Ability of mouse embryos to degranulate mast cells *in vitro*

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Summary. Mouse embryos flushed from the reproductive tract on Day 4 or 5 *post coitum* degranulated peritoneal mast cells *in vitro*. The degranulating activity of embryos developed with age of embryos: it was absent with Day-3 embryos, present with Day-4 embryos and was increased with Day-5 embryos. Day-4 embryos cultured for 24 h also exhibited degranulating activity. Such activity was even greater for embryos cultured for 48 h. As the degranulating activity of the incubated embryos increased, it was accompanied by an increase in the degranulating activity of the culture medium.

Introduction

Prostaglandins (Lau, Saksena & Chang, 1973; Kennedy, 1977; Kennedy & Lukash, 1982) and histamine (Shelesnyak, 1957; Johnson & Dey, 1980) have been considered to be involved in the process of implantation and decidualization. These substances are stored and/or produced amongst others in mast cells (Uvnas, 1974; Lewis *et al.*, 1982). The number of uterine mast cells decreases during oestrus (Harvey, 1964; Le Vier & Spaziani, 1966; Maraspin & Bo, 1971; Brandon & Evans, 1983) and around the time of implantation (Shelesnyak, 1960; Brandon & Bibby, 1979). The decrease of the numbers of uterine mast cells during oestrus and around the time of implantation is believed to be the result of degranulation of the mast cells caused by oestrogens (review: Spaziani, 1975). Although the number of uterine mast cells decreases during implantation, little is known of the effect of peri-implantation embryos on degranulation of mast cells.

In this study the degranulating activity of mouse embryos at different stages of development and the medium in which embryos were cultured was assessed by their action on the peritoneal mast cells of rats.

Materials and Methods

Mouse embryos recovered from the reproductive tract, mouse embryos cultured *in vitro* and the culture medium were used in the present study.

Embryos and culture conditions

The embryos were obtained after hormonally induced ovulation in mice of an outbred Swiss albino strain. The mice received 7.5 i.u. PMSG (Gestyl: Organon, Oss, The Netherlands) and 7.5 i.u. hCG (Pregnyl: Organon) 48 h apart. Nulliparous females were caged with outbred Swiss albino males of proven fertility and inspected for copulation plugs the next morning. The day on which the copulation plug was found was considered as Day 1 of pregnancy.

Early morulae were flushed with PBS from the oviduct on Day 3, later morulae and early blastocysts were flushed from the uterus on Day 4 and mature, zona-free blastocysts, probably

from attachment sites (Finn & McLaren, 1967; Theiler, 1972) were flushed from the uterus on Day 5 of pregnancy.

To test the degranulating activity of embryos which developed *in vitro*, Day-4 embryos were cultured for 24 or 48 h in a siliconized Petri dish, under paraffin oil gassed with a 5% mixture of CO₂ with air at 37°C. The medium for the 24 h culture was that described by Whitten (1971). After 24 h the embryos had developed to the blastocyst stage and had a rounded shape within the zonae pellucidae. The 48-h culture comprised two periods: the first 24 h was in Whitten's medium, and the second 24 h was in Eagle's medium supplemented with 1 mM-L-glutamine, 1 mM-sodium pyruvate (Sigma, St Louis, MO, U.S.A.), and 10% fetal calf serum (Gibco, Grand Island, New York, U.S.A.). The change of culture medium for the second 24-h period is essential for obtaining the further development of blastocysts (Hsu, 1978). By the end of culture the zonae were shed and blastocysts showed a significant increase in size due to elongation and spreading.

To test whether embryos release the degranulating factor into the environment, the medium in which embryos were cultured was investigated. In each 0.05 ml medium, 40 Day-4 embryos were placed under paraffin oil. They were cultured in Whitten's medium for 24 h (embryo culture medium I), and in the supplemented Eagle's medium for the next 24 h (embryo culture medium II). For the control experiments 0.05 ml Whitten's or Eagle's medium was placed under paraffin oil in similar conditions but no embryos were used (control medium).

Materials and incubation procedure

The embryos were washed in cooled PBS and twice-distilled water, placed on a watchglass in 0.05 ml twice-distilled water and freeze-dried. Under these conditions the zonae pellucidae and cells were disrupted. The dried material was covered with 0.05 ml Whitten's medium and thoroughly mixed to provide an homogenate of embryo cells. The homogenates were prepared from 30 embryos in separate experiments. For early morulae (Day 3 of gestation) 5 additional experiments were conducted, in each of which 150 embryos were used for each homogenate. For the control experiments 0.05 ml of twice-distilled water was placed in the watchglass and then freeze-dried. The dried material was covered with 0.05 ml Whitten's medium and thoroughly mixed (control medium).

Degranulating activity was studied on mixed rat peritoneal cells, of which mast cells constitute about 40%. Mast cells were obtained by washing the peritoneal cavity of female rats with 5 ml PBS

Table 1. Mast cells degranulated by homogenates of mouse embryos developed *in vivo*

Material	No. of exps	No. of embryos	Mean $\pm$ s.e.m. no. of mast cells degranulated by:	
			Embryos	Control medium
Day-3 embryos (morulae)	10	300	14.5 $\pm$ 1.6*	12.6 $\pm$ 1.2
Day-4 embryos (morulae and early blastocysts)	9	270	40.0 $\pm$ 2.5*†	8.5 $\pm$ 1.1
Day-5 embryos (blastocysts from attachment areas)	8	240	60.3 $\pm$ 3.1†	8.4 $\pm$ 0.8

* $P < 0.001$.

† $P < 0.001$.

supplemented with 1 mg streptomycin, 1 mg penicillin (Polfa, Warsaw, Poland) and 0.1 mg nystatin (E. R. Squibb & Sons, London, U.K.). The liquid collected from one donor was centrifuged for 2 min at 18 g. The supernatant was decanted and the residue was diluted with PBS so as to obtain 700–900 mast cells sedimented on a coverslip. Then, on the two opposite edges of a silicone-coated coverslip, 0.1 ml PBS was placed to form two high drops. On the surface of each drop 0.05 ml peritoneal fluid suspension was layered with a micropipette. A sediment formed within 90 sec and then the drops were poured off or delicately blotted with filter paper. Mast cells and a few other cells from the peritoneal fluid remained on the slides. They were covered with 0.05 ml embryo homogenate (or embryo culture medium) on one edge, and with 0.05 ml control medium on the other. The whole was then incubated in closed vessel, saturated with water vapour and a mixture of 5% CO₂ with air at 37°C. The reaction was stopped by adding 0.05 ml 20% formalin in PBS. After 20 min the supernatant was poured off and the mast cells remaining on the slides were stained with 0.02% acidified (pH 5) toluidine blue solution in PBS for 3 min.

By this method the mast cells were placed on a coverslip by sedimentation, thus avoiding the need of manipulation with a brush, and they remained there throughout the experiment. In this way any accidental influences of physical factors were eliminated. The sensitivity and repeatability of the method were checked by the incubation of mast cells with protamine sulphate (Biomed, Warsaw, Poland) (Choroszevska & Mańkowska, 1983). The degranulated mast cells were counted under a light microscope. A mast cell was considered to be degranulated if there were at least 15 liberated granules nearby. The values were expressed as the mean $\pm$ s.e.m. number of mast cells/0.05 ml peritoneal fluid suspension and were compared by Student's *t* test.

Results

There were no significant differences between the mean number of mast cells degranulated by homogenates prepared from 30 Day-3 embryos and by control medium (Table 1). A similar result (not shown) was also obtained when the homogenates were prepared from 150 Day-3 embryos (18.2 ± 1.1 and 12.6 ± 1.2 degranulated mast cells/0.05 ml peritoneal fluid).

The degranulating activity was detectable in Day-4 and Day-5 embryos and was greater for Day-5 than for Day-4 embryos (Table 1).

Table 2. Mast cells degranulated by homogenates of cultured mouse embryos and by the culture medium

Material	No. of exps	No. of embryos	Mean $\pm$ s.e.m. no. of mast cells degranulated by:	
			Embryos or culture medium	Control medium
Day-4 embryos	9	270	40.0 ± 2.5	8.5 ± 1.1
Day-4 embryos cultured for 24 h	8	240	$53.7 \pm 2.8^*$	10.1 ± 0.7
Day-4 embryos cultured for 48 h	8	240	$70.5 \pm 3.3^*$	9.0 ± 0.4
Culture medium I†	8		49.1 ± 2.4	9.1 ± 0.8
Culture medium II‡	8		$64.5 \pm 1.0^*$	8.7 ± 0.6

†Whitten's medium in which Day-4 embryos were cultured for the first 24 h.

‡Enriched Eagle's medium in which embryos were cultured for the second 24 h.

**P* < 0.01 compared to the value above.

The degranulating activity was also detectable for embryos developed *in vitro* and in the culture medium (Table 2). In all experiments the mean number of mast cells degranulated by embryo homogenates or culture medium was significantly greater than that by control medium. The degranulating activity of embryos increased with the duration of embryo culture and it was accompanied by an increase in the degranulating activity of the culture medium.

Discussion

The present study revealed that homogenates of Day-4 and Day-5 mouse embryos degranulate mast cells *in vitro*. This seems to indicate that embryos in the peri-implantation period contain a factor(s) which reacts with mast cells and causes extrusion of cytoplasmic granules. The degranulating activity of embryos correlates with their age, but the increase in the degranulating activity is not connected with the increase in the number of blastomeres because the degranulating activity was not found in homogenates from 30 or from 150 Day-3 embryos. The activity is also not dependent on the protein content because in morulae and late blastocysts the total protein stays constant in spite of their increased volume (Brinster, 1967). The degranulating factor is not accumulated from the intraluminal environment because it occurs in embryos developing in the uterus and *in vitro*.

The degranulating activity of cultured embryos correlates with their stage of development; it is lower in rounded blastocysts still encased in the zona (cultured for 24 h) and greater in elongated blastocysts that have spread and lost the zona (cultured for 48 h). Degenerate embryos are inactive (Choroszevska & Mañkowska, 1983). The interpretation of the above findings is that the degranulating activity may be the result of the biochemical differentiation of embryos and of the formation of new, biologically active compounds.

Peri-implantation mouse embryos also contain proteolytic enzymes (Andary, Dabich & Van Winkle, 1972; Sherman, 1976) and enzyme systems which could synthesize oestrogens (Dickmann, Dey & Sen Gupta, 1976; Sen Gupta, Roy & Manchanda, 1982). Other studies indicate that trypsin degranulates mast cells (Archer, 1959), and local application of oestrone in the skin induces mast cell degranulation (Winer, Bierman & Sternberg, 1963).

We conclude that (1) peri-implantation embryos are able to degranulate peritoneal mast cells *in vitro*, and (2) when embryos are cultured degranulating factor(s) is released into the medium. It therefore seems possible that a degranulating factor may be released from embryos *in vivo* into the uterus.

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