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Chromosome analysis of single-pronuclear haploid parthenogenetic blastocysts and their inner cell mass derivatives

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SUMMARY

Single-pronuclear haploid parthenogenetically activated mouse embryos were transferred to the oviducts of suitable recipients. One group of embryos was isolated at the morula stage and subsequently allowed to develop to the expanded blastocyst stage *in vitro*. Intact embryos were either analysed by the air-drying technique at that stage to determine their total cell number and ploidy, or treated by immunosurgery to isolate their inner cell mass. These were either analysed to establish their total cell number and ploidy, or retained in culture for an additional 24 h or 72 h. The inner cell mass derivatives were then analysed to establish the total cell number and ploidy. A second group of recipients was ovariectomized on the 4th day of pseudopregnancy, treated with Depo-Provera and blastocysts recovered 5 or 6 days later. The 'delayed' blastocysts recovered were treated by immunosurgery, and the inner cell masses isolated and either analysed at this time or transferred to culture for 72 h, 96 h or 144 h. As in the previous groups, the inner cell mass derivatives were analysed to establish the total cell population present and their ploidy. The analysis of this material was found to be technically particularly difficult, though in general the non-'delayed' embryos and their inner cell mass derivatives yielded higher success rates than the 'delayed' inner cell mass derivatives. The 'delayed' inner cell masses initially contained on average about twice the number of cells compared to the number present in those isolated from the non-'delayed' expanded blastocysts. Cellular proliferation occurred in all the groups retained in culture, though only a small proportion of the cells analysed gave 'scorable' mitotic cells in which the ploidy could be unequivocally determined. In general, in both the non-'delayed' and 'delayed' groups, the proportion of diploid mitotic cells observed increased with their duration in culture, though this effect was clearly more marked in the 'delayed' series. The present study indicated that the chance of obtaining haploid mouse cell lines in the future might be increased by using inner cell masses derived from non-'delayed' rather than 'delayed' blastocysts despite their initial reduced cell number at the time of explantation into tissue culture..

INTRODUCTION

It has recently been reported that a successful attempt has been made to establish pluripotential cell lines from 'delayed' haploid-derived parthenogenetic mouse blastocysts (Kaufman, Robertson, Handyside & Evans, 1983). Direct chromosomal analyses of even the early passages from these lines, however, revealed that they all appeared to contain only diploid mitotic cells. It was also

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reported that over 80 % of 129/SvE embryos isolated at the morula stage following oviduct transfer of recently activated one-pronuclear haploid eggs contained only haploid mitotic cells. The present study was therefore undertaken in order to try to establish whether blastocysts derived from the same class of haploid embryos might have a greater chance of giving rise to haploid cell lines than similar blastocysts that had been retained for between 3 and 6 days in the 'delayed' state *in vivo*. Equally, it was hoped that it might be possible to develop a technique that enabled the direct chromosome analysis of inner cell mass (ICM) derivatives that had been retained in culture for various periods of time as this has not previously been successfully achieved (see Kaufman *et al.* 1983). Such a technique might also provide useful information on the approximate timing of diploidization from the haploid cell population.

MATERIALS AND METHODS

8- to 12-week-old 129/SvE female mice were superovulated with an intraperitoneal injection of 5 i.u. PMSG followed 48 h later by a similar injection of 5 i.u. HCG. The females were autopsied 17 h later and their eggs activated following a 4·5 min exposure to a freshly prepared 7 % (v/v) solution of Analar-quality ethanol in phosphate-buffered saline (PBS). A more detailed description of this activation technique has been published elsewhere (Cuthbertson, Whittingham & Cobbold, 1981; Kaufman, 1982). As in a recent study on the isolation of pluripotential cell lines from haploid embryos (Kaufman *et al.* 1983), only those activated oocytes that developed a single haploid pronucleus following second polar body extrusion were used in this study.

All of the pronucleate-stage one-pronuclear haploid eggs were transferred to the oviducts of recipients (Tarkowski, 1959) anaesthetized with Avertin on the afternoon of the first day of pseudopregnancy (i.e. on the day that the vaginal plug had earlier been observed, following mating of the female with a vasectomized male).

The recipients were divided into two groups. The first group of recipients were autopsied at about midday on the 4th day of pseudopregnancy and the reproductive tract flushed with PBS. The recovered embryos, which were mostly at the morula stage, were then incubated for 24 h in standard mouse embryo culture medium (Whittingham, 1971). By this time all had achieved the expanded blastocyst stage. Some of these embryos were then examined at this stage by the air-drying technique (Tarkowski, 1966) and the preparations stained with Giemsa. The ICMs from additional groups of expanded blastocysts that had been retained in culture for a total of 24 h or 48 h, respectively, from the time of their initial isolation at the morula stage, were isolated by the technique of immunosurgery (Solter & Knowles, 1975; Handyside & Barton, 1977) and their total cell population established using a modification of the air-drying technique described by Evans, Burtenshaw & Ford (1972). In an additional group retained in culture for a total of 96 h, the blastocysts attached to the culture dish surface, outgrew and individual ICM-derived 'lumps' were subsequently isolated and disaggregated.

All of the blastocysts, ICMs and ICM derivatives in this series were incubated in medium containing 1 $\mu g\,ml^{-1}$ Colcemid for 6 h prior to air-drying. The two air-drying techniques employed also enabled those embryos with 'scorable' mitotic cells to be classified into various groups, namely (i) those with only haploid mitotic cells, (ii) haploid–diploid mosaics, (iii) those with only diploid mitotic cells, and (iv) haploid–diploid–tetraploid mosaics.

The second group of recipients was ovariectomized on the afternoon of the 4th day of pseudopregnancy and, while still under the influence of the anaesthetic, they were given a subcutaneous injection of 1 mg Depo-Provera (Upjohn). This group of females was subsequently autopsied 5 or 6 days later and the 'delayed' blastocysts recovered were immediately subjected to immunosurgery in order to isolate their ICM. While one group of ICMs was analysed by the modified air-drying technique of Evans *et al.* (1972) at this stage after injection of recipients with Colcemid, the remaining ICMs were explanted into standard embryo culture

medium supplemented with 10 % foetal calf serum and retained in culture for a period of either 72 h, 96 h or 144 h. In the latter three groups, the ICM-derived cells were exposed to medium containing 1 $\mu g\,ml^{-1}$ Colcemid for between 6–10 h prior to air-drying. The cells derived from individual ICMs were then isolated from tissue culture by mild trypsinization and subjected to air-drying to establish both their ploidy and the total number of cells derived from the individual ICMs.

RESULTS

(1) *Analysis of non-'delayed' intact blastocysts and ICMs derived from them*

Ten embryos that were retained in culture for 24 h after their isolation at the morula stage developed to the intact blastocyst stage and were then examined by the air-drying technique. Each embryo at this time contained, on average, a total of about 52 cells, of which approximately 13 % were in mitosis. Of the 27 % of the latter that were 'scorable', just over 60 % were haploid and the rest were diploid (Table 1, group 1). Information from the ICMs isolated by immunosurgery at about the same time from a similar group of blastocysts suggested that the ICMs contained on average about 17 cells. A slightly higher proportion of these cells was in mitosis than in the intact group; about 55 % of the mitotic preparations were 'scorable' and 90 % of the latter were still haploid (Table 1, group 2).

When the immunosurgically isolated ICMs comparable to those examined in group 2 (see above) were retained in culture for a further period of 24 h, it was apparent that the population of cells derived from individual ICMs had on average increased by just over 50 % to a total of about 26 cells per embryo (Table 1, group 3). Following an additional period of 48 h in culture, the average number of cells derived from each ICM almost doubled, to a total of about 47 cells per embryo (Table 1, group 4). In both of the latter groups, the analyses indicated that a high proportion (group 3, 72 %; group 4, 85 %) of the 'scorable' mitotic cells was still haploid.

(2) *Analysis of cell populations derived from ICMs isolated from 'delayed' blastocysts recovered at various times after the initiation of the 'delayed' state*

With the exception of the nine ICMs obtained from the 'delayed' blastocysts analysed immediately after their isolation from recipients, when all the cells were successfully disaggregated and the average number of cells present was about 27 (Table 1, group 5), the disaggregation procedure carried out on the cells derived from the ICMs from the 'delayed' group that had been retained in culture for various periods of time was always found to be incomplete. Small clumps containing groups of cells which failed to disaggregate were often observed. Only the cells that were successfully disaggregated were counted, and therefore the figures quoted in Table 1 necessarily represent mean minimum numbers of cells derived from individual ICMs.

After a 72 h period in culture, individual ICMs from 'delayed' blastocysts gave rise on average to about 54 cells per embryo (Table 1, group 6). After a further

Table 1. *A summary of the results of the disaggregation procedures carried out on intact blastocysts and isolated ICMs maintained in culture for various periods of time in order to establish their ploidy*

Group	Description	Non-'delayed' (ND) or 'delayed' (D)	Total duration in culture (h)	No. embryos	Total cells counted*	Mean cells/ embryo ± S.E.	Total cells in metaphase (%)	Total 'scorable' metaphases (%)	Ploidy		
									Haploid	Diploid	Tetraploid
1	intact blastocysts	ND	24 h	10	523	52·3 ± 7·9	70 (13·4)	19 (27·1)	11	8	—
2	isolated ICMs	ND	24 h	10	170	17·0 ± 2·4	36 (21·2)	20 (55·6)	18	2	—
3	isolated ICMs	ND	48 h	35	916	26·2 ± 1·8	138 (15·1)	81 (58·7)	58	23	—
4	isolated ICMs	ND	96 h	3	140	46·7 ± 25·2	20 (14·3)	13 (65·0)	11	2	—
5	isolated ICMs	D (5 days in 'delay')	Nil	9	240	26·7 ± 3·2	25 (10·4)	2 (8·0)	1	1	—
6	isolated ICMs	D (5 or 6 days in 'delay')	72 h	44	2366+	53·8 ± 4·2	300 (12·7)	77 (25·7)	34	33	10
7	isolated ICMs	D (6 days in 'delay')	96 h	8	543+	67·9 ± 14·6	58 (10·7)	28 (48·2)	9	18	1
8	isolated ICMs	D (5 days in 'delay')	144 h	4	348+	87·0 ± 23·4	58 (16·7)	7 (12·1)	1	6	—

* In groups 6, 7 and 8, numerous small 'clumps' of cells were often present due to incomplete disaggregation of the ICMs. In these groups, therefore, the numbers given necessarily only refer to the total number of disaggregated cells counted.

24 h incubation period, the average number of cells derived from each ICM was about 68 (Table 1, group 7) and after an additional period of 48 h in culture, each ICM gave rise to, on average, about 87 cells (Table 1, group 8). However, in contrast to the findings in the non-'delayed' series, considerably higher proportions of the 'scorable' mitotic cells encountered were either diploid or tetraploid. In these three groups, the haploid cells only constituted about 44 %, 32 % and 14 %, respectively, of the 'scorable' mitotic spreads.

DISCUSSION

One of the first observations that emerged during this study was the considerable difficulty encountered in making chromosome spreads from ICM derivatives that would allow the ploidy of individual mitotic cells to be unequivocally established. If only the non-'delayed' material is considered, the overall incidence of 'scorable' mitotic cells in this study was found to be in the region of 59 % of all the chromosome spreads examined, and even this figure was higher than the situation encountered with the 'delayed' material in which the overall incidence of 'scorable' mitotic cells was closer to 29 %. It should be noted, however, that the overall incidences of cells in mitosis in the non-'delayed' and 'delayed' series were quite similar, namely 15·1 % and 12·8 %, respectively, of all the disaggregated cells examined in these two series.

The other interesting information to emerge from this study relates to the total number of cells present in the intact non-'delayed' blastocysts, in the ICMs (from non-'delayed' and 'delayed' embryos) and in the ICM derivatives (from non-'delayed' and 'delayed' embryos) at various stages during their incubation in culture. In each of the non-'delayed' groups studied, the disaggregation procedure was completely successful and enabled an accurate estimate to be made of the mean total cell population at each time interval.

The total cell population of the haploid-derived expanded blastocysts and the approximate number of cells present in their ICMs indicated here, were surprisingly high and not greatly different from published figures for comparable stages of fertilized mouse embryonic development. For example, Horner & McLaren (1974) reported that the total cell population present in 12 blastocysts analysed was 55·3 ± 4·4, of which 16·1 ± 1·1 cells were in the ICM. Similar findings have been reported by Barlow, Owen & Graham (1972), Handyside (1978), Magnuson, Jacobson & Stackpole (1978), Rossant & Lis (1979), and Spielmann, Jacob-Müller & Beckord (1980) using different strains of mice (for detailed discussion of this topic, and growth kinetics of blastocysts, see Kaufman, 1983).

On the assumption that about one-third of the total cell population of the intact blastocysts studied consisted of inner cell mass cells, at the expanded blastocyst stage each embryo contained on average about 17 inner cell mass cells. This finding would appear to confirm previous experience with haploid-derived embryos examined at about the same stage of development, namely that the total blastocyst cell population as well as the ICM cell population present could vary

quite considerably between embryos. Whereas fertilized embryos and diploid-derived parthenogenetic blastocysts that developed from the 1-cell stage entirely in culture always contained ICMs, those isolated from immediate cleavage-derived (haploid) blastocysts that developed under similar conditions were generally reduced in size, and in a few cases appeared to be entirely devoid of ICM cells. This was, however, more likely to be encountered in those embryos that only achieved the blastocyst stage by the 6th rather than the 5th day of culture (Kaufman, 1978, 1981). The fact that the embryos in the present study had been transferred to recipients shortly after activation and developed to the morula stage *in vivo*, were genetically dissimilar to the earlier embryos studied and furthermore were of the one-pronuclear rather than immediate cleavage class, did not appear to greatly influence this finding.

In the case of the 'delayed' blastocysts, the total ICM cell population present at the time of their isolation from the recipients was significantly greater than that present in the non-'delayed' group (one-sided *t*-test against expected value, $P < 0{\cdot}05$), so that their ICM cell population became almost identical to that of the non-'delayed' expanded blastocysts that had been retained in culture for a period of 24 h. The latter finding would certainly seem to confirm the earlier contention that this procedure provides a means of substantially increasing the ICM cell population of rodent blastocysts before implantation (Surani, 1975). In the remaining 'delayed' groups, however, because small numbers of cells invariably remained in clumps despite the disaggregation procedure, estimates of the mean cell number present at each stage necessarily only refer to those cells that were disaggregated into single cells. The latter values therefore represent mean minimum estimates of the total number of cells present at intervals during the culture period.

Certain interesting trends may be discerned from the cytogenetic findings reported here. It was of interest, for example, that the relatively high incidence of diploid mitotic cells observed in the intact non-'delayed' blastocysts was not seen in the ICMs isolated from a similar group of embryos. The simplest and most likely explanation for this observation is that the ICMs at the time of their isolation, principally contained haploid cells, whereas a considerable proportion of the diploid cells observed in the intact group were in all probability trophectodermal in origin. Following an additional period in culture, the proportion of haploid mitotic cells observed in the ICM derivatives remained fairly stable. Initially, haploid mitotic cells accounted for 90 % of all 'scorable' preparations. Comparable figures after an additional 24 h and 72 h of culture were 72 % and 85 %, respectively. These figures are, however, statistically not significantly different.

In the case of the 'delayed' material analysed, no information was gained on the ploidy of these embryos at the time of their isolation from recipients. What was apparent, however, was the fact that the proportion of haploid cells encountered diminished markedly as their duration in culture increased. About one-half of the 'scorable' mitotic cells were still haploid after 72 h; after 96 h in culture this diminished to about one-third of all the mitotic cells scored, whereas after 144 h in

culture, only one out of seven mitotic cells scored was still haploid. Because of the relatively small numbers involved, it was not possible to establish whether this reduction in the proportionate incidence of haploid compared to diploid mitotic cells between the 96 h and 72 h groups, and between the 144 h and 96 h groups was statistically significantly different. The reduction in the proportionate incidence of haploid mitotic cells observed between the 144 h and the 72 h groups, however, was statistically significant ($P < 0{\cdot}05$). Similar findings were also reported when egg-cylinder-stage haploid-derived embryos were analysed at a stage approximately equivalent to fertilized embryos of between 6·5 and 7·5 days of gestation (Kaufman, 1978). The embryos in this series had also previously been held in 'delay' (for about 4 days) prior to their implantation.

Because of the relatively small proportion of 'scorable' mitotic cells encountered, only a limited degree of extrapolation is possible from these cytogenetic analyses, though we have no reason to believe that the data presented here do not provide a representative cross-section of the ploidy of the cell populations studied. A more useful approach which could be employed in future studies would be to analyse all of the disaggregated cells by nuclear densitometry. This technique might be expected to provide a more accurate means of assessing the ploidy of a higher proportion of the ICM-derived cells than was possible in the present study. Despite these reservations, we believe that the findings reported here indicate that it may eventually be possible to establish haploid mammalian cell lines, as reasonable numbers of haploid mitotic cells were still encountered at least 96 h following the explantation of ICMs into tissue culture. The present study would, however, appear to indicate that the chance of obtaining haploid cell lines in the future may be increased by using as source material ICMs derived from non-'delayed' blastocysts despite their initial reduced total cell number at the time of isolation compared to ICMs isolated from 'delayed' blastocysts.

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