Effects of fetal genotype and uterine environment on placental development in equids

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Measurement of the concentrations of equine chorionic gonadotrophin (eCG) in the serum of pregnant mares and Jenny donkeys carrying normal intraspecies and hybrid interspecies pregnancies suggested that the production of this hormone may be influenced by parental gene imprinting. Specifically, a differential expression of maternal and paternal genes may control the size and secretory activity of the structures that secrete eCG, the fetal endometrial cups. However, bisection of an interspecies mule embryo followed by transfer of the resulting demi-embryos and other intact mule embryos to horse and donkey recipients resulted in striking differences in the size, secretory activity and lifespan of the endometrial cups in the two types of surrogate mother. This finding has therefore demonstrated the ability of uterine factors to alter profoundly the development and characteristic phenotype of the specialized invasive chorionic girdle portion of the equine trophoblast that gives rise to the endometrial cups.

Introduction

Equine chorionic gonadotrophin (eCG) is a highly glycosylated heterodimeric protein that expresses both FSH-like and LH-like biological activities in non-equine species (Stewart *et al.*, 1976). It first appears in the peripheral blood of pregnant equids between 37 and 41 days after ovulation and concentrations rise steeply to a peak between days 55 and 70. Thereafter they decline steadily until gonadotrophic activity disappears from the serum between days 100 and 140 (Cole and Hart, 1930; Day and Rowlands, 1940; Allen 1969a). The hormone is secreted by unusual fetal structures known as endometrial cups (Cole and Goss, 1943) which comprise a circle of ulcer-like protuberances in the endometrium of the gravid uterine horn. Each cup consists of a densely packed mass of large, epithelioid, binucleate cells interspersed with dilated endometrial glands and occasional blood vessels (Clegg *et al.*, 1954; Amoroso, 1955). The large binucleate cells secrete eCG and are fetal in origin, stemming from a discrete annulate portion of the trophoblast, the chorionic girdle, which invades the overlying endometrium between days 36 and 38 after ovulation (Allen and Moor, 1972; Allen *et al.*, 1973). As the resulting endometrial cups mature, increasing numbers of lymphocytes and other leucocytes accumulate in the endometrial stroma at the periphery of each cup. This reaction has many hallmarks of a cellular immune response to a foreign tissue graft and it appears to hasten the degeneration of the cups and their eventual desquamation from the surface of the endometrium at about day 100–140 (Allen 1975; Antczak and Allen, 1984).

In earlier reports we described the marked influence of fetal genotype on the secretion rates of eCG in pregnant mares and Jenny donkeys carrying intraspecies horse and donkey pregnancies, respectively, or interspecies hybrid mule and hinny pregnancies, respectively (Allen, 1969b; Stewart and Allen, 1981). For example, peak serum CG concentrations are high (80–200 iu ml⁻¹) in mares carrying intraspecific horse fetuses and in Jenny donkeys carrying interspecific hinny fetuses in which the horse (*Equus caballus*, 2n = 64) is the sire in both cases. Conversely, peak serum CG concentrations are much lower (5–40 iu ml⁻¹) in Jenny donkeys carrying intraspecific donkey conceptuses and in mares carrying interspecific mule conceptuses in which the Jack donkey (*E. asinus*, 2n = 62) is the sire in each case (Fig. 1).

Further experiments carried out subsequently have uncovered one reason for the big differences in endometrial cup development and eCG secretion rates. The width and overall development of the progenitor chorionic girdle appears to be closely governed by paternal genotype (Fig. 2). Thus, on horse and hinny conceptuses (horse father) a broad, thick chorionic girdle develops between days 25 and 36 which gives rise, following invasion of the maternal endometrium at day 36–38, to large, active endometrial cups that secrete large amounts of CG into the maternal circulation. Conversely, a much narrower chorionic girdle develops on donkey and mule conceptuses (donkey father) which, in turn, results in smaller endometrial cups after invasion. The amount of eCG entering maternal blood is correspondingly less (Allen, 1975). This influence of fetal genotype on endometrial cup development has also been demonstrated in extraspecific equine pregnancies created by embryo transfer. For example, transfer of horse embryos to

Fig. 1. Mean ($\pm$ SEM) concentrations of equine chorionic gonadotrophin (eCG) measured by haemagglutination inhibition assay in the serum of (a) 30 horse mares (*Equus caballus*, $2n = 64$) carrying intraspecies horse pregnancies; (b) 11 mares carrying interspecies mule pregnancies; (c) 14 Jenny donkeys (*E. asinus*, $2n = 62$) carrying intraspecies donkey pregnancies and (d) 6 Jenny donkeys carrying interspecies hinny pregnancies. Peak gonadotrophin concentrations are high (80–200 iu ml⁻¹) when the horse is the father and low (5–40 iu ml⁻¹) when the donkey is the father (Redrawn from Stewart and Allen, 1981).

Jenny donkey recipients results in the development of broad chorionic girdles and very large and active endometrial cups, just as in donkeys carrying interspecies hinny pregnancies. Conversely, transfer of donkey embryos to horse mares is followed by the development of extremely small chorionic girdles on the donkey conceptuses which fail completely to invade the surrogate horse endometrium at day 36–38 to form endometrial cups (Allen, 1982a; Allen *et al.*, 1987).

There has been increasing evidence in recent years which suggests that the relative contributions of homologous maternal and paternal chromosomes to development in mammals are unequal. Support for this concept of genetic imprinting has come from embryological experiments in mice (Barton *et al.*, 1985), and from studies of genetically determined human diseases such as hydatidiform mole (see Solter, 1988; Hall, 1990 for reviews). Our findings of the apparent influence of paternal

genotype on endometrial cup development and eCG secretion would seem to be a good example of parentally imprinted genes controlling placental development. To test this hypothesis further we have examined the influence of maternal uterine environment on endometrial cup development by transferring intact and bisected F_1 hybrid mule embryos to the uteri of the two parental species, the horse and donkey.

Materials and Methods

Embryo transfers

Three mule embryos were flushed non-surgically on day 6 after ovulation from the uteri of maiden pony mares that had been inseminated with Jack donkey semen on alternate days

Fig. 2. (a) Intraspecies horse and (b) interspecies mule conceptuses at 35 days of gestation. The annulate chorionic girdle (cg) of the horse conceptus, situated at the junction of the enlarging allantochorion (ac) and regressing yolk sac (ys), is much broader and thicker than that of the mule; bo: bilaminar omphalopleure, e: embryo. The diameter of the conceptus is approximately 75 mm at this stage of pregnancy.

during the preceding oestrus. Two embryos were already at the early expanding blastocyst stage and were transferred intact, as described by Allen (1982b), to the uteri of unmated synchronized Jenny donkey recipients. The third embryo was a compact morula and this was bisected using an extruded glass knife in a micromanipulator (Research Instruments, Devon) as described by Skidmore *et al.* (1989). The resulting monozygotic demi-embryos were maintained *in vitro* for 2 h to allow re-compaction of the blastomeres and were then transferred surgically to unmated recipients, one to a mare and the other to a Jenny

Fig. 3. Concentration of eCG measured by amplified enzyme-linked immunoassay (AELIA) in the sera of a horse mare (○—○) and a Jenny donkey (●—●), each carrying one half of the same interspecies mule embryo bisected as a morula prior to transfer.

donkey. Pregnancy in the four recipients was diagnosed initially at day 16–18, and monitored at fortnightly intervals thereafter, by transrectal realtime ultrasound scanning of the uterus (Simpson *et al.*, 1982).

eCG assay

Peripheral blood samples were recovered three times a week from day 20 onwards from all the pregnant recipients. The serum was decanted and assayed subsequently for serum eCG concentrations in an amplified enzyme-linked immunoassay (Stanley *et al.*, 1986) using two polyclonal anti-eCG sera raised in rabbits (MSIB and MSIIC) against highly purified extracts of eCG (MSI, 16 500 iu mg⁻¹; MSII, 14 800 iu mg⁻¹; M. J. Stewart, Cambridge) and the Second International Standard for Serum Gonadotrophin (2nd IRP-PMSC; Bangham and Woodward, 1966) as standard. The limit of sensitivity was 0.6 iu eCG ml⁻¹ in unextracted serum and the intra- and inter-assay coefficients of variation were, respectively, 8 and 11%.

Tissue sampling

Pregnancy was not interrupted in one of the Jenny donkeys that received an intact blastocyst. The other two donkey recipients were killed on days 59 (intact embryo) and 62 (bisected demi-embryo) of gestation, respectively. Their uteri were opened and, after gross examination of the endometrial cups, small pieces of cup tissue with surrounding normal

Fig. 4. Comparison of the gross (a and b) and histological (c and d) appearances of the endometrial cups at day 62 of gestation in the uteri of a horse mare (a and c) and a Jenny donkey (b and d), each carrying one half of an interspecies mule embryo bisected as a morula before transfer. The endometrial cups (arrowed) in the mare (a) are very small and narrow compared to those in the Jenny donkey (b). Histologically, the cups in the mare (c) are completely necrotic and the surrounding endometrial stroma is densely packed with maternal leucocytes (arrowed). Those in the donkey (d) are still fully viable, with the typical dense accumulation of large eCG-secreting trophoblast cells and pockets of leucocytes (arrowed) restricted to the surrounding stroma. Bar, 500 μm .

endometrium and attached allantochorion were fixed in Bouin's fluid for conventional histological sectioning and staining with haematoxylin and eosin. In the recipient mare carrying the other half of the bisected mule embryo, the conceptus was removed surgically on day 62 by ventral midline hysterotomy performed under general anaesthesia. The endometrial cups and the non-invasive allantochorion–endometrium interface were biopsied for histological examination.

Results

eCG production

In all three donkeys carrying transferred mule conceptuses, serum CG profiles were much more like those in donkeys carrying interspecies hinny conceptuses than the typical profiles in either mares carrying interspecies mule conceptuses or donkeys carrying intraspecies donkey conceptuses (see Fig. 1). In the donkey that continued to term, a peak concentration of 224 iu ml^{-1} was recorded on day 69 and CG could be detected until day 147; she gave birth spontaneously to a healthy male mule foal after 357 days of gestation. In the other two donkey

recipients, serum CG concentrations had reached 164 and 224 iu ml^{-1} , respectively, on days 59 and 62, and were still increasing when the animals were killed. In marked contrast, serum CG concentrations in the horse mare carrying the other demi-mule embryo rose slowly from day 37 to reach a maximum of only 16 iu ml^{-1} on day 58. The serum CG concentrations fell again thereafter to a concentration of only 11 iu ml^{-1} on day 62 when the conceptus was removed (Fig. 3).

Endometrial cup development

Gross and histological comparison of the mule endometrial cups that developed in the uteri of the two different species identified the reason for the great disparity in serum CG concentrations measured in the mare and Jenny donkey when each was carrying half of the same embryo. In the mare, the endometrial cups were typically narrow, small and flattened (Fig. 4a) and, histologically, the cup tissue was already degenerate and heavily infiltrated by maternal leucocytes (Fig. 4c). Thus, the picture mirrored the situation in conventional interspecies mule pregnancy as described previously (Allen, 1975). In both the Jenny donkeys, by contrast, a circle of much larger, protruberant and active-looking endometrial cups was

Table 1. Theoretical model of the control of endometrial cup development in equine pregnancies

	Components		Total ascribed numerical value	Observed size of endometrial cups
	Embryonic	Uterine		
Intraspecies pregnancies				
Horse	$P^H + M^H$	$+ U^H$	+ 18	Large
Donkey	$P^D + M^D$	$+ U^D$	+ 14	Small
Interspecies pregnancies				
Mule	$P^D + M^H$	$+ U^H$	+ 14	Small
Hinny	$P^H + M^D$	$+ U^D$	+ 18	Large
Transferred extraspecies pregnancies				
Mule→Horse	$P^D + M^H$	$+ U^H$	+ 14	Small
Mule→Donkey	$P^D + M^H$	$+ U^D$	+ 18	Large
Horse→Donkey	$P^H + M^H$	$+ U^D$	+ 22	Very large*
Donkey→Horse	$P^D + M^D$	$+ U^H$	+ 10	Very small*

A. Let the embryonic derived growth factor, P , derived from the paternal gene, be stimulatory and have a nominal numerical value of +14 for the horse ligand and +10 for the donkey ligand (i.e. $P^H = +14$; $P^D = +10$).

B. Let the embryonic growth factor receptor, M , expressed from the maternal gene, be stimulatory and have nominal numerical values of +12 for the horse and +8 for the donkey (i.e. $M^H = +12$; $M^D = +8$).

C. Let the assumed uterine effect, in the form of an inhibitory factor, have nominal numerical values of -8 in the horse and -4 in the donkey (i.e. $U^H = -8$; $U^D = -4$).

*Details in Allen (1982a).

present at the base of the gravid horn (Fig. 4b). Histologically, most of the eCG-secreting cup cells were still viable and there were fewer accumulated leucocytes and they remained clustered in the surrounding endometrial stroma (Fig. 4d). Thus, the picture in these two animals reflected the situation in conventional interspecies hinny pregnancy (Allen, 1975).

Discussion

Mammalian hybrids have great potential value for studying the phenomenon of gene imprinting and the already reported biochemical (Hamerton *et al.*, 1971) and phenotypic (Tegetmeier and Sutherland, 1895) differences between the mule and its reciprocal hybrid, the hinny, may result from the action of imprinted genes (Chandley, 1989). Similarly, the major endocrinological differences exhibited by mares and Jenny donkeys when carrying interspecies mule and hinny pregnancies (see Fig. 1) would appear initially to provide a good example of imprinted genes controlling placental development and the production of placental hormones (Allen, 1969a; Stewart and Allen, 1981).

In the experiment reported here, the striking reversal of endometrial cup size and maternal serum eCG concentrations that occurred when mule embryos were made to develop in the uteri of Jenny donkeys instead of horse mares did so both with intact embryos and with a bisected demi-embryo. Hence, although only one mule embryo was bisected and transferred in the study, the results are consistent with the findings in conventional mule pregnancy and following the transfer of intact mule embryos to donkey recipients; namely, chorionic girdle and endometrial cup development are greatly enhanced in the

donkey uterus compared to the horse uterus. This is consistent with normal hinny pregnancies (donkey mother) in which endometrial cup development is likewise greatly enhanced. It also accords with extraspecies donkey-in-horse pregnancy (i.e. horse uterus) in which chorionic girdle development is so diminished that endometrial cups do not form and the pregnancy usually fails before day 100 of gestation (Allen, 1982a). The horse uterus therefore appears to be inhibitory to any conceptus that contains a donkey component, whereas the donkey uterus is stimulatory to any conceptus with a horse component. This suggests that the donkey uterus is more 'permissive' than the horse uterus in terms of accepting 'foreign' inter- and extraspecies pregnancies. Thus, the apparent paternal (imprinted) effect on eCG values in intraspecies horse and donkey, and in interspecies mule and hinny pregnancies (see Fig. 1), can be modified greatly by transferring conceptuses to the 'wrong' uterus. This does not, however, eliminate the possibility that gene imprinting may influence placental development in equids, perhaps by means of reciprocal imprinting of a growth factor and its receptor, as has been demonstrated in the mouse for insulin-like growth factor 2 (Dechiara *et al.*, 1990) and its receptor (Barlow *et al.*, 1991).

Taking all our observations into account, and assuming that the imprinting effect involves a growth factor and its receptor, it is possible to create a model to explain our observations on the marked variations in endometrial cup development across the different types of equine pregnancy (see Table 1). If the stimulatory effects on chorionic girdle development of the proposed reciprocally imprinted growth factor ligand and receptor genes are ascribed higher numerical values in the horse than in the donkey, and if the suppressive effects of a proposed uterine factor are likewise given a more negative value in the horse

than in the donkey, summation of the values in each type of pregnancy gives a figure that agrees closely with the observed differences in endometrial cup development and eCG secretion rate. For example, addition of the stimulatory effects of the parentally imprinted conceptus factors with the negative effects of the uterine factor gives the same total for intraspecies horse, interspecies hinny and extraspecies mule-in-donkey pregnancy, all three of which are associated with a broad and active chorionic girdle that gives rise to large endometrial cups and high serum eCG concentrations. Similarly, addition of the three values for intraspecies donkey, interspecies mule and extraspecies mule-in-horse pregnancies gives the same lower total in each case and all three types of pregnancy are associated with a narrower, less active chorionic girdle and correspondingly smaller endometrial cups. But perhaps most significant is the finding that summation of the numerical values for the transferred donkey-in-horse and horse-in-donkey pregnancy models gives a very low total for the former and a very high total for the latter. This too accords well with previous observations (Allen, 1982a) of an exceptionally small and poorly developed chorionic girdle and the complete absence of endometrial cups in the horse carrying a donkey conceptus, compared to particularly large and hyperactive endometrial cups in donkeys carrying transferred horse conceptuses.

In conclusion, it appears that development of the equine chorionic girdle and its invasion of the maternal endometrium to form gonadotrophin-secreting endometrial cups can be affected by fetal genotype and, to an even greater extent, by maternal uterine environment. Awareness of these interacting factors has come about as a result of the apparently unique ability of equids to conceive a wide range of interspecific hybrid fetuses and to carry true xenogeneic extraspecies pregnancies created by embryo transfer.

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