Correlation between cytological and morphogenetic effects of acute X-irradiation of preimplantation mouse embryos cultured *in vitro*

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Summary. Inhibition of cavitation by acute X-irradiation of 2-cell stage eggs was due to the early killing of the cells. Eggs in which cavitation did take place had a smaller number of cells due mainly to mitotic delay, leading to lack of inner cell mass (ICM) and its derivatives in further development. A similar effect is responsible for the inhibition of ICM formation after irradiation of morulae.

Introduction

It has been shown that X-irradiation of preimplantation mouse embryos *in vivo* leads to a high rate of prenatal deaths either just before or soon after implantation (see review by Russell, 1965). More recently, studies *in vitro* have shown that X-irradiation of 2-cell or 4-cell stages inhibits blastulation, the effect being dose-proportional (Fisher & Smithberg, 1973; Alexandre, 1974; Kirkpatrick, 1974; Goldstein, Spindle & Pedersen, 1975) and related to the time of the cell cycle (DuFrain, 1976). In contrast, irradiation of morulae does not prevent cavitation but it does impair the hatching process (Alexandre, 1974; Goldstein *et al.*, 1975) and the growth of the inner cell mass (ICM) *in vitro* (Goldstein *et al.*, 1975).

The apparently selective radiobiological effect of X-rays on the growth of the ICM, which suggests a lack of totipotency, has also been reported by Snow (1973a, b), who cultured 2-cell mouse eggs for long periods in low concentrations of [³H]thymidine. In this case, the failure of ICM formation seems to be correlated with a delay in mitotic rate occurring at the 16–32-cell stage. However, Kelly & Rossant (1976) have clearly shown that 8-cell mouse blastomeres, short-term labelled with [³H]thymidine, are able to colonize the ICM after aggregation with unlabelled cells.

Since hatching *in vitro* is almost certainly mechanical, the expansion of the blastocyst causing the zona pellucida to split, the failure of spontaneous hatching after X-irradiation (Alexandre, 1974) may be ascribed to a reduction in the number of cells in the blastocyst. Tarkowski & Wroblewska (1967) have clearly established that morulae with a reduced cell number develop into vesicular structures consisting purely of trophoblast and it is conceivable therefore that the impaired formation and growth of the ICM after X-irradiation could result from a random reduction in the cell number of embryos undergoing cavitation.

The present work was performed to correlate more precisely the morphogenetic effects of acute X-irradiation with some of the cytological effects.

Materials and Methods

Random-bred albino female mice, 6–10-week-old virgins, were induced to superovulate by intraperitoneal injections of 5 i.u. PMSG (Gestyl: Organon) at 17:00–18:00 h followed by 5 i.u. hCG (Pregnyl: Organon) 48 h later. They were then caged with males overnight. Fertilization was assumed to occur at 06:00 h on the following day (Day 1 of pregnancy, time 0 of embryonic development). The

2-cell eggs were removed on Day 2 of pregnancy by flushing the oviducts and placed in organ culture dishes (Falcon Plastics) in drops of culture medium (Whittingham, 1971) under paraffin oil (Brinster, 1963) for incubation at 37°C in a humidified atmosphere of 5% CO₂ in air.

Irradiation conditions were as previously described (Alexandre, 1974); the exposure rate was 600 R/min.

The cell number was ascertained by counting nuclei, including mitotic figures, in preparations fixed on slides with ethanol:acetic acid (3:1 v/v) and air-dried (Tarkowski, 1966). All the preparations were stained with Giemsa stain.

The cell numbers of control and irradiated embryos were first analysed after development for 70 h *in vitro* (95-h-old embryos). The embryos were irradiated at two different stages of development: (1) soon after removal from the oviducts, at the 2-cell stage (35-h-old) embryos, and (2) at the late morula stage *in vitro* (76-h-old embryos). Doses of 50, 200 and 500 R were applied because they are known to have a very small, a 50% and a total effect respectively on cavitation (Alexandre, 1974).

Results and Discussion

As shown in Table 1 there was a large heterogeneity in the distributions of the blastocysts according to their cell number. This has also been shown by Barlow, Owen & Graham (1972) and Smith & McLaren (1977) and can be partly correlated with an asynchrony in fertilization time and mitosis (Barlow *et al.*, 1972). Control embryos with less than 16 cells correspond mainly to the normal proportion of embryos which fail to develop to the blastocyst stage *in vitro*, but the effect of increasing radiation dose is clear (Table 1), and indicates that the inhibition of cavitation corresponds to an early cell death before the onset of cavitation. Cytological observations support this belief: most of the blastomeres of the irradiated embryos (mainly after 500 R and, to a lesser extent, 200 R) were aneuploid, as shown by the presence of chromatin bridges, subnuclei and fragmented metaphase chromosomes. Similar abnormalities have been described by Russell (1965) and Snow (1973a, b).

Table 1. Number of cell nuclei in 95-h-old embryos developed from control and X-irradiated 2-cell stages and morulae of mice

Treatment	No. of embryos	No. of cells							Cell number (mean $\pm$ s.e.m.)
		≤ 16	32	48	64	96	112	128	
None (control)	70	2	15	19	17	9	5	3	46.6 $\pm$ 2.7 ^a
2 cell stage									
50 R	46	11	9	13	10	3	—	—	36.2 $\pm$ 2.9 ^b
200 R	40	12	16	7	5	—	—	—	26.8 $\pm$ 2.5 ^c
500 R	45	41	4	—	—	—	—	—	12.8 $\pm$ 0.9
Morula									
50 R	22	3	3	6	4	6	—	—	46.1 $\pm$ 4.4 ^d
200 R	33	3	7	13	8	2	—	—	39.9 $\pm$ 2.8 ^e
500 R	23	3	9	9	2	—	—	—	30.5 $\pm$ 2.6

For each sample, mean values were significantly different from each other at $P < 0.01$, except for *b* versus *c* ($0.01 < P < 0.02$), *a* versus *d* ($0.08 < P < 0.09$) and *d* versus *e* ($0.07 < P < 0.08$).

Although the irradiation of morulae had no effect on cavitation, Table 1 shows that a significant dose-proportional reduction in cleavage rate occurred and after 500 R cleavage was almost totally arrested. Cytological examination suggested that cell death occurred soon after cavitation, an event known to occur in healthy embryos irrespective of the cell number (Tarkowski & Wroblewska, 1967). However, blastocoele formation is apparently determined either by a certain nucleo-cytoplasmic ratio

or by adequate DNA and chromosome replications just before the fifth cleavage division (Smith & McLaren, 1977). Most of the embryos in the present study had reached this stage at the time of irradiation. However, Smith & McLaren (1977) have shown that, under normal conditions, embryos which are just beginning to cavitate contain a variable number of cells (24–35). It is possible therefore that in irradiated embryos cavitation mainly occurs in those embryos containing the lowest numbers of cells and the observations are not inconsistent with the views of Smith & McLaren (1977). A similar explanation can be proposed for the 2-cell stages because the embryos which survive irradiation with 200 R and cavitate slightly later than the controls (Alexandre, 1974), have probably reached the 20–25-cell stage at a time when the controls already contain more than 30 cells.

To determine the state of the embryos at the precise time when cavitation is initiated, embryos irradiated at the 2-cell stage were air-dried for cell counting at the late morula–early blastocyst stage (84-h-old embryos), when cavitation is initiated in about 15% of the controls. Table 2 shows that irradiation with 200 and 500 R induced a statistically significant dose-proportional reduction in the cell number of the embryos, even before cavitation. This confirms the results reported above and indicates that the delay in cleavage is the immediate response to acute irradiation at whatever stage of development.

Table 2. Number of cell nuclei in 84-h-old embryos developed from control and irradiated 2-cell eggs of mice

Treatment	No. of embryos	No. of cells								Cell number (mean $\pm$ s.e.m.)
		4	8	12	16	20	24	28	32	
None (control)	124	1	21	14	42	17	5	10	14	16.8 $\pm$ 0.7
200 R	117	5	25	24	41	11	5	3	1	12.7 $\pm$ 0.5*
500 R	105	10	62	21	12	5	—	—	—	8.4 $\pm$ 0.3*

* Values significantly different from the control value and each other ($P < 0.001$).

Thus, after an acute X-irradiation at the 2-cell stage, all mouse embryos are affected to different degrees. Some die before they reach the normal time of cavitation; the others, with a reduced number of cells, differentiate into vesicular forms with an abnormally small, if any, ICM. These findings are in agreement with the epigenetic theory of primary differentiation (Tarkowski & Wroblewska, 1967; Rossant, 1975). The same conclusion can be drawn from acute X-irradiation of later stages of cleavage, before blastocyst formation. It is therefore suggested that the response of preimplantation mouse embryos to acute X-irradiation does not have the same explanation as that of chronic irradiation resulting from the uptake and/or incorporation of [^3H]thymidine, for which a selective action on the stem cells of the ICM at the 16-cell stage has been proposed (Snow, 1973a, b).

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