Placental mRNA in maternal plasma: Prospects for fetal screening

Malcolm A. Ferguson-Smith*

Cambridge University Centre for Veterinary Science, Madingley Road, Cambridge CB3 0ES, United Kingdom

Increasingly, over the past 30 years, couples at risk of bearing children with certain types of severe handicaps have had the option of fetal diagnosis, followed by selective termination of pregnancy, with the prospect of trying for a healthy baby later. In a society where financial and other help is seldom available to provide adequate support to the parent caring for a severely disabled infant, it is understandable that there is an increasing demand for obstetric services to provide prenatal screening to determine risks, and safe tests to confirm, or exclude, the diagnosis. Modern obstetric ultrasonography brings the fetus into sight and is one of the main screening tests for developmental malformations. Nuchal translucency is a measurable ultrasound feature associated with Down's syndrome and, when used in conjunction with maternal serum screening, increases the detection rate in the first trimester to >85% for a false-positive rate of 5% (1). The key maternal serum markers are the levels of the β subunit of human chorionic gonadotrophin (βhCG), and pregnancy-associated plasma protein-A (PAPP-A). Ultrasound bears no hazard for the fetus, but the procedures used for definitive diagnosis, namely chorion villus sampling (CVS) and amniocentesis, carry a risk of miscarriage of ≈1%. Almost all current tests for fetal chromosome, genetic, and biochemical disorders depend on either of these two invasive procedures to provide fetal cells for culture or DNA analysis.

The search for a noninvasive diagnostic procedure that avoids the risk of miscarriage has centered in recent years on attempts to isolate fetal cells from the maternal circulation and, more recently, on attempts to assay for free nucleic acids of fetal origin in maternal serum or plasma. In this issue of PNAS, Ng *et al.* (2), provide important new insight on mRNA levels in maternal plasma of the genes coding for βhCG and human placental lactogen (hPL). Their results indicate that the investigation of placental RNA in maternal plasma may have considerable potential for noninvasive prenatal screening and diagnosis.

It has been known for a long time that small numbers of nucleated fetal cells pass into the maternal circulation (3). It is estimated that there are, on average, one to two such cells per milliliter in the second trimester (4). They are composed of trophoblast cells, lymphocytes, and nucleated red blood cells (NRBCs). Most attention has been paid to NRBCs because they are, at present, more consistent throughout pregnancy, disappear rapidly after delivery (unlike lymphocytes), and, because of their fetal origin, can be predicted to some extent from the quantity of cytoplasmic fetal and embryonic hemoglobin. Unfortunately, maternal erythroid precursors containing smaller amounts of these hemoglobins are also present. Clear distinction between fetal and maternal cells is possible only when the presence of paternal DNA sequences can be demonstrated; in 50% of fetuses the Y chromosome is a reliable paternal marker, whereas female fetuses require tests for a paternal DNA polymorphism. It is disappointing that multicenter studies on fetal sexing from maternal blood, based on the isolation and analysis of fetal cells by fluorescence *in situ* hybridization (FISH) with Y-specific probes, have yielded too many false results to warrant application in obstetric practice (5). However, in Down's syndrome pregnancies and in preeclampsia the numbers of fetal cells in the maternal circulation is increased, and this has been a factor in the successful FISH diagnosis of several cases of Down's syndrome. In other pregnancies, microdissection of Y-bearing single cells followed by PCR amplification and assay of DNA have been successful in the diagnosis of sickle-cell anemia, β-thalassemia (6), and spinal muscular atrophy (7). These results have encouraged attempts to find better strategies for fetal cell isolation and enrichment.

Almost all current tests for fetal chromosome, genetic, and biochemical disorders depend on procedures to provide cells for analysis.

A more promising possibility for noninvasive fetal diagnosis became apparent in 1997 when Lo *et al.* (8) demonstrated that Y-chromosome sequences could be detected by PCR assay of DNA in boiled plasma and serum of pregnant women carrying male fetuses. This study was prompted by earlier observations on DNA in the serum of patients with systemic lupus erythematosus (9) and cancer (10). In the cancer patients the DNA contained oncogene mutations that were present in the tumors, indicating the tumor source of the serum DNA. Lo *et al.* followed up their original observation by developing a quantitative PCR assay using primers for a single-copy sex-determining (SRY) sequence that could detect male pregnancies from 7 weeks onward with 100% reliability, using as little as 50 μl of maternal plasma or serum (11). Female pregnancies were identified by exclusion, as SRY sequences were absent in these pregnancies as well as in nonpregnant controls. It was estimated that fetal DNA constituted ≈3.4% of plasma DNA in early pregnancy, increasing to 6.2% in late pregnancy. It was absent before conception and was cleared in a matter of hours after delivery. The amount of fetal DNA in plasma was >20 times the amount estimated from the mean number of fetal cells in maternal blood. This observation raises the question about the source of the fetal DNA in maternal plasma. Lysis of circulating fetal cells by the maternal immune system, apoptosis of cells during fetal development, and apoptosis of the trophoblast seem the likely possibilities. The steep rise in fetal DNA levels in the weeks before delivery suggests a major contribution from the aging placenta.

It seems that fetal sex can be determined in early pregnancy with greater reliability by PCR assay of DNA in the maternal plasma than by FISH studies on isolated cells. This finding has application in the management of pregnancies at risk of severe X-linked recessive

See companion article on page 4748.

*E-mail: maf12@mole.bio.cam.ac.uk.

www.pnas.org/cgi/doi/10.1073/pnas.0830966100

disease such as Duchenne's muscular dystrophy. Male fetuses of carrier women are at a 50% risk and require diagnostic tests by chorion villus sampling, whereas female fetuses are unaffected but have a 50% risk of carrier status. Other applications of this approach depend on the distinction between paternal and maternal alleles at polymorphic loci. A valuable development has been the identification of the paternal *RHD* allele in the plasma of rhesus-negative mothers carrying a rhesus-positive fetus at risk of hemolytic disease of the newborn (12). Several other autosomal dominant traits have been diagnosed in the fetus from the identification of the paternal mutation in maternal plasma. These include mytonic dystrophy (13), achondroplasia (14), and Huntington's disease: in each case the father was known to be affected. For autosomal recessive disease, identification of the paternal allele, or a closely linked marker, serves to increase the *a priori* risk of an affected fetus from 25% to 50%; absence of the paternal allele excludes disease in the fetus. The prenatal exclusion of β-thalassemia has been achieved in this way (15).

As with fetal NRBCs, fetal DNA in maternal plasma is increased in Down's syndrome pregnancies (16) and in preeclampsia, even before clinical signs are apparent (17). Levels are also raised in preterm labor (18) and in association with hydramnios (19). Taken together, these observations seem to confirm an association between placental pathology and elevated fetal DNA in maternal blood and support the view that the placenta is an important source of this DNA in normal pregnancy. It remains to be seen whether fetal DNA assay can be used as an early indicator of other abnormalities in obstetric practice, for example, in intrauterine growth retardation and in impending fetal loss.

Traffic of nucleated cells between mother and fetus is known to occur in both directions. Indeed, it has been shown as a cause of microchimerism, with possible significance in the etiology of autoimmune disease (20). The same bidirectional traffic seems to occur with plasma DNA, because maternal DNA has been found in cord blood in $\approx$30% of samples tested (21). The levels of maternal DNA in cord blood seem much lower than the levels of fetal DNA in maternal blood.

If DNA assays in maternal plasma can be exploited for fetal genotyping, perhaps RNA assays in maternal plasma may reveal levels of gene expression, which are diagnostic for fetal pathology. And so it transpires. Shortly after the observation in 1999 that the plasma of cancer patients contained tumor RNA, as well as DNA (22), Poon *et al.* (23) reported the presence of fetal RNA in the plasma of pregnant women. The paper by Ng *et al.* (2) takes this observation several steps further by measuring placental RNA levels throughout gestation by using a quantitative RT-PCR assay for mRNA transcripts of two placenta-expressed genes coding for βhCG and hPL. Placental mRNA was detected in all samples from all pregnant women. The first surprising finding was the stability of both mRNAs over 24 h at room temperature, in view of the acknowledged lability of RNA in other circumstances. The authors show by filtration through 5-μm filters that this finding is not because of an origin from residual fetal cells. However, a significant reduction in mRNA levels occurred after the plasma was passed through a 0.45-μm filter. This result suggests that the stability is most likely because of the association of mRNA in subcellular particles.

Ng *et al.* (2) find that hPL mRNA levels increase with gestation whereas βhCG levels fall. This result mirrors the protein plasma levels of hPL and βhCG. Clearance of hPL mRNA was rapid after delivery; 70% of subjects had no detectable mRNA 2 h after delivery, and after 24 h it was undetectable in all cases. No hPL mRNA was detected in cord blood immediately after cesarean section, suggesting that placental mRNA is not transferred to the fetal circulation. As mentioned above, this result contrasts with the finding of maternal cells and DNA in cord blood.

It is appropriate to consider the relevance of these placental RNA studies for prenatal screening and diagnosis. It is clear from the above that the expression of placental genes can be measured in maternal plasma. Here lies the advantage of assaying mRNA transcripts, rather than DNA. One of the two genes investigated codes for βhCG, which is one of the proteins widely used in maternal serum screening for Down's syndrome. Raised levels of serum βhCG in the first trimester are associated with affected pregnancies as are decreased levels of PAPP-A. Also, βhCG seems to be higher in female than in male pregnancies (24). If maternal plasma levels of mRNA for each of these genes accurately reflect levels of gene product at each gestation, then it is possible that quantitative RT-PCR could be used instead of immunological protein assay to determine the risk of Down's syndrome and to give an indication about fetal sex. There could be an advantage in using a single multiplex RT-PCR assay for these markers, rather than using independent immunoassays.

The results suggest that abnormal patterns of gene expression are likely to be associated with placental pathology, not only in Down's syndrome but also in a variety of obstetric disorders, including preeclampsia. Careful measurement of relevant mRNA levels in maternal plasma in normal and abnormal pregnancies throughout gestation will be required to establish the feasibility of this approach for screening and diagnosis. Prenatal diagnosis of single-gene defects may depend on whether the relevant gene is transcribed in placenta. This concern may prove theoretical as it is known that low levels of mRNA for tissue-specific genes may be revealed by RT-PCR in unrelated tissues. As with prenatal diagnosis from fetal DNA in the maternal circulation, autosomal dominant disorders transmitted from the father may be detectable, and prenatal exclusion of autosomal recessive disorders may also be possible. The relative advantages of fetal DNA and fetal RNA in this respect are yet to be determined. While allele dropout is a potential hazard with both nucleic acids, placental mRNA could pose additional problems because of the rare occurrence of uniparental disomy and confined placental mosaicism.

Perhaps RNA assays in maternal plasma may reveal levels of gene expression, which are diagnostic for fetal pathology.

The choice of Ng *et al.* (2) to study mRNA transcribed from genes for two well-characterized placental proteins proved to be a good choice as a potential measure of placental function. It also provides evidence of release of placental mRNA into the maternal circulation throughout pregnancy, and circumstantial evidence that the same is likely to occur for placental DNA. The relative contributions of other sources of fetal nucleic acid may prove more difficult to determine.

1. Wald, N. J. & Hackshaw, A. K. (1997) *Prenat. Diagn.* **17,** 821–829.
2. Ng, E. K. O., Tsui, N. B. Y., Lau, T. K., Leung, T. N., Chiu, R. W. K., Panesar, N. S., Lit, L. C. W., Chan, K.-W. & Lo, Y. M. D. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 4748–4753.
3. Walknowska, J., Conte, F. A. & Grumbach, M. M. (1969) *Lancet* ***i,*** 1119–1122.
4. Bianchi, D. W., Williams, J. M., Sullivan, L. M., Hanson, F. W., Klinger, K. W. & Shuber, A. P. (1997) *Am. J. Hum. Genet.* **61,** 822–829.
5. Bianchi, D. W., Simpson, J. L., Jackson, L. G., Elias, S., Holzgreve, W., Evans, M. I., Dukes, K. A., Sullivan, L. M., Klinger, K. W., Bischoff, F. Z., *et al.* (2002) *Prenat. Diagn.* **22,** 609–615.
6. Cheung, M.-C., Goldberg, J. D. & Khan, Y. W. (1996) *Nat. Genet.* **14,** 264–268.
7. Chan, V., Lau, K., Yip, B., Sin, S. Y., Cheung, M.-C. & Kan, Y. W. (1998) *Lancet* **352,** 1196–1198.
8. Lo, Y. M. D., Corbetta, N., Chamberlain, P. F., Rai, V., Sargent, I. L., Redman, C. W. G. & Wainscoat, J. S. (1997) *Lancet* **350,** 485–487.
9. Tan, E. M., Schur, P. H., Carr, R. I. & Kunkel, H. G. (1966) *J. Clin. Invest.* **45,** 1732–1740.
10. Chen, X. Q., Stroun, M., Magnenat, J. L., Nicod, L. P., Kurt, A. M., Lyautey, J., Lederrey, C. & Anker, P. (1996) *Nat. Med.* **2,** 1033–1035.
11. Lo, Y. M. D., Tein, M. S. C., Lau, T. K., Haines, C. J., Leung, T. N., Poon, P. M. K., Wainscoat, J. S., Johnson, P. J., Chang, A. M. Z. & Hjelm, N. M. (1998) *Am. J. Hum. Genet.* **62,** 768–775.
12. Faas, B. H., Beuling, E. A., Christiaens, G. C., von dem Borne, E. A. & van der Schoot, C. E. (1998) *Lancet* **352,** 1196 (lett.).
13. Amicucci, P., Gennarelli, M., Novelli, G. & Dallapiccola, B. (2000) *Clin. Chem.* **46,** 301–302.
14. Saito, H., Sekizawa, A., Morimoto, T., Suzuki, M. & Yanaihara, T. (2000) *Lancet* **356,** 1170 (lett.).
15. Chiu, R. W. K., Lau, T. K., Leung, T. N., Chow, K. C. K., Chui, D. H. K. & Lo, Y. M. D. (2002) *Lancet* **360,** 998–1000.
16. Lo, Y. M. D., Lau, T. K., Zhang, J., Leung, T. N., Chang, A. M., Njelm, N. M., Elmes, R. S. & Bianchi, D. W. (1999) *Clin. Chem.* **45,** 1747–1751.
17. Leung, T. N., Zhang, J., Lau, T. K., Chan, L. Y. & Lo, Y. M. D. (2001) *Clin. Chem.* **47,** 137–139.
18. Leung, T. N., Zhang, J., Lau, T. K., Hjelm, N. M. & Lo, Y. M. D. (1998) *Lancet* **352,** 1904–1905.
19. Zhong, X. Y., Holzgreve, W., Li, J. C., Aydinli, K. & Hahn, S. (2000) *Prenat. Diagn.* **20,** 838–841.
20. Nelson, J. L., Furst, D. E., Maloney, S., Gooley, T., Evans, P. C., Smith, A., Bean, M. A., Over, C. & Bianchi, D. W. (1998) *Lancet* **351,** 559–562.
21. Lo, Y. M. D., Lau, T. K., Chan, L. Y., Leung, T. N. & Chang, A. M. (2000) *Clin. Chem.* **46,** 1301–1309.
22. Kopreski, M., Benko, F. A., Kwak, L. W. & Gocke, C. D. (1999) *Clin. Cancer Res.* **5,** 1961–1965.
23. Poon, L. L., Leung, T. N., Lau, T. K. & Lo, Y. M. D. (2000) *Clin. Chem.* **46,** 1832–1834.
24. Yaron, Y., Lehavi, O., Orr-Urtreger, A., Gull, I., Lessing, J., Amit, A. & Ben-Yosef, D. (2002) *Hum. Reprod*. **17,** 485–489.