AUTORADIOGRAPHIC STUDIES ON [^3H]OESTRADIOL LOCALIZATION IN THE BLASTOCYSTS AND UTERUS OF RATS DURING DELAYED IMPLANTATION

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Summary. The present studies were undertaken to obtain information about the localization of oestradiol in rat blastocysts and to elucidate the interrelationship between hormone localization and function. Tritium-labelled oestradiol was administered intraluminally into the uterus or subcutaneously in rats in which implantation was delayed by ovariectomy on Day 3 *post coitum* followed by treatment with progesterone. Rats were killed 5, 15 and 30 min after intraluminal instillation or 5, 15 and 60 min after subcutaneous injection of [^3H]oestradiol. Autoradiograms of flushed-out blastocysts as well as a blastocyst *in situ* showed no retention and subcellular concentration of radioactivity while, in uterine stromal, glandular and muscle cells, characteristic nuclear concentration and retention of the steroid were observed. Since blastocysts are metabolically activated by treatment with oestradiol, several possible mechanisms are discussed to explain the activation of blastocysts in the apparent absence of oestradiol-binding proteins in cells of the blastocysts.

INTRODUCTION

Metabolic activities characterized by DNA, RNA and protein synthesis are markedly decreased in blastocysts during delayed implantation in lactating mice (Weitlauf & Greenwald, 1965) or during experimentally induced delayed implantation in rats (Prasad, Dass & Mohla, 1968; Gulyas & Daniel, 1969; Jacobson, Sanyal & Meyer, 1970) and are enhanced following administration of oestrogen (Weitlauf & Greenwald, 1965; Prasad *et al.*, 1968; Dass, Mohla & Prasad, 1969; Gulyas & Daniel, 1969; Jacobson *et al.*, 1970; Mohla & Prasad, 1971). These studies indicate that oestrogens, which act directly on the blastocysts or indirectly through a uterine factor, stimulate synthetic activities in preimplantation rat blastocysts. Oestrogenic activation of the blastocysts in diapause is demonstrable within 5 min of a subcutaneous administration of oestradiol in increased incorporation of [^3H]uridine into RNA (Mohla & Prasad, 1971). The present studies were, therefore, undertaken to determine

whether [^3H]oestradiol could be localized by autoradiographic techniques to provide evidence for the direct action of oestrogen on the blastocysts.

MATERIALS AND METHODS

Adult virgin female rats of the Holtzman strain, ranging in weight from 200 to 220 g, were used. They were housed in an air-conditioned room and fed a balanced diet. Females were caged with males. Mating was confirmed by the presence of spermatozoa in the vaginal smear; the day on which spermatozoa were found in the smear was regarded as Day 1 of pregnancy. Delayed implantation was induced according to the method of Cochrane & Meyer (1957). Mated females were bilaterally ovariectomized on Day 3 of pregnancy. On the day of ovariectomy and thereafter, each rat was injected subcutaneously with 4 mg progesterone in oil/day until the termination of the experiment. Surgical procedures were carried out under light ether anaesthesia using semi-sterile conditions.

In the first experiment, 2,4,6,7- ^3H oestradiol-17 β , specific activity 95 Ci/mmol, obtained from New England Nuclear Corp., was evaporated to dryness over a stream of nitrogen, dissolved in absolute ethanol and diluted to the appropriate concentration with isotonic saline. The steroid was injected subcutaneously in a dose of 0.2 μg /100 g body weight. Rats were killed 5, 15 and 60 min after the administration of oestradiol. Thirty-six blastocysts were collected from the uteri of fourteen rats.

In another series of experiments, 0.005 μg ^3H oestradiol in 0.05 ml of physiological saline was instilled into the lumen of each uterine horn by means of a 26-gauge needle. The uteri were exposed by a mid-ventral incision and the steroid was instilled into the uterine horn from the cervical end which was ligated after withdrawal of the needle to avoid leakage of the oestrogen into the peritoneal cavity. Rats were killed 5, 15 and 30 min after exposure to ^3H -oestradiol; fifty-four blastocysts were collected from the uteri of seventeen rats. The uteri were removed and dissected free of fat and connective tissue. The uterine horns were flushed from the cervical end with physiological saline. The blastocysts were collected, immediately washed two or three times with isotonic saline at room temperature, and transferred to slides pre-coated with photographic emulsion, Kodak NTB 3. A few blastocysts were mounted unwashed on emulsion-coated slides. The respective uteri were also washed with saline and cut into small pieces of 1 to 2 mm in length, placed on a tissue holder, and frozen in liquefied propane cooled by liquid nitrogen. The tissues were cut in a Wide-Range cryostat (Harris Mfg. Co., Cambridge, Mass.) at 1 or 2 μm , freeze-dried with a Cryopump (Thermovac Inc., N.Y.), and then mounted on desiccated emulsion-coated slides (Kodak NTB 3). The mounted slides were exposed in a desiccator box at -15°C . The autoradiographic exposure time ranged from 3 months to over a year. After photographic exposure, the slides were developed, fixed and stained with methylgreen-pyronin. The procedure has been described in detail (Stumpf, 1970). From two of the rats, which were treated subcutaneously with ^3H oestradiol and bearing blastocysts, the uteri were removed without flushing and processed for autoradiography as described above.

RESULTS

Blastocyst

Blastocysts collected from the uteri of rats treated subcutaneously or intraluminally with [³H]oestradiol are shown in Pl. 1, Figs 1 to 7. None of the blastocysts showed any cellular or subcellular concentration or retention of radioactivity at any of the time intervals studied, even after prolonged exposure up to 12 months. Differences were evident in the autoradiograms of blastocysts mounted without (Pl. 1, Figs 1, 3, 5 and 6) or with (Pl. 1, Figs 2 and 4) prior washing in saline. Unwashed blastocysts, both after subcutaneous and intraluminal administration of [³H]oestradiol, showed diffusely distributed radioactivity similar in intensity to that in the surrounding fluid. The washed blastocysts showed no radioactivity even after a year's exposure. No differences existed between the inner cell mass and the other portions of the blastocysts. Pyronin-positive processes arising from the surface could be seen in blastocysts (see Pl. 1, Figs 1 and 2) after staining with methylgreen-pyronin for DNA and RNA.

Uterus

After intraluminal injection of [³H]oestradiol, the radioactivity disappeared from the lumen so that no retention in the uterine lumen was visible at 5 min and thereafter. As early as 5 min, the luminal epithelium, including the luminal contents, showed less radioactivity than the structures of the substantia propria. Although the radioactivity was reduced in the luminal region, a gradient of radioactivity existed in the substantia propria and muscularis, decreasing towards the outer structures of the uterus, being maximal at 5 min. The subcellular distribution of radioactivity was most heavy, at all time intervals, in the nuclei of the glandular epithelium in contrast to the luminal epithelium, where no such concentration and retention of radioactivity appeared. Nuclear concentration existed also in the cells of the substantia propria and muscularis, more heavily in the former and in the cells immediately surrounding the blood vessels. After subcutaneous injection of [³H]oestradiol, the pattern of distribution of radioactivity at 5, 15 and 60 min essentially resembled the one seen after intraluminal instillation at 30 min, with lack of retention of radioactivity in the luminal epithelium but maximal concentration in the nuclei of the glandular epithelium, followed in intensity by cells of the substantia propria and the muscularis. In one instance, at 5 min after subcutaneous injection of [³H]oestradiol, sections of a blastocyst, still *in situ*, could be prepared (Pl. 1, Fig. 7). In these sections, the blastocyst *in situ* and the adjacent luminal epithelium showed no concentration of radioactivity, in contrast to the retention and concentration of radioactivity in nuclei of substantia propria cells (Pl. 1, Fig. 7) and glands (Pl. 1, Fig. 8). The dry-mount autoradiograms of the blastocyst *in situ* confirm the findings of the smear-mount autoradiograms with the isolated blastocysts.

DISCUSSION

Although blastocysts in diapause were exposed to oestradiol, instilled directly

into the uterine lumen or diffused into the lumen following subcutaneous administration, there was no obvious subcellular concentration and retention of radioactivity in cells of the blastocyst in contact with the photographic emulsion. By contrast, uterine tissues obtained from the treated animals showed varying degrees of uptake and retention of radioactivity in glandular, stromal and muscle cell nuclei. The luminal epithelium, similar to the blastocysts, was devoid of a selective subcellular retention of radioactivity. Such a lack or reduction of oestrogen uptake in luminal epithelium has been observed after progesterone pretreatment (W. E. Stumpf and M. Sar, unpublished observation), but not in the castrated and untreated immature intact rat (Stumpf, Baerwaldt & Sar, 1971).

Diffusely distributed radioactivity observable in the unwashed blastocysts corresponded with the radioactivity in the surrounding fluid. Unbound radioactivity adsorbed to or contained in the unwashed blastocysts was removed through washing with isotonic saline. A comparison of the uptake of oestradiol by the blastocyst with the uptake by the uterus shows that the uterus concentrates oestradiol even within 5 min of the administration of oestradiol and increases in intensity with time up to 60 min, whereas the outer cells of the blastocysts do not show uptake and concentration of oestradiol at any time interval studied. Mohla & Prasad (1971) showed that the blastocysts in diapause lying free in the uterine lumen were activated and exhibited marked increase in RNA synthesis within 5 min of a subcutaneous administration of oestradiol; RNA synthesis in the uterine epithelium was optimally stimulated at the same time. These results suggest a concurrent activation of the blastocyst and uterine epithelium within 5 min following treatment with oestradiol. In view of the lack of detectable concentration and retention of radioactivity in the blastocysts, it is questionable whether activation of the blastocyst is due to a direct action of oestradiol on the blastocyst or whether oestrogen

EXPLANATION OF PLATE 1

Figs 1 to 8. Autoradiograms of blastocysts (Figs 1 to 7) and uterine tissues (Figs 7 and 8) from pregnant rats after injection of 2,4,6,7- ^3H oestradiol-17 β . The unwashed blastocysts, at 5 min after subcutaneous (Fig. 1) or intraluminal (Fig. 3) injection, showed a high amount of diffusely distributed radioactivity. Cellular and subcellular concentration of radioactivity was not observed at any time interval in the smear-mounted blastocysts (Figs 1 to 6) or in the blastocyst cut *in situ* (Fig. 7), even after prolonged exposure times. Similarly, the luminal epithelium of the endometrium did not concentrate radioactivity. This is in contrast to the accumulation of radioactivity in the nuclei of glandular (Fig. 8) and stromal cells (Figs 7 and 8). All autoradiograms were stained with methylgreen-pyronin for DNA (blue) and RNA (red).

FIG. 1. At 5 min after subcutaneous injection, unwashed. Exposure time, 376 days. $\times 320$.

FIG. 2. At 5 min after subcutaneous injection, washed. Exposure time, 372 days. $\times 590$.

FIG. 3. At 5 min after intraluminal injection, unwashed. Exposure time, 41 days. $\times 410$.

FIG. 4. At 5 min after intraluminal injection, washed. Exposure time, 42 days. $\times 320$.

FIG. 5. At 15 min after subcutaneous injection, unwashed. Exposure time, 126 days. $\times 330$.

FIG. 6. At 1 hr after subcutaneous injection, unwashed. Exposure time, 134 days. $\times 400$.

FIG. 7. Uterine tissue with blastocyst *in situ*, 5 min after subcutaneous injection. Exposure time, 222 days. $2\ \mu\text{m}$. $\times 690$.

FIG. 8. Uterine tissue, 1 hr after subcutaneous injection. Exposure time, 422 days. $2\ \mu\text{m}$. $\times 610$.

PLATE I

action is brought about through stimulation of the uterus and, if so, how this action is mediated.

The effects of oestrogen (oestrone or oestradiol) in stimulating synthetic activities in the dormant blastocysts have been demonstrated in the rat and mouse in a series of experiments *in vitro* and *in vivo* (Weitlauf & Greenwald, 1965; Prasad *et al.*, 1968; Smith, 1968; Weitlauf & Greenwald, 1968; Dass *et al.*, 1969; Sanyal & Meyer, 1969, 1970; Jacobson *et al.*, 1970; Wu & Meyer, 1970; Mohla & Prasad, 1971; Smith & Smith, 1971; Weitlauf, 1971). In all studies involving exposure of blastocysts *in vivo* to oestrone or oestradiol, the direct effects of oestrogen on the blastocyst cannot be distinguished from the effects of oestrogen mediated through the uterus (Weitlauf & Greenwald, 1965, 1968; Prasad *et al.*, 1968; Dass *et al.*, 1969; Sanyal & Meyer, 1969, 1970; Jacobson *et al.*, 1970; Wu & Meyer, 1970; Mohla & Prasad, 1971). However, treatment of blastocysts *in vitro* with oestrogen demonstrates a direct stimulatory effect on the blastocysts (Smith, 1968; Smith & Smith, 1971). Smith (1968) treated mouse blastocysts with [³H]-oestradiol and showed that the radioactivity was taken up and retained by the cultured blastocysts for at least 1 hr. Such treated blastocysts implanted better than blastocysts not treated with oestradiol on transfer to uteri of ovariectomized mice treated with progesterone. The manner in which these oestradiol-treated blastocysts initiated a decidual cell response in the uterus is not clear. The possibility that some substances which are released from the blastocyst stimulate the decidual cell response needs to be considered. The rat blastocysts have been shown to release some substance into the uterine horn (Psychoyos, 1967a). Our studies with blastocysts, stained with methylgreen-pyronin (Pl. 1, Figs 1 and 2), show pyronin-positive processes arising from the oestradiol-treated blastocysts. Pyronin is used as a stain for RNA.

Several explanations are possible for the activation of blastocyst cells in the apparent absence of concentration and retention of oestradiol.

(a) The blastocyst may be deficient in subcellular macromolecules to which oestradiol binds. The blastocyst represents a mass of cells in which differentiation of germinal cell layers has not yet begun. It is likely that it represents a stage in which oestrogen-binding proteins are not yet differentiated.

(b) Binding of oestradiol, even if it occurs, may be so transient as not to be detectable at the earliest time interval of 5 min, as in the present study.

(c) The action of oestradiol in activating the blastocyst may be through an initial activation of RNA synthesis not necessarily involving the continuing binding of oestradiol to the cells of the blastocysts, similar to the action of oestrogen on the uterus postulated by Segal, Davidson & Wada (1965). They showed that oestrogen-induced uterine RNA mimics the action of oestrogen in stimulating morphological changes in the uterus and blastocysts in a manner similar to implantation evoked by oestradiol. They postulated that the regulatory action of oestradiol on the uterus was primarily on the biosynthesis of RNA; after this primary effect, the hormone was no longer needed for the manifestation of oestrogenic action. Unhjem (1971) and other investigators showed that tissue specific RNA molecules may act as templates for further RNA synthesis in cells, resulting in characteristic growth responses. However, Psychoyos

(1967b) was unable to demonstrate effects on blastocysts with RNA extracted from pseudopregnant rat uteri.

(d) Action of oestradiol may be mediated through the uterus by stimulation of a uterine factor.

(e) Activation of blastocysts by oestradiol may be through alteration of cell permeability.

(f) Oestradiol may act on the blastocysts through activation of cyclic AMP (Mohla & Prasad, 1970). As in the present autoradiographic studies with [^3H]oestradiol, however, no subcellular concentration in blastocysts could be observed in similar experiments after administration of tritium-labelled cyclic AMP (M. R. N. Prasad, W. E. Stumpf and M. Sar, unpublished observation).

It should be noted here that the autoradiographic approach with collapsed unsectioned blastocysts, as used previously by other investigators (Dass *et al.*, 1969; Jacobson *et al.*, 1970; Mohla & Prasad, 1971) and in this study, probably does not record localization of tritium-derived radioactivity from the inner cell mass. Superimposition of structures also impairs the precise attribution of silver grains. Despite these disadvantages, the reports just cited have demonstrated the usefulness of unsectioned blastocysts and the present results can be related to these studies published in the literature. Since only one blastocyst was sectioned in the present experiments, these studies of blastocysts *in situ*, although difficult, will have to be extended, including earlier and later time intervals. Oestrogen target cells in the inner cell mass with oestrogen binding and, perhaps, indirect oestrogenic effects on the various cells of the blastocysts, remain a distinct possibility.

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