Binucleate blastomeres in preimplantation human embryos *in vitro*: failure of cytokinesis during early cleavage

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The nuclei of disaggregated blastomeres from two hundred preimplantation human embryos were examined between days 2 and 4 after insemination *in vitro* by vital labelling with a polynucleotide-specific fluorochrome. Although the majority of blastomeres had a single nucleus, binucleate blastomeres containing two nuclei of equal size were common and other blastomeres had fragmented nuclei or were anucleate. Seventeen per cent of normally fertilized embryos at the two- to four-cell stage had at least one binucleate blastomere, and this increased to 65% at the nine- to 16-cell stage when individual embryos had between one and six binucleate blastomeres. The proportion of binucleate blastomeres in normally fertilized embryos increased from 5 to 10% over this period, whereas in abnormally fertilized, polyspermic or parthenogenetic, embryos the proportion was significantly higher during early cleavage stages but decreased at the nine- to 16-cell stage when the majority of these embryos arrest (25 and 6%, respectively). The incidence of anucleate blastomeres in normally fertilized embryos was also high, especially in those of poor morphology. In contrast, blastomeres with fragmented nuclei were relatively uncommon and the incidence was variable among classes and stages of development. Estimates of the volume of binucleate blastomeres based on measurement of their diameters and comparison with mononucleate blastomeres at various cleavage stages indicated that these blastomeres arise from a failure of cytokinesis between the second and fourth cleavage divisions. On this basis, assignment of binucleate blastomeres to particular cleavage stages in normally fertilized day 4 embryos suggests that at least some of these blastomeres arising during early cleavage persist without further cell division for up to 48 h. At the cellular level, therefore, blastomeres with either binucleate or abnormal nuclei contribute to cleavage stage arrest *in vitro*.

Introduction

In humans, after *in vitro* fertilization (IVF), normally fertilized embryos develop to the blastocyst stage *in vitro* by day 5 or 6 after insemination and by day 7 have about 125 cells, two-thirds of which form the outer trophoctoderm and one-third the inner cell mass (Hardy *et al.*, 1989a). However, only about half reach the blastocyst stage, while the other half remain arrested predominantly at eight-cell and earlier cleavage stages (Hardy *et al.*, 1989a, b). Even after transfer to the uterus on day 2 or 3, only 15–30% of embryos implant and establish pregnancies (ILA report, 1991; Hardy, 1993).

The reasons why so many human embryos undergo cleavage arrest *in vitro* or fail to develop *in vivo* are not fully understood. A high incidence of gross genetic defects is likely to be responsible for some of the losses (Plachot *et al.*, 1987). In addition, the simple culture media used routinely for IVF are based on those that have been used successfully in other animal species, particularly mice, and may not be optimal or sufficient for development beyond early stages *in vitro*. However, a feature of the preimplantation human embryo *in vitro* is the prevalence of

morphological abnormalities, including uneven cleavage, cytoplasmic fragmentation and degenerate cells. In particular, various nuclear abnormalities have been reported, including pseudo-nuclei (Tesarik *et al.*, 1987) and either fragmented, swollen and 'flocculent' nuclei, or total absence of a nucleus (Hardy *et al.*, 1989a). In addition, blastomeres with two or more nuclei have been observed in embryos fertilized both *in vivo* and *in vitro* (Hertig *et al.*, 1954; Sathananthan *et al.*, 1982; Lopata *et al.*, 1983; Trounson and Sathananthan, 1984; Plachot *et al.*, 1986; Braude *et al.*, 1990; Winston *et al.*, 1991) and tetraploid and polyploid nuclei have been detected by microfluorimetry and *in situ* hybridization at cleavage and blastocyst stages (Angell *et al.*, 1987; West *et al.*, 1988; Griffin *et al.*, 1991).

Whatever their respective causes, many of these morphological and nuclear abnormalities clearly contribute to failure of early human development. Binucleate, multinucleate and polyploid blastomeres, however, may be induced by culture *in vitro*, or, alternatively, represent stages in the formation of polyploid trophoblast cells or multinucleate syncytiotrophoblast (West *et al.*, 1989). During early mouse development, binucleate cells occur in the outer cells of morulae (Soltynska *et al.*, 1985) and in trophoblast cells of the ectoplacental cone (Ilgren, 1981), both of which form trophoblast giant cells which later become

progressively more polyploid through a process of endoreduplication in which repeated DNA replication occurs in the absence of cell division.

To contribute additional information, particularly in relation to binucleate blastomeres, we have examined the nuclei of disaggregated blastomeres from normally and abnormally fertilized cleavage stage human embryos, by vital labelling with a DNA-specific fluorochrome, between days 2 and 4 over the period of cleavage arrest *in vitro*. On day 4, the diameter of each blastomere was measured to calculate the cytoplasmic volume. Since cleavage divisions successively subdivide the cytoplasm of the zygote and there is no cellular growth, blastomeres become proportionately smaller at later stages. Thus, by reference to the volume of blastomeres from normal embryos that had uniformly and evenly completed particular cleavage divisions, it has been possible to determine how many divisions a blastomere has undergone.

We conclude that binucleate cells are formed by a failure of cytokinesis in the second, third or fourth cleavage divisions, with a peak at the third division, and that the persistence of some binucleate blastomeres for one or two days after their initial formation suggests that they may have undergone cleavage arrest. However, the causes of this are unknown.

Materials and Methods

Human preimplantation embryos

The work reported here has been approved by the Human Fertilization and Embryology Authority for Human In-Vitro Fertilization and the Research Ethics committee of the Royal Postgraduate Medical School. Surplus human embryos were obtained with permission from patients undergoing *in vitro* fertilization (IVF), using a method of superovulation described by Rutherford *et al.* (1988). After pituitary–gonadal suppression with an LHRH agonist (Buserelin, Hoechst, Hounslow), patients were superovulated with human menopausal gonadotrophin (hMG; Pergonal, Serono, Welwyn Garden City). Human chorionic gonadotrophin (hCG, Pregnyl, Organon, Cambridge), 10 000 iu was given 34 h before egg collection.

Oocytes were collected, preincubated, inseminated (day 0) and checked for pronuclei the following day, as described by Hillier *et al.* (1984). Embryos were cultured in 1 ml of Earle's balanced salt solution (Gibco, Paisley) supplemented with 25 mmol sodium bicarbonate l^{-1} (BDH, Lutterworth) and 0.47 mmol pyruvic acid l^{-1} (Sigma, Poole), containing 10% heat-inactivated maternal serum under a gas phase of 5% CO₂, 5% O₂ and 90% N₂. On day 2, each fertilized embryo was examined and up to three were selected on the basis of their morphology for embryo transfer on day 2 or 3. After confirming the patients' consent, the surplus embryos were allowed to develop *in vitro* in the original medium.

Classification and grading

Oocytes and embryos were classified initially according to the number of pronuclei visible on day 1 (16 h after insemination) as follows: (i) fertilized embryos (with two pronuclei); (ii)

polyspermic embryos (with three or more pronuclei); and (iii) unfertilized oocytes (with one or no pronucleus). Later on day 2, embryos were graded according to evenness of blastomeres, fragmentation and presence of cellular debris from perfectly symmetrical embryos with no fragmentation (grade I) to embryos having one intact blastomere with gross fragmentation (grade IV) or being totally degenerate (grade V). In addition, unfertilized oocytes that cleaved were reclassified as parthenogenetic embryos.

Disaggregation of embryos

Embryos on days 2, 3 and 4 had their zonae pellucidae removed using acid Tyrodes' solution (Nicolson *et al.*, 1975), pH 2.4, and were washed in Medium M2 supplemented with 4 mg BSA ml^{-1} (Crystalline, ICN Biochemicals, High Wycombe) (Quinn *et al.*, 1982). They were disaggregated by gentle pipetting with a flame polished pipette with an internal diameter just smaller than the embryo. With day 4 embryos that were starting to compact, embryos were incubated for 15 min in Hanks' balanced salt solution without calcium and magnesium (Imperial Laboratories, Andover) supplemented with 6 mg BSA ml^{-1} . Preincubation under calcium- and magnesium-free conditions causes decompaction, facilitating easy disaggregation.

Vital labelling of nuclei

The nuclei were vitally labelled with the polynucleotide-specific fluorochrome Hoechst 33342, by a modification of the method of Critser and First (1986). Isolated blastomeres were incubated in M2 plus BSA containing 5 μg Hoechst 33342 ml^{-1} (Sigma) for a minimum of 5 min. All the blastomeres from a single embryo were then mounted in a group in M2 plus BSA in a chamber constructed from a coverslip raised 1 mm from the surface of a microscope slide with small amounts of Blu-Tack (Bostik, Leicester). Labelled nuclei were examined by fluorescence microscopy with epifluorescent UV illumination using the appropriate filters (A2: Leica UK, Milton Keynes). The number of blastomeres with (i) a single normal sized nucleus (mononucleate); (ii) two (binucleate) or more (multinucleate) equally sized nuclei; (iii) two or more nuclei of various sizes (mainly fragmenting) or (iv) no nucleus (anucleate) were recorded. Groups of blastomeres from each embryo were viewed with a $\times 20$ objective and photographed with an Olympus OM2 camera and Ilford HP5 film, both under fluorescence and under Hoffman contrast optics. This method allowed mononucleate and binucleate blastomeres to be identified to correlate cell size with the number of nuclei. Negatives showing labelled nuclei were mounted as slides and viewed with a Wotan Diastar 320 slide viewer, which enlarges the image, and enabled the accurate measurement of the diameter of the nuclei.

Staging of blastomeres

During cleavage, blastomeres become progressively smaller, as there is no cellular growth. Embryos at intermediate cleavage stages consist of a mixture of blastomeres from two consecutive cleavage stages. For example, a six-cell embryo will consist of

Table 1. Number of cells in cleavage stage human embryos between days 2 and 4 after insemination

Day (h after insemination)	Number of pronuclei	Morphology ^a	Number of embryos	Mean number of cells (range)
2 (46–50)	2	Good	13	4.0 ± 0.3 (2–7)
		Poor	18	4.3 ± 0.2 (3–6)
	> 2	All	4	6.0 ± 0.9 (4–8)
		All	1	5.0
3 (65–76)	2	Good	51	6.9 ± 0.3 (2–15)
		Poor	20	6.9 ± 0.5 (3–11)
	> 2	All	13	8.2 ± 0.7 (4–13)
		All	7	5.1 ± 1.0 (2–8)
4 (90–97)	2	Good	35	12.1 ± 0.7 (5–23)
		Poor	10	10.2 ± 0.7 (8–16)
	> 2	All	6	10.8 ± 1.3 (6–14)
		All	2	12.0 ± 1.0 (11–13)

^a'Good embryos' are those that are scored as grades 1 and 2 on day 2; 'poor' embryos are those that are scored lower than grade 2.

two larger 'four-cell' blastomeres which still have to divide, and four smaller 'eight-cell' blastomeres. The embryonic stage which individual blastomeres within an embryo had attained was calculated as follows. The expected volume of two-, four-, eight- and 16-cell blastomeres was ascertained by photographing intact and disaggregated grade I embryos with even blastomeres and no fragments at these stages and the diameter of the blastomeres were measured as described above. The range and mean of volumes of two-, four-, eight- and 16-cell blastomeres were then calculated. Binucleate and mononucleate blastomeres identified as described in the previous section could be similarly measured and, on the basis of whether their volumes fell within the range of volumes of reference blastomeres at a particular cleavage stage, assigned to that stage. A minority of blastomeres that fell outside these ranges were assigned on the basis of which mean reference volume they were closest to.

Statistical analysis

The differences between distributions of numbers and sizes of blastomeres were analysed using the Wilcoxon Rank-Sum (Mann-Whitney) test.

Results

Cell number and cleavage rate

Two hundred surplus embryos from 37 patients (5.4 ± 0.5 per patient, range 1–16) were disaggregated into individual blastomeres, where possible, or small groups of blastomeres. Nineteen embryos that were so fragmented that it was impossible to determine cell number accurately and one embryo which was fertilized a day late were excluded from analysis.

For normally fertilized embryos between days 2 and 4, numbers of blastomeres ranged between 2 and 7 on day 2 to between 5 and 23 on day 4 (Table 1). Thus, blastomeres from all stages between the two- and 32-cell stages, resulting from the

first to the fifth cleavage divisions, were represented. Cleavage rates in normally fertilized embryos of different morphological grades were similar. Polyspermic embryos on days 2 and 3 appeared to have more blastomeres possibly resulting from some embryos dividing into three blastomeres at the first cleavage division because of the formation of a tripolar spindle (Kola *et al.*, 1987; Plachot *et al.*, 1989).

Analysis of blastomere nuclei

Although the majority of blastomeres had single nuclei, binucleate blastomeres were common (Figs 1a and 2a), as were anucleate blastomeres (Fig. 2c). A minority of blastomeres had abnormal, multiple nuclei (Figs 2b, c and d), which varied in morphology. Some of these blastomeres had three unevenly sized nuclei which either had a similar labelling intensity to binucleate blastomeres (Fig. 2b) or were swollen, with less intense 'flocculent' labelling (Fig. 2c). Finally, there were blastomeres in which the nuclei were clearly fragmenting (Fig. 2d).

The nuclei in binucleate blastomeres were of the same size as those in mononucleate cells from the same embryo. In nine day-4 embryos, the diameters of the nuclei were $16.7 \pm 0.4 \mu\text{m}$ ($n = 39$) and $16.6 \pm 0.3 \mu\text{m}$ ($n = 67$) in binucleate and mononucleate blastomeres, respectively. Multinucleate blastomeres with more than two nuclei of equal size were rarely observed.

The incidence of binucleate blastomeres was similar in embryos of good and poor morphology (Table 2). The only major difference between normally fertilized embryos of different grades was a significantly higher proportion of anucleate blastomeres in those classified as having poor morphology. The proportion of blastomeres with single nuclei declined between the two- to four-cell stages and later cleavage stages indicating a continued increase in the numbers of binucleate blastomeres and those with nuclear abnormalities (Fig. 3). In contrast, abnormally fertilized embryos had a considerably lower proportion of blastomeres with single nuclei than did normally fertilized embryos at early cleavage stages, mainly as a result of large

Fig. 1. (a) Fluorescence and (b) Hoffman contrast micrographs of a disaggregated normally fertilized 14-cell human embryo cultured *in vitro*, of good morphology (grade 1.5), on day 4 after insemination. The nuclei have been labelled with Hoechst 33342. Binucleate blastomeres (arrows) are approximately twice the volume of blastomeres with one nucleus. (Bar = 100 μm)

numbers of binucleate blastomeres and those with fragmenting nuclei, but this proportion increased as cleavage progressed.

Seventeen per cent of embryos had one or more binucleate cells at the two- to four-cell stage and this increased to 65 per cent at the nine- to 16-cell transition (Fig. 4a). The numbers of binucleate blastomeres in each embryo was variable; four-cell embryos never had more than a single binucleate blastomere, whereas embryos at later stages could have two, three or more. The maximum number was six in an eight-cell embryo on day 4 (Fig. 2a). In parallel with the overall increased proportion of anucleate blastomeres in embryos graded as having poor morphology, their distribution was also more widespread in these embryos compared with those of good morphology (Fig. 4b). The percentage of embryos with blastomeres with fragmented nuclei increased but remained low at later cleavage divisions (Fig. 4c).

Fewer abnormally fertilized embryos were analysed, but the pattern of distribution of the blastomeres of different types was similar (Table 3). The only exception was that about twice the number of polyspermic embryos at the five- to eight-cell stage had one or more binucleate blastomeres.

Staging of blastomeres

Binucleate and mononucleate blastomeres were identified in fifteen disaggregated day 4 embryos that were normally fertilized (mean cell number 12.00 ± 1.17 , range 6–23 blastomeres) (Table 4). The volumes of a total of 158 blastomeres were measured of which 34 were binucleate and 124 were mononucleate. A further 14 blastomeres were not included: nine were lysed, three had fragmented nuclei and two were anucleate. The mean volume of binucleate blastomeres ($8.68 \times 10^4 \pm 6.3 \times 10^3 \mu^3$) was significantly greater than the mean volume of mononucleate blastomeres ($3.79 \times 10^4 \pm 2.1 \times 10^3 \mu^3$) ($P = 0.0001$). The majority (89%) of blastomeres were assigned to cleavage stages according to which reference range of volumes they matched (Table 4). Eighteen out of 158 blastomeres (11%) were not within these ranges and therefore were assigned on the basis of which mean reference volume for a particular cleavage stage they were closest to. Although the majority of mononucleate blastomeres (69%) were at the 16-cell stage and a further 10% had reached the 32-cell stage, the majority of binucleate blastomeres (65%) were only at the equivalent of the eight-cell stage with only 15% reaching the 16-cell stage (Fig. 5).

Fig. 2. Fluorescence micrographs of nuclei of disaggregated human embryos cultured *in vitro* labelled with Hoechst 33342. (a) Normally fertilized grade 2 eight-cell embryo, on day 4 after insemination, showing six binucleate blastomeres. (b) Parthenogenetic grade 3 eight-cell embryo, on day 3 after insemination, showing two binucleate blastomeres and two blastomeres with three nuclei. (c) Partial spread of a normally fertilized grade 2.5 ten-cell embryo, on day 4 after insemination, clearly showing one blastomere with three swollen, flocculent nuclei. Note the anucleate cell (arrow). (d) Partial spread of a normally fertilized grade 2 seven-cell embryo, on day 3 after insemination showing a blastomere containing multiple small, pale labelled nuclear fragments (arrow). (Bar = 100 μ m)

The difference in cleavage stage between the binucleate and mononucleate blastomeres is clearly shown (Fig. 1). All twelve mononucleate blastomeres are at the 16-cell stage, whereas the two binucleate blastomeres are double the volume and are still at the eight-cell stage.

Discussion

Analysis of the nuclei of disaggregated blastomeres from normally and abnormally fertilized human cleavage stage embryos between

days 2 and 4 revealed a high incidence of binucleate and anucleate blastomeres and other nuclear abnormalities (mainly fragmentation) with a widespread distribution between embryos. Anucleate blastomeres and those with fragmenting nuclei or other abnormalities clearly lack the potential for further development and are likely, therefore, to contribute to the process of cleavage arrest *in vitro* and the low pregnancy success rate *in vivo* after transfer. However, the combined incidence of these anomalies in normally fertilized embryos, even in those of poor morphology, affected a maximum of 16% of cells at the nine- to 16-cell stage. Embryos lacking one or two blastomeres at about

Table 2. The percentage of binucleate and anucleate blastomeres and blastomeres with fragmented nuclei in normally fertilized human preimplantation embryos

Morphology ^a	Stage (cell)	Number of embryos	Number of blastomeres				Total binucleate or abnormal (%) ^b
			Total	Binucleate (%) ^b	Anucleate (%) ^b	Fragmented (%) ^b	
Good	2-4	19	71	3 (4)	2 (3)	1 (1)	6 (8)
	5-8	47	331	27 (8)	18 (5)	11 (3)	56 (17)
	9-16	29	345	38 (11)	17 (5)	10 (3)	65 (19)
	> 16	4	79	7 (9)	5 (6)	1 (1)	13 (16)
Poor	2-4	16	59	3 (5)	7 (12)	0	10 (17)
	5-8	21	143	8 (6)	22 (15)	2 (1)	32 (23)
	9-16	11	117	16 (14)	13 (11)	3 (3)	32 (27)

^a'Good embryos' are those that are scored as grades 1 and 2 on day 2; 'poor' embryos are those that are scored lower than grade 2.

^b% of total number of cells.

Fig. 3. Percentage of blastomeres in all preimplantation human embryos cultured *in vitro*, either normally fertilized of good or poor morphology, or abnormally fertilized, which have one nucleus per blastomere (□); two nuclei per blastomere (■); more than two nuclei per blastomere or nuclei of varying sizes (▨) and no nuclei per blastomere (anucleate) (□). Numbers in parentheses indicate the total number of blastomeres examined.

the eight-cell stage, either after cryopreservation or embryo biopsy, can give rise to pregnancies (Trownson and Mohr, 1983; Handyside *et al.*, 1990), as can embryos known to have multinucleated blastomeres (on the basis of light microscope observations) at the time of transfer (Mohr *et al.*, 1983). Nevertheless, these anomalies are likely to contribute to reduced viability and may reflect other disturbances of development.

Binucleate blastomeres were common. The significance of cells with two apparently normal nuclei of equal size is not clear. Various mechanisms have been described for the formation of binucleate and multinucleate cells, including cell fusion,

nuclear amitotic splitting and acytokinesis. Cell fusion is typical of the formation of osteoclasts and striated muscle, and may also be involved in the formation of syncytiotrophoblast. Although the possibility that cell fusion is involved cannot be excluded, it is considered unlikely. In mice, electrophoretic separation of the dimeric enzyme marker glucose phosphate isomerase (GPI) in decidual giant and multinucleated cells from a mouse chimaeric for two electrophoretic variants failed to show an intermediate band indicative of heterokaryon formation between two or more cells of different GPI variants, indicating that cell fusion was not the mode of formation of

Fig. 4. Percentage of normally fertilized human embryos of (■) good morphology and (□) poor morphology with (a) one or more binucleate, (b) anucleate or (c) multinucleate blastomeres at advancing preimplantation stages *in vitro*. Nineteen, 47 and 29 embryos of good morphology and 16, 21 and 11 embryos of poor morphology at the two- to four-cell, five- to eight-cell and nine- to sixteen-cell stages, respectively, were examined.

binucleate cells (Ansell *et al.*, 1974). However, this approach is not possible with human embryos. An alternative would be to use time lapse cinematography to examine whether cell fusion is involved with the formation of binucleate cells in human preimplantation embryos. In cultured hepatocytes, such a technique was used successfully to demonstrate that mononucleate cells proceed through telophase, but fail to undergo cytoplasmic cleavage, producing cells with two nuclei (Sattler *et al.*, 1988).

Nuclear amitotic splitting involves division of the nucleus with no chromosome replication, and has been observed in liver parenchyma cells as constricted and haploid nuclei (reviewed by Carriere, 1969). However, the failure to observe any constricted nuclei in all of the 1406 blastomeres examined, coupled with the similarity in size between nuclei from binucleate and mononucleate cells, suggests that this mechanism is not involved.

Our results strongly suggest that binucleate blastomeres in human preimplantation embryos arise through acytokinesis, related to cleavage stage arrest. The volume of binucleate blastomeres was almost always greater than the mononucleate blastomeres in the same embryo indicating that those particular blastomeres are still at earlier cleavage stages. The presence of binucleate blastomeres which are still at the four- and eight-cell stages along with mononucleate blastomeres at the 16- and even 32-cell stages within the same embryo shows that these blastomeres persist for at least 48 h and suggests that they

have arrested. Similarly, in mice, binucleate blastomeres were larger (Soltynska *et al.*, 1985; Hillman and Hillman, 1975) indicating that a similar mechanism operates. In addition, binucleate blastomeres are characteristic of both homozygous ℓ^{w32}/ℓ^{w32} and ℓ^{12}/ℓ^{12} embryos, the majority of which die at the morula stage (Hillman and Hillman, 1975; Hillman *et al.*, 1970), further implicating binucleate blastomeres in developmental arrest. However, although binucleate blastomeres in both mouse and human cleavage stage embryos may be symptomatic of the underlying cause for arrest, they cannot themselves be entirely responsible since only in rare cases did embryos have a majority of these blastomeres. Furthermore, large cells resembling blastomeres at the 16-cell stage have been observed in the blastocoel of human blastocysts (Hardy, 1993), indicating that development to the blastocyst stage can continue despite the arrest of one or more cells at cleavage stages.

Alternatively, binucleate and multinucleate or polyploid cells may be interrelated (Fig. 6) and it has been suggested that polyploid cells, detected at cleavage and blastocyst stages (Angell *et al.*, 1987; West *et al.*, 1988; Griffin *et al.*, 1991) derived through an intermediate binucleate stage, represent an early stage in the development of the syncytiotrophoblast (West *et al.*, 1989). Binucleate blastomeres have been observed in mouse embryos at the morula stage and it was suggested that these blastomeres were the precursors for mural-trophoblast giant cells (Soltynska *et al.*, 1985). Giant cells in mice are formed in the mural trophoblast of the blastocyst at the time of implantation, by a process of endoreduplication involving DNA replication without cytokinesis (Barlow *et al.*, 1972). Giant cells are also formed at the periphery of the ectoplacental cone which is derived from polar trophoblast. Ilgren (1981) found both binucleate and polyploid cells in ectoplacental cone cultures and suggested that binucleate cells gave rise to polyploid cells by nuclear fusion and endoreduplication. However, it seems unlikely that this is the case in humans since binucleate cells arise predominantly in the early cleavage divisions, before trophoblast differentiation, and multinucleate cells were very rarely observed. It seems more likely that syncytiotrophoblast arises by fusion of mononucleate cytotrophoblast cells as originally suggested by Boyd and Hamilton (1970).

The peak incidence for binucleation occurred during the third cleavage division in the study reported here. This peak coincides with the onset of embryonic gene expression in humans (Braude *et al.*, 1988; Tesarik, 1987, 1988). In mice, embryos from outbred strains arrest at the two-cell stage *in vitro* and this phenomenon is known as the 'two-cell block', when embryonic gene expression is initiated in mice (Flach *et al.*, 1982; West and Green, 1983; Bolton *et al.*, 1984). Injection of cytoplasm from nonblocking strains to blocking strains at the two-cell stage allowed a higher proportion of embryos that normally block at the two-cell stage to develop to the blastocyst stage, showing that a cytoplasmic factor is responsible for this arrest (Pratt and Muggleton-Harris, 1988). Furthermore, there is evidence in *Drosophila* that early cleavage divisions depend on maternally derived transcripts (O'Farrell *et al.*, 1989). It is possible that, in humans, deficiencies in maternal macromolecules, possibly as a result of inadequate oocyte maturation, result in a failure of cytokinesis in some blastomeres during early cleavage before embryonic transcription takes over. Potential candidate molecules involved in cytokinesis include cytoskeletal components

Table 3. Proportion of abnormally fertilized human embryos having one or more binucleate or anucleate blastomeres or blastomeres with fragmented nuclei

Abnormality of fertilization	Stage (cell)	Number of embryos			
		Total number	With binucleate blastomeres (%)	With anucleate blastomeres (%)	With blastomeres with fragmented nuclei (%)
Polyspermic	2–4	2	1	1	1
	5–8	12	10 (83%)	3 (25%)	4 (33%)
	9–16	9	5 (56%)	5 (56%)	1 (11%)
Parthenogenetic	2–4	3	1	0	1
	5–8	5	2 (40%)	3 (60%)	0
	9–16	2	1	2	0

Table 4. Volumes of blastomeres from grade I ‘reference’ human embryos with even blastomeres and no fragments

Stage (cell)	Number of blastomeres	Mean blastomere volume (μ ³)	Range (μ ³)
2	5	$3.17 \times 10^5 \pm 1.5 \times 10^4$	2.7×10^5 – 3.6×10^5
4	21	$1.42 \times 10^5 \pm 6.8 \times 10^3$	1.2×10^5 – 2.2×10^5
8	27	$7.47 \times 10^4 \pm 2.1 \times 10^3$	5.8×10^4 – 1.0×10^5
16	14	$2.97 \times 10^4 \pm 2.4 \times 10^3$	2.0×10^4 – 4.5×10^4
32*	—	1.29×10^4	

*Extrapolated from a least squared fit to the observed volumes of two-, four-, eight-, and 16-cell blastomeres.

Fig. 5. Embryonic stage of 34 individual binucleate and 124 mononucleate blastomeres in fifteen day 4 normally fertilized human embryos. (□) mononucleate blastomeres and (■) binucleate blastomeres. One of the mononucleate four-cell blastomeres was in mitosis. Nine lysed, three multinucleate (at the four-, eight- and sixteen-cell stage) and two anucleate (at the 32-cell stage) blastomeres were excluded.

crucial for polarization, cell division and compaction (Johnson and Maro, 1986).

Finally, failure of cytokinesis may result from defects at the cell surface level, affecting cell–cell interactions and therefore the shape and behaviour of the cell. After disaggregation of 16-cell mouse embryos and further culture, binucleate blastomeres were observed, the majority of which failed to divide (Sherman and Atienza-Samols, 1979). Culturing fibroblasts or certain epithelial cells under conditions that inhibit cell spreading causes an inhibition of cytokinesis and the formation of binucleate cells. When reattachment to the substrate is then allowed, the cells extrude their extra nuclei and become mononucleated (reviewed by Ben-Ze’ev, 1985). It is possible that certain blastomeres in embryos growing *in vitro* fail to form the necessary interactions with neighbouring blastomeres, resulting in binucleation. Further evidence, in mouse embryos, for the importance of cell interactions in cytokinesis is provided by the observation that a reversible uncoupling of gap junctions between blastomeres occurs during mitosis, an event thought to be crucial in cell division (Goodall and Maro, 1986; Larsen and Wert, 1988).

In conclusion, we suggest that both binucleate blastomeres and blastomeres with nuclear abnormalities result from a process of developmental arrest at the cellular level, which may contribute to the overall low pregnancy rate after transfer

Fig. 6. Hypothetical model demonstrating the possible inter-relationships among binucleate, anucleate, multinucleate and polyploid blastomeres in human preimplantation embryos. 2c: two-cell; 4c: four-cell.

following IVF. It is not known whether these various abnormalities are caused independently or whether they are inter-related. Various possible interrelationships are suggested (Fig. 6). Under optimal conditions, blastomeres undergo normal cell division and cytokinesis, producing two mononucleate daughter blastomeres. However, if the blastomere is subject to suboptimal conditions, either in the form of less than adequate culture conditions, chromosomal abnormalities, defective cell surface properties or lack of the molecular components that trigger cytoplasmic division, cytokinesis fails and a binucleate cell is formed, which arrests and persists for some time. Multinucleate blastomeres, although rarely observed, could be formed by continued karyokinesis. Fragmenting nuclei may represent a form of cell death similar to apoptosis, and further degradation of these fragments would produce an anucleate cell. An alternative route for the production of anucleate and binucleate blastomeres would be that cytokinesis does occur, but that it is asymmetric, so that one of the daughter blastomeres has two nuclei and the other, smaller one, is anucleate. The polyploid blastomeres seen in cleavage stage embryos (Angell *et al.*, 1987; West *et al.*, 1988) could be produced by endoreduplication of mononucleate blastomeres, fusion of nuclei in binucleate blastomeres, or by the formation of one mitotic spindle during possible division of a binucleate cell, forming two mononucleate daughter blastomeres with polyploid nuclei. Further work needs to be done to follow the fate of binucleate blastomeres in human embryos and to ascertain which of these pathways operates.

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