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Differential aromatase (CYP19) expression in human arteries from normal and neoplasic uterus : an immunohistochemical and *in situ* hybridization study

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TABLE OF CONTENTS

1. ABSTRACT

Aromatase CYP19 catalyzes the synthesis of estrogen from androgens in a tissue-specificc manner. This enzyme is present in several tissues, including gonads, brain and fatty tissue. More recently, its presence has been described in vessels. Here, we describe the expression of aromatase in human uterine artery and compare its expression with that found in arteries of estrogen-dependent uterine leiomyomata from women. To do this, we employed immunohystochemical and *in situ* hybridization techniques. We used, a polyclonal antibody raised against the carboxyl terminus of aromatase (ARO) and RNAm probes, of the exon 1 of ARO. We found an increased immunoreactivity of ARO in uterine arteries of patients with leiomyoma as compared with control group. Probe showing positive signal in skin fibroblasts (1b), showed positive hybridization signal in normal artery, while probes with positive signal in placenta (1a), ovary (1c) and testis (1d) were over-expressed in arteries of leiomyomas.

2. INTRODUCTION

Aromatase (ARO) is a member of cytochrome P450 (CYP19A1) that synthesizes estrone and estradiol from androstenedione and testosterone, respectively (1,2).

The gene encoding ARO is localized on chromosome 15q21.2. This gene encodes nine tissue-specific promoters: two placental types (major and minor species) (3), adipose/breast cancer, skin/adipose, fetal tissues, bone, testis, ovary/breast cancer/endometriosis, and brain (4). The ARO gene has at least 10 exons, where only exon 1 is variable, giving several tissue-specific transcripts: the exon 1a (placenta), 1b (liver fetal/ skin fibroblast), 1c (ovary) (5), and 1d (testis) (6). In animals, ARO has been identified in equine and porcine embryos (7,8), in smooth muscle cells and coronary microvascular endothelial cells of rat, as well as in bovine aorta (9,10,11). In humans the enzyme is present in several tissues, such as the gonads, skin fibroblasts, cirrhotic liver, brain, fatty tissue, macrophages, and lymphocytes (12,13,14,15,16,17).

Various reports have shown that overexpression of ARO may be related with the development of a number of pathologies, such as gynecomastia, endometriosis, uterine leiomyoma, and cancer of the breast, liver, and uterus (18,19,20,21,22,23).

Recently, the presence of ARO in human vascular regions, such as aorta (24) and pulmonary artery

have been determined (25), it has been suggested *in situ* synthesis and a paracrine or intracrine function of the estrogen, such as occurred in vascular smooth muscle and breast cancer cells (26,27). On the other hand, it has been proposed that the rate of estrogen synthesis is lower in normal tissue in comparison with that found in some diseases, especially those that are estrogen-dependent, where the need of angiogenesis or vascular growth is mandatory in order to maintain the growth of the tissue.

Based on the aformentioned information, the aim of this study was to determine the expression of ARO (semi quantitatively) in normal human uterine artery, and compare it with the obtained in uterine artery from leiomyoma, an estrogen dependent uterine smooth muscle pathology. Furthermore, we study the relative expression of transcripts from exon 1: 1a, 1b, 1c, 1d by *in situ* hybridization.

3. Material And Methods

The study was approved by Institutional Research and Ethics Committees, informed consent was obtained and signed from all subjects.

3.1. Tissue Samples

Uterine arteries were obtained from six women (30-43 years old), undergoing hysterectomy for leiomyoma, as well as from three women undergoing uterus removal due to uterine prolapse. All patients in the study were in the proliferative phase of the menstrual cycle at the time of surgery. Samples with evidence of adenomyosis, endometriosis, or treated with radiotherapy or hormonal replacement treatment were excluded.

After surgery, the tissue samples were dissected quickly, and the proximal portion of the uterine artery was collected.

3.2. Immunohystochemistry

In order to determine ARO expression, polyclonal antibodies against a polypeptide (DDVIDGYPVKLGINIILNIG) coupled to hemocyanin, were developed in rabbit; this peptide has immunogenic properties and is a constant segment in ARO from rat, human and chicken (28). The specificity of the antibody was validate by inhibition by haptene (29), and was demonstrated in previous work (10).

3.3. Tissue Treatment

General procedures of tissue treatment were as follow: immediately after dissection, each uterine artery was fixed in paraformaldehyde (4%), dehydrated and paraffin-embedded, posteriorly, sections of 5 μm were obtained, deparaffined, rehydrated, and permeabilized 5 min with Triton X100, 0.5%. The tissues were incubated (30 min) with normal fetal bovine serum to block unspecific binding, and then, they were incubated (18 h, at RT), with rabbit antihuman ARO antiserum (1:500), after washing (3X5´PBS) the tissues were incubated for 2 h at room temperature with a fluorescein- labeled goat antirabbit secondary antibody (1:250). An immunostaining control was performed excluding ARO antiserum. Finally, the immunoreactivity for ARO antiserum was evaluated through an epifluorescent microscopy analysis (Nikon Eclipse E600).

3.4. *In Situ* Hibridization

Tissue sections were prepared as referred above and subsequently incubated at 37°C as follows: 10 min in hydrochloric acid (0.02 M), 90 sec in triton/PBS (0.01%), 10 min in proteinase K (100 μg/ml of buffer TE, Tris-HCl 10mM; EDTA 1mM at 37°C), and 5 min in glycine (2 mg/ml), next, the tissue was treated 30 sec with 20 % cold acetic acid, and 30 min with levamisole (20 mM) at room temperature. The slides were then incubated at 60° C for 10 min in hybridization buffer (SSC20X, Denharts IX, salmon DNA sperm, and dextran sulphate), which was diluted 1:1 with formamide, the *in situ* hybridization was carried out at 60° C for 18 h.

The fluorescein labeled oligonucleotide probes (0.002 μg/ml,) were comprised of the following: exon 1a of placenta (5´-TTC TTC ACC TTC CTG TTT GCC-3´; BCO20767), exon 1b of skin fibroblast (5´- TCA GGC TCC AGT TGG TCA CGT-3´D21240), exon 1c of ovary (5´- TTA CAA GTC AAA ACA AGG AAG CC-3´ D21241), exon 1d of testis (5´-CAA AGG GAC AGG AAA ATT ACA GAA-3´D2124). As experimental sequence controls, we used globin in erythrocytes (5´-CAA CTT CAT CCA CGT TCA CC-3´; AY356351), and actin (5´-GGA TCT TCA TGA GGT AGT-3´BCO13835).

After of incubation with glycine the control slides several types of controls were performed. Experimental controls: slides were treated with DNAse, free of RNAse (1mM/ml buffer TE) and RNAse, (10 μl/200 ml buffer TE, 20 min, 37°C). Another set of control slides were incubated with sense and antisense RNAm probes. Also, sequences of globin and actin wer used as probes of experimental references as negative and positive controls, respectively.

3.5. Statistical Analysis

For the statistical analysis the median values ± SE of each probe were determined, and the non parametric Kruskal Wallis test ($p<0.05$) was applied.

4. RESULTS

4.1. Immunohystochemistry

The analysis of the immunoreactivity of ARO (antibody raised against a 20 residue peptide near the carboxyl terminal) gave a positive staining, mainly associated with vascular smooth muscle (figure 1). We can not exclude ARO immunoreactivity in endothelial cells; the intense signal in the muscle masked direct visualization of this cell type; however immunoreactivity of von Willebrand factor in the same areas (not show) suggest the presence of ARO in endothelial cells. The results obtained in arteries from pathologic uterus, showed a more intense signal (41%) than normal. Figure 1, shows the result of a semiquantitative analysis of the immunohistochemical staining, which was normalized against the tissue

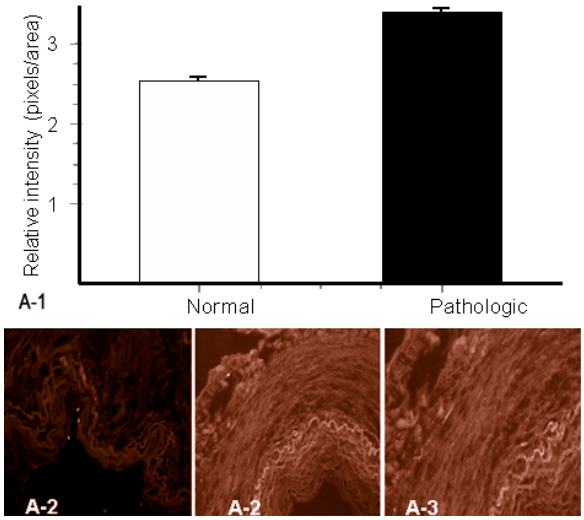

Figure 1. Immunohystochemical localization of $P_{450AROM}$ in human uterine artery: A-1) negative control (samples without primary antibody; A-2) Artery from normal uterus and; A-3) Artery from leiomyomatosis. Graph represents relative intensity in samples normalized against control

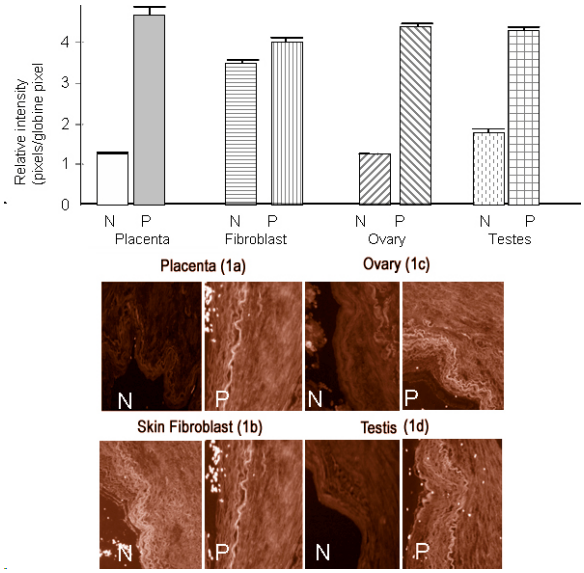

Figure 2. *In situ* hybridization of RNAm exon 1 probes. Each probe is compared in arteries from normal and pathological uterus. The relative intensity in samples normalized against globin intensity is shown in graph.

autofluorescence, obtained from the slides treated excluding the antibody against aromatase. The staining shows an overexpression of immunopositivity in the arteries from leiomyoma tissues.

4.2. *In Situ* Hybridization

In this assay, we used probes of globin and actin of known sequence, as negative and positive controls, respectively. In the case of globin, no positive signal in any assayed tissues was detected. In the assays using actin probe, an intense fluorescent signal was obtained (data not shown).

The expression of specific-tissue transcripts in normal arteries is shown in figure 2. The detected expression (relative) of the transcripts was as follows: skin fibroblast>testis>ovary and placenta. On the other hand, arteries obtained from pathologic uterus showed a differential expression, there is an increase in the relative expression of all transcripts assayed. Hybridization of transcript 1b with an increase of 12% ($p<0.05$) was not significant. Probe from testis/prostate show an increase of 128 % in the expression of its transcript, exon 1c increases in 255% and exon 1a increases 268%.

5. DISCUSSION

Our results showed ARO expression in human uterine artery, including vascular smooth muscle and possibly in the endothelium, this fact, to our best knowledge, is the first description of this expression in human uterine artery.

The ARO expression in the arteries of the estrogen-dependent pathology leiomyoma was increased, showing an overexpression of the tissue-specific probes in comparison with the normal arteries. This may be related to a growth stimulus of pathologic tissues.

We developed an antibody against a 20 residue polypeptide which is located near the carboxyl terminal of ARO (28), and used it to analyze the immunoreactivity of the ARO protein in arteries from normal and from estrogen dependent myomatosis. Immunoexpression was identified in vascular smooth muscle and apparently in endothelium. Our results agree with studies showing ARO immunoreactivity in human male and female coronary arteries (30) (vascular smooth muscle and endothelium), in smooth muscle from atherosclerotic (24).

As mentioned, ARO has been detected in normal and pathologic tissues; one of the aims of this study was to explore a possible difference on level of expression of ARO, between normal and pathologic arteries. Our results showed a higher ARO protein content in leiomyoma arteries compared with those from normal uterus. This increase may be related with changes induced by the pathology by itself, or with a stimulus that promotes the increase of synthesis of estrogen, raising the possibility of the estrogen, being synthesized in uterine artery, as an inducer of mitotic effects. These results point out the importance of the androgen metabolism, through intracrine or paracrine pathways, as a source of estrogens in vascular tissues in estrogen-dependent pathologies, and may also be related with the growth of the neoplasm, as reported in other studies (31). In this regard, it has been proposed that ARO expression and its activity in vascular tissues (including vascular smooth muscle and endothelium), can be related with an abnormal growth of the tissue irrigated by the specific artery (26).

On the other hand, although there is controversy about the stimuli that originates the neoplasic growth of tissues, it is generally accepted the involvement of absence

of negative control, or an overexpression of the transcription of specific DNA promoters (32).

On regard to aromatase gene, it is composed by at least 10 exons. Exon 1 through a splicing process, originates tissue-specific transcripts regulated in turn by specific promoters, may be exist local factors that produce the increased synthesis of ARO and consequently the production of estrogens (333). It has been shown that in normal uterine smooth muscle, aromatase expression is minimal or nonexistent, contrasting with the increased expression in muscle from myomatosis (34), where the enzyme has an apparent Km of 3 nM, value that is close related with the plasma concentration of androstendione (35) with these data in mind we reasoned that ARO transcription in arteries from these pathologic tissues may be also altered, and this, ARO related with neoplasm may synthesize estrogens even with low androgen concentrations in plasma, thus also might be related with changes in enzymatic kinetics, however, more work is necessary in order to clarify this possibility.

Our *in situ* hybridization studies showed that probes related with exon 1b (skin fibroblasts) were the main responsible for the ARO expression in arteries from normal tissues, followed by for the 1d (testis), 1c (ovary) and 1a (placenta). This fact may be relevant, because, it shows that the exon 1b may be constitutive and possibly does not cooperates to neoplasm development, in contrast, in the uterine artery from leiomyoma, the main exon used was 1a, followed by 1c, and 1d and finally 1b. The differential use of transcripts has been described in other situations, for example in normal mammary gland tissue that uses exon 1b, whereas mammary gland neoplasm uses exon 1c (36).

These results, together with the immunochemical analysis, shown that arteries from estrogen-dependent leiomyoma, suffer development changes perhaps related with an increase in a demand of estrogens, being by this, increased the local synthesis, changes may also be related with growth of arteries by themselves.

In conclusion, since normal arteries, mainly through exon 1d express aromatase, and arteries from pathologic uterus show an overexpression of several exons, it is clear that there are stimuli, still unknown, that are responsible for the lack of restriction, or for the overexpression of synthesis of aromatase. Vascular tissues can be sites of estrogen synthesis and by this, a source of steroids that in turn, may favor the growth of neoplasic tissues.

However, more work is necessary in order to clarify, in dept, these phenomena.

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