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The effects of retinoic acid on mmp-2 production, proliferation and ultrastructural morphology in rat uterus

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ABSTRACT

Aims: Retinoic acid (RA) has a vital importance in order to ensure continuity and morphology in many tissues. Matrix metalloproteinases (MMPs) have significant roles in proliferation, the formation of cancers, and metastasis. In this study the effects of RA on MMP-2 production in cells of rat uterus were investigated.

Methods: Twenty-four adult Spraque Dawley rats were divided into two groups, the experimental group was treated with 40 mg/kg/day 13-cis RA for 5 days by gavage. Uterine tissue sections were treated with BrdU and MMP-2 antibodies, evaluated using light microscopy. Tissues were fixed with 2.5% glutaraldehyde and evaluated using transmission electron miroscopy.

Results: MMP-2 immunoreactivity decreased in the stromal cells compared with the control group and no staining of MMP-2 was observed in glandular epithelium in the experimental group. BrDU labeling of cells showed significant decrease in RA-treated group versus control group cells. Based on the electron microscopy evaluation, the surface epithelial cells of the experimental group showed vacuolization, and an accumulation of lipofuscin bodies was also observed in the gland epithelium. Cells involving autophagic vacuoles contained excess lipid granules in the entire uterus layers especially localized at the border of the endometrium and myometrium.

Conclusion: RA had negative effects on cell proliferation and cell morphology and inhibited MMP-2 expression.

1. Introduction

MMPs are zinc-containing proteolytic enzymes that play a role in the breakdown of extracellular matrix components. In many adult tissues the level of expression of MMPs is very low, but is increased during the active tissue remodelling. Uterus, organ of the female reproductive system, shows some cyclic changes depending on steroid hormones. This process is called as uterine cycle. For human beings, MMPs have important roles in remodelling the endometrium during the 28-day uterine cycle period (Curry and Osteen, 2003; Goffin et al., 2003). Regulation of endometrial MMP expression has a critical importance in normal tissue remodelling (Curry and Osteen, 2003). MMPs are divided into groups depending on their substrates and similarities in their structures. The gelatinase group including MMP-2 is responsible for the clevage of collagen fibers and basement membrane components such as type IV collagen, laminin and fibronectin (Monaco et al., 2006; Rodríguez et al., 2010). Most MMPs show maximal expression before menstruation, nonetheless, some types like MMP-2 are produced throughout the whole cycle (Goffin et al., 2003).

Retinoic acid is a low molecular weight lipophilic metabolite in terms of Vitamin A. It is necessary for the maintenance of female reproductive system functions. RA regulates many biological functions such as cell proliferation and differentiation (Grenier et al., 2007). RA is necessary for the integrity of many surfaces and duct epithelium structures, like those in the reproductive system. Deficiency of vitamin A causes morphological changes of surface and duct epithelia, irregular menstrual cycle, failure of pregnancy and fetal malformations (Zheng et al., 2000). Overcoming the deficiency facilitates gaining normal features of uterus morphology and fertility (Li et al., 2004). It is claimed that RA has a vital role in controlling the expression of MMP produced by stromal cells in the endometrium of the uterus during decidualization (Zheng et al., 2000; Osteen et al., 2003). Retinoic acid (RA) has regulatory effects on various tissues and organs. Low and high levels of RA could affect cells functions in many different ways. Protective effects of RA on uterine tissue have been reported in many studies. It is reported that, RA treatment in endometrial cancer cell growth has given

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a positive result (Saidi et al., 2006). A recent study has supported this finding by showing the inhibitory effect of RA on endometrial epithelial cell proliferation and regulatory effect on cell cycle and apoptosis using molecular genetic methods. (Cheng et al., 2011).

In this study, we investigated the effects of RA on cell proliferation, morphology, and on the production of MMP-2.

2. Materials and method

2.1. Experiment protocol

A total of 24 adult female Spraque Dawley obtained from Istanbul University, Institute for Experimental Medical Research (DETAE). Animals were housed in metallic cages under regular light- dark conditions. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Istanbul University, Institute for Experimental Medical Research. Rats were randomly divided into two groups; control and experimental. In the experimental group, 40 mg/kg/day 13-*cis*-retinoic acid (Isotretionin, Roaccutane®, Hoffmann La Roche Ltd. Basel, Switzerland) was administered by gavage for 5 days. Studies on reproductive effects about 13-*cis*-RA showed no significant effects on spermatogenesis via 30 mg/kg (Kuhlwein and Schutte, 1985) but 50 mg/kg showed testicular weight reduction and decreased spermatogenesis (Kamm, 1982). Also Hixson et al. (1979) used 40 mg/kg of 13-*cis*-retinoic acid in Spraque Dawley rats and did not report over toxicity. Control rats were treated with normal saline. The rats were injected 50 mg/kg BrdU (Sigma B-5002) an hour before the dissection. After the experiment the rats were sacrificed by inhalation of ether overdose and uterine tissues were taken. For immunohistochemistry studies, 10% formaldehyde solution was used for fixation of tissues. A portion of the uterus was fixed with 2.5% glutaraldehyde for electron microscope evaluation.

2.2. Cell proliferation

The 10% formaldehyde fixed tissues were processed and embedded into paraffin blocks using a routine light microscopy processing schedule. 2–3 μm thick sections were taken using a microtome and put on charged slides. Sections were incubated overnight at 56° C. Rehydration was performed in a decreasing concentration alcohol series. Sections were incubated in darkness for 15 min in 5% hydrogen peroxide (Merck 108597) in methanol (Riedel-de Haen 24229) to block the endogenous peroxidase reaction. Sections were washed for 5 min by phosphate buffered saline (PBS). For BrdU staining, sections were incubated for 30 min in trypsin at 37° C after washing with PBS, the sections were incubated in 4N HCL for 30 min at 37° C. After 20 min incubation with Ultra V block (Zymed 85–9043) at ambient temperature, sections were treated with the 1/50 diluted BrdU antibody (Mouse monoclonal BrdU Antibody, Neomarkers, Ab-4) for an hour at ambient temperature. After washing with PBS, biotinylated secondary antibody (Zymed 85–9043) was applied for 20 min. Sections were washed with PBS, incubated for 20 min with streptavidin-peroxidase (Zymed 85–9043). AEC chromogen solution (Zymed 002007) was applied and the sections were kept for 7–11 min in darkness. Sections were washed with distilled water and background staining was performed using Mayer's hematoxylin for 5 min. After washing with tap water, sections were coversliped using ultra-mount (Zymed 008010). We used Bologna-Molina et al. (2011) cell counting method for immunohistochemistry. Each slide was observed under the ×40 objective and digital pictures were taken and stored as jpeg files. We opened each file using Microsoft Office PowerPoint and using the 'Table' function, a 6 × 6 grid was placed over and covered the image and we divided the images into 36 squares with the same dimentions. Both negative and positive cells were counted manually in each frame. We calculated the percentage of positive cells as follows: % positive cells: positive cells/total cell nuclei ×100. Areas not belonging to the tissue of interest were visually excluded. The results were analyzed statistically with paired *t*-test using Graph Pad software and graphs were plotted using Excel software.

2.3. MMP-2 immunoreactivity

The 10% formaldehyde fixed tissues were processed and embedded into paraffin blocks using a routine light microscopy processing schedule. 2–3 μm thick sections were taken using a microtome and put on charged slides. Sections were incubated overnight at 56° C. Rehydration was performed in decreasing concentration alcohol series. Sections were incubated in darkness for 15 min in 5% hydrogen peroxide (in methanol) to block endogenous peroxidase reaction. Sections were washed with PBS for 5 min. For antigen retrival, sections were incubated in a microwave 3 times for 5 min in a 10% citrate buffer. Then sections were left for cooling at ambient temperature for 20 min. After treatment with Ultra V block for 20 min sections were incubated with 1/200 diluted MMP-2 primary antibody (Santa Cruz sc-13595) for an hour at ambient temperature. After washing with PBS, sections were covered with biotin-labeled secondary antibody for 20 min. Sections were washed with PBS, incubated with streptavidin-peroxidase for 20 min. For visualization, the immunreactivity sections were incubated in AEC chromogen solution for 7–11 min, washed with distilled water and background staining was performed with Mayer's hematoxylin. After washing with tap water sections were coversliped, examined and scored under light microscopy. Because of MMP-2 reaction is cytoplasmic or extracellular, MMP-2 antibody staining intensity were scored according to the overall intensity and classified into 4 levels: 0: no staining, +: weak staining, ++ moderate staining, +++: strong staining.

2.4. The study of transmission electron microscopy

For the study using electron microscopy, the tissues were fixed with 2.5% glutaraldehyde at +4° C, secondary fixation completed in 1% osmium tetroxide for an hour at + 4° C. After washing with PBS, the tissues were treated with 1% uranyl acetate at + 4° C for 10 min. After the increasing acetone concentration series (30, 50, 70, 90, 100, 100%), the tissues were treated with 1:1 acetone: epon, 1:3 acetone: epon mixtures and pure epon at ambient temperature for an hour then embedded into capsules filled with pure Epon (Fluka 45359). For polymerization, the capsules were incubated at 60° C for 18 h. Semi-thin sections were taken and then stained with toluidine blue and evaluated under a light microscope. Thin sections were taken and floated on to nickel grids and stained with drops of uranyl acetate for 30 min, and drop of lead nitrate for 6 min. Sections were evaluated using a Jeol Jem 1011 transmission electron microscope.

3. Results

Cell proliferation: In the control group 26,75%, 28,8% and 16,55% of positive staining cells in the surface and glandular epithelia and stroma were detected respectively (Fig. 1a). In the experimental group, staining of the surface epithelium was 2,7%, in glandular epithelial 7,9% of cell was positive and 4,1% of stromal cells showed positive staining (Fig. 1b).

Statistical analysis demostrated extremely significant quantitative changes in the BrDU immunoreactivity between the control group surface epithelium versus the RA-treated surface epithelium, and control group glandular epithelium versus RA-treated glandular epithelium ($P < 0.0001$), and also the control group stroma versus the Ra-treated group stroma ($P = 0.0003$) (Table 1).

MMP-2 immunoreactivity: In the control group, weak (+) MMP-2 immunoreactivity was detected in endometrial surface epithelium whereas moderate (++) labeling was observed in glandular epithelium. Uterine stromal cells revealed very strong (+++) immunoreactivity (Fig. 2a). No staining (−) was observed on the