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Evidence for selection against tetraploid cells in tetraploid ↔ diploid mouse chimaeras before the late blastocyst stage

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Summary

Tetraploid (4n) cells do not contribute equally to all tissues of midgestation mouse chimaeras and mosaics. Our previous studies of early blastocysts showed that 4n cells are preferentially allocated to the mural trophectoderm of the early blastocyst and this may contribute to the restricted distribution pattern seen at later stages. In this study of later-stage blastocysts we found evidence for selection against 4n cells. The contribution of 4n cells to 4n ↔ 2n chimaeric blastocysts decreased between E3·5 and E4·5 days, whereas the composition of 2n ↔ 2n controls changed little over this period. These results suggest that, prior to implantation, blastocysts have already lost some tetraploid cells from their embryonic and extra-embryonic lineages due to a combination of preferential allocation of 4n cells to the mural trophectoderm and selection against 4n cells throughout the embryo.

1. Introduction

Previous studies have demonstrated that tetraploid cells show a restricted distribution pattern when combined with diploid cells in midgestation mouse chimaeras and mosaics (Tarkowski *et al.*, 1977; Lu & Markert, 1980; James *et al.*, 1995). Their poor contribution to embryonic lineages during development has been utilized in both the production of embryonic stem (ES) cell chimaeras, to achieve maximum ES cell contribution to the fetus (Nagy *et al.*, 1990, 1993) and the rescue of a mutant affecting placental development (Guillemot *et al.*, 1994).

Studies of tetraploid ↔ diploid (4n ↔ 2n) conceptuses at E12·5 and E7·5 days have revealed that tetraploid cells are not simply restricted to the extra-embryonic tissues, but, more specifically, to trophoblast and primitive endoderm lineage derivatives (James *et al.*, 1995). In contrast, it was found that, as a group, E3·5 4n ↔ 2n chimaeric blastocysts contained 4n cells in all three developmental lineages, but had a significantly greater contribution in the mural trophectoderm (mTE) than in either the polar trophectoderm (pTE) or inner cell mass (ICM): a phenomenon which appeared to be related to the ploidy of the blastomere and not cell size at aggregation (Everett & West, 1996).

In this study we have analysed the contribution made by tetraploid cells to the E4·5 blastocyst and found that the contribution has already been reduced compared with that at E3·5. Selective disadvantage of 4n cells is evident even at this early stage.

2. Materials and methods

(i) *Mice*

Chimaeras were made by aggregating embryos from two genetically distinct stocks of mice, one carrying a reiterated β-globin transgenic sequence TgN(Hbb-b1)83Clo (Lo, 1986; West *et al.*, 1995) (*Tg*) that can be identified by DNA–DNA *in situ* hybridization on histological sections (Katsumata & Lo, 1988; Thomson & Solter, 1988). The two stocks of mice used were (BF1 × BF1) and (BF1 × TGB) embryos where BF1 is the (C57BL/Ola × CBA/Ca)F1 hybrid. TGB is a random-bred stock of predominantly BF1 genetic background, homozygous for *Tg*, that was originally derived from strain 83 (Lo, 1983, 1986). Some BF1 females were obtained from the Department of

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Medical Microbiology, University of Edinburgh; all other mice were bred and maintained under standard laboratory conditions in the Centre for Reproductive Biology.

(ii) *Collection of embryos*

BF1 females were superovulated by injecting 5 IU pregnant mares' serum gonadotrophin (PMSG) at approximately 12 noon followed 48 h later by 5 IU human chorionic gonadotrophin (hCG). Females were then caged with either BF1 or TGB males and mating was verified the next morning by the presence of a vaginal plug; the day of the vaginal plug was designated E0·5. Embryos produced this way were either hemizygous for the transgene (BF1 × TGB) or lacked the transgene (BF1 × BF1). Embryos were flushed from the oviducts of BF1 females at E1·5 with HEPES-buffered M2 handling medium (Quinn *et al.*, 1982) at the 2-cell stage.

(iii) *Tetraploidy induction and chimaera production*

Tetraploid embryos were produced by electrofusion, as previously described (Kaufman & Webb, 1990; James *et al.*, 1992). The exposure of 2-cell diploid (BF1 × TGB) embryos to electric field pulses caused degradation of the cell membrane and cell fusion to produce 1-cell tetraploid embryos. These 1-cell tetraploid embryos were incubated overnight in drops of M16 culture medium (Whittingham, 1971) under paraffin oil at 37 °C in 5% CO_2 in air. BF1 × BF1 embryos were also collected at the 2-cell stage and incubated overnight. On the day following collection of the 2-cell embryos and electrofusion of the 4n embryos, aggregation chimaeras were produced as previously described (James *et al.*, 1995).

(iv) *Blastocyst fixation and analysis*

Two series of chimaeras were produced: 4n ↔ 2n chimaeras and control 2n ↔ 2n chimaeras. Fifty-four hours after aggregation, healthy looking chimaeric blastocysts (E4·5) were introduced into molten agar, fixed in acetic alcohol (3 ethanol:1 acetic acid) and processed for analysis by *in situ* hybridization to check for the presence of hemizygous, diploid ($Tg/-$) or tetraploid cells ($Tg/Tg/-/-$) bearing one or two copies of the β-globin transgene respectively (James *et al.*, 1992). Paraffin wax serial sections were cut at 7 μm and the probe pM$\beta\partial$2 (Lo, 1983, 1986) was used to detect the β-globin sequences. The probe was labelled using digoxygenin-labelled deoxyuridine triphosphate (non-radioactive DNA Labelling and Detection Kit; Boehringer Mannheim) and *in situ* hybridization was carried out on batches of 10–15 slides as previously described (Keighren & West, 1993) using a diaminobenzidine endpoint. A positive control was included in each *in situ* experiment.

The blastocyst sections were scored for presence of hybridization signal. Several sections were not scored because of poor morphology, so some analysed blastocysts were incomplete. Data were included if more than five sections were scored and if the inner cell mass proportion was greater than 10%. In the analysis no distinction was made between cells with one hybridization signal and cells with two signals: nuclei were scored as either *Tg* positive or negative. The nuclei were allocated to either trophectoderm (TE) or inner cell mass (ICM).

(v) *Statistical analysis*

Chi-square (χ^2) tests with 1 degree of freedom were performed on an Apple Macintosh computer using a routine established on the spreadsheet Microsoft Excel (Microsoft Corporation).

3. Results

Analysis of E4·5 blastocyst sections after *in situ* hybridization revealed the mean percentages of *Tg*-positive nuclei to be 10·0% and 27·8% for the 4n ↔ 2n and 2n ↔ 2n chimaera groups respectively (see Table 1). Ideal frequencies resulting from the 4-cell ↔ 8-cell and 8-cell ↔ 8-cell aggregations in the production of 4n ↔ 2n and 2n ↔ 2n chimaeras are 33% and 50% respectively, due to the numbers of contributing cells at aggregation. A reduction from these ideal frequencies was expected, due to an underestimate of the proportion of *Tg*-positive nuclei in 7 μm sections (Everett & West, 1996).

At E3·5 the corresponding frequencies for the 4n ↔ 2n and 2n ↔ 2n chimaera groups were 25·1% and 29·6% respectively (Everett & West, 1996). Although there was no significant difference in the *Tg*-positive cell contribution in E3·5 and E4·5 2n ↔ 2n chimaeras, the frequency was very significantly reduced from 25·1% to 10·0% for 4n ↔ 2n chimaeras ($\chi^2 = 200{\cdot}0$; $P < 0{\cdot}001$). It is considered unlikely that a suboptimal *in situ* hybridization technique was the cause of such a large reduction, as a good-quality hybridization experiment (measured by a high % *Tg* signal in positive controls) was a prerequisite for blastocyst scoring. We therefore regard the decrease in numbers of *Tg*-positive nuclei to reflect a genuine loss of 4n cells between E3·5 and E4·5.

From the results presented in Table 1, it is apparent that the reduction in *Tg*-positive 4n cell contribution occurred in both the ICM and TE. In the ICM the percentage of *Tg*-positive 4n cells was significantly reduced by 6·7% from 21·0% to 13·3% ($\chi^2 = 14{\cdot}2$; $P < 0{\cdot}001$); in the TE the reduction, from 26·8% to 8·2%, was much greater ($\chi^2 = 210{\cdot}6$; $P < 0{\cdot}001$). This

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Table 1. *Observed* (*uncorrected*) *percentages of Tg-positive cells in the inner cell mass* (*ICM*) *and trophectoderm* (*TE*) *from each of the E3·5 and E4·5 chimaera groups*

Chimaeras		Number of embryos	Blastocyst		Inner cell mass		Trophectoderm		ICM (%)
			Tg+ve	*n*	*Tg*+ve	*n*	*Tg*+ve	*n*	
E3·5[a]	4n↔2n	21	25·1	1756	21·0	495	26·8	1261	28·2
	2n↔2n	17	29·6	1976	30·5	693	29·1	1283	35·1
E4·5	4n↔2n	23	10·0	3229	13·3	1121	8·2	2108	34·7
	2n↔2n	11	27·8	1913	22·7	636	30·2	1277	33·2

[a] Data taken from Everett & West (1996).

resulted in the TE having a lower level of tetraploidy than the ICM ($\chi^2 = 21{\cdot}6$; $P < 0{\cdot}001$), in contrast to the pattern found at E3·5 ($\chi^2 = 6{\cdot}8$; $P < 0{\cdot}01$; Everett & West, 1996).

The ICM of the 2n↔2n chimaeras also showed a 7·8% decrease in the *Tg*-positive component between E3·5 and E4·5 from 30·5% to 22·7% ($\chi^2 = 9{\cdot}5$; $P < 0{\cdot}01$), which resulted in a small (7·5%) but significant difference in the contribution between the TE and ICM ($\chi^2 = 11{\cdot}3$; $P < 0{\cdot}001$).

4. Discussion

Between E3·5 and E7·5, the 4n cell distribution pattern in 4n↔2n conceptuses alters considerably (James *et al*., 1995; Everett & West, 1996). At E3·5, 4n cells can contribute to all lineages, albeit at a higher frequency in the mTE. At E7·5, however, 4n cells are largely restricted to the primitive endoderm and trophectoderm lineages. This study of 4n↔2n chimaeras at the late blastocyst stage was an attempt to identify how the E7·5 pattern could be derived from that at E3·5.

Evidence for a reduction in overall numbers of *Tg*-positive cells in 4n↔2n but not 2n↔2n chimaeras provided evidence to support the hypothesis that selection against 4n cells contributes to the distribution pattern seen later in development.

In contrast to expectations, the reduction in 4n component was greater in the TE than the ICM. The mechanisms responsible for the reduction could be either selective cell death of 4n cells or a failure of 4n cells to proliferate. Certainly cell death in the ICM has been proposed as a regular feature of development (Copp, 1978; Handyside & Hunter, 1986; Pierce *et al*., 1989). In E4·5 4n↔2n chimaeric blastocysts, cell death in the ICM could be predominantly a feature of 4n cells. The slight reduction in *Tg*-positive cells in the 2n↔2n chimaeras probably also reflected the occurrence of cell death. The reason for the preferential reduction of *Tg*-positive cells is uncertain given the evidence for the neutrality of the transgene during development (West *et al*., 1996).

The mechanism of reduction in 4n cell proportion in the TE may be different. Indeed it is possible that some of this reduction may be an artifact of the larger size of TE cells at E4·5 compared with those at E3·5. The larger the cells the more false negative results would be obtained, reducing the observed frequency of *Tg*-positive cells.

Selective cell death may also play a role in modifying the composition of unbalanced chimaeras, where one strain predominates (Mullen & Whitten, 1971). For example, BALB/c cells were consistently underrepresented in fetal and extra-embryonic tissues of E12·5 BALB/c↔(C57BL×CBA)F2 chimaeras (West & Flockhart, 1994). This is compatible with generalized selection against BALB/c cells or their preferential allocation to the mTE lineage, which contributes little by this stage. Generalized selection against BALB/c cells seems favoured by the observations of Dvorak *et al*. (1995). They reported approximately equal contributions of BALB/c and C3H cells in E3·5 BALB/cA↔C3H/HeN chimaeric blastocysts but, by E7·5, BALB/c cells were again significantly underrepresented in both embryonic and extra-embryonic tissues. In this case, cell selection could occur at any time between E3·5 and E7·5. For example, size regulation of double-embryo aggregates occurs between E5·5 and E6·0 (Buehr & McLaren, 1974) and may provide an opportunity for genotype-specific cell death. However, in our present study, selection against 4n cells in cultured 4n↔2n chimaeras began between E3·5 and E4·5.

We have shown that E4·5 4n↔2n chimaeric blastocysts showed a reduced contribution of 4n cells to each of the two developmental lineages identified. Unfortunately, due to technical difficulties, the primitive endoderm and primitive ectoderm tissues of the ICM could not be distinguished, so it is not known whether 4n cells are present in both these tissues. This still leaves the reduction in 4n cell contribution to the primitive ectoderm between E4·5 and E7·5 unexplained. It is possible that 4n cells are displaced from the primitive ectoderm to the primitive endoderm but, as yet, there is no evidence for this. Alternatively, a further round of selection may occur. Selective death of 4n cells in the primitive ectoderm lineage may account for the smaller retarded E7·5 embryos

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reported by James *et al*. (1995). In a few embryos 4n cells may not be excluded from primitive ectoderm derived tissues. These, however, have been reported to be morphologically abnormal at E7·5 and are generally eliminated by E12·5 days by embryonic selection (James *et al*., 1995).

In summary, each of the three mechanisms – preferential allocation of 4n cells, cell selection and selective embryonic death – acts to produce the final pattern of 4n cell distribution evident at midgestation.

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