Control of uterine stromal mitosis in relation to uterine sensitivity and decidualization in mice

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The relationship between mitosis, ovarian hormones, decidual stimuli and decidualization was investigated using progestagen-treated ovariectomized mice. Oestradiol, or the intraluminal instillation of oil or saline, all stimulated stromal mitosis. When oil or saline was instilled following oestradiol, the response depended on the dose of oestradiol, the interval between the oestradiol and the instillation, and the time when the mice were killed. After 20 ng oestradiol, the instillation of oil 7 h later induced large mitotic and decidual responses that were evident within 17 h of instillation and increased with time. Smaller mitotic and decidual responses were obtained when the interval between oestradiol and oil was 24 h; there was no response when the interval was 42 h. When a higher dose (100 ng) of oestradiol was given, oil injected 7 h later initially stimulated mitosis (at 17 h), but this effect was reduced at 24 h and no decidualization occurred. After instilling oil 24 or 42 h after 100 ng oestradiol, the mitotic response was limited, and there was no decidual response. Regardless of the dose of oestradiol, saline induced only a transient mitotic response and no decidualization occurred. It is concluded that there are three stimuli that can cause stromal mitosis in the progestagen-treated mouse uterus: oestrogen, an intraluminal stimulus (blastocyst, oil or saline) and factors associated with decidualization. Oestradiol not only induces mitosis, but also produces a period of heightened sensitivity to the mitotic effects of intraluminal stimuli. In addition, low doses of oestradiol induce a period of sensitivity to decidual stimuli. When these phases of uterine sensitivity to intraluminal stimuli have passed, decidualization and mitosis within the stroma can continue, presumably stimulated by local signals from the decidua in a self-propagating cascade of cellular proliferation and differentiation.

Introduction

The cells of the endometrial stroma undergo mitosis just before implantation in mice. After attachment of the blastocyst, there is decidualization of some of the stromal cells and further division of others (Finn and Martin, 1967). The initial wave of mitosis is controlled by progesterone and oestrogen (Martin and Finn, 1968). Progesterone, given alone to ovariectomized mice, causes little or no mitosis in the stroma. However stromal mitosis is induced when progesterone is given to mice previously primed with oestradiol (Finn and Martin, 1970). Oestradiol treatment of progesterone-dominated animals induces large numbers of stromal cells to undergo mitosis, with maximum cell division occurring within 24 h of oestradiol treatment.

In addition to stimulating stromal mitosis, sequential exposure to progesterone and oestrogen induces a period of uterine sensitivity during which the uterus will respond to the blastocyst with decidualization and further mitosis. A similar

decidual response can be obtained following an intraluminal injection of oil, but this response is not induced by saline introduced into the uterine lumen. The dose of oestradiol is critical for the decidual response to oil: if large doses of oestradiol are given, decidualization is prevented (Finn, 1966).

Little is known about the control of blastocyst-stimulated stromal cell division, partly because it is superimposed on endocrine-induced changes. The injection of a small quantity of oil into the uterine lumen of ovariectomized, hormone-treated mice causes a similar response to that of a blastocyst (Finn and Keen, 1963) and has been widely used to study the cellular and endocrine parameters of implantation (Finn and Hinchliffe, 1964). Das and Martin (1978) showed that after the injection of oil into a sensitized mouse uterus, some stromal cells go into S phase and then undergo decidualization while other cells undergo mitosis. In their experiments, an interval of 32 h occurred between the beginning of oestrogen treatment and the instillation of the oil and it can, therefore, be assumed that the oestrogen-induced wave of mitosis had already occurred. The aim of the present work was to investigate the relationship between stromal mitosis and sensitivity

to decidualization by undertaking a systematic study of the stromal mitotic response in relation to the timing of the administration of oestradiol and oil.

Materials and Methods

Swiss mice, 2–3 months old, from a random-bred, closed colony, were used. All surgical procedures were carried out under tribromoethanol anaesthesia. Ovariectomized mice were rested for a least a week before hormone treatment.

Schedule of treatments

Hormones were given (s.c. in 0.1 ml arachis oil) according to the following regimen: days 1 and 2: 100 ng oestradiol (Sigma Chemical Co., Poole); days 3–5: no hormones; day 6: 1 mg medroxyprogesterone acetate (a long acting progestagen; Sigma: Finn and Martin, 1978); day 8: 20 or 100 ng oestradiol (nidatory oestrogen) as indicated in the Results section. An intraluminal injection of 0.02 ml arachis oil or physiological saline (0.154 mol l^{-1}) was injected through the utero–tubal junction of one or both uterine horn at times indicated in the Results section.

Assessment of mitosis and decidual reaction

Colchicine (Sigma; 0.1 mg in 0.1 ml aqueous solution) was injected s.c. 1 h before the animals were killed by cervical dislocation. Early decidualizing areas were located using i.v. Pontamine Sky Blue (0.3 ml of 1% aqueous solution) given 15 min before autopsy (Psychoyos, 1960) and later decidualization in the stroma was confirmed by the presence of alkaline phosphatase activity in cryosections (Finn and Hinchliffe, 1964). Sections of uteri (randomly chosen from along the length of the uterus or stained positive with Pontamine Sky Blue) were fixed in cold calcium formal infiltrated with sucrose and frozen and 10 μm sections cut on a cryostat. These were either processed for the demonstration of alkaline phosphatase activity as an indicator of decidualization, or stained with haematoxylin and eosin and cells undergoing mitosis counted in one cross-section taken at random. Previous work has shown that this gives a good estimate of the extent of mitosis in the endometrium: counting several slides from one horn gives an estimate of the within-animal variability, but this is very small compared with the between-animal variability (C. A. Finn and L. Martin, unpublished observations).

Statistical analyses

Results were analysed using Student's paired or unpaired *t* tests as appropriate.

Results

Timing of mitotic response to nidatory oestradiol alone

The aim of this experiment was to confirm in our present colony of mice the timing of the mitotic response to a single

injection of oestradiol (Martin *et al.*, 1973) in animals pretreated with priming oestrogen and progestagen. Mice were killed 17 or 24 h after an injection of 20 ng oestradiol (controls received no oestradiol) and the number of endometrial cells undergoing mitosis counted. In the controls, 8.2 ± 2.4 mitoses per section ($n = 5$) were counted; after administration of oestradiol, there were 22.6 ± 2.5 17 h after treatment ($P < 0.01$; $n = 5$) and 54.4 ± 11.7 , 24 h after treatment ($P < 0.01$; $n = 5$).

Mitosis in response to an intraluminal injection in the absence of nidatory oestradiol

Oil or saline was injected into one uterine horn of progestagen-treated, ovariectomized mice and the animals were killed 17, 24 or 31 h later (Table 1). At 17 h, there was a small, but significant difference between the treated and the control horns (Group 1); at 24 h, the number of mitoses was considerably higher in the injected horn (Group 11) but had fallen again ($P < 0.01$) by 31 h (Group 21). This fall between 24 and 31 h was confirmed in a later experiment (Table 2, Groups 3 and 11; $P < 0.05$).

Mitosis and decidualization in response to combined nidatory oestrogen and intrauterine instillation

Low dose (20 ng) of oestradiol. Low doses of oestradiol induce a transient period of sensitivity to a decidual stimulus (for example, introduction of oil) in progestagen-treated mice. The time course of the mitotic response to oil during and after this period of sensitivity was investigated by instilling oil into one uterine horn 7, 24 or 42 h after the s.c. injection of 20 ng oestradiol and the mice were killed 17 or 24 h after the introduction of oil.

When oil was instilled into one horn of the uterus 7 h after the oestradiol injection, there was a significant increase in the number of mitoses in the uteri of animals killed 17 or 24 h later (Table 1, Groups 3 and 13). This response was not seen when the oil was introduced 12 h after oestradiol (Group 4). When the oil was injected 24 h after oestradiol, the oil-injected horns showed higher mitotic counts than did the control horns (Groups 5 and 14), although the response was lower than when the oil was injected 7 h after the oestradiol (Group 3 versus Group 5: $P < 0.01$; Group 13 versus Group 14: $P < 0.01$). The injection of oil at 42 h caused no increase in the number of mitoses (Group 15). Injection of saline, 7 or 24 h after oestradiol treatment, also induced an increase in the number of mitoses within 24 h of instillation (Groups 6, 16 and 17: $P < 0.01$; Group 7: NS).

To determine whether the effects of oestradiol and intra-luminal oil were independent, additive or synergistic, the effects of a number of combinations of the two treatments were studied. Oil was injected 7 h before, at the same time as, or 7 h after the oestradiol (Table 2). When oil was injected 7 h before the oestradiol (Group 9), the mitotic response was higher than the responses to either oestradiol alone (Group 10; $P > 0.05$) or oil alone (Group 11; $P < 0.05$) but similar to the combined response to the two treatments. When oil and oestradiol were given at the same time (Group 5), the response at 24 h was not statistically higher than either of the individual

Table 1. Mitotic response of uterine stroma in mice to oil or saline instilled at various times after oestrogen treatment*

Group number	Treatment		Time (h) between oestradiol and oil or saline instillation		Time (h) between oil or saline instillation and autopsy		Mean number of mitoses ($\pm$ SEM)		Statistical difference between horns	n
	Nidatory oestradiol						Treated horn	Untreated horn		
1	No oestradiol	Oil	—	—	17		7.5 $\pm$ 2.7	3.4 $\pm$ 0.6	$P < 0.05$	18
2		Saline	—	—	17		3.4 $\pm$ 1.5	1.4 $\pm$ 0.5	NS	7
3	After 20 ng oestradiol	Oil	7	24	17		114.8 $\pm$ 12.4	50.4 $\pm$ 5.9	$P < 0.01$	24
4		Oil	12	29	17		46.1 $\pm$ 9.0	47.5 $\pm$ 4.2	NS	8
5		Oil	24	41	17		17.9 $\pm$ 6.2	5.7 $\pm$ 0.6	$P < 0.01$	19
6		Saline	7	24	17		65.9 $\pm$ 13.9	33.7 $\pm$ 5.7	$P < 0.01$	11
7		Saline	24	41	17		6.0 $\pm$ 1.0	6.2 $\pm$ 1.2	NS	5
8	After 100 ng oestradiol	Oil	7	24	17		113.6 $\pm$ 22.1	35.5 $\pm$ 8.6	$P < 0.01$	8
9			12	29	17		59.1 $\pm$ 6.3	61.7 $\pm$ 14.2	NS	8
10			24	41	17		8.2 $\pm$ 2.4	4.8 $\pm$ 1.9	NS	5
11	No oestradiol	Oil	—	—	24		55.6 $\pm$ 10.6	7.9 $\pm$ 2.4	$P < 0.01$	17
12		Saline	—	—	24		31.0 $\pm$ 13.1	3.4 $\pm$ 0.6	$P < 0.01$	7
13	After 20 ng oestradiol	Oil	7	31	24		199.0 $\pm$ 24.2	47.0 $\pm$ 6.1	$P < 0.01$	12
14		Oil	24	48	24		85.2 $\pm$ 29.1	5.1 $\pm$ 1.6	$P < 0.01$	12
15		Oil	42	66	24		0.6 $\pm$ 0.6	1.7 $\pm$ 0.6	NS	7
16		Saline	7	31	24		60.7 $\pm$ 10.5	36.2 $\pm$ 5.9	$P < 0.01$	10
17		Saline	24	48	24		27.8 $\pm$ 12.0	2.0 $\pm$ 0.4	$P < 0.01$	5
18	After 100 ng oestradiol	Oil	7	31	24		56.6 $\pm$ 6.4	38.0 $\pm$ 2.7	$P < 0.01$	13
19		Oil	24	48	24		15.6 $\pm$ 7.0	2.6 $\pm$ 0.6	$P < 0.01$	13
20		Oil	42	66	24		1.6 $\pm$ 0.7	1.4 $\pm$ 0.8	NS	5
21	No oestradiol	Oil	—	—	31		11.4 $\pm$ 3.6	1.4 $\pm$ 0.6	$P < 0.01$	5
22		Saline	—	—	31		13.2 $\pm$ 3.4	4.6 $\pm$ 1.5	$P < 0.01$	5

*Oil or saline was instilled into the lumen of one horn of each uterus; the other horn acted as control.

treatments (Group 5 versus Groups 6 and 7: $P > 0.05$) and was lower than the sum of these responses. However, when oil was injected 7 h after oestradiol (Group 1), the mitotic response was about double the combined response to the individual treatments (Group 1 versus Groups 2 and 3: $P < 0.05$). These results suggest that, while both oestradiol and oil provide a mitotic stimulus, there is a synergistic effect between the separate stimuli only when the oil is given during a limited period after the oestradiol.

There was no evidence of a synergistic effect between two injections of oestradiol given 7 h apart (Group 13); the number of uterine mitoses induced by two injections of oestradiol 7 h apart was similar to the sum of the mitoses in animals receiving single injections (Groups 14 and 15) and killed at an equivalent time.

The relationship between the onset of decidualization and the mitotic response was investigated by processing Pontamine Sky Blue-positive areas (indicating an increase in vascular permeability associated with the onset of the decidual response) for alkaline phosphatase activity. Animals received an injection of 20 ng oestradiol, followed by intraluminal instillation of oil into both uterine horns 7 or 24 h later; animals were killed 17 or 24 h after the introduction of the oil. Most of

the animals (38 of 40) showed a positive Pontamine Sky Blue reaction. Alkaline phosphatase was also present in these uteri, but the intensity was greater in those killed 24 h after the injection of oil. As before, mitotic counts were high when the oil was given 7 h after the oestradiol (81.0 ± 16.1 , and 196.8 ± 25.5 , at 17 and 24 h after introduction of the oil, respectively). Few mitoses (3.3 ± 0.7 , $n = 12$) were seen in uteri in which oil was introduced 24 h after the oestradiol and animals killed after a further 17 h, although these animals still showed an alkaline phosphatase-positive decidual reaction. The mitotic response was considerably higher 24 h after the oil instillation (108.8 ± 18.3 , $n = 7$).

Eight animals were injected with oil either 22 h after the nidatory oestrogen or in the absence of oestrogen and were killed 48 h later to investigate how the mitotic response increased as decidualization progressed. Seven of the eight oestrogen-treated animals had large Pontamine Sky Blue-positive decidual swellings in the oil-injected horns. The number of mitoses in these horns was 410 ± 50.5 , while the control horns had 6.3 ± 3.4 ($P < 0.001$). The eight animals not treated with oestradiol had no Pontamine Sky Blue areas in the oil-injected horns and the mitotic count was 4.5 ± 1.3 , compared with 2.75 ± 0.7 in the control horns (P : NS).

Table 2. Mitosis in uterine stroma of mice after intraluminal instillation of oil into one uterine horn^a and/or subcutaneous injection of 20 ng oestradiol

Group	n	Timing of treatments (h)				Mitotic counts Mean $\pm$ SEM
		0	7	24	31	
1	6	E	Oil		K	184.3 $\pm$ 59.3
2	6	E			K	53.2 $\pm$ 9.3
3	8		Oil		K	41.2 $\pm$ 14.3
4	8				K	6.1 $\pm$ 1.1
5	6	E + oil		K		64.5 $\pm$ 6.2
6	6	E		K		49.8 $\pm$ 8.0
7 ^b	8	Oil		K		41.2 $\pm$ 14.3
8 ^b	8			K		6.1 $\pm$ 1.1
9	4	Oil	E		K	63.0 $\pm$ 17.9
10	4		E		K	45.3 $\pm$ 9.0
11	5	Oil			K	15.4 $\pm$ 9.7
12	5				K	3.6 $\pm$ 1.2
13	6	E	E	K		65.8 $\pm$ 5.4
14	6		E	K		29.5 $\pm$ 3.0
15	6	E		K		33.3 $\pm$ 5.2

^aOdd-numbered groups are the treated horn; even-numbered groups are the control horn.

^bSame groups as 3 and 4, repeated for clarity.

E = 20 ng oestradiol. K = autopsy.

High dose (100 ng) of oestradiol. High doses of oestradiol do not support the induction of decidualization by intraluminal oil instillation. To investigate this effect in relation to the uterine mitotic response, oil was injected into one uterine horn 7 h after a single injection of 100 ng oestradiol and the animals killed 17 or 24 h later. At 17 h, the number of mitoses in the oil-treated uteri was higher than in the control horns (Table 1, Group 17), but this difference was considerably reduced by 24 h (Group 18). When oil was introduced 24 or 48 h after the administration of 100 ng oestradiol and the animals killed after a further 17 or 24 h, mitotic counts were low (Table 1, Groups 20–22) and no Pontamine Blue-positive areas were found in any of the uteri.

Discussion

Martin *et al.* (1973) showed that oestrogen treatment of progesterone-treated, ovariectomized mice induced a wave of cell division in a population of stromal cells, with G1 lasting about 10–12 h, with an S phase of about 8–10 h, and a short G2 of 1–2 h (giving a cell cycle of 20–24 h). The initial experiments of the present study were consistent with these observations and showed that parenteral oestradiol and intraluminal injections of oil or saline independently stimulate mitosis of the endometrial stromal cells in the progestagen-treated mouse, with some cells undergoing mitosis within 17 h and a maximum response by 24–31 h. As the introduction of oil did not stimulate decidualization in the absence of nidatory oestrogen, and saline did not induce decidualization, even after oestrogen sensitization, these results indicate that intraluminal injection of a substance acts as a mitotic stimulus independently of any role as a decidual stimulus.

The response to combined treatment with nidatory oestrogen and intraluminal stimuli was complex and depended on the dose of oestradiol, the interval between the oestrogen and intraluminal injections, and the nature of the material instilled into the uterus. Figure 1 summarizes the relationship between these variables, standardized according to the time of intraluminal instillation. After the injection of oestradiol, the endometrium not only responded with increased mitosis as a result of the oestrogen per se (not shown), but also went through a short period (< 12 h) of heightened sensitivity to the additional mitosis-inducing effects of intraluminal injections. Thus the administration of oil during this initial mitosis-sensitive period initiated a burst of mitosis which coincided with, and augmented, the mitotic response to oestradiol. After treatment with 20 ng oestradiol, the endometrium also went through a period during which it could respond with decidualization to a suitable stimulus. When oil, but not saline, was injected during this decidual-sensitive phase, decidualization was induced and was associated with further mitosis. The decidual-sensitive phase had a later onset, and lasted longer than did the mitosis-sensitive period. It was followed by a period in which decidualizing stimuli were ineffective. The decidual-sensitive phase did not occur following a higher dose (100 ng) of oestradiol.

Work using slow-release hormone preparations has suggested that decidual sensitivity starts about 12 h after oestradiol treatment and may last for about 24 h (Finn *et al.*, 1992). In practice, however, interpretation of the response of the uterus to intraluminal stimuli is complicated by the fact that oil remains in the uterus for some time after its instillation and can provide an effective stimulus for a long period. Thus, when oil was administered soon after oestradiol, it provided a stimulus in the initial mitosis-sensitive phase and, by its persistence in the lumen, in the decidual-sensitive phase. The mitotic response to oil (but not to saline) therefore continued as the decidual reaction developed. As the cell division accompanying decidualization occurred at a time when the uteri would not respond directly to the instillation of oil, it appears that the events associated with decidualization provide a different stimulus from that produced by the presence of oil in the lumen.

These various changes in uterine sensitivity that follow the administration of oestradiol appear to be related (at least temporally) to the stage of the cell cycle reached by the stromal cells. Martin *et al.* (1973) showed that, after an injection of oestradiol, stromal cells entered G1 and then the S phase, with the uptake of thymidine beginning at 11 h, increasing up to 16 h, falling at 21 h and low at 24 h. Mitosis follows the S phase, is high at 21 and 25 h, still present at 30 h, and is low at 35 h. In the present experiments, therefore, the mitosis-sensitive period would correspond to the time when the cells are in G1, while the decidual-sensitive period would correspond to a later time, possibly when the stromal cells are in S and G2. It is possible that the phase of the cell cycle of the stromal cells may determine their response to decidual stimuli: the initial response to oestradiol may provide a population of cells in a phase of the cell cycle (S or G2) during which appropriate stimulation can divert them towards decidualization. The process of decidualization of these cells then stimulates neighbouring cells to continue the cascade of cellular proliferation and differentiation.

Fig. 1. Summary of the relationship between oestradiol and timing of oil instillation and the mitotic and decidual responses of uterine stroma of mice (standardized according to the time of intraluminal instillation). DCR: decidual response. E₂: oestradiol. (- - -) Uterus sensitive to direct mitotic effects of oil; (—) increased uterine sensitivity after oestradiol; (◁ ▷) uterus sensitive to decidual inducing effects of oil; (—) uterus insensitive to the mitotic effects and to the decidual inducing effects of oil.

Martin *et al.* (1973) suggested that a terminal mitosis of stromal cells may be necessary before decidualization. The relationship between differentiation and terminal mitosis is still an active area of interest in cell biology (for example, Rohrer and Thoenon, 1987). However, while it is apparent that the decidual reaction plays a role in stimulating mitosis in the stromal cells, the present results cast doubt on whether mitosis is a necessary prelude for the earliest steps of decidualization. In the present work, when oil was injected 7 h after oestradiol, there was both mitosis and evidence of decidualization at 17 h. This short time is not sufficient to allow the decidualization at 17 h to have been the result of mitosis followed by the induction of decidualization in the daughter cells.

The number of stromal cells undergoing mitosis increases in the area surrounding an implanting blastocyst (Finn and Martin, 1967). However, it is not clear whether this is due to an

effect of the blastocyst via the uterine epithelium, or secondary to the formation of decidual cells. The present results suggest that it is due to both. The initial mitotic effect of an intraluminal stimulus is presumably mediated via the luminal epithelium and can be augmented or inhibited depending on when the stimulus is applied relative to the time of oestradiol administration. Once decidualization has been initiated, further signals are produced that stimulate neighbouring cells to divide and differentiate. Thus, once started, there is a self-propagating chain reaction of proliferation and differentiation of stromal cells to produce the decidual cell reaction. The separation of the early mitotic response from later decidualization was evident in the uterine response to saline and oil introduction after a high dose of oestradiol: both induced an initial mitotic response but no decidualization and no continued mitosis.

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