Lysis of the zona pellucida and attachment of embryos to the uterine epithelium in ovariectomized mice treated with oestradiol-17 β and progesterone

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Summary. Preimplantation mouse embryos with intact zonae pellucidae were transferred into the uteri of ovariectomized females treated with progesterone, oestradiol-17 β plus progesterone, or oestradiol-17 β alone; the disappearance of zonae from the uterine lumen was used to determine the presence of 'zona-lytic' activity *in situ*. Lysis of zonae did not occur in animals treated with progesterone or oestradiol-17 β alone. However, lysis did occur when oestradiol-17 β was combined with progesterone; 'zona-lytic' activity reached peak levels within 12 to 24 h, then decreased. Attachment of embryos to the uterine epithelium occurred only in animals treated with oestradiol-17 β plus progesterone and was initiated at about the time of peak 'zona-lytic' activity. It is suggested that the chymotrypsin-like enzyme activity present in uterine fluid is involved with the initiation of implantation.

Introduction

The mechanism that is responsible for removal of the zona pellucida in mice at the time of implantation has not been established, but most evidence suggests that a 'zona-lytic' factor from the uterus is involved (Orsini & McLaren, 1967; McLaren, 1969, 1970; Mintz, 1971). In addition, it has been suggested that the factor responsible for lysis of the zona pellucida, possibly a proteolytic enzyme or enzyme complex (Bowman & McLaren, 1970; Mintz, 1972; Pinsker, Sacco, & Mintz, 1974), is also responsible for altering the surface of the blastocysts and thereby initiating attachment of embryos to the uterine epithelium (Mintz, 1972; Pinsker & Mintz, 1973).

The concentration of a chymotrypsin-like enzyme in the uterine lumen of ovariectomized mice is influenced by treatment with both oestradiol-17 β and progesterone (Hoversland & Weitlauf, 1978, 1981): values are low after treatment with progesterone alone (i.e. conditions for delayed implantation) and elevated transiently after injection of oestradiol-17 β in combination with progesterone (i.e. conditions for termination of delayed implantation). Although it was suggested that the enzyme might be involved in zona lysis and initiation of embryo attachment, one of the questions that remained unanswered was whether changes in the concentration of the chymotrypsin-like enzyme activity follow the same temporal course as 'zona-lytic' activity and the initiation of embryo attachment *in utero*. The following experiments were undertaken to examine this question.

Materials and Methods

An egg transfer technique was used in the first two experiments to examine the effects of oestradiol-17 β and progesterone on lysis of zonae pellucidae *in situ*. In the third experiment the effects of these hormones on attachment of embryos to the uterine epithelium were studied.

Experiment 1

Embryo donors. Virgin female white Swiss mice (6–8 weeks old) were selected at random stages of the oestrous cycle and induced to ovulate with intraperitoneal injections of 5 i.u. PMSG followed 48 h later with 5 i.u. hCG (Fowler & Edwards, 1957) and were immediately placed with fertile males. Mating was confirmed the following morning by the presence of a vaginal plug (designated Day 1 of pregnancy). Embryo donors were killed at 10:00 h on Day 4 of pregnancy by cervical dislocation. The uterine horns were excised, and embryos with intact zonae pellucidae were recovered by flushing a stream of Eagle's basal medium (GIBCO, containing Earle's salts in addition to 280 ng glutamine/ml, 1 mg bovine serum albumin/ml, 6 μ g penicillin/ml, and 50 μ g streptomycin/ml) from a blunt hypodermic needle through the uterus. Embryos were pooled and rinsed twice in fresh medium before transfer into recipient mice.

Recipients. Virgin white Swiss mice were selected at random stages of the oestrous cycle, bilaterally ovariectomized via dorsolateral incisions, and ligatures were placed, avoiding uterine vein and artery, at the cervical end of each uterine horn via a midventral incision. Counting the day of ovariectomy as Day 1, animals were allowed to recover for 10 days and allotted randomly to one of three hormone treatment groups and treated as follows: Group 1, 2.0 mg progesterone per day on Days 11–14 (i.e. conditions for delayed implantation); Group 2, 2.0 mg progesterone per day on Days 11–12 followed by 2.0 mg progesterone combined with 25.0 ng oestradiol-17 β per day on Days 13–14 (i.e. conditions for implantation: Weitlauf & Greenwald, 1968a); Group 3, 0.1 ml sesame seed oil on Days 11–12 followed by 25.0 ng oestradiol-17 β per day on Days 13–14. All hormones were dissolved in 0.1 ml sesame seed oil and injected s.c. at 12:00 h on the days indicated.

Five or six embryos with intact zonae pellucidae were transferred into the uteri of recipients at 12:00 h on Day 13 as described previously (Weitlauf & Greenwald, 1968b). At various times between 12 and 24 h after the transfer (see Table 1), recipients were killed and the embryos and zonae pellucidae were recovered by flushing the uteri with a stream of normal saline (9.5 g NaCl/l) from a blunt needle and syringe. The resultant flushings were stained with 5 μ l 1% toluidine blue in normal saline, to enhance the visibility of zonae, and examined under a compound microscope to determine the numbers of embryos and zonae recovered. Differences in the ratios of recovered zonae to embryos at the various times were tested for statistical significance with contingency tables (Snedecor & Cochran, 1967).

Experiment 2

Embryo donors and recipients were treated as described in Exp. 1 with the exception that all recipients were injected with progesterone followed by oestradiol-17 β in combination with progesterone (i.e. as in Exp. 1, Group 2). For purposes of reference, 12:00 h on Day 13 (i.e. the time of the first injection of oestradiol-17 β combined with progesterone) is considered 0 h. Five or six embryos with intact zonae pellucidae were transferred into recipients at –24, –12, 0, +12, +18, +21 or +24 h and recovered 24 h after the transfer procedure.

Experiment 3

Virgin white Swiss mice were induced to ovulate and mate. They were bilaterally ovariectomized and ligatures were placed at the cervical end of each uterine horn on Day 4 of pregnancy

(10:00–11:00 h). The animals were then allowed to recover for 10 days and randomly allotted to treatment groups to receive progesterone followed by a combination of oestradiol-17 β and progesterone (Group 4, same treatment as Group 2 in Exp. 1), or sesame seed oil followed by oestradiol-17 β alone (Group 5, same treatment as Group 3 in Exp. 1). Considering the time of the hormone injection on Day 13 as 0 h, the animals were killed at 0, 18, 24, 30 or 36 h. At 10 min before death, each animal was injected i.v. with 0.2 ml 1% Evans' blue in normal saline (via the tail vein). The numbers of dye reaction sites (Psychoyos, 1966) were counted and then each uterine horn was flushed with a stream of normal saline to recover unattached embryos.

Results

Experiment 1

Lysis of zonae pellucidae occurred only in animals treated with oestradiol-17 β in combination with progesterone, and this process was essentially complete by 18 h (Group 2, Table 1).

Table 1. The effect of oestradiol-17 β and progesterone on 'zona-lytic' activity in the uteri of ovariectomized mice

Group*	Time in utero (h)	No. of embryos with zona pellucida transferred	Total no. recovered		Ratio of zonae recovered to embryos
			Zonae pellucidae	Embryos	
1 (progesterone)	12	91	41	45	0.93
	24	45	27	31	0.87
2 (progesterone + oestradiol)	12	45	15	22	0.68
	15	72	13	32	0.40
	18	56	1	25	0.04
	24	63	0	25	0.00
3 (oestradiol)	12	45	23	26	0.88
	24	50	22	24	0.91

* See text. Embryos with intact zonae pellucidae were transferred into recipients at 12:00 h on Day 13, the time of the third hormone injection.

Table 2. The level of 'zona-lytic' activity during different 24-h intervals in mice treated with oestradiol-17 β in combination with progesterone

Time (h)* of:		No. of embryos with zona pellucida transferred	Total no. recovered:		Ratios of:	
Transfer	Recovery		Zonae pellucidae	Embryos	Zonae recovered to embryos	Zonae 'lysed' to embryos
-24	0	72	34†	38	0.87	0.13
-12	+12	107	9	24	0.38	0.62
0	+24	63	0	25	0.00	1.00
+12	+36	50	1	18	0.05	0.95
+18	+42	117	15	44	0.34	0.66
+21	+45	124	26	51	0.50	0.50
+24	+48	150	24	35	0.68	0.32

* 0 h is 12:00 h on Day 13, the time of the first injection of oestradiol-17 β in combination with progesterone (see text).

† Includes zonae pellucidae that had become free from embryos.

Experiment 2

The level of 'zona-lytic' activity appeared to increase transiently, with peak levels occurring at 0 to +24 h and +12 to +36 h (Table 2).

Experiment 3

Attachment of embryos occurred only in mice treated with the combination of both hormones; this process was initiated by 18–24 h after the addition of oestradiol-17 β to the progesterone regimen and was essentially completed by 36 h (Group 4, Table 3). The failure of embryos to attach in animals treated with oestradiol-17 β alone (Group 5, Table 3) was not due to the presence of zonae, as all embryos were zona free.

Table 3. The effect of oestradiol-17 β and progesterone on attachment of mouse embryos to uterine epithelium

Group*	Time after oestradiol (h)†	Total no. of:			Mean no. of:	
		Horns examined	Horns + blue dye (%)	Horns + embryos (%) ‡	Blue reactions in horns with dye reaction	Embryos recovered from horns with embryos§
4	0	36	0	18 (50)	0	4.6 $\pm$ 0.6
	18	28	16 (57)	16 (58)	3.7	3.6 $\pm$ 0.7
	24	46	25 (54)	20 (43)	3.6	2.7 $\pm$ 0.4
	30	32	22 (68)	13 (40)	5.3	1.6 $\pm$ 0.3
	36	26	15 (57)	2 (7)	4.4	0.2 $\pm$ 0.1
5	0	24	0	16 (66)	0	4.5 $\pm$ 0.5
	18	28	0	18 (67)	0	4.6 $\pm$ 0.6
	24	35	0	19 (54)	0	3.4 $\pm$ 0.5
	30	30	0	18 (60)	0	4.1 $\pm$ 0.7
	36	42	0	21 (50)	0	4.7 $\pm$ 0.4

* See text.

† 0 h is the time of the hormone injection on Day 13 (i.e. 12:00).

‡ Unattached embryos.

§ Mean $\pm$ s.e.m.

Discussion

The present experiments demonstrate that lysis of zonae pellucidae occurs in the uteri of ovariectomized mice treated with oestradiol-17 β combined with progesterone and that treatment with either hormone alone is insufficient for this effect. Peak levels of 'zona-lytic' activity were found between 12 and 24 h after the injection of oestradiol-17 β combined with progesterone, and this time coincided with the initiation of embryo attachment to the uterine epithelium.

These results are in agreement with earlier reports that dissolution of the zona pellucida in mice does not occur during experimental delayed implantation (Orsini & McLaren, 1967; McLaren, 1971), delayed implantation during lactation (McLaren, 1967, 1968), or in prepubertal mice (Mintz, 1971, 1972). These findings also agree with reports that lysis of zonae is induced within 18 h after removal of the sucking young or an injection of oestradiol-17 β into mice with lactational delay (McLaren, 1968, 1970; Mintz, 1971). In addition, premature lysis of zonae occurs when pregnant mice are given an injection of oestradiol-17 β before Day 4 of pregnancy (Mintz, 1971). These previous observations have led to the hypothesis that the appearance of 'zona-lytic' activity in the uterus is oestrogen-dependent. Although this hypothesis is supported by the present results, the finding that lysis of zonae does not occur following

treatment with either hormone alone suggests that, although oestradiol-17 β may act as a stimulus for the appearance of 'zona-lytic' activity, actual lysis of the zonae is dependent upon both hormones.

The results of Exp. 1 demonstrate that there is a progressive loss of zonae pellucidae following treatment with oestradiol-17 β in combination with progesterone, and therefore the appearance of 'zona-lytic' activity. However, the design of the experiment does not allow the distinction between two possible explanations: (i) that there were increasing concentrations of 'lytic' activity and zonae were lost when the concentration reached an effective level; or (ii) that there were constant levels of 'lytic' activity and zonae were lost with increasing lengths of exposure. In Exp. 2 the length of time that zonae were exposed to the uterine environment was held constant so that comparisons of the levels of 'zona-lytic' activity could be made between different or overlapping intervals. Because the length of exposure was constant, differences in the ratio of zonae recovered to embryos could only represent differences in the level of 'zona-lytic' activity. Comparison of the data obtained at -12 to +12, 0 to +24 and +12 to +36 h clearly indicates that maximal activity occurs later than +12 h, and comparison of the data obtained at 0 to +24 and +24 to +48 h demonstrates that peak levels of activity occurred before +24 h. From the data in Table 3, therefore, it appears that peak levels of activity are between +12 and +24 h and most probably maximum at about +18 h. A second injection of oestradiol-17 β in combination with progesterone at 24 h did not result in further increases of 'zona-lytic' activity.

Experiment 3 results demonstrate, as expected, that there is a significant effect of hormone treatment on attachment of embryos to the uterine epithelium. Attachment of embryos occurred only in mice treated with both hormones, it was initiated by 18–24 h and essentially completed by 36 h, as indicated by the decrease in the number of embryos that could be recovered by flushing the uterus. All embryos were free of zonae pellucidae at the time animals were killed and empty zonae were recovered from animals treated with oestradiol-17 β alone. This confirms the observation that embryos in ovariectomized mice escape from the zona by a non-lytic process (probably 'hatching': Orsini & McLaren, 1967). Because zona-free embryos were recovered, it is clear that attachment is dependent upon more than simply the removal of the zona, possibly an enzyme-dependent alteration in the glycoproteins on the surface of the embryo, as suggested by Pinsker & Mintz (1973).

Mintz (1971, 1972) has proposed that 'zona-lytic' activity in the uterine lumen is involved in implantation and that the factor responsible for lysis of zonae is also responsible for altering the surface of embryos and thus initiating embryo attachment. The present results are compatible with this hypothesis. However, the finding reported by McLaren (1971) that zonae are lysed during the experimental delay of implantation caused by ovariectomy on Day 4 of pregnancy is not. The reason for this discrepancy is not clear.

The factor responsible for lysing the zonae pellucidae and altering the embryo surface is thought to be a proteolytic enzyme or enzyme complex in uterine fluid (Bowman & McLaren 1970; Mintz, 1972; Pinsker *et al.*, 1974). The similarity in the endocrine regulation of 'zona-lytic' activity and initiation of embryo attachment reported here, and that reported for chymotrypsin-like protease activity in uterine fluid (Hoversland & Weitlauf, 1978) suggests that this enzyme may be involved in lysis of zonae and/or initiation of embryo attachment. However, before the chymotrypsin-like enzyme activity in uterine fluid can be implicated in these events, it will be necessary to demonstrate that the purified enzyme *in vitro* has both 'zona-lytic' activity and the ability to alter the surface of the embryo.

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