The extended survival of \( t^{w5} / t^{w5} \) mouse embryo cells \textit{in vitro}

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Summary

The \( t^{w5} \) haplotype is a recessive mutation which is lethal when homozygous in mouse embryos following implantation. This series of studies was undertaken to determine the effect of the \( t^{w5} / t^{w5} \) genotype on embryos developing \textit{in vitro}. Blastocyst embryos from \(+/t^{w5}\) inter se matings were compared with control blastocysts obtained from matings between \( T/+ \) and \(+/+\) females and \(+/t^{w5}\) males for their abilities to continue development \textit{in vitro} in two culture media. The data show that there are no significant differences between the percentages of experimental and control blastocyst embryos which attach and outgrow or which contain inner cell masses on any day of culture up to equivalent gestation day 21 in either media. These findings show that the life span of cells from \( t^{w5}/t^{w5} \) embryos can be extended significantly by \textit{in vitro} culture.

1. Introduction

Embryos homozygous for the \( t^{w5} \) haplotype die between gestation days 6 and 11 \textit{in vivo} (Bennett & Dunn, 1958). Wudl & Sherman (1976) found that the \( t^{w5}/t^{w5} \) embryos are arrested in development on equivalent gestation days (e.g.d.) \textit{in vitro}. The observed numbers of embryo outgrowths on e.g.d. 11 to 14 fit the numbers expected if the \( t^{w5}/t^{w5} \) genotype were lethal \textit{in vitro} between e.g.d. 6 and 11. Hogan et al. (1980) reported between 30 and 50% of the inner cell mass cells (ICMs) immunosurgically removed from blastocyst embryos obtained from \(+/t^{w5}\) inter se matings to be morphologically abnormal after seven days in culture. These abnormal ICMs were presumed to be homozygous for the \( t^{w5} \) haplotype. Although the stage(s) of death of the ICMs is not reported, the endodermal cells of some of these presumed \( t^{w5}/t^{w5} \) ICMs attached to the culture dish and outgrew into flat sheets of cells which ceased to grow after a few additional days in culture. Wudl & Sherman (1976) also found that \( t^{w5}/t^{w5} \) embryos could not be rescued when combined with wild-type embryos to produce chimeric blastocyst embryos which were allowed to continue development \textit{in vitro}. All of the above studies suggest that cells from \( t^{w5}/t^{w5} \) blastocyst embryos do not survive \textit{in vitro} beyond their normal lethal period \textit{in vivo}.

Previous studies showed that embryos homozygous for two other lethal phenotypes, \( t^{12} \) and \( r^{e} \), die \textit{in vitro} at gestational stages comparable to their respective lethal stages \textit{in vivo} (Hillman et al. 1970; Nadijcka & Hillman, 1975). These lethal phenotypic expressions have been used to determine the effects of various types of insemination on the transmission frequencies of the respective haplotypes (McGrath & Hillman, 1980a, b).

Recently, we attempted to utilize the reported \textit{in vitro} lethality for \( t^{w5}/t^{w5} \) embryos as a means of determining the transmission frequencies of the \( t^{w5} \) haplotype following normal and delayed matings and \textit{in vitro} fertilization. The data presented here show that the numbers of viable outgrowths with ICMs developing \textit{in vitro} from the blastocyst embryos obtained from \(+/t^{w5}\) inter se matings, and expected to include \( t^{w5}/t^{w5} \) embryos, do not differ from those found developing from control blastocysts up to e.g.d. 21. Therefore, \textit{in vitro} embryonic death cannot be used to determine the transmission frequency of this haplotype. More importantly, however, the data show that the life span of cells from \( t^{w5}/t^{w5} \) embryos can be extended \textit{in vitro} beyond their lethal period \textit{in vivo}.

2. Materials and methods

BALB/c females and \( T/t^{w5} \) males were mated to obtain normal tailed \(+/t^{w5}\) and short tailed \(+/T^{+}\) offspring. The \(+/t^{w5}\) animals were mated \textit{inter se} to obtain the experimental embryos \(++/+, +/t^{w5}, t^{w5}/t^{w5}\), and the \(+/t^{w5}\) males were mated with their
3. Results

On the basis of the distorted transmission ratio of the \( r^{05} \) haplotype, 0.95, the expected genotypic distribution of embryos from \( +/r^{05} \) inter se matings would be: 2.5% +/+; 50% +/r^{05}; and 47.5% \( r^{05}/r^{05} \). Based on the findings of Wudl and Sherman (1976), and on the transmission ratio of the \( r^{05} \) haplotype, no more than 52.5% of the blastocyst embryos placed into NCTC-109 medium should be viable on e.g.d. 14. Moreover, the percentage of viable embryos might be even lower depending upon the level of background death. The data of the present studies show, however, that 95.8% (366/382 embryos) of the outgrowths from the experimental crosses are viable on e.g.d. 14 in this medium (Fig. 1). This percentage is the same as the percentage of viable outgrowths from +/+ x +/+ \( r^{05} \) matings (98.8%; 165/167) and from \( T/+ \) x +/+ \( r^{05} \) matings (100%; 257/257) on e.g.d. 14.

Also, there are no significant differences among the percentages of outgrowths with attached ICMs from experimental and control blastocysts on e.g.d. 14 in this medium: experimental crosses (98.8%; 165/167) and from +/+ x +/+ \( r^{05} \) control matings (98.8%; 165/167) (Fig. 2). The data show that 93.1% (311/334 embryos); +/+ x +/+ \( r^{05} \) control matings (38.9%; 65/167 embryos); and \( T/+ \) x +/+ \( r^{05} \) control matings (56.0%; 130/237 embryos).

Similarly, there are no significant differences among the percentages of viable outgrowths from any of the matings on e.g.d. 14 in modified Eagle’s medium (MEM) (Fig. 2). The data show that 93.1% (311/334) of the outgrowths originating from +/+ \( r^{05} \) inter se matings, 89.6% (172/192) of those from the \( T/+ \) x +/+ \( r^{05} \) matings and 94.2% (114/121) of those from the +/+ x +/+ \( r^{05} \) matings are viable on this day of culture. On e.g.d. 14 the percentage of outgrowths with ICM cells from experimental \( r^{05} \) inter se matings is 56% (187/334 embryos), from \( T/+ \) x +/+ \( r^{05} \) matings, 58.9% (113/192 embryos) and from +/+ x +/+ \( r^{05} \) matings, 60.3% (73/121 embryos). There are no significant differences among these percentages.

The data show that there are no significant differences between the percentages of experimental outgrowths with or without ICM cells when compared with their counterparts from either of the control crosses on any equivalent day of gestation in either

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On the basis of the distorted transmission ratio of the \( r^{05} \) haplotype, 0.95, the expected genotypic distribution of embryos from \( +/r^{05} \) inter se matings would be: 2.5% +/+; 50% +/r^{05}; and 47.5% \( r^{05}/r^{05} \). Based on the findings of Wudl and Sherman (1976), and on the transmission ratio of the \( r^{05} \) haplotype, no more than 52.5% of the blastocyst embryos placed into NCTC-109 medium should be viable on e.g.d. 14. Moreover, the percentage of viable embryos might be even lower depending upon the level of background death. The data of the present studies show, however, that 95.8% (366/382 embryos) of the outgrowths from the experimental crosses are viable on e.g.d. 14 in this medium (Fig. 1). This percentage is the same as the percentage of viable outgrowths from +/+ x +/+ \( r^{05} \) matings (98.8%; 165/167) and from \( T/+ \) x +/+ \( r^{05} \) matings (100%; 257/257) on e.g.d. 14. Also, there are no significant differences among the percentages of outgrowths with attached ICMs from experimental and control blastocysts on e.g.d. 14 in this medium: experimental crosses (41.1%; 157/382 embryos); +/+ x +/+ \( r^{05} \) control matings (38.9%; 65/167 embryos); and \( T/+ \) x +/+ \( r^{05} \) control matings (56.0%; 130/237 embryos).

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Outgrowths with ICMs in MEM. Outgrowths: ○, +/r°5 x +/r°5; △, T/+ x +/r°5; □, +/+ x +/r°5.
Outgrowths with ICMs: •, +/r°5 x +/r°5; ■, +/+ x +/r°5; ▲, T/+ x +/r°5.

Outgrowths from +/+ x +/r°5 crosses is significantly higher in NCTC-109 than in MEM on e.g.d. 13 (98.8%, 165/167 vs. 95.9%, 116/121; 0.05 > P > 0.02) and on e.g.d. 14 (98.8%, 165/167 vs. 94.2%, 114/121; 0.02 > P > 0.001). On e.g.d. 16, the percentage of viable outgrowths with ICMs is significantly higher (0.02 > P > 0.01) among the embryos developing in MEM (45.4%, 55/121) than among those developing in NCTC-109 (20.3%, 34/167). Otherwise there are no significant differences between the control outgrowths or outgrowths with ICMs in the two media.

4. Discussion

The current studies show that homozygosity for the r°5 haplotype which results in prenatal lethality between gestation days 6 to 11 is not lethal when embryos with this genotype are maintained in vitro from early developmental stages to e.g.d. 21. These observations contrast with those of Wudl & Sherman (1976) who reported that r°5/r°5 embryos die in vitro before e.g.d. 14, i.e. a time equivalent to their in vivo lethal period. In their experiments, Wudl and Sherman noted that the observed percentage of dead embryos from +/r°5 inter se matings was not significantly different from that expected on the basis of the transmission frequency of the r°5 haplotype. From these data they concluded the r°5 haplotype when homozygous to be a generalized cell lethal and that all cells of the embryo expressed the lethal phenotype at a specific stage or time; furthermore, neither embryos nor embryonic cells with this genotype could be rescued by in vitro culture. The discrepancy between the current results and those of Wudl & Sherman (1976) who also used NCTC-109 as the culture medium explains some of the observed differences. On the other hand, two separate studies, Magnuson et al. (1982) and Martin et al. (1987), succeeded in establishing pluripotent stem cell lines from the inner cell mass of blastocyst embryos that were, based on karyotypic and gene marker analyses, homozygous for the r°5 haplotype. They concluded that the r°5 haplotype does not cause generalized cell lethality and that cells from such embryos can be rescued. The data from the present study support the latter conclusion and clearly show that cells from homozygous r°5 embryos originating from in vivo matings survive in vitro beyond their in vivo lethal developmental period.

The rescue of cells from embryos homozygous for other lethal r haplotypes by explanting them to a different environment before developmental arrest has been reported by several laboratories. Artzt & Bennett (1972) found that r°16/r°16 embryos, which normally die around gestation day nine, could be rescued by...
transplanting them into the testes of mice where they develop into tumours. The observations of Wudl et al. (1977) support these results. The latter investigators also reported that $t^{e18}/t^{e18}$ embryos remained viable under in vitro conditions. On e.g.d. 22, the percentage of viable outgrowths originating from the experimental embryos ($+/t^{e18} \times +/t^{e18}$) was the same as that originating from embryos from control matings ($+/t^{e18}$). Axelrod et al. (1981) found that explanted $t^{e12}/t^{e12}$ embryos, which have a protracted lethal period ranging between gestation days nine and 20, can also be rescued if they are explanted to mouse testes. Moreover, cells obtained from the tumours developing from these explanted embryos have been successfully maintained in vitro.

Collectively, the results from the studies by Magnuson et al. (1982), Martin et al. (1987), Artzt & Bennett (1972), Wudl et al. (1977), Axelrod et al. (1981) and those from the present studies show that individual cells from embryos homozygous for some $t$ haplotypes can survive past their in vivo lethal period if they are placed into specific culture media or into appropriate ectopic sites. The fact that cells from homozygous lethal embryos can be rescued is not surprising since the results of light and electron microscopic studies of $t^{12}/t^{12}$, $t^{e12}/t^{e12}$ and $+/t^{e}$ embryos show that embryos which are developmentally arrested as a result of their lethal genotype contain both pycnotic and healthy cells, many of which are undergoing mitoses (Hillman et al. 1970; Hillman & Hillman, 1975; Nadijcka & Hillman, 1975). Thus not all cells of the embryo die simultaneously. Initial cell death of $t/t$ embryos occurs either at random in cleavage staged embryos (Hillman et al. 1970; Hillman, 1972; Hillman & Hillman, 1975) or in specific cell types in later staged embryos following implantation and cellular differentiation (Artzt & Bennett, 1972; Bennett, 1975; Nadijcka & Hillman, 1975). Axelrod et al. (1981). The exact stage of in vivo embryonic death can be related to the time of onset of cell death, to the proportion and relative position of necrotic cells to healthy cells (Hillman, 1975) and to the type(s) of cells which express the mutant phenotype (e.g. overgrowth of the primitive streak; Artzt & Bennett, 1972). In utero, those embryos which contain necrotic cells or are phenotypically aberrant stop development and ultimately all cells become necrotic. Thus, in utero, the $t/t$ genotype interferes with the developmental sequence, ultimately producing a cascade of effects resulting in embryo death.

In spite of the fact that those $t/t$ embryos ($t^{12}$, $t^{e12}$) which die during the preimplantation stages and those that die at implantation (e.g. $+/t^{e}$) contain both normal and necrotic cells at the time of embryo developmental arrest, there have been no reports showing that the embryos or cells from these embryos can be rescued either by allowing them to develop in vitro, by combining them with wild-type embryos to produce chimeric embryos, or by transplanting them to ectopic sites (Mintz, 1964; Hillman, 1975; Wudl et al. 1977). Only cells from $t/t$ embryos which die at later stages of gestation have been rescued. However, since the $t$-lethal mutations are not allelic (Artzt et al. 1982; Shin et al. 1983) it is highly unlikely that the cause of lethality is the same for all $t/t$ embryos and, consequently, it is not reasonable to expect that cells from all $t/t$ embryos can be rescued.

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