Role of mast cells in oestradiol effects on the uterus of ovariectomized rats

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The role of mast cells and their main secretory products in the effects of oestradiol on the uterus was investigated. Ovariectomized rats were treated with a single injection of oestradiol (10 µg per rat, i.m.) or vehicle together with drugs affecting the activity of mast cells, cromoglycate (10 mg per rat, i.m.), which diminishes the degranulation of mast cells, or compound 48/80 (0.5 mg per rat, i.m.), which enhances this process. Oestradiol or vehicle was also administered with two important secretory products of mast cells, heparin (0.4 mg per rat, i.m.) or histamine (2 mg per rat, i.m.). All drugs were injected simultaneously with oestradiol (first injection) and then every 6 h until the animals were killed. Observations were performed at 24, 36 and 48 h after oestradiol or vehicle injection. The condition of mast cells was determined by the percentage of degranulated mast cells in sections stained with toluidine blue. Oestradiol-induced effects in the uterus were estimated by the mitotic index, proliferating cell nuclear antigen-labelling index, DNA content, volumes of cells, nuclei and nucleoli in the luminal epithelium, glandular epithelium and stroma cells of the endometrium. Cromoglycate treatment resulted in a decrease in both mast cell degranulation and all examined oestradiol effects in the uterus at all periods of observation. Compound 48/80 increased mast cell degranulation and expression of one aspect of oestradiol effects on the volumes of cell compartments. Histamine or heparin led to a marked increase in the cell, nucleus and nucleolus volumes in all uterine structures. However, heparin produced a depression in proliferation, whereas histamine had a weak transient stimulating action on this process. No effects of the protocols were found in the absence of oestradiol treatment. These results suggest that mast cells are involved in the realization of oestrogen action, including the stimulation of cell growth and proliferation in the uterus, and that the effect of mast cells is mediated by both histamine and heparin.

Introduction

Many studies have investigated the inter-relationships between mast cells and the action of oestrogens on the uterus. Oestrogens increase the functional activity of mast cells (Iversen, 1964; Maraspin and Bo, 1971; Brandon and Evans, 1983; Huang *et al.*, 1995; Jeziorska *et al.*, 1995) and increase the concentration of histamine in the uterus (McKercher *et al.*, 1973; Hine *et al.*, 1985; Gunin, 1989; Cocchiara *et al.*, 1992). Although contradictory data have also been obtained (Szego, 1965; Wordinger *et al.*, 1985; Padilla *et al.*, 1990; Drudy *et al.*, 1991; Gosden *et al.*, 1993), it has been suggested that mast cells play a role in the realization of oestradiol effects in the uterus.

Mast cells and histamine have been shown to be involved in the development of several oestrogen-like (Spaziani, 1963; Pankova *et al.*, 1985) and oestrogen-induced effects (Mohla and Prasad, 1969; Castro Vazquez *et al.*, 1976; Marshall and Senior,

1986) in the uterus, although some oestrogen-dependent and oestrogen-like processes remain unaffected (Brody *et al.*, 1975; Clark *et al.*, 1977; Terada *et al.*, 1985; Wordinger *et al.*, 1985). There are no data demonstrating the role of mast cells in oestrogen-induced proliferation and cell growth in the uterus, or in oestrogen action on the uterus under blockade of functional activity of mast cells. Nor are there data about involvement of histamine and heparin, the two most important mast cell secretory products, in oestrogen-induced cell growth and division in the uterus. Hence, a role of mast cells in the action of oestradiol on the uterus remains unclear. The present study was designed to investigate the role of mast cells and their main secretory products in the realization of the most important oestradiol effects, including the activation of growth and proliferation in the uterus.

Some of the data reported here on the effects of heparin have been published (Gunin, 1997); however, it was considered useful to include those results in this paper to allow comparison between heparin and histamine action and better understanding of the role of mast cells in oestradiol effects on the uterus.

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Materials and Methods

Animals

All procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals (Universities Federation for Animal Welfare, Potters Bar, Herts).

White female mature unmated rats weighing 150–180 g were obtained from Central Nursery for laboratory animals of the Russian Academy of Medical Sciences (Moscow), and were housed under the same conditions with free access to water and food. One month before the experiment, all the rats were ovariectomized under anaesthesia with ketamine and droperidol (7 mg per rat; 0.1 mg per rat, i.m., respectively; Gedeon Richter, Budapest).

Treatment schedule

Two experimental series were designed. In Expt 1, the rats were treated with oestradiol together with drugs affecting mast cell degranulation, to compare the action of oestradiol on the uterus with a background of either high or low activity of mast cells. Disodium cromoglycate was chosen as a drug that stabilizes mast cells and prevents their degranulation (Tainsh *et al.*, 1991) and compound 48/80 was chosen as an agent that produces a strong degranulation of mast cells (Barker, 1967; Josefsson, 1968). In Expt 2, rats were treated with oestradiol together with the two most important secretory products of mast cells: heparin and histamine.

The rats were treated with a single injection of oestradiol dipropionate (10 µg per rat; i.m.; Minmedprom, Rostov-Don) or vehicle (olive oil; 0.1 ml per rat, i.m.) together with injections of disodium cromoglycate (10 mg per rat, i.m., Fisons Ltd, Loughborough) or compound 48/80 (0.5 mg per rat, i.m., Sigma Chemical Co., St Louis, MO), or heparin (molecular weight approximately 16 kDa; 0.4 mg (50 iu) per rat, i.m.; Minmedprom, Moscow), or histamine dihydrochloride (2 mg per rat, i.m.; Merck, Darmstadt), or saline vehicle (0.15 mol sodium chloride l⁻¹, 0.1 ml per rat, i.m.).

All drugs were dissolved in saline and injected simultaneously with oestradiol or olive oil (first injection) and then every 6 h until the animals were killed. Observations were made at 24, 36 and 48 h after oestradiol or olive oil injection. There were ten rats per oestradiol treated group, and five rats per olive oil treated group.

Manipulations with tissue specimens

The middle segments of uterine horns were removed from animals under deep ether anaesthesia and placed immediately in fixing fluids. The animals were then killed by exsanguination. Part of the tissue specimens from each animal was fixed in Carnoy's fluid (1.5 h at room temperature) and another was fixed in Bouin's fluid (24 h at room temperature). All material was then dehydrated and embedded in paraffin wax and transverse sections (5–6 µm) were prepared.

Analysis of mast cells

The effectiveness of action of the drugs on mast cells was estimated by calculating the percentage of degranulated mast

cells in three uterine regions: the endometrium, myometrium and mesometrial triangle (Gibbons and Chang, 1972). For this, sections fixed in Bouin's fluid were stained with 0.1% (w/v) toluidine blue in 40% (v/v) ethanol for 15 min. Degranulated and non-degranulated mast cells in each compartment were counted separately under a light microscope. Mast cells with more than three granules outside of the cell shape or with empty cavities in the cytoplasm were considered as degranulated (Gibbons and Chang, 1972). The counting procedure was performed in each third section in a series of sections and at least ten sections were viewed for each rat.

Analysis of oestradiol effects

The action of oestradiol on the uterus was assessed on the basis of mitotic index, proliferating cell nuclear antigen (PCNA)-labelling index, DNA content, and the volumes of cells, nuclei and nucleoli. These parameters reflect two of the most important aspects of oestrogen action on the uterus: tissue proliferation and growth.

Mitotic index. Mitotic index was determined for the luminal epithelium, glandular epithelium and stroma cells of the endometrium in the sections prepared from tissue fixed in Carnoy's fluid and stained with iron haematoxylin (Gunin, 1996). Mitotic index is the proportion of cells in any phase of mitosis out of the total number of cells examined. At least 3000 cells were viewed in each structure for each animal.

PCNA immunoreactivity, PCNA-labelling index. Sections from tissue fixed in Bouin's fluid were hydrated, and non-specific binding was blocked with 1% normal goat serum in Tris-buffered saline (TBS) pH 7.2–7.6 for 1 h. Tissues were incubated for 2 h with primary anti-PCNA mouse monoclonal antibody (DAKO, Glostrup) diluted 1:50 in TBS with 1% normal goat serum and with 0.1% (v/v) Triton X-100. After rinsing in TBS for 3 × 5 min, sections were incubated for 1 h with secondary goat anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (Jackson Immuno-Research Laboratories Inc., West Grove, PA) diluted 1:50 in TBS with 0.1% Triton X-100, and then rinsed in TBS for 3 × 5 min. Alkaline phosphatase activity was revealed using naphthol AS-BI-phosphate and new fuchsin as chromogens. All procedures were performed at room temperature. In control sections, the primary antibody was replaced with normal goat serum. The slides were examined under a light microscope at × 400–1000 magnification. Positive staining by primary antibody was indicated by a red precipitate localized in the nuclei. PCNA-labelling index was determined in the luminal epithelium, glandular epithelium and stroma cells of endometrium by counting the proportion of PCNA-positive cells of the total number of cells examined. At least 3000 cells in each structure were examined for each animal.

DNA content. DNA content in luminal epithelium, glandular epithelium and stroma cells of the endometrium was determined by Feulgen's reaction in sections prepared from tissue fixed in Carnoy's fluid. Feulgen staining was quantified by photometric measurement of light absorbance (*D*) for the

nuclei. Photometry was performed using a light microscope and FMEL-1A microspectrophotometer (LOMO, St Petersburg) by measuring the intensity of light passed through the equal areas occupied by the nucleus (F) and occupied by the structures other than nucleus (F_0). Absorbance (light absorption) was calculated from the formula: $D = 1g(F_0/F)$, multiplied by the nucleus volume, and the DNA content was expressed in arbitrary units. At least 100 nuclei were analysed for each structure in each rat. All procedures for evaluation of DNA content were made as described by Gunin (1996).

Volumes of cells, nuclei and nucleoli. These volumes were determined in the luminal epithelium, glandular epithelium and the cells of endometrial stroma in uterine sections fixed in

Carnoy's fluid and stained with iron haematoxylin. The calculation of the volumes was based on results of morphometry performed using an ocular micrometer and light microscope. The sizes of cells, nuclei and nucleoli were determined and the volumes calculated, as described by Gunin (1996). At least 100 measurements were performed for each object examined from each animal.

Statistical analysis

Arithmetic means and standard errors were calculated in each data group. The significance of differences was evaluated by Student's *t* test. The significance of the influence of the treatments at different time points after oestradiol or vehicle injection was determined by two-way analysis of variance.

Results

Condition of mast cells

Treatment with oestradiol caused an intensive degranulation of mast cells for all the uterine compartments, compared with those for animals treated with olive oil (Fig. 1). All statistical comparisons among corresponding data from control rats treated with oestradiol and olive oil with saline were significant ($P < 0.05$, Student's *t* test). However, there are no significant differences among the data from different time groups. Injections of cromoglycate to rats treated and untreated with oestradiol led to a decrease in the proportion of degranulated mast cells in all the uterine compartments at all points of observation (Fig. 1). In contrast, treatment with compound 48/80 enhanced mast cell degranulation in rats that received either oestradiol or olive oil. In this case, almost all results were significant (Fig. 1). Two-way analysis of variance showed that cromoglycate or compound 48/80 markedly increased action on mast cells degranulation (Fig. 1).

Oestradiol effects under treatment with cromoglycate

Treatment with cromoglycate resulted in a decrease in all uterine parameters including mitotic and PCNA-labelling indices, DNA content, and cell, nucleus and nucleolus volumes in all structures, compared with those of rats treated with

Fig. 1. The effects of cromoglycate and compound 48/80 on mast cell degranulation in the uteri of ovariectomized rats treated and untreated with oestradiol. Data from rats treated with (a) oestradiol (a single injection, 10 µg per rat, i.m.) or (b) vehicle (olive oil, 0.1 ml per rat, i.m.) together with cromoglycate (●, ○) or compound 48/80 (▲, △) are compared with the data from control rats treated with oestradiol (■) or olive oil (□) together with saline. Cromoglycate disodium (10 mg per rat, i.m.) or compound 48/80 (0.5 mg per rat, i.m.) was injected at intervals of 6 h after oestradiol injection. Measurements were made at 24, 36 and 48 h after oestradiol or olive oil injection. Values are means ± SEM. The significance of the influence of cromoglycate or compound 48/80 on the mast cell degranulation in the uterus of ovariectomized rats at 24, 36 and 48 h after oestradiol or olive oil injection (two-way analysis of variance) is indicated.

oestradiol (Fig. 2). However, differences were not so marked and only some were significant.

Oestradiol effects under treatment with compound 48/80

In rats treated with oestradiol plus compound 48/80, all data for cell, nucleus and nucleolus volumes in all structures at all time-points were higher than in control animals (Fig. 2). Most of these differences were significant. The parameters reflecting the proliferative component of oestradiol action (mitotic index, PCNA-labelling index and DNA content) did not change after treatment with compound 48/80 (Fig. 2). However, the mitotic index for glandular epithelium at 36 h and the DNA content in stroma cells at 24 and 48 h were significantly greater than control values.

Oestradiol effects under treatment with histamine or heparin

Treatment with oestradiol plus histamine led to an increase in all tested parameters in the uterus (Fig. 3). Only the mitotic indices for luminal epithelium at 48 h and for glandular epithelium and stroma cells at 36 and 48 h after oestradiol injection were lower than in controls.

Treatment with oestradiol plus heparin led to a reduction in mitotic activity and DNA content for all uterine structures examined compared with control oestradiol-treated rats (Fig. 3). In the luminal and glandular epithelia of rats treated with oestradiol and heparin, the PCNA-labelling index was almost unchanged at all time points compared with that of oestradiol-treated animals. In the stroma cells for rats treated with oestradiol plus heparin, the PCNA-labelling index was higher than in rats treated with oestradiol only at all time points (Fig. 3). Cell, nucleus and nucleolus volumes in all uterine structures at all time points were much higher after treatment with oestradiol plus heparin, and most comparisons were statistically significant (Fig. 3). In comparison with histamine, the effect of heparin on volume parameters is similar but more marked. In contrast to histamine, which stimulates oestradiol-induced proliferative activity, heparin produces a decrease in these reactions.

In ovariectomized rats that were not treated with oestradiol, none of the treatment protocols used produced any significant changes compared with animals treated with olive oil. The parameters of rats treated with olive oil and saline 24 h after olive oil injection are presented (Figs 2 and 3 at 0 h). The data of rats treated with olive oil and cromoglycate, compound 48/80, histamine, heparin, or saline were very similar (data not shown).

Treatment with cromoglycate results in a decrease in mast cell degranulation and all tested oestradiol effects in the uterus. The action of cromoglycate on cell proliferation and growth is approximately equal. In contrast, compound 48/80 increases mast cell degranulation and expression of one aspect of oestradiol effects on cell growth. Histamine and heparin also affect the action of oestradiol on the uterus. Their effects on volume parameters are similar and include marked increases in cell, nucleus and nucleolus volumes in all the uterine structures. However, heparin produces a marked reduction of proliferation, whereas histamine has a weak transient stimulating

action on oestradiol-induced proliferative processes in the uterus. No effect of any of the protocols used was found in the absence of oestradiol treatment.

Discussion

The present study was designed so that oestradiol could act on the uterus in the conditions of blockade or activation of mast cells. For this purpose two drugs known to modulate the functional activity of mast cells were used: disodium cromoglycate, which decreases mast cell secretion (Tainsh *et al.*, 1991), and compound 48/80, which stimulates mast cell degranulation (Barker, 1967; Josefsson, 1968). The effectiveness of action on uterine mast cells of both of these drugs has been proved by previous investigations (Barker, 1967; Josefsson, 1968; Tainsh *et al.*, 1991). Treatment with cromoglycate also led to a decrease in the proportion of degranulated mast cells in all uterine compartments, whereas compound 48/80 had the opposite effect. Therefore, oestradiol injected together with either cromoglycate or compound 48/80 acted with the background of diminished or abolished, respectively, activity of mast cells.

The results of the present study and those of other workers (Westin, 1955; Gibbons and Chang, 1972; Popova, 1989) showed that oestradiol produces an increase in mast cell degranulation compared with untreated controls. However, the mechanism by which oestradiol acts on the mast cells has not been established.

The three uterine structures, luminal epithelium, glandular epithelium and stroma cells, were chosen for study because they have different numbers of oestrogen receptors (Janssens *et al.*, 1983). In addition, epithelial and stromal tissues have different embryonic sources and, therefore, may be expected to respond differently to treatment.

In the present study, two aspects of oestradiol action on the uterus were analysed. The first of these is an enhancement of proliferative activity. It is known that oestrogen hormones reduce the duration of all the periods of the cell cycle and drive cells from the G0 to the G1 phase (Quarmby and Korach, 1984; Evans *et al.*, 1990; Galand and de Maertelaer, 1992). As a result, the number of cells passing through and remaining in the G1 and S phases, the number of PCNA-labelled cells and the number of dividing cells are increased (Quarmby and Korach, 1984; Evans *et al.*, 1990; Galand and de Maertelaer, 1992; Rumpel *et al.*, 1995). PCNA is an auxiliary protein of DNA polymerase δ which is required for DNA synthesis and repair, and is present in cells in the G1, S, G2 and M phases of the cell cycle (Woods *et al.*, 1991; Sawtell *et al.*, 1995). The other important aspect of oestrogen action on the uterus is an increase of cell growth, and this was analysed by measurement of the volume of cells, their nuclei and nucleoli. It is clear that an enlargement in the volumes of cells, nuclei and nucleoli is caused by an activating influence of this hormone on numerous intracellular processes such as an intensification of transcription (Aronica and Katzenellenbogen, 1993), including that of the nucleolar organizer region (Brantley and Whelly, 1990), an increase of the RNA content and in the products of RNA processing in the nucleus (O'Malley and Means, 1974; Brantley and Whelly, 1990) and an activation of biosynthetic reactions

Fig. 2. The effect of treatment with cromoglycate or compound 48/80 on oestradiol-induced increases in mitotic indices, proliferating cell nuclear antigen (PCNA)-labelling indices, DNA content, and the volumes of cells, nuclei and nucleoli in the uteri of ovariectomized rats. Data from rats treated with oestradiol together with cromoglycate (▨) or compound 48/80 (■) are compared with data from control rats treated with oestradiol and saline (▨). Oestradiol was administered singly (10 µg per rat, i.m.). Cromoglycate disodium (10 mg per rat, i.m.) or compound 48/80 (0.5 mg per rat, i.m.) was injected at intervals of 6 h after oestradiol injection. Measurements were made at 24, 36 and 48 h after oestradiol injection. Values are means $\pm$ SEM. Data from rats treated with olive oil and saline at 24 h after oil injection are shown at 0 h (□). No changes were observed among data from all olive oil-treated time-groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's t test, compared with the data from rats treated with oestradiol.

Fig. 3. The effects of treatment with histamine or heparin on oestradiol-induced increase in mitotic indices, proliferating cell nuclear antigen (PCNA)-labelling indices, DNA content, and volumes of cells, nuclei and nucleoli in the uteri of ovariectomized rats. Data from rats treated with oestradiol together with histamine (■) or heparin (▨) are compared with the data from control rats treated with oestradiol and saline (□). Oestradiol was administered singly (10 µg per rat, i.m.). Histamine dihydrochloride (2 mg per rat, i.m.) or heparin (0.4 mg (50 iu) per rat, i.m.) was injected at intervals of 6 h after oestradiol injection. Measurements were made at 24, 36 and 48 h after oestradiol injection. Values are means ± SEM. Data from rats treated with olive oil and saline at 24 h after oil injection are shown at 0 h (□). No changes were observed among data from all olive oil-treated time-groups. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; Student's *t* test, compared with the data from rats treated with oestradiol.

in the cytoplasm (Abdi Dezfuli and Poyser, 1993). The sizes of these structures are closely and positively correlated with their functional activity (Martin *et al.*, 1973; Lavia *et al.*, 1985; Murray, 1992) and reflect an intensity of oestrogen influence on the uterus. Therefore, the changes in these parameters observed in the present study after different treatment protocols are probably caused by changes in the above mentioned processes in the uterine cells. All the treatment protocols, except injection of heparin, led to one-directional shifts in proliferative parameters. However, heparin produced a decrease in DNA concentration and in the mitotic indices and a slight increase in PCNA-labelling index. Since the mitotic index reflects the number of cells in the M phase and the DNA content reflects the number of cells in the S, G2 and M phases, these data indicate a decrease in the number of cells passing through the S and M phases. However, PCNA presents in cells in the G1, S, G2 and M phases but not in cells in the G0 phase (Woods *et al.*, 1991; Sawtell *et al.*, 1995), indicating that the number of cyclic cells in the G1, S, G2 and S phases is increased. Therefore, heparin treatment appears to prevent the passing of cells from the G1 to the S phase of the cell cycle, causing these cells to remain in the G1 phase. It is probable that uterine cells in the G1 phase are able to prolong their biosynthetic processes and accumulate a greater variety of products after increases in the volumes of the cell, nucleus and nucleolus (Gunin, 1997).

Treatment with oestradiol against a background of diminished mast cell activity (caused by injection of cromoglycate) led to a decrease in proliferative activity and volume parameters of cells. This finding suggests that mast cells are involved in oestradiol action on the uterus and require oestrogens to realize their action fully. Treatment with oestradiol against a background of increased mast cell degranulation (caused by injection of compound 48/80) produced an increase in volume parameters, but a less marked effect on the proliferative component of oestradiol action. Hence, this protocol supports or provides oestrogen action only partially. These results support the contention that proliferative and growth processes are controlled by different regulatory pathways and, therefore, may be regulated independently (Janssens *et al.*, 1983; Cheng *et al.*, 1985).

Experiments with histamine and heparin help to define and explain mechanisms involved in effects obtained under treatment with cromoglycate or compound 48/80. Both histamine and heparin induce a marked increase in cell, nucleus and nucleolus volumes in all uterine structures. However, heparin produces a decrease in proliferation, whereas histamine has a weak transient stimulating action on oestradiol-induced proliferative processes in the uterus. Since histamine and heparin have the same effect on the volume parameters, and cromoglycate and compound 48/80 have the same effect on oestradiol action, it may be concluded that an effect of mast cells on the oestradiol-induced increase in volumes of the cell compartments in all the uterine structures is realized via synergistic action of histamine and heparin. The situation with proliferative activity is more complex. Heparin diminishes, histamine has a weak stimulating action and compound 48/80 has almost no effect on oestradiol-induced proliferative processes. It is possible that the effect of mast cells on proliferation depends on the action of both histamine and heparin; however, their

effects are opposite and, therefore, their combined influence produces almost no result. Experiments with cromoglycate, which showed that uterine parameters analysed are diminished under treatment with this drug, do suggest that mast cells are also involved in the development of oestrogen-induced proliferation in the uterine tissues.

The mechanisms by which histamine and heparin bring about their action are unclear. It may be that histamine realises its action through H₂-histamine receptors, which are known to be present in the uterus (Goyal and Verma, 1982; Pankova *et al.*, 1985). In addition, numerous processes involved in a regulation of proliferative activity are mediated by this type of histamine receptor (Pankova *et al.*, 1985; Gunin, 1989). The action of heparin may be realized via binding with heparin-binding epidermal growth factor-like growth factor which is probably involved in oestrogen action in the uterus (Wang *et al.*, 1994; Zhang *et al.*, 1994), or via inhibiting the activity of different enzymes required for DNA synthesis (Cook and Aikawa, 1973; Fisher and Blumental, 1982). Heparin has been shown to bind oestrogen receptors *in vitro* (Molinari *et al.*, 1977; Tong *et al.*, 1983) and it is possible that this property is present *in vivo*. Mast cells, heparin and histamine probably realize their own actions on the oestradiol effects via changes in the mechanism of oestrogen action in the uterus, because in ovariectomized rats untreated with oestradiol, no changes were found under the treatments.

In conclusion, the present study showed that mast cells are involved in the realization of oestrogen action, including a stimulation of cell growth and proliferation in the uterus, and that the effect of mast cells is mediated by both histamine and heparin. However, the mechanisms of mast cells, histamine and heparin action are not so clear. Moreover, other mast cell-derived cytokines not investigated in the present study may also be involved in realization of oestradiol effects in the uterus. Further experiments are needed to elucidate the inter-relationships between mast cells and oestrogen hormones in the uterus.

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