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CONTRASTING ACTION OF ANTIESTROGEN (ICI-182780) FOR PREVENTING INITIATION OF EMBRYO IMPLANTATION BY ESTRADIOL OR EPIDERMAL GROWTH FACTOR (EGF)

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Summary

The pure estrogen antagonist ICI-182780, at doses above 50 µg/kg, effectively inhibited the initiation of embryo implantation in rats when administered on day 4 of pregnancy (day 1=sperm positive). The same dose inhibited the implantation initiating effect of intravenous 25 ng of estradiol-17ß in delayed implanting progesterone-primed hypophysectomized rats. In contrast, the anti-estrogen at a dose of 1 mg/kg was ineffective at inhibiting the initiation of implantation induced by intrauterine plus intravenous administration of murine epidermal growth factor to delayed implanting rats. The growth factor also initiated implantation of blastocysts transferred from donor animals injected with the anti-estrogen to progesterone-primed hypophysectomized recipients. The results clearly demonstrate that the implantation initiating effect of the growth factor is not inhibited by a pure estrogen antagonist, and therefore this estrogenic function does not appear to require action initiated by the classical estrogen receptor.

Although not clearly defined, an action of estrogen on the progesterone-primed uterus is necessary for initiating implantation of the embryo into the rodent uterus (reviewed in 1 & 2). Recent evidence indicates that estrogen action, at least as applied to mitogenic activity of the steroid, may involve synthesis of growth factors and their receptors, particularly epidermal growth factor (EGF) and transforming growth factor alpha (TGFα)(3). Furthermore there is one report that exogenous administration of EGF mimicked the effect of estrogen on the uterine and vaginal epithelium of the mouse (4). This effect of EGF was attenuated by co-administration of a so-called "pure", i.e. only antagonistic action, anti-estrogen (ICI-164,384)(5). Other studies have also shown that anti-estrogens can reduce or inhibit the effect of growth factors, suggesting an interaction between the signal transduction system of the classical estrogen receptor and the growth factors (5,6).

Recently we have shown that exogenous EGF can replace estrogen for the initiation of embryo implantation in the rat (7,8). We do not know whether this implantation-initiating effect of EGF is related to its mitogenic action, but the apparent requirement for arachidonic acid metabolites for both effects (8,9,10) suggests a similarity. Increases in arachidonic acid and its metabolites has long been associated with the estrogenic action involved with embryo implantation (1,2). With these relationships in mind we tested the ability of a "pure" anti-estrogen (ICI-182,780), which is structurally similar to that of ICI-164,384 (11), to inhibit the initiation of implantation brought about by EGF in the delayed implanting, progesterone-primed, hypophysectomized rat model. The results clearly demonstrate that while the anti-estrogen inhibits the implantation associated with endogenous or exogenous estradiol it does not inhibit this action of EGF.

Methods

Chemicals: Progesterone, estradiol-17ß, culture medium and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO.). Receptor grade murine epidermal growth factor (EGF) was purchased from Harlan Bioproducts for Science

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(Indianapolis, IN) and was dissolved in sterile phosphate-buffered (pH 7.4) saline just prior to use. The anti-estrogen ICI-182,780 [7α(9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl)estra-1,3,5(10)-triene-3,17ß-diol] was provided by ICI Pharmaceuticals (Cheshire, UK), through the courtesy of Dr. B. Vose, and was dissolved in sesame seed oil: benzyl benzoate 80:20 (v/v).

Animals: Young adult virgin female (225±25 g) rats of the Holtzman strain (Harlan, Sprague-Dawley, Madison, WI) were maintained in temperature (23±1°C) and light (lights on 0600-2000 h) controlled quarters with free access to Purina Laboratory Chow and tap water. On the afternoon of proestrus, as determined by examination of the vaginal lavage, a female was placed in the home cage of two proven male breeders of the same strain. Presence of vaginal spermatozoa on the following morning was taken as day 1 of pregnancy. Delayed implantation was induced by hypophysectomy, using the parapharyngeal approach with ether anesthesia, on the morning of day 3. Following this operation the animals were provided with 5% glucose water and softened food. Pregnancy was maintained in the hypophysectomized animals by daily injection (sc) of 2 mg progesterone dissolved in 0.1ml of sesame seed oil:benzyl benzoate (80:20, v/v). Females used as recipients for embryo transfers were hypophysectomized without regard to the stage of the estrous cycle. These animals were untreated for 72 h before receiving (sc) 2 mg progesterone daily for at least 2 days before receiving embryos. On the day of embryo transfer the hair was removed from the flank region on one side, the skin washed with alcohol and an incision made through the skin and peritoneum. After exposure of a uterine horn blastocysts were transferred with a micropipette into the lumen via a puncture made with a sterile 25 gauge needle. After replacing the uterus within the peritoneum the skin was closed with 11 mm wound clips. The blastocysts for transfer were obtained by flushing the uteri of normal day 5 pregnant rats with Dulbecco's modified Eagle's medium containing 1% bovine serum albumin (RIA grade) but no phenol red. The blastocysts were washed in the same medium before transfer.

For initiating implantation with EGF animals were anesthetized with ether and one uterine horn exposed as described above. Murine EGF (1.5µg in 3µl) was injected into the uterine lumen using a 10µl syringe with a fixed needle (Hamilton Company Reno NV), the uterus returned in the peritoneum and the wound closed with 11 mm clips. Two h later 0.2ml of saline containing 100 ug of the same EGF was injected via a lateral tail vein using light ether anesthesia. Forty-eight h later the animals were anesthetized with ether and injected (iv) with 0.5ml of a 1% solution of Chicago Blue B in 0.15 molar NaCl 15 min before killing with an overdose of ether. For testing the effect of ICI-182 on normal implantation the animals were injected (sc) with the anti-estrogen on the morning of day 4 and injected with the macromolecular dye on the morning of day 6. The uteri were examined for implantation sites, which were evident at places of embryo-uterine interaction due to increased capillary permeability (1). If no sites were present the uterine horns were flushed with saline to determine the presence and number of blastocysts. If neither sites nor blastocysts were found the animal was assumed not to be pregnant and was discarded from the study.

All procedures involving animals were reviewed and approved by the University of Kansas Medical Center Animal Care and Use Committee and followed guidelines set forth by the Public Health Service for the care and use of laboratory animals.

Results

The effect of giving ICI-182 on day 4 of pregnancy on implantation is shown in Table I. The anti-estrogen inhibits the effects of endogenous estrogen at a dose of about 50µg/kg. Even with a dose as large as 1 mg/kg blastocysts were recovered in the uteri 7 days later; implantation could not be initiated in these animals by 100 ng of estradiol benzoate given (sc) 48 h earlier.

Delayed implanting hypophysectomized control animals (group 1, Table II) received an intrauterine injection of 3µl of saline followed 2 h later by an iv injection of 0.2 ml saline. Although all animals had blastocysts there were no implantation sites. Thus the trauma of anesthesia, surgery, and intrauterine injection was insufficient to initiate implantation in progesterone-primed animals. In contrast, the animals of group 2, treated with a single iv injection of 25 ng of estradiol in 0.25 ml saline, had 50 implantation sites. This effect of estradiol, however, could be prevented by subcutaneous injection of 0.1mg/kg ICI-182 one h before giving the estrogen (group 3). The addition of intrauterine

trauma by injecting 3µl of saline and the injection of 0.2ml of saline 2 h later did not alter the inhibiting action of ICI-182 upon estradiol (group 3A). Implantation sites were missing also in animals that received 0.05 mg/kg ICI-182 one h before estradiol (group 4). Reducing the dose of ICI to 0.025mg/kg resulted in implantation being initiated in 2 of the 5 rats treated with estradiol (group 5).

TABLE I

Inhibition of Implantation by the Anti-estrogen ICI-182,780.

Dose of ICI-182 (mg/kg bd wt)	*Number with sites/ Total number*	*Number of Blastocysts or Implant sites/rat*	
1.0	*0/3*	*10.3±0.9**	*0*
0.5	*0/6*	*10.8±1.0*	*0*
0.25	*0/2*	*14*	*0*
0.125	*0/2*	*11*	*0*
0.0625	*0/2*	*10.5*	*0*
0.047	*0/2*	*11*	*0*
0.03125	*2/2*	*0*	*15*
0.0156	*2/2*	*0*	*12*

*Pregnant rats were injected (sc) on the morning of day 4 (sperm+ = day 1) and given 0.5ml of a 1% solution of Chicago Blue B 15 min before autopsy on the morning of day 6. * Blastocysts were recovered on day 11 and had not implanted after the animals received 100 ng of estradiol benzoate in oil (sc) on day 9.*

Intrauterine and intravenous dosing with EGF initiated 31 implantation sites in the 9 rats of group 6 (Table II). Administration of 1 mg/kg ICI-182 one h before the intrauterine and 3 h before the intravenous injection of EGF resulted in 50 sites in 8 of the 10 animals of group 7. The same doses of EGF initiated 22 sites in 4 of 5 rats in which the effect of 25ng of estradiol had been inhibited by 0.1 mg/kg ICI-182 (compare groups 3 and 8).

TABLE II

Effect of Antiestrogen on Initiation of Implantation Induced by Epidermal Growth Factor (EGF).

Group	*No of animals*	*ICI-182 mg/kg*	*EGF µg*	*E2 ng*	*No with implant sites/total No (percent)*	*No of sites*	*Blastocysts*
1	*5*	*0*	*0*	*0*	*0 (0)*	*0*	*7.2±1.1*
2	*5*	*0*	*0*		*5 (100)*	*50*	*---*
3	*3*	*0.1*	*0*	*25*	*0 (0)*	*0*	*9.3±0.7*
3A	*3*	*0.1*	*0*	*25#*	*0 (0)*	*0*	*9.7±1.4*
4	*4*	*0.05*	*0*	*25*	*0 (0)*	*0*	*8.2±1.4*
5	*5*	*0.025*	*0*	*25*	*2 (40)*	*9*	*7.7±1.2*
6	*9*	*0*	*1.5+ 100*	*0*	*9 (100)*	*31*	*---*
7	*10*	*1*	*1.5+ 100*	*0*	*8 (80)*	*50*	*15,2*
8	*5*	*0.1*	*1.5+ 100*	*25*	*4 (80)*	*22 6*	

Rats were hypophysectomized on day 3 of pregnancy (day 1= sperm positive) and given 2mg progesterone (sc) daily. After 6 days of delay ICI-182,780 dissolved in oil was injected (sc) 1 h before estradiol (E2) 25ng (iv) in saline or intrauterine injection of 1.5µg (3µl) EGF and 3 h before 100µg (0.2 ml,) EGF in saline (iv). Animals in groups 1 and 3A (#) received intrauterine and iv saline. Implantation sites were counted 48 h after treatment and visualized 15 min after injection of 0.5ml of Chicago Blue B in normal saline. Animals without implantation sites or blastocysts were discarded as not pregnant.

In preliminary experiments blastocysts from normal day 5 pregnant rats were transferred to the uteri of progesterone-primed hypophysectomized animals

that had received 1mg/kg ICI-182 one h before transfer. Only 2 of 9 animals had a total of 7 sites but 33 of 74 (44%) of the transferred embryos were recovered. In those studies, however, the iv dose of EGF was given at the time of the embryo transfer. Subsequent experiments revealed that EGF was more effective when given after a period of recovery from an intra-uterine injection and therefore the study was repeated using such a regimen.

TABLE III

Initiation of Implantation of Embryos Transferred to Progesterone-primed Hypophysectomized Recipients Treated with EGF.

Group	*Treatment of Recipient*	*Donor*	*Number with sites/ total number*	*Number of sites/total number transferred*
1	*EGF*	*oil*	*4/5 (80%)*	*11/42(26.2%)*
2	*EGF*	*ICI 1mg/kg*	*4/5 (80%)*	*18/39(46.1%)*
3	*ICI 1mg/kg+ EGF*	*---*	*4/5 (80%)*	*19/56(33.9%)*

Recipients received progesterone (2mg/day) for at least 2 days before transfer. ICI-182, dissolved in oil, was given to the recipient one h before embryo transfer, but it was given to the donor on the morning of day 4 of pregnancy. Blastocysts were harvested from donor animals on the morning of day 5 of pregnancy. EGF was injected (iv) 2 h after embryo transfer.

Four of the 5 hypophysectomized progesterone-primed recipients that received day 5 blastocysts from donors that were injected (sc) with oil one h before transfer had implantation sites (group 1, table III). The same result was obtained when the blastocysts were recovered from donor animals that had been treated with ICI-182 (1 mg/kg) 24 h earlier (group 2). The percentage of sites per transfer was somewhat improved in this group. Injection of ICI-182 to the recipient one h before it received day 5 blastocysts from normal untreated donors did not prevent initiation of implantation in 4 of the 5 animals injected with EGF.

Discussion

The present results clearly demonstrate that ICI-182 effectively blocks initiation of implantation when administered in a dose of about 50μg/kg on day 4 of pregnancy, before the time of the presumed endogenous estrogen surge (discussed in 2). On the other hand 1 mg/kg of the anti-estrogen given on the morning of day 5 or day 6 had no effect upon implantation (data not shown). In the delayed implanting hypophysectomized rat model, which is extremely sensitive to exogenous estrogen (12), about 50μg/kg ICI-182 blocked the effect of 25ng (≈100ng/kg) estradiol even when given only one h in advance of the latter. Although some controversy exits concerning the mechanism of action of ICI-182 (13) there is universal agreement that it functions by competitive binding to the nuclear estrogen receptor and inhibits the activation by the estrogen response element.

Intravenous administration of EGF initiates implantation in the delayed implanting rat model if the uterus is also exposed to a small amount of trauma (7,8). The latter can be provided either by the technique of embryo transfer or by intrauterine injection. The uterine trauma can be omitted if multiple doses of prostaglandins (PGE2 or PGF2α) or histamine dihydrochloride are administered (ip) before and after EGF (Chatterjee and Johnson, unpublished data). On the other hand, injection of the cyclooxygenase inhibitor indomethacin (8) but not the lipoxygenase inhibitor nordihydroguaiaretic acid (Chatterjee and Johnson, unpublished data) inhibits the effect of EGF on implantation suggesting that prostaglandins are required for the response. The mitogenic activity of EGF in BALB/c3T3 cells also requires the production of prostaglandin (9), but leukotrienes appear to be involved with EGF action in A431 cells (10).

The actions of several growth factors have been shown to be inhibited by anti-estrogens. Increases in progesterone receptor, an estrogenic response in the rat uterus that is also induced by cyclic AMP and insulin-like growth factor I was inhibited by ICI-164384 (6). The mitogenic activity of EGF in reproductive tract tissues was inhibited by the same anti-estrogen (5). However, conflicting reports regarding the effects of anti-estrogens on growth factor action in breast cancer cells have appeared. Vignon et al (14) reported that anti-

estrogens inhibited the mitogenic action of EGF and IGF-1 on MCF-7 breast cancer cells. These results were recently confirmed (15). In contrast, Cormier and Jordan (16) reported that anti-estrogens, tamoxifen or ICI-164384, had no effect upon the mitogenic action of EGF in MCF-7 cells. The latter authors also found that with mixtures of EGF and estradiol, only the action of the steroid was inhibited by the anti-estrogens.

ICI-182 certainly did not inhibit the implantation initiating effect of EGF in the present experiments. Actually there is a suggestion that the anti-estrogen enhanced the action of EGF. Comparing the results of group 6 and 7 (Table II) it is apparent that with EGF alone the average number of implantation sites per rat was 3.4 but when ICI-182 was administered an hour before intrauterine EGF the rate was 6.2. Furthermore, only 1 of the 9 rats that did not receive ICI-182 had implantation sites in both uterine horns, i.e. the injected and non-injected sides, whereas with ICI-182 5 of the 8 animals had implantations in both horns.

Previous studies have shown that estrogenic effects on the uterine epithelium and the embryo trophoblast are required for initiating implantation (12). That is, delayed implanting embryos transferred into a progesterone-primed uterus that had been exposed to intravenous estradiol one h earlier do not implant. If on the other hand, the embryos were obtained from a uterus that had been exposed to estrogen one h before transfer then implantation occurs. Intravenously administered EGF is also more effective at initiating implantation of transferred blastocysts obtained one h after the donor received estradiol (7). In the present study non-delayed embryos, i.e. normal day 5 blastocysts that had been exposed to endogenous estrogen action, were used for the transfer experiments. EGF initiated implantation of these embryos (group 1, Table III) but only 26% of those transferred implanted. The growth factor also initiated implantation of normal day 5 blastocysts when the recipient was injected with the anti-estrogen. ICI-182 was given on day 4 of pregnancy to the donor of the embryos with the thought of inhibiting the effect of endogenous estrogen and thus possibly preventing the implantation initiating action of EGF. However, the growth factor was at least as effective at inducing implantation of these embryos as those from the control animals. This suggests that the effect of ICI-182 does not mimic the effect of hypophysectomy in producing delayed implanting embryos.

In summary, the purely antagonistic anti-estrogen, ICI-182 effectively prevents the effect of endogenous and exogenous estradiol for initiating implantation but even at doses 20 times those effective against the steroid it does not inhibit the effect of EGF for the same response.

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