

Site of action of indomethacin on implantation in the rabbit*

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Summary. Blastocysts recovered from oil- or indomethacin-treated donor rabbits between 5½ and 6 days after insemination and hCG injection were transferred to oil- or indomethacin-treated recipients between 135 and 147 h after hCG injection. Indomethacin treatment of donor rabbits (10 mg/kg s.c.) given every 6 h during the day before transfer had no effect on subsequent implantation of the blastocysts. However, indomethacin treatment of the recipients (10 mg/kg s.c. every 6 h from 120 to 168 h after hCG) prevented implantation of all transferred blastocysts, although 6 of the 8 rabbits died between Days 9 and 16 of (pseudo)pregnancy. Restriction of the indomethacin treatment of the recipients to only 3 injections of 10 mg/kg s.c. between 128 and 140 h after hCG injection had no effect on the implantation of the transferred blastocysts. It is concluded that indomethacin exerts its inhibitory influence on implantation via an action on the endometrium rather than on the blastocyst.

Introduction

The evidence for a role for prostaglandins (PGs) in the implantation process in several species is increasing (Lau, Saksena & Chang, 1973; Saksena, Lau & Chang, 1976; Kennedy, 1977, 1979; Biggers, Leonov, Baskar & Fried, 1978; Evans & Kennedy, 1978; Hoffman, 1978; Hoffman, DiPietro & McKenna, 1978; Oettel, Koch, Jurishko & Schubert, 1979; Holmes & Gordashko, 1980; Kennedy, Barbe & Evans, 1980; Biggers, Baskar & Torchiana, 1981; Phillips & Poyser 1981).

Implantation of blastocysts can be blocked by indomethacin treatment in mice (Lau *et al.*, 1973; Saksena *et al.*, 1976) and in rats (Kennedy, 1977; Phillips & Poyser, 1981) but not in rabbits (Hoffman, 1978). However, in these rabbit experiments the indomethacin treatment on Days 4-7 of pregnancy did reduce the number of implantation sites giving rise to viable fetuses on Day 14, and this action, while not prevented by concomitant administration of oestradiol, progesterone, LH or LH + FSH, was partly reversed by PGE-2 and PGF-2 α treatment (Hoffman, 1978). Further, Hoffman *et al.* (1978) found that one major action of indomethacin was to prevent the uterine blueing response (indicative of increased capillary permeability) at the implantation site and, associated with this, a reduced size of the implantation swellings. Similar findings have been recorded for hamsters (Evans & Kennedy, 1978). Studies of the rabbit and rat have shown that there is a close correlation between the diameter of the implantation site and subsequent fate of the embryo (Adams, 1960; Harper, 1964). Kennedy (1979) showed that the increased vascular permeability that accompanies the decidual reaction in rats is associated with increased levels of PGE and PGF in endometrial tissue. Intraluminal administration of PGE-2, but not PGI-2, was able to increase endometrial vascular permeability in indomethacin-treated, progesterone-primed

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rats (Kennedy *et al.*, 1980). These findings led Kennedy (1979, 1980) to conclude that PGs of the E series were likely to be the key mediators of increased vascular permeability at the implantation site. Specific binding of PGE-2 to endometrial membranes of stromal, but not epithelial cells, from ovariectomized rats sensitized for the decidual cell reaction, has now been demonstrated (Kennedy, Martel & Psychoyos, 1983). Similarly, in rabbits endometrial epithelial cells on Day 6 of pregnancy do not bind PGs specifically, but exhibit non-saturable uptake (Jones & Harper, 1983).

However, the source of the PGs during the implantation process has not been determined. Unlike the rat (Kennedy & Armstrong, 1981) and mouse (Racowsky & Biggers, 1983), rabbit blastocysts contain, just before implantation, large quantities of PGE and PGF, some of which may be synthesized by the blastocysts themselves (Dickmann & Spilman, 1975; Dey, Chien, Cox & Crist, 1980; Sharma, 1980; Harper, Norris & Rajkumar, 1983; Racowsky & Biggers, 1983). An important question is whether, in the rabbit, it is the PGs from the blastocyst or the endometrium which trigger the capillary permeability changes at the implantation site. The present experiments were therefore designed to inhibit PG synthesis in donor and recipient animals, and to examine the effect of such inhibition in blastocysts and endometrium on subsequent implantation by means of a blastocyst transfer technique. A preliminary report has been published (Snabes & Harper, 1984).

Materials and Methods

Mature, New Zealand White-Cambridge-strain female rabbits (> 3.0 kg) were caged individually in a controlled environment with a photoperiod of 14 h light per 24 h. They were fed with 170 g of rabbit pellets per day and water was supplied *ad libitum*. The rabbits were induced to superovulate using the method of Mukherjee, Dey, Gupta, Ramadoss & Dickmann (1978). Donor and recipient oestrous rabbits were injected subcutaneously (s.c.) with 0.1 U FSH (Sigma Chemical Company, St Louis, MO) twice daily on Days -3, -2 and -1. On Day 0, the donor rabbits were artificially inseminated with 0.5 ml of mixed sperm suspension collected, via an artificial vagina, from fertile bucks immediately before use. After insemination, animals were injected intravenously (i.v.) with 100 i.u. hCG (APL; Ayerst Laboratories, New York, NY) to induce ovulation. At the same time, the recipient rabbits also received an ovulating injection of 100 i.u. hCG.

Experiment 1. Four of the 8 donor rabbits and 8 of the 16 recipient rabbits were injected with indomethacin (10 mg/kg s.c.) in peanut oil (stock solution was 80 mg/ml) starting at 120 h after the hCG injection. Injections were continued at 6-h intervals until 132 h for donors (3 injections) and until 168 h for recipients (9 injections). The remaining rabbits served as controls and received s.c. injections of oil at the same times.

Between 135 and 139 h after hCG, the donor rabbits were killed by an i.v. overdose of pentobarbitone sodium (Diabotal; Diamond Labs, Des Moines, IA) and the uteri removed. These were then flushed by sterile technique using 10 ml warm (37°C) Brinster's medium (Brinster, 1963) with 20% of decomplexed (56°C for 30 min) rabbit serum added. Blastocysts were transferred within 20 min of recovery (135-139 h after hCG) into the uterine lumina of oil- or indomethacin-treated recipients laparotomized under general anaesthesia with pentobarbitone sodium. A 5-mm incision was made in the uterine wall near the uterotubal junction, through which a teflon tube (5 cm long; 5 mm o.d.; 3 mm i.d.) fitted to a 500 µl Hamilton syringe (Hamilton Company, Whittier, CA) containing the blastocysts was introduced. After expulsion of the contents of the tube and its withdrawal, the uterine incision was closed with two small stitches. The peritoneum, abdominal wall and skin were closed using 2-0 silk. After recovery, the animals were returned to their original cages. The design of the experiment was such that half of the blastocysts from any one donor (oil- or indomethacin-treated) were transferred into an oil-treated recipient and the rest into an indomethacin-treated recipient.

Experiment 2. This experiment was similar to Exp. 1 except that it was modified as follows: indomethacin (10 mg/kg s.c. in oil) was given only at 128, 134 and 140 h after hCG to the 8 donors and 14 recipients. No animals died with this regimen. The blastocyst transfers in this experiment were done between 143 and 147 h after hCG injection.

Observations after transfer. In both experiments, the animals were again anaesthetized and subjected to laparotomy on Day 10 of pregnancy. The external diameters of the implantation sites were measured and the number of implantations recorded. The incisions were then closed and the rabbits allowed to recover. On Day 17 in Exp. 1 and on Day 24 in Exp. 2, the rabbits were killed by an overdose of pentobarbitone sodium i.v. and the uteri removed. The diameters of the uterine swellings on Day 17, and the crown-rump lengths of the fetuses and the fetal weights were measured in both groups.

Statistical analysis. Differences in implantation rates were assessed by the χ^2 test using Yates's correction, and differences amongst means were tested by Student–Newman–Kuels procedure with $P < 0.05$ considered significant (Sokal & Rohlf, 1969).

Results

Experiment 1

As shown in Table 1, 102 out of 108 blastocysts recovered were transferred to recipients. One oil-treated donor rabbit was not used for provision of blastocysts and was allowed to maintain pregnancy until Day 17. This provided control data additional to that of Group 1 and data taken from the extensive work of Adams (1960) on normal pregnancies in Cambridge-strain rabbits. Treatment of the donors with oil or indomethacin did not affect the subsequent fate of the blastocysts, whereas indomethacin treatment continued up to the time of implantation in the recipients caused an almost complete inhibition of implantation (as assessed on Day 10). The implantations measured on Day 10 were of normal size, except for the indomethacin-treated recipients in which the diameters were significantly smaller. At Day 17, no differences existed amongst mean diameters of surviving implantation sites. Survival of embryos to Day 17 was not different in the untreated animal compared to the animals in Groups 1 and 2 receiving transferred blastocysts and treated only with oil. No embryos survived in the recipients (Groups 3 and 4) that received indomethacin to the time of implantation. Fetal weights and lengths of surviving fetuses on Day 17 were within normal limits and did not differ amongst groups.

This treatment regimen of indomethacin had a delayed toxic reaction, since 1 rabbit died on Day 9, 4 between Days 10 and 13, and a final one on Day 16 of pregnancy.

Experiment 2

Out of 88 blastocysts recovered, 84 were transferred to recipients. Implantation in all groups was entirely normal, as assessed by survival and mean diameter on Day 10 (Table 2). There was a heavy post-implantation embryonic loss, but this was not different amongst the groups, irrespective of treatment of donors or recipients. Fetal lengths and weights were within normal limits on Day 24 for all groups.

Discussion

Given the now extensive evidence that rabbit blastocysts contain large quantities of PGE and PGF on Days 6 and 7 of pregnancy (Dickmann & Spilman, 1975; Dey *et al.*, 1980; Sharma, 1980; Harper *et al.*, 1983; Racowsky & Biggers, 1983), and can also, to some extent, synthesize PGs *de novo* (Dey *et al.*, 1980; Harper *et al.*, 1983; Racowsky & Biggers, 1983), it is possible that it is these blastocyst

Table 1. Fate of blastocysts transferred to recipient rabbits between 135 and 139 h of pregnancy (Exp. 1)

| Group | Treatment | | No. of recipients | No. of blastocysts | | Diam. of implantation sites (mm) | | Viable fetuses on Day 17 | | | |
|-----------------------------|-----------|-------------|-------------------|--------------------|------------------------|----------------------------------|-------------------------|--------------------------|-------|-------------|-------------|
| | Donors | Recipients | | Transferred | Implanted (%) | Day 10 | Day 17 | No. | % | Length (mm) | Weight (g) |
| — | Untreated | Not transf. | 1* | 14† | 12 (85.7) | 12.5 ± 0.5 ^c | 28.1 ± 0.7 ^e | 11 | 100.0 | 20.2 ± 0.4 | 0.82 ± 0.02 |
| 1 | Oil | Oil | 4 | 25 | 15 (60.0) ^b | 12.3 ± 0.6 ^c | 23.7 ± 2.0 ^e | 15 | 8 | 20.7 ± 0.4 | 0.84 ± 0.01 |
| 2 | Ind.‡ | Oil | 4 | 24 | 14 (58.3) ^b | 14.0 ± 1.1 ^c | 25.5 ± 1.4 ^e | 12 | 9 | 20.3 ± 0.6 | 0.83 ± 0.05 |
| 3 | Oil | Ind.§ | 4 | 28 | 4 (14.3) ^b | 5.0 ± 0.7 ^d | 0 | 0 | 0 | — | — |
| 4 | Ind.‡ | Ind.§ | 4 | 25 | 2 (8.0) ^b | 6.8, 5.8 ^d | 0 | 0 | — | — | — |
| Normal control pregnancies¶ | | | 56 | — | 510 | 15.7 ± 0.1 | — | — | — | — | — |
| Normal control pregnancies¶ | | | 2 | — | — | — | 25.2 ± 0.5 | 22 | — | — | — |

Values are mean ± s.e.m.

* One animal, not a recipient.

† Number of corpora lutea, not blastocysts transferred.

‡ Indomethacin (10 mg/kg s.c.) 120, 126 and 132 h after hCG (3 injections).

§ Indomethacin (10 mg/kg s.c.) every 6 h starting at 120 h and continuing until 168 h after hCG (9 injections).

¶ Data from Adams (1960).

^a Significantly different at $P < 0.01$ by χ^2 analysis (Groups 3 and 4 combined). Percentages with the same superscript did not differ significantly from each other.

^b Significantly different at $P < 0.05$ by Student-Newman-Kuels test.

^c Mean diameters of Day-17 implantation sites did not vary amongst groups.

Table 2. Fate of blastocysts transferred to recipient rabbits between 143 and 147 h of pregnancy (Exp. 2)

| Group | Treatment | | No. of recipients | No. of blastocysts | | Diam. of implantation sites on Day 10 (mm) | | Viable fetuses on Day 24 | | |
|-----------------------------|-----------|------------|-------------------|--------------------|---------------|--|---|--------------------------|------------|------------|
| | Donors | Recipients | | Transferred | Implanted (%) | No. | % | Length (mm) | Weight (g) | |
| 1 | Oil | Oil | 3 | 16 | 12 (75.0) | 14.0 ± 1.2 | 6 | 50 | 58.8 ± 2.0 | 13.8 ± 0.6 |
| 2 | Ind.* | Oil | 4 | 26 | 15 (57.7) | 15.7 ± 0.6 | 5 | 33 | 58.2 ± 1.3 | 15.7 ± 0.4 |
| 3 | Oil | Ind.* | 3 | 16 | 11 (68.8) | 18.7 ± 2.2 | 3 | 27 | 56.7 ± 0.7 | 13.4 ± 0.5 |
| 4 | Ind.* | Ind.* | 4 | 26 | 15 (57.7) | 14.5 ± 1.2 | 5 | 33 | 59.7 ± 1.8 | 16.1 ± 0.3 |
| Normal control pregnancies† | | | 56 | — | 510 | 15.7 ± 0.1 | — | — | — | — |

Values are mean ± s.e.m.

* Indomethacin (10 mg/kg s.c.) at 128, 134 and 140 h after hCG.

† Data from Adams (1960).

PGs that are responsible for the prostaglandin-mediated actions locally induced at the implantation site. The more rapid release of PGE-2 compared to that of PGF-2 α on Days 5–6–8, and on Day 6–8 only compared to that of itself on Days 5 and 6 of pregnancy (Jones & Harper, 1984), suggests a possible differential release of PGE-2 (the PG implicated in the local changes of vascular permeability (Kennedy *et al.*, 1980)) during the period when such changes at the implantation site are first observed (Hoffman *et al.*, 1978). If this were the case, it seems possible that exposure of blastocysts to a milieu in which PG synthesis by both endometrium and blastocysts was inhibited up to the time of transfer at Day 6 of pregnancy should render the blastocysts deficient in PGs and unable to induce the prostaglandin-induced changes at the implantation site in untreated recipients. We know from other experiments that the treatment regimen with indomethacin (given to the donors) reduces PG levels in Day-6 blastocysts from the usual 0.67 ± 0.45 ng PGF and 0.21 ± 0.05 ng PGE/blastocyst ($n = 3$ experiments with 10 blastocysts pooled from 3 rabbits) to 0.03 ± 0.03 ng PGF and no detectable PGE/blastocyst ($n = 3$ experiments with 10 blastocysts pooled from 3 rabbits) (M. A. Jones, C. J. Norris & M. J. K. Harper, unpublished). Most of the PG in the blastocyst is found in the blastocoelic fluid (88% of PGF-2 α and 92% of PGE-2), where it is not subjected to metabolism (Jones & Harper, 1984), and therefore, theoretically, is easily released to the exterior when properly stimulated. Indeed, during in-vitro culture, 6-day blastocysts do release substantial quantities of radioimmunoassayable PGF and PGE into the culture medium— 0.18 ± 0.05 and 0.07 ± 0.01 ng/blastocyst, respectively, during a 1-h period (Harper *et al.*, 1983).

We feel certain, therefore, that the blastocysts from the indomethacin-treated rabbits contained essentially no prostaglandins at the time of transfer to recipients. The recipients were treated with two separate protocols. In Exp. 1, the indomethacin treatment was continued after transfer every 6 h up until the time of implantation. This dose was clearly toxic to the rabbits, because 6 out of 8 died after Day 9 of pregnancy. Therefore, the almost complete failure of either oil- or indomethacin-treated blastocysts to implant in these animals may not have been due to inhibition of endometrial prostaglandins, but to generalized toxicity. Indomethacin has also been reported to have other pharmacological actions (Bito, Davson & Salvador, 1976). However, none of these deaths occurred until well after actual implantation, and so this toxic effect was clearly not immediate. In addition, implantation of oil- or indomethacin-treated blastocysts in oil-treated recipients was completely normal, the success rate being greater than in previously published results (Chang, 1950). The mean diameters of the implantation sites on Days 10 and 17 were not different from previously published data (Adams, 1960), irrespective of the source of the blastocysts. In this first experiment, transfers were done when blastocysts were only a little more than 5½ days old, so it could be argued that there was sufficient time for them to sequester PGs from uterine fluid and then release them at the critical time 12–24 h later. In other experiments, we have found that blastocysts must be in the uterus for more than 6 h but not more than 20 h to induce the uterine blueing reaction at Day 6–8 of pregnancy in rabbits.

To obviate the problems of toxicity and to reduce the time for recovery of blastocyst PG levels, in Exp. 2 indomethacin treatment ceased after transfer and blastocysts were transferred at 6 days of pregnancy. The results for blastocysts transferred from oil- or indomethacin-treated donors to oil-treated recipients were similar to those in Exp. 1. Blastocyst implantation and development were not influenced by the treatment of the donors. In contrast to Exp. 1, however, blastocyst implantation in indomethacin-treated recipients was completely normal, as was embryonic growth to Day 10. Furthermore, no recipients died.

These results led to the conclusion that indomethacin treatment which affects the endometrium must be continued to the time of implantation to be effective. It is still not certain that the blastocysts also may not have had time between the time of transfer (Day 6) and Day 6–5 to synthesize and/or sequester sufficient prostaglandins and then to release them locally to initiate the vascular permeability changes normally seen at Day 6–5 of pregnancy. However, El-Banna (1980) has reported that, in rabbits, administration of 8 mg indomethacin/kg s.c. twice daily on Days 5 and 6, but not on Day 6 only or on the evening of Day 5 and morning of Day 6 only, was able to reduce the

percentage of blastocysts implanting. Given these results and those of the present experiments, the balance of probabilities would suggest that it is the endometrium rather than the blastocysts which is responsible for the production of the prostaglandins implicated in the implantation process; the mechanism localizing this action to the area adjacent to the blastocyst remains obscure.

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