Morphological demonstration of the failure of *Mus caroli* trophoblast in the *Mus musculus* uterus*

M. A. Crepeau, S. Yamashiro and B. A. Croy

Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Summary. A histological study of *Mus caroli* embryos gestating in the *Mus musculus* uterus was undertaken at Day 8.5 of gestation, 1 day after such embryos are reported to be normal and 1 day before the earliest events associated with death of the xenogeneic embryos. In comparison to control *M. caroli* embryos recovered from *M. caroli* and to control *M. musculus* embryos recovered from *M. musculus*, the xenogeneically transferred embryos showed intrauterine growth retardation that was associated with trophoblastic insufficiency. Trophoblast cell degeneration was observed, in the absence of lymphocytic infiltration. Therefore, loss of trophoblast cell function rather than lymphocyte-mediated destruction of trophoblast appears to underlie the death of *M. caroli* embryos in the *M. musculus* uterus.

Keywords: trophoblast; mouse; pregnancy failure/abortion; xenogeneic embryo transfer; embryonic chimaeras

Introduction

The biological interactions between mother and fetus that ensure successful mammalian pregnancy are numerous and complex. Trophoblast plays a crucial, but incompletely defined, role in the maintenance of pregnancy (Rossant *et al.*, 1983; Surani *et al.*, 1987). Early in its development, trophoblast surrounds the embryo and is actively involved in nutrient provision (Snell & Stevens, 1966). Later in gestation, trophoblast anchors the conceptus to the uterine wall and forms the bulk of the definitive placenta. Trophoblast has been shown to secrete hormones and growth factors (Sherman, 1983; Linzer & Nathans, 1985; Adamson, 1987) and is considered to be an immunological barrier between the mother and her immunogenic conceptus (Chaouat *et al.*, 1983; Hunziker & Wegmann, 1987). Whether any single function of trophoblast controls the success of pregnancy is unclear.

That trophoblast alone can control the success or failure of pregnancy has been demonstrated by successful gestation of chimaeras, composed of xenogeneic embryonic cells protected by trophoblast, species-homologous to the embryo transfer recipient (Rossant *et al.*, 1982, 1983; Fehilly & Willadsen, 1986; Polzin *et al.*, 1987; Anderson, 1988). In the murine model of interspecies pregnancy, involving *Mus caroli* and *Mus musculus*, fully xenogeneic *M. caroli* are viable in *M. musculus* following transfer of chimaeric blastocysts composed of a *M. caroli* inner cell mass within a *M. musculus* trophoblast vesicle (Rossant *et al.*, 1983). Non-manipulated *M. caroli* embryos gestating in the *M. musculus* uterus fail to recruit intrauterine suppressor cells (Clark *et al.*, 1983), become infiltrated by cytotoxic lymphocytes (Croy *et al.*, 1982), then die and resorb during the second half of pregnancy (Croy *et al.*, 1982, 1985). While such studies suggest that immunoprotection could be a major role played by trophoblast, transfer of *M. caroli* embryos to immunocompromised and immunodeficient animals failed to prolong gestation. *M. caroli* embryos die at mid-gestation in transfer recipients treated with the immunosuppressive drug cyclosporin A, in

*Reprint requests to Dr B. A. Croy.

T-lymphocyte-deficient mice of *nu/nu* genotype, in natural killer cell-deficient mice of *bg/bg* genotype and in B- and T-cell deficient mice of *scid/scid* genotype that were unresponsive to antigenic stimulation by *M. caroli* cells *in vivo* and *in vitro* (Croy *et al.*, 1985; Crepeau & Croy, 1988). One interpretation of these observations is that involvement of the immune system in the death and resorption of *M. caroli* in the *M. musculus* uterus is secondary to other, non-immunological problems.

Development of *M. caroli* embryos transferred to *M. musculus* recipients is reported to be normal until Day 7 of gestation (Frels *et al.*, 1980). In a preliminary study assessing chorioallantoic fusion in *M. caroli* embryos transferred to *M. musculus*, abnormalities of trophoblast were detected at Day 8.5 of gestation (Crepeau *et al.*, 1988). An expanded study has therefore been undertaken to characterize *M. caroli* development in the *M. musculus* uterus at Day 8.5 of gestation and to compare it with normal development of *M. caroli* in the *M. caroli* uterus and *M. musculus* in the *M. musculus* uterus.

Materials and Methods

Mice. Random-bred *M. musculus* (CD1) were purchased from Charles River Laboratories, St Constant, Quebec, Canada. Random-bred *M. caroli* were bred and maintained at the University of Guelph, Guelph, Ontario, Canada. The *M. caroli* breeding colony was established from stocks obtained in 1978 from Dr V. Chapman, Roswell Park Memorial Institute, Buffalo, NY, USA. Mice were housed under standard conditions and used in experiments at 8–12 weeks of age.

Matings. To obtain *M. musculus* embryos, *M. musculus* females in oestrus were paired overnight with *M. musculus* males. Detection of a vaginal plug the following morning was deemed evidence of successful mating. Pseudopregnant *M. musculus* females were obtained by overnight pairing of oestrous females with vasectomized males, followed by selection of females with vaginal plugs. The morning of plug detection was designated Day 0.5 of pregnancy.

To obtain *M. caroli* embryos, *M. caroli* females were paired with *M. caroli* males after hormone-induced ovulation as previously described (Croy *et al.*, 1982). Since these matings do not produce copulation plugs, all hormone-treated *M. caroli* females were used for embryo collection. Day 0.5 of gestation was 24 h after administration of human chorionic gonadotrophin (hCG).

Embryo collection and transfer. Blastocysts were collected from *M. musculus* females at Day 3.5 of gestation, and from *M. caroli* females 76 h after hCG treatment. Mice were killed by cervical dislocation and the uteri excised and flushed with medium. Blastocysts were transferred to anaesthetized *M. musculus* females at Day 2.5 of pseudopregnancy as previously described (Croy *et al.*, 1982, 1985). Depending upon the number of embryos recovered from the uterine flushings, 3–6 *M. caroli* blastocysts were transferred into the lumen of one uterine horn and 3–6 *M. musculus* blastocysts to the opposite horn.

Histological analysis of implantation sites. Transferred *M. caroli* and *M. musculus* embryos were recovered from *M. musculus* uteri at Day 8.5 of gestation. Stage-matched untransferred *M. caroli* embryos were recovered from *M. caroli* on Day 7.5 of gestation (Frels *et al.*, 1980).

For light microscopy, implantation sites were individually fixed in 10% phosphate-buffered formalin for 72 h, and then processed and embedded in glycol methacrylate (Bennett *et al.*, 1976). Serial transverse sections of the implantation sites, 2–3 µm thick, were cut on a JB-4 microtome (Sorvall, Newtown, CN, USA) using glass knives, and then stained with Harris' haematoxylin and eosin or reacted with the periodic acid–Schiff technique (Fisher Scientific, Unionville, Ontario, Canada).

For electron microscopy, small pieces of implantation sites were immersed in a modified Karnovsky's fixative (Karnovsky, 1965) consisting of 4% paraformaldehyde (w/v) and 2% glutaraldehyde (w/v) in phosphate-buffered saline pH 7.4 (PBS) for 6 h. The samples were then washed in PBS and post-fixed in 1% (w/v) osmium tetroxide (Sigma, St Louis, MO, USA) in PBS, for 2 h. Samples were dehydrated in ethanol and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT, USA). Sections (1 µm) were cut with an OMU3 ultramicrotome (Reichert, Vienna, Austria), and areas from these specimens were selected and sectioned for electron microscopy.

Fig. 1. Photomicrographs of transverse sections of implantation sites illustrating development of *M. caroli* embryos; $\times 51$. (a) In *M. caroli* uterus at Day 7.5, mid-somite stage; (b) in *M. musculus* uterus at Day 8.5, early somite stage with chorio-allantoic fusion (arrow); and (c) in *M. musculus* uterus at Day 8.5, mid-somite stage with rotation.

Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM 100S transmission electron microscope.

Results

Stage of embryo development in allogeneic and xenogeneic uteri

Five *M. caroli* embryos and 4 *M. musculus* embryos recovered from 3 different *M. musculus* recipients, as well as 4 control *M. caroli* embryos recovered from *M. caroli*, were studied using serial sections. An additional 2 *M. caroli* embryos and 2 *M. musculus* embryos recovered from a single *M. musculus* recipient were processed for transmission electron microscopy.

All of the transferred *M. musculus* embryos had developed normally to the early or mid-somite stage characterized by fusion of the allantois to the chorion and rotation of the embryo (Theiler, 1972). Development of control *M. caroli* embryos gestating in *M. caroli* appeared uniform and normal: 3 were at the mid-somite stage and had rotated while development of the 4th was only slightly retarded (Fig. 1a). In contrast, the stage of development of transferred *M. caroli* embryos was not uniform: 1 embryo was presomite stage with an emerging allantois while another *M. caroli* embryo, from the same uterus, was very early somite stage. In this embryo, the allantois was clearly fused with the chorion (Fig. 1b). The other *M. caroli* embryos, recovered from 2 different *M. musculus* recipients, had developed further and 2 had reached the mid-somite stage with rotation (Fig. 1c).

Trophoblast development in allogeneic and xenogeneic uteri

Trophoblast development in *M. musculus* embryos was extensive; ectoplacental cones were covered by several layers of polar trophoblast giant cells (Fig. 2a). Mural trophoblast giant cells formed an uninterrupted meshwork between Reichert's membrane and the maternal decidua. In control *M. caroli* embryos, trophoblast was somewhat less abundant than in *M. musculus* but mural giant cells, polar giant cells and ectoplacental cone trophoblast cells could be distinguished (Fig. 2b). By comparison, trophoblast development of transferred *M. caroli* embryos recovered from *M. musculus* uteri was deficient. A few irregular layers of polar trophoblast giant cells were observed above the ectoplacental cones. Numbers of trophoblast giant cells were reduced. In one implantation site, the paucity of trophoblast was drastic and the characteristic ectoplacental cone, seen in normal pregnancies, consisted of only a few islands of cells (Fig. 2c).

Morphological features of trophoblast

M. musculus ectoplacental cone cells and trophoblast giant cells were densely packed with intensely staining, eosinophilic granules of variable size (Fig. 3a). Similar granules were also abundant in ectoplacental cone cells of control *M. caroli* embryos (Fig. 3b) but were less numerous in the mural and polar trophoblast giant cells. These granules were also stained, although less intensely, using the periodic acid-Schiff's technique. In contrast, granules were markedly deficient in ectoplacental cone cells and trophoblast giant cells from all *M. caroli* embryos recovered from a *M. musculus* uterus (Fig. 3c).

Transmission electron microscopy was used to characterize further the nature of these cytoplasmic granules. The size and appearance of the granules in *M. musculus* trophoblast varied;

Fig. 2. Photomicrographs of ectoplacental cone (E) in region of the chorionic plate (C); $\times 128$. (a) *M. musculus* in *M. musculus* at Day 8.5 with well developed ectoplacental cone; (b) *M. caroli* in *M. caroli* at Day 7.5; and (c) *M. caroli* in *M. musculus* at Day 8.5 showing few trophoblast cells.

homogeneous electron-dense granules of variable size were most abundant (Fig. 4a). Granules of various electron densities and variable sizes which contained vacuolations were less numerous. Granules of uniform size, composed of fine particulate material of medium electron density, and granules of similar size and appearance, but also containing fine electron-opaque regions, were observed. These appeared to be phagocytosed erythrocytes, not yet fused with lysosomes, and partly digested erythrocytes respectively. Granules present in *M. caroli* trophoblast giant cells were different. Most of those present were large, irregularly shaped and of light to medium electron density, resembling lipid inclusions (Fig. 4b). Intact phagocytosed erythrocytes were also observed. The electron-dense inclusions, abundant in *M. musculus* trophoblast giant cells, were infrequent in trophoblast cells from transferred *M. caroli* embryos.

A second difference was observed between trophoblast giant cells from control and experimental embryos at the light microscopic level. In each of the *M. caroli* embryos recovered from *M. musculus* uteri many giant cells appeared to be in a degenerative state, characterized by ill-defined cellular outlines, vacuolated cytoplasm, pycnosis or karyorrhexis. Mildly eosinophilic exudates were found in areas that appeared devoid of trophoblast giant cells and were next to giant cells that appeared to be dying (Fig. 5). The exudative foci were infiltrated by polymorphs (Fig. 5b) and abutted the yolk sac, particularly in the posterior neural tube region. In the most severely growth-retarded *M. caroli* embryo studied, an engorged blood vessel appeared to disrupt the giant cell barrier. In this implantation site, lymphocytes were detected within blood spaces but not within embryonic tissues. In control embryos, neither giant cells with a degenerative appearance nor large exudative foci were observed.

Discussion

Studies of chimaeric mammalian embryos derived from different species have shown that trophoblast genotype is critical for embryonic success (Rossant *et al.*, 1982, 1983; Fehilly & Willadsen, 1986; Polzin *et al.*, 1987). While trophoblast has been identified as the protective tissue, the mechanism by which protection is afforded is unknown. New observations obtained from this histological study support the hypothesis that in the *M. caroli*—*M. musculus* model system, degenerative events affecting *M. caroli* trophoblast precede involvement of immune effector cells in the failure and resorption of *M. caroli* embryos in the xenogeneic uterus.

At Day 8.5 of gestation, retarded, asynchronous development of *M. caroli* embryos in *M. musculus* was detected in comparison to the stage of development of *M. caroli* or *M. musculus* embryos from intraspecies pregnancies. The inner-cell mass-derived structures of the embryo proper appeared normal in all xenogeneic pregnancies. From earlier studies it is known that these tissues become disorganized then necrotic and collapse before haemorrhagic resorption between Days 10 and 15 (Croy *et al.*, 1982). Such a sequence of events suggests that the lesions present at Day 8.5 of gestation lead progressively to failure of the entire conceptus and excludes mechanisms of generalized embryo toxicity. Variations in the stages of early *M. caroli* development no doubt contribute to the asynchrony of grossly detectable embryonic death within and between *M. musculus* recipients and to the relatively long time interval over which resorptions appear to be initiated (Croy *et al.*, 1985).

Fig. 3. Differences in the appearance of granules within trophoblast cells (T); $\times 320$. (a) *M. musculus* in *M. musculus* at Day 8.5, with abundant granules of variable size in cytoplasm of trophoblast cells; (b) *M. caroli*, in *M. caroli* at Day 7.5, with granules similar to those of *M. musculus*; and (c) *M. caroli* in *M. musculus* at Day 8.5, showing a relative absence of granules compared to control embryos.

Failure to establish a proper chorioallantoic circulation between Days 7.5 and 8.5 of gestation (Theiler, 1972) would offer a simple explanation for death of *M. caroli* embryos in *M. musculus*. However, our studies at the light microscopic level suggest that the chorion had fused normally with the allantois. Similarly, the yolk sac placenta, an important nutritive and respiratory organ during early gestation, appeared to develop normally.

Trophoblast giant cells and ectoplacental cone cells in implantation sites from transferred *M. caroli* embryos were reduced in number compared to control embryos. Since ectoplacental cone cells eventually differentiate into spongiotrophoblast cells (Snell & Stevens, 1966; Gardner *et al.*, 1973; Rossant *et al.*, 1983), paucity of these cells at Day 8.5 of gestation could adversely affect placental development and, subsequently, function. A deficiency of phagocytic trophoblast giant cells would limit the nutrients provided to the embryo (Robertson *et al.*, 1971; Poelmann & Mentink, 1982), while lack of a cohesive network of trophoblast giant cells would readily allow cells and potentially harmful substances to cross Reichert's membrane and reach the embryo. This could include substances found in the uterus during normal pregnancy that are usually bound or modified by trophoblast cells. Alternatively, substances could escape from the embryonic cavity, altering embryo metabolism and disrupting proper materno-fetal interactions.

At Day 8.5 of gestation, numerous trophoblast cells, principally mural giant cells surrounding transferred *M. caroli* embryos, were degenerate as indicated by pycnosis, karyorrhexis and ill-defined, vacuolated cytoplasm. This observation is significant because it precedes the earliest known participation of immune effector cells in *M. caroli* embryo failure (Croy *et al.*, 1982; Clark *et al.*, 1983). Tachi & Tachi (1979) observed that trophoblast cells from rat embryos transferred to the *M. musculus* uterus degenerate and die shortly after penetration of the basement membrane of the uterine luminal epithelium. As in the present study, immune effector cells did not appear to be responsible for degeneration of xenogeneic rat trophoblast cells and similar mechanisms may be responsible for embryo death in both model systems.

The differences in the quantity and quality of cytoplasmic granules between trophoblast cells of *M. musculus* and *M. caroli* embryos recovered from *M. musculus* uterus were striking and are likely to be due to metabolic differences. The abundance of lipid inclusions in *M. caroli* trophoblast suggests low levels of metabolic activity consistent with an hypothesis of functional impairment of trophoblast. The substances contained within the granules of healthy *M. caroli* and *M. musculus* trophoblast cells are not known but most appear to be lysosomes or secretory bodies. The absence of histochemically detectable acid phosphatase activity in these trophoblast cells (data not shown) may indicate that lysosomal activity is not elevated in trophoblast as suggested by Welsh & Enders (1987). The granules appear to be proteinaceous since they stain intensely with eosin but only moderately with periodic acid-Schiff. Since peptide hormones are known to be secreted by trophoblast throughout gestation (Robertson *et al.*, 1982; Linzer & Nathans, 1985; Linzer *et al.*, 1985; Soares *et al.*, 1985), many granules may contain hormones. The relationship between granule paucity in trophoblast from transferred *M. caroli* embryos and pregnancy failure is unclear. The contents of the granules may be essential for pregnancy, in which case failure to produce them would contribute directly to embryonic death. Alternatively, impairment of trophoblast development and function may result in reduced metabolic activity and reduction in the number of granules as a secondary problem.

Fig. 4. Electron micrographs of trophoblast cells. (a) *M. musculus* in *M. musculus* at Day 8.5 showing two trophoblast cells with granules of various sizes and appearance. Electron-dense granules are the most abundant. Arrow indicates phagocytosed erythrocyte. $\times 5100$. (b) *M. caroli* embryo from the same *M. musculus* recipient at Day 8.5. The upper trophoblast cell contains few inclusions, most of which are of intermediate electron density (arrow), while the lower cell has very few inclusions but many noticeable mitochondria. $\times 3400$.

Fig. 5. Photomicrographs of *M. caroli* embryo in *M. musculus* uterus at Day 8.5 showing (a) exudative foci (F) in the region of the posterior neural tube ($\times 128$); and (b) degenerating trophoblast cells (large arrow) adjacent to exudate (F) and polymorphs (small arrow) ($\times 320$).

Trophoblast giant cells are known to be resistant to immune cell-mediated lysis (Simmons & Russell, 1962), even under conditions of enhanced immune effector activity (Zuckerman & Head, 1985) or xenotransfer (Kirby, 1962; Hancock *et al.*, 1968; Croy *et al.*, 1984). Consequently, the observed degeneration of trophoblast giant cells around transferred *M. caroli* embryos is probably not mediated by immunological effector mechanisms. A role for lymphokines, particularly granulocyte-macrophage colony stimulating factor, has been postulated in trophoblast proliferation (Athanasakis *et al.*, 1987) and it is possible that failure of *M. caroli* embryonic cells to stimulate *M. musculus* lymphokine production or to bind lymphokines of *M. musculus* origin remains as an immunological explanation for gradual degeneration of *M. caroli* trophoblast cells in xenogeneic pregnancy.

However, the hypothesis that death of *M. caroli* embryos in *M. musculus* uteri is not immunologically mediated but initiated by trophoblast failure offers an explanation for lack of success in prolonging the survival of *M. caroli* embryos in immunodeficient compared to immunocompetent *M. musculus* recipients (Croy *et al.*, 1985). Necrosis of trophoblast would recruit specific and non-specific immune effector cells and phagocytes to implantation sites and lack of trophoblast continuity would readily permit access of these cells to the embryo proper, before onset of gross degenerative changes.

Primary trophoblast failure and cell death also explain other observations made in this model system. Some *M. caroli* $\leftrightarrow$ *M. musculus* aggregation chimaeras transferred to *M. musculus* uteri survive to term (Rossant *et al.*, 1982). The trophoblast giant cells of the survivors are solely of *M. musculus* genotype by Day 9 of gestation (Rossant *et al.*, 1983). Many chimaeras die during gestation and it can be suggested that the dying embryos are those which differentiate trophoblast cells wholly or largely of *M. caroli* genotype, while in the survivors gradual death and loss of *M. caroli* trophoblast cells from the mixed population results in a conceptus having trophoblast cells of the genotype of the embryo transfer recipient and survival. A relative deficiency of healthy *M. caroli* trophoblast cells could also account for the deficit in recruitment of decidua-associated, trophoblast-recruited, suppressor cells (Clark *et al.*, 1983) at *M. caroli* implantation sites in *M. musculus*.

In many studies, lymphocytes have been deemed to play an important role in initiation of interspecies and intraspecies embryo death (Allen, 1982; Allen *et al.*, 1986; Clark *et al.*, 1986; Gronvik *et al.*, 1986; Gendron & Baines, 1988; Tartakovsky & Gorelik, 1988). Failure to prolong *M. caroli* embryo survival in immunodeficient *M. musculus* recipients, including xenogeneically unresponsive recipients of *scid/scid* genotype (Croy *et al.*, 1985; Crepeau & Croy, 1988), and demonstration of trophoblastic failure early during gestation indicate that immunological differences between *M. musculus* and *M. caroli* are not responsible for initiation of pregnancy failure in this model system. Rather, immune effector cells appear to be recruited following degeneration of *M. caroli* trophoblast cells in the xenogeneic uterus and participate in embryonic resorption as a secondary event. Further detailed studies of other reported models of immunologically-mediated pregnancy failure, including histological, time course studies, may also show that presently unsuspected events underlie some or all of these embryonic deaths.

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