Survival and normal function of glycolysis-deficient mouse oocytes

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Mouse embryos homozygous for a null allele of *Gpi1* which encodes the glycolytic enzyme glucose phosphate isomerase fail to complete gastrulation and die at about embryonic day 7.5, but mutant cells can survive in fetal chimaeras in which they are mixed with wild-type cells. An adult female mouse chimaera, composed of wild-type cells and homozygous *Gpi1*^{-/-} null mutant cells, was produced to test whether the presence of wild-type cells in the ovary allowed mutant oocytes to survive and function. This mouse produced 28 offspring, eight of which were derived from homozygous *Gpi1*^{-/-} null oocytes. DNA *in situ* hybridization also showed that some *Gpi1*^{-/-} follicle cells

were able to survive in chimaeric ovarian follicles. It is likely that the survival of mutant follicle cells and fully functional mutant oocytes was mediated by the presence of wild-type cells that could provide metabolic intermediates and so bypass the block in the glycolytic pathway. Wild-type cumulus cells probably supported the growing GPI-deficient oocytes via metabolic co-operation, by passing ATP and other glycolytic products through gap junctions. It was concluded that female mouse germ cells and ovarian follicle cells do not need an intact endogenous glycolytic pathway if they can obtain appropriate metabolites from an exogenous source.

Introduction

Mouse embryos homozygous for a null mutation (*Gpi1*^{-s^a-m^{1H}}, abbreviated to *Gpi1*⁻) in the gene encoding the glycolytic enzyme, glucose phosphate isomerase (GPI; E.C. 5.3.1.9) die early in development (West *et al.*, 1990) and fail to complete gastrulation (Kelly and West, 1996). Although the homozygous *Gpi1*^{-/-} null genotype is lethal to embryos it need not be lethal to cells. Homozygous *Gpi1*^{-/-} null cells co-existing with wild-type *Gpi1* cells in chimaeric *Gpi1*^{-/-}↔*Gpi1*^{+/+} conceptuses survive, usually in small numbers, to at least embryonic day 12.5 and have the developmental potential to contribute to a wide range of embryonic and extraembryonic tissues (Kelly and West, 2002). The aim of the present study was to determine whether *Gpi1*^{-/-} null cells can survive in *Gpi1*^{-/-}↔*Gpi1*^{+/+} chimaeras until adulthood and whether homozygous *Gpi1*^{-/-} null female germ cells can produce fully functional oocytes.

Materials and Methods

Mice

The *Gpi1*^{-s^a-m^{1H}} mutant null allele (abbreviated to *Gpi1*⁻) was maintained by mating *Gpi1*^{+/+} × *Gpi1*^{+/+} and *Gpi1*^{+/+} × *Gpi1*^{+/+} mice in alternate generations. Two *Gpi1*⁻ stocks ('GN' and 'NUL') were used in the present study as

described by Kelly and West (2002). Stock 'GN' was pigmented and *Gpi1*^{+/+}, and stock 'NUL' was pigmented, *Gpi1*^{+/+} and homozygous (*Tg*^{+/+}) for the reiterated β-globin transgene TgN(Hbb-b1)83Clo (Lo, 1986; Lo *et al.*, 1987).

Production of chimaeras

Chimaeras were produced by aggregating pairs of pre-implantation embryos produced from superovulated females using the method developed by Tarkowski (1961), as fully described by West and Flockhart (1994). Eight-cell stage, genetically pigmented embryos from *Gpi1*^{+/+}, *Tg*^{-/-} × *Gpi1*^{+/+}, *Tg*^{+/+} (GN × NUL) crosses were flushed from the uterus at embryonic day 2.5 and aggregated with eight-cell stage, genetically albino *Gpi1*^{+/+} embryos (designated 'CF₂') produced by intercrossing (C57BL-*Gpi1*^{+/+}, *c*/Ws × BALB/c-*Gpi1*^{+/+}, *c*/Ws) F₁ hybrids ('CF₁' hybrids). After culture overnight, the embryonic day 3.5 chimaeric aggregates were transferred to the uteri of pseudopregnant 'CF₁' (homozygous *Gpi1*^{+/+}) females 2.5 days after mating to vasectomized males and allowed to go to term. This procedure resulted in pigmented↔albino chimaeras of four genotype combinations: *Gpi1*^{+/+}↔*Gpi1*^{+/+}, *Gpi1*^{+/+}↔*Gpi1*^{+/+}, *Gpi1*^{+/+}↔*Gpi1*^{+/+} and *Gpi1*^{+/+}↔*Gpi1*^{+/+}.

Analysis of chimaeras

The percentage of pigment in the coats and eyes of adult chimaeras was estimated subjectively. Mice were killed by exposure to carbon dioxide and their eyes were removed and examined under a dissecting microscope for overall pigmentation in the superimposed choroid and retinal

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Fig. 1. Homozygous $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaera AK2. (a) Chimaera AK2 at 2 weeks. (b,c) Two views of the right eye. Scale bar represents 1 mm.

pigment epithelium. Various organs were removed and samples were prepared for analysis by GPI electrophoresis and DNA *in situ* hybridization. Cellulose acetate electrophoresis of GPI and quantitative densitometry were performed as described by West and Flockhart (1994). A small blood sample was taken from the tail vein of anaesthetized live mice at 5 weeks for GPI analysis and to prepare blood smears for DNA *in situ* hybridization. Smears were air dried on clean microscope slides, fixed in ethanol:acetic acid (3:1) for 60 min, air dried, immersed in acetone for 10 min and dehydrated through a graded series of alcohol before *in situ* hybridization.

DNA *in situ* hybridization to the reiterated β -globin transgene TgN(Hbb-b1)83Clo was detected in tissue sections and blood smears by diaminobenzidine staining for a peroxidase-conjugated antibody bound to the digoxigenin-labelled DNA probe (Keighren and West, 1993). Slides were counterstained with haematoxylin and eosin, and examined by bright-field microscopy to identify $Tg^{+/-}$ cells derived from the GN $\times$ NUL component in the chimaera by the presence of a brown spot (hybridization signal) in the nucleus. For each tissue section or blood smear, approximately 300 nuclei were scored for the presence of the hybridization signal.

Results

Identification of an adult $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaera

Eleven live mice were born after embryo aggregation and nine were identified as chimaeras by coat colour variegation

(Fig. 1a). GPI electrophoresis of peripheral blood identified one $Gpi1^{al-/-} \leftrightarrow Gpi1^{dc}$, three $Gpi1^{bl-/-} \leftrightarrow Gpi1^{dc}$ and three $Gpi1^{alb-/-} \leftrightarrow Gpi1^{dc}$ chimaeras. The remaining two chimaeras (females AK1 and AK2) each had some coat pigment but their blood samples produced only GPI1-C activity. It is possible that these were $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaeras but it is difficult to distinguish $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaeras from other chimaeras with a very high proportion of $Gpi1^{dc}$ cells.

Electrophoresis of mixtures of GPI1-B and GPI1-C tissue homogenates showed that as little as 3% GPI1-B (by tissue mass) was consistently detected (Kelly, 1995). Thus, (GN $\times$ NUL) cells should be detectable in blood samples from chimaeras by GPI1 electrophoresis, if they contributed at least 3% to the blood. The composition of different tissues in individual chimaeras tends to be similar (Falconer *et al.*, 1981), so usually (GN $\times$ NUL) cells should be detectable in the blood if they contribute significantly to other tissues. Chimaera AK1 had < 1% pigment in the coat and eyes, so its genotype remains unknown. It could have been either $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ or another chimaeric genotype with undetectable concentrations of GPI1-A, -B or -AB in the blood. However, the coat of chimaera AK2 contained a significant amount of pigment (Fig. 1a) and was classified as 10% pigmented in the adult. Therefore, AK2 was provisionally classified as a $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaera, implying that homozygous $Gpi1^{-/-}$ null coat melanocytes can survive to adulthood.

DNA *in situ* hybridization was performed on blood smears from chimaera AK2 at 5 weeks and 31/399 (8%) nucleated blood cells carried the transgenic marker. The presence of 8% (GN $\times$ NUL) cells in the absence of (GN $\times$ NUL) GPI activity provides more direct evidence for the conclusion that AK2 is a genuine $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaera. This finding also demonstrates that homozygous $Gpi1^{-/-}$ leucocytes survived for at least 5 weeks.

Homozygous $Gpi1^{-/-}$ null germ cells can produce viable and functional oocytes

Chimaera AK2 was mated to an albino $Gpi1^{dc}$ male (stock CF₁) to confirm that it was a $Gpi1^{-/-} \leftrightarrow Gpi1^{dc}$ chimaera and to test whether gametes derived from homozygous $Gpi1^{-/-}$ mutant germ cells were functional. The progeny were classified for coat colour and GPI phenotype. Both $Gpi1^{dc}$ and $Gpi1^{-/-}$ offspring produced a single GPI1-C band by electrophoresis but the genotypes of the GPI1-C offspring could be deduced without test breeding (for example, GPI1-C offspring from the $Gpi1^{bl-/-}$ component of a pigmented $Gpi1^{bl-/-} \leftrightarrow$ albino $Gpi1^{dc}$ chimaera mated to a $Gpi1^{dc}$ mouse would be pigmented and $Gpi1^{dc}$, whereas those from the $Gpi1^{dc}$ component would be albino and $Gpi1^{dc}$).

Chimaera AK2 produced 28 offspring, 20 of which were albino and GPI1-C ($Gpi1^{dc}$), and eight of which were pigmented and GPI1-C ($Gpi1^{dc}$). The eight pigmented offspring were distributed among all three litters which yielded 2/8, 3/8 and 3/12 pigmented offspring, respectively. Pigmented

Gpi1^{d/-} offspring could be produced from matings with albino *Gpi1*^{dc} males if the pigmented component of the chimaera was *Gpi1*^{al/-}, *Gpi1*^{bl/-} or *Gpi1*^{-/-}. If the chimaera was either *Gpi1*^{al/-}↔*Gpi1*^{dc} or *Gpi1*^{bl/-}↔*Gpi1*^{dc}, 50% of the pigmented offspring should be *Gpi1*^{al/c} or *Gpi1*^{bl/c}, whereas if it was *Gpi1*^{-/-}↔*Gpi1*^{dc} all of the pigmented offspring should be GPI1-C (*Gpi1*^{dc}). The probability that all eight pigmented offspring were GPI1-C if the chimaera was either *Gpi1*^{al/-}↔*Gpi1*^{dc} or *Gpi1*^{bl/-}↔*Gpi1*^{dc} is only (0.5)⁸ (that is, *P* = 0.004), which supports the conclusion that AK2 is a *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera.

The genotypes of albino and pigmented GPI1-C offspring of the mating between chimaera AK2 and the albino *Gpi1*^{dc} male were confirmed by test-mating some of the offspring to pigmented *Gpi1*^{bl/b} (C57BL/Ws × CBA) F₁ hybrid mice. Four albino GPI1-C offspring (putative *Gpi1*^{dc} genotype) each produced only GPI1-BC (*Gpi1*^{bl/c}) progeny (90 in total), as expected for a *Gpi1*^{dc} × *Gpi1*^{bl/b} cross. Five of the eight pigmented GPI1-C offspring (putative *Gpi1*^{d/-} genotype) were similarly test-mated and all five produced some GPI1-B (*Gpi1*^{bl/-}) and some GPI1-BC (*Gpi1*^{bl/c}) progeny (66 GPI1-B and 66 GPI1-BC in total), as expected for a *Gpi1*^{d/-} × *Gpi1*^{bl/b} cross. This finding confirms that all the AK2 pigmented GPI1-C progeny that were test-mated were *Gpi1*^{d/-} rather than *Gpi1*^{dc}. The results of the genetic test-crosses confirm that AK2 is a genuine *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera and demonstrate that homozygous *Gpi1*^{-/-} null oogonia can produce viable and functional oocytes that result in viable offspring when fertilized with wild-type spermatozoa.

GPI analysis of other tissues

After completion of the test breeding, the chimaeras were killed and GPI electrophoresis was performed on > 20 tissue samples. Chimaera AK2 died while giving birth (aged 21 weeks), but a full set of samples was analysed successfully for GPI. All samples were entirely GPI1-C (in agreement with the 5 week blood analysis), which is consistent with the conclusion that this mouse was a genuine *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera.

Tissue composition of *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera AK2

After chimaera AK2 died, its eyes were removed and the percentage of eye pigment was estimated as approximately 15%. Although this is a crude estimate because variegation in the retinal pigment epithelium may be obscured by pigment in the overlying choroid, pigmented homozygous *Gpi1*^{-/-} null cells survived at least in the choroid of *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera AK2 until 21 weeks (Fig. 1b,c).

Although chimaera AK2 died, DNA *in situ* hybridization was performed successfully on a range of tissues and the transgenic lineage marker, present in the *Gpi1*^{-/-} null cells, was detected in the right ovary, brain, uterus, liver, thymus, small intestine and bladder. Generally the number of mutant cells was small but one ovarian follicle with a mutant oocyte and a high proportion of *Gpi1*^{-/-} follicle cells was found (Fig. 2). *Gpi1*^{-/-} null cells were not detected by *in*

Fig. 2. Ovarian follicle from *Gpi1*^{-/-}↔*Gpi1*^{dc} chimera AK2 after DNA *in situ* hybridization to detect the reiterated β-globin transgene in *Gpi1*^{-/-} cells, which were hemizygous (*Tg*^{+/+}) for the transgene. Hybridization to the transgene (visualized as a brown spot in the nucleus) can be seen in the oocyte (arrow) and in many of the surrounding follicle cells, indicating that they are mutant *Gpi1*^{-/-} cells. Other follicle cells are wild type and lack the brown hybridization signal. Scale bar represents 20 μm.

situ hybridization in heart, right kidney, pancreas, spleen, lung, large intestine and caecum plus appendix, but it is not clear whether this indicates that mutant cells were excluded from these tissues or simply reflects the poor preservation of the tissues from the dead mouse. For this reason no attempt was made to quantify the *Gpi1*^{-/-} contribution to the other tissues.

Discussion

The results of the present study demonstrate that one adult female mouse (AK2) was a genuine *Gpi1*^{-/-}↔*Gpi1*^{dc} chimaera and that some homozygous *Gpi1*^{-/-} null cells can survive in a range of adult tissues, including coat melanocytes, choroidal melanocytes, leucocytes, brain, uterus, liver, thymus, small intestine, bladder, ovarian follicle cells

and oocytes. Homozygous *Gpi1*^{-/-} cells would be unable to generate energy via glycolysis, unless wild-type cells provided substrates downstream of GPI, but they could produce energy by the tricarboxylic acid cycle (TCA cycle or Krebs cycle) if appropriate substrates (for example, pyruvate or glutamine) were available. The low survival seen in several fetal *Gpi1*^{-/-} ↔ *Gpi1*^{dc} chimaeras reported by Kelly and West (2002) indicates that most *Gpi1*^{-/-} cells are at a selective disadvantage and need an intact glycolytic pathway to function optimally.

The *in situ* hybridization analysis revealed that mutant follicle cells and oocytes can survive in chimaeric ovarian follicles composed of a mixture of mutant and wild-type cells. The genetic crosses demonstrate that such oocytes can be ovulated and fertilized, and will produce normal fertile offspring.

Mouse oocytes use pyruvate rather than glucose as a major energy source, whereas follicle cells actively metabolize glucose to pyruvate and rely predominantly on glycolysis for energy provision between mid-preantral and preovulatory stages (Biggers *et al.*, 1967; Donahue and Stern, 1968; Boland *et al.*, 1994; Downs and Utecht, 1999). Pyruvate is secreted by cultured follicle cells (Donahue and Stern, 1968; Leese and Barton, 1985; Downs and Mastropolo, 1994) and if this occurs *in vivo* pyruvate may diffuse through the intercellular space and be available to rescue *Gpi1*^{-/-} null follicle cells and oocytes. Downs *et al.* (2002) reported that most of the ATP used by oocytes in cultured oocyte-cumulus complexes is generated by glycolysis (in follicle cells) rather than from oxidation of pyruvate. Follicle cells communicate with each other and with oocytes via gap junctions (Anderson and Albertini, 1976; Gilula *et al.*, 1978) and probably pass metabolites, such as glucose-6-phosphate (Saito *et al.*, 1994) and ATP (Downs and Mastropolo, 1994; Downs, 1995), to the oocytes via the gap junctions.

Mutant *Gpi1*^{-/-} null oocytes could produce energy by metabolizing pyruvate in the TCA cycle, so that surrounding wild-type follicle cells could rescue the oocytes by providing either ATP or pyruvate. Pyruvate could diffuse via the intercellular space, and both pyruvate and ATP could be transferred to mutant oocytes via gap junctions and so rescue them by metabolic co-operation. Mutant GPI-deficient follicle cells could produce energy by glycolysis downstream of the blocked GPI step that converts glucose-6-phosphate to fructose-6-phosphate. Thus, transfer of ATP or glycolytic products downstream of glucose-6-phosphate through gap junctions would allow wild-type follicle cells to rescue neighbouring *Gpi1*^{-/-} null follicle cells by metabolic co-operation.

It also follows that mutant *Gpi1*^{-/-} primordial germ cells and oogonia were able to survive in the chimaera. It is likely that these earlier stage mutant germ cells were also dependent on metabolites produced by wild-type cells in the chimaera. Survival of embryos produced by fertilization of null oocytes with wild-type spermatozoa can also be explained because preimplantation mouse embryos do not

use glucose as an energy source until the eight-cell stage (Brinster, 1965; Gardner and Leese, 1986), which is about the time that the wild-type paternal *Gpi1*⁺ allele would be activated, thereby restoring an intact glycolytic pathway (Chapman *et al.*, 1971; Brinster, 1973; West and Green, 1983; Duboule and Burki, 1985; Gilbert and Solter, 1985; West *et al.*, 1986).

By a combination of DNA *in situ* hybridization and genetics the present study has shown that oocytes from homozygous *Gpi1*^{-/-} null germ cells in a female *Gpi1*^{-/-} ↔ *Gpi1*^{dc} chimaera are both viable and functional, and that mutant ovarian follicle cells can also differentiate and survive. This finding demonstrates that neither female mouse germ cells nor follicle cells need an intact endogenous glycolytic pathway provided that they can obtain appropriate exogenous metabolites.

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