Identification of a mammalian 10-kDa heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase *in vitro*. Proc Natl Acad Sci USA 1992;89:3394–3398
9. Summers KM, Murphy RM, Webb GC, Peters GB, Cassady AI, Morton H, Cavanagh AC: The human early pregnancy factor/chaperonin 10 gene family. Biochem Mol Med 1996;58:52–58
10. Ellis RJ, van der Vies SM: Molecular chaperones. Annu Rev Biochem 1991;60:321–347
11. Lindquist S, Craig EA: The heat-shock proteins. Annu Rev Genet 1988;22:631–677
12. Zeilstra-Ryalls J, Fayet O, Georgopoulos C: The universally conserved groE (hsp60) chaperonins. Annu Rev Microbiol 1991;45:301–325
13. Athanasas S, Quinn KA, Wong TY, Rolfe BE, Cavanagh AC, Morton H: Passive immunization of pregnant mice against early pregnancy factor (EPF) causes loss of embryonic viability. J Reprod Fert 1989;87:495–502
14. Quinn KA, Athanasas-Platsis S, Wong T-Y, Rolfe BE, Cavanagh AC, Morton H: Monoclonal antibodies to early pregnancy factor perturb tumour cell growth. Clin Exp Immunol 1990;80:100–108
15. Athanasas-Platsis S, Morton H, Dunglison GF, Kaye PL: Antibodies to early pregnancy factor retard embryonic development in mice *in vivo*. J Reprod Fert 1991;92: 443–451
16. Quinn KA, Morton H: Effect of anti-EPF monoclonal antibodies on the *in vivo* growth of transplanted murine tumours. Cancer Immunol Immunother 1992:34:265–271
17. Athanasas-Platsis S: Early Pregnancy Factor: A Study of its Immunomodulatory and Growth Regulatory Properties During Pregnancy, PhD thesis. Brisbane: University of Queensland, 1993
18. Cavanagh AC, Morton H: Chaperonin 10. International Patent Application PCT/AU94/00740, 1995
19. Morton H, Cavanagh AC: Antagonists to chaperonin 10. International Patent Application PCT/AU94/00742, 1995
20. Love SG, Engelbretsen DR, Gamham B, Cavanagh AC, Morton H, Chemusevich I, Standing K, Alewood PF: Observation of protein heptamer formation for chemically ligated

early pregnancy factor. Proceedings of NATOARW on New Methods for the Study of Molecular Aggregates. NATO ASI, Kluwer, 1997 (in press)
21. Kaye PL, Corcoran CM, Pantaleon M, Cavanagh AC, Morton H: Early pregnancy factor/chaperonin 10 in preimplantation mouse embryos. J Reprod Fert 1996; Abstr Ser 17:p6, abstr 11
22. Clarke FM, Orozco C, Perkins AV, Cock I, Tonissen KF, Robins AJ, Wells JRE: Identification of molecules involved in the "early pregnancy factor" phenomenon. J Reprod Fert 1991;93:526–539

Alice C. Cavanagh
University of Queensland
Department of Surgery
Clinical Sciences Building
Royal Brisbane Hospital 4029
Brisbane, Australia