Regulation of Expression of Secretory Leukocyte Protease Inhibitor by Progesterone in BeWo Choriocarcinoma Cells

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Abstract

Secretory leukocyte protease inhibitor is a multifunctional protein with a variety of activities attributed to it. A significant increase in the expression of Secretory leukocyte protease inhibitor was noticed in syncytiotrophoblasts following differentiation of cytotrophoblasts in to syncytiotrophoblasts by addition of Forskolin. Using the BeWo cells which are derived from choriocarcinoma, the effect of addition of progesterone and estradiol on the expression of Secretory leukocyte protease inhibitor by Reverse Transcription Polymerase Chain Reaction was assessed. It was found that while addition of low concentration of progesterone resulted in a significant increase in expression of Secretory leukocyte protease inhibitor, addition of estradiol even at high concentration had no effect. The specificity of effect of progesterone was established by the observation that addition of Progesterone along with progesterone receptor antagonist (RU484) resulted in decrease in the level of expression of Secretory leukocyte protease inhibitor. These results suggest that Secretory protease leukocyte protease inhibitor is a progesterone regulated gene.

Keywords: Progesterone; Estrogen; Regulation; SLPI; BeWo cells.

Introduction

Secretory Leukocyte Protease Inhibitor (SLPI) is a nonglycosylated hydrophobic cationic 12 kDa protein with a variety of activities attributed to it. During our studies on the profiling of genes during differentiation of unineucleated cytotrophoblasts (CT) into multinucleated syncytiotrophoblasts (ST) by DDRT-PCR and micro array analysis of RNA from CT and ST we found several fold increase in the expression of SLPI in the ST [1]. It is also known that it exhibits a variety of activities, which includes anti-protease, anti-inflammatory, anti-bacterial activities [2]. SLPI is a highly conserved protein and murine SLPI appears to be structurally highly similar to human SLPI [3]. However, it is reported that while the human SLPI is regulated by progesterone (P4) [4], in the case of rat, it is regulated by estrogen [5]. In view of the apparent differential regulation of SLPI in rodents and human and considering the fact that SLPI structure is highly conserved, it was of interest to investigate the regulation of SLPI by P4 in BeWo cells which is derived from human choriocarcinoma.

Materials and Methods

Cell culture and induction of differentiation in human trophoblastic BeWo cells

BeWo cells (a human choriocarcinoma cell line, generously gifted by Dr. Susan Fisher, University of California, San Francisco, California) were cultured in DMEM/Ham’s F-12 medium (Sigma Chemical Co., St. Louis, MO) containing 10 % FCS (Hyclone laboratories Inc., UT) and antibiotics (100 U/ml Penicillin, 50μg/ml Gentamycin and 5 U/ml Nystatin), at 37°C, in an atmosphere of 5% CO₂. For experiments involving induction of differentiation, cells were maintained in serum-free medium as described by Taylor et al. [6], with minor modifications prepared with DMEM/Ham’s F-12 medium supplemented with insulin (1μg/ml), transferrin (5 μg/ml), Bovine Serum Albumin (500 μg/ml) and antibiotics (100μU/ml penicillin, 50 μg/ml Gentamycin and 5U/ml Nystatin).

The following chemicals Insulin, Transferrin, MEM, Ham’s F12 medium; Bovine serum; Albumin were obtained from Sigma chemicals Co., St. Louis, MO, USA. P4 and Estradiol 17β (E2) were obtained from Steraloids Inc, Wilton, NH, USA, ICI 182780 (a pure antiestrogen is a gift from Dr. A.E. Waking of Zeneca Pharmaceuticals, UK). RU486 (17α-hydroxy-11β-(4-dimethylamino-phenyl)-17β-(1-propynyl) extra-4, 9-dien-3-one) was obtained from Roussel-Uclaf, Paris, France. Fadrazole (aromatase inhibitor) CGS 16949A was a gift from CIBA Geigy Ltd. Basel, Switzerland.

Hormonal regulation of SLPI expression in BeWo cells

0.5X10⁵ cells/ml/well were plated in 6 well multi-dishes and after overnight culture, the cells were incubated in the presence of 1μM of P4 or 2. 5μM of E2 or 1μM RU486 or 1μM of Fadrazole (Aromatase inhibitor) in serum free medium supplemented with insulin, transferrin and BSA. After different duration of incubation i.e., 0, 6, 12, 24h, cells were harvested for RNA isolation using Trizol reagent (Sigma Chemical Co., St. Louis, MO, USA), according to manufacturer’s instructions. Parallel control experiments were performed with cells incubated with vehicle control (ethanol). MTT assay was carried out to check the viability of the cells before and after the treatment (Data not provided). The integrity of the isolated RNA was checked on a 1% MOPS-HCHO agarose gel and the quantity of RNA estimated spectrophotometrically. RT-PCR was performed as described earlier [7]. The PCR reaction mix contained 0.4 μM of the forward and reverse primers, 200 μM of the dNTP mix, in 10 mM Tris-1 mM MgCl2 buffer and 1 unit of Taq Polymerase [Bangalore Genie, India]. cDNA amplifications used highly specific forward and reverse primers (for SLPI or GAPDH) with an initial heating at 94°C for 3 min, followed by the required number of cycles of 94°C for 1 min, annealing temperature for 1 min and 72°C for 2 min, on a PCR Thermal Cycler (MJ Research Inc., USA). PCR reactions were performed within the linear range of amplification.

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for each amplicon (which ranged from 25-35 cycles) to facilitate quantitation. To ensure accurate quantification, PCR products were assessed by melt curves and also electrophoresed on 2% agarose gels to ensure a single product of the correct size was being amplified. A 12 μl aliquot of the PCR products was electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide (EtBr). Each figure is representative of three independent experiments. Signal intensities of various bands were determined using the Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120). The expression levels of specific transcripts were inferred upon normalizing their signal intensities to that of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), which served as an internal control in this semi-quantitative analysis. Values so obtained were used to determine mean ± S.E. for a graphical representation. ‘Significance or P-value’ was determined using standard statistical test. The authenticity of the RT-PCR products was confirmed by sequencing, following their purification from low-melting agarose gels using the commercially available GFXTM PCR DNA/Gel Band Purification kit (Amersham Pharmacia Biotech., UK). Sequencing was performed at the DNA Sequencing Facility, IISc, using the ABI Prism 377 automated DNA sequencer.

The effect of addition of P4 was also tested at a concentration ranging from 0.01μM to 1μM and a time course study was also carried out. The effect of addition of P4 receptor antagonist RU486 on the expression of SLPI was also monitored. A dose dependent study was carried out to assess the expression of SLPI by RT-PCR in the linear range of amplification. A 12 μl aliquot of the PCR products was electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide (EtBr). Each figure is representative of three independent experiments. Signal intensities to that of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used as an internal control (values expressed as fold increase over control mean ± S.E. from three independent experiments). RT-PCR analysis for expression of SLPI 1) control, (2) P4 (1μM), (3) RU486 (1μM), (4) P4 + RU486 (both at 1μM) and incubations were carried out for 24 h. RNA was reverse transcribed and cDNA was subjected to semi-quantitative PCR in the linear range of amplification with GAPDH as an internal control. Graphical representation of results presented in C (values expressed as fold increase over control; mean ± S.E. from three independent experiments). * Significantly different from group 2 p<0.001.

Results

Regulation of SLPI expression by P4 and E2

Initially the effect of addition of P4 or E2 to BeWo cells on the expression of SLPI after 24h of addition was examined. It can be seen from the results presented in Figure 1 (A&B) that addition of 1μM P4 resulted in a very significant increase in the level of expression of SLPI gene while E2 at 2.5 μM had no effect.

The effect of P4 was also tested at concentrations ranging from 0.01μM to 1μM. It can be seen from the results presented in the Figure 1 (C&D). P4 was effective in stimulating the expression of SLPI gene at the concentrations tested and even 0.01μM was quite effective in stimulating the expression of SLPI.

A time course study on the effect of incubation of BeWo cells with and without P4 (1 μM) for 0, 6, 12 and 24h on the expression of SLPI was carried out to assess the expression of SLPI by RT-PCR. It can be seen from the results presented in the Figure 2 (A&B) that by as early as 6 h there was a distinct increase in expression of SLPI gene.

The specificity of P4 effect was established by incubating the BeWo cells along with a specific P4 receptor antagonist RU486. It can be seen from the results presented in the Figure 2 (C&D) that incubation of P4 along with RU486 decreased the stimulatory effect of P4 on SLPI gene expression.

Regulation of SLPI gene expression by E2 in BeWo cells

As placenta has high expression of aromatase enzyme, it was essential to rule out the possibility that increase in SLPI expression is not due to conversion of P4 into E2. This was established by addition of Fadrazole (Aromatase inhibitor AI) along with P4 and it can seen from the results presented in the Figure 3 (A&B) that incubation of BeWo cells with P4 along with AI also had no effect on the increase in SLPI gene expression as assessed by RT-PCR analysis.
that pregnancy hormones like P4 and E2 control gene expression in SLPI starting from 24 h of incubation with Forskolin. It is well known that during differentiation, there was an increase in the system employed [14]. The appearance of ST specific markers like β hCG and endoglin as assessed by RT-PCR thus validating markers like PCNA and Cyclins and the appearance of ST specific clear multinucleated syncytia were observed in the treated groups.

Before proceeding with DD-RT-PCR and microarray analysis we found that SLPI is one of the transcripts is highly expressed in BeWo cells. A & B: Effect of Aromatase inhibitor (fadrazole) on progesterone (12 h) Induced SLPI expression. C & D: Effect of different concentrations of E2 on expression of SLPI mRNA. A) Cells were incubated with P4 (1μM) or P4 (1μM) + Fadrazole (1μM) or with vehicle (ethanol) control for 12 h. RNA was reverse transcribed and cDNA was subjected to semi-quantitative PCR in the linear range of amplification with GAPDH as an internal control. B) Graphical representation of results presented in A. C) Cells were incubated with different concentrations of E2 ranging from (0-10μM) for 12 h. RNA was reverse transcribed and cDNA subjected to semi-quantitative PCR in the linear range of amplification with GAPDH as an internal control. D) Graphical representation of results presented in C, (values expressed as fold increase over control mean ± S.E. from three independent experiments).

The results of study in which the effect of addition of different concentration of E2 also revealed that even at a very high concentration of 10 μM, E2 had no effect on the expression of SLPI in BeWo cells as assessed by RT-PCR (Figure 3 C&D).

Discussion
Over the years the differentiation of proliferating mononuclear cytotrophoblasts into non-proliferative multi nucleated but highly functional syncytiotrophoblasts has been employed to address several questions related to regulation of growth, stage specific expression of genes as well as cytoskeletal changes that occur during the differentiation process. We have successfully employed both primary cell culture of cells isolated from first trimester and term placenta as well as the BeWo choriocarcinoma cell line to address several questions regarding regulation of telomerase as well as role of E2 in differentiation of CT into ST [1,7,8].

In our attempts to understand the molecular players of trophoblastic differentiation using two approaches of DD-RT-PCR and microarray analysis we found that SLPI is one of the transcripts is highly expressed following differentiation of CT into ST. Before proceeding with DD-RTPCR analysis we have validated the system both at morphological as well as at the functional level. By 72 h, following addition of Forskolin, clear multinucleated syncytiota were observed in the treated groups. We have also observed that by 48 h there is a decrease in proliferation markers like PCNA and Cyclins and the appearance of ST specific markers like β hCG and endoglin as assessed by RT-PCR thus validating the system employed [1].

SLPI expression increased during differentiation in a time dependent manner and as the cells differentiated, there was an increase in SLPI starting from 24 h of incubation with Forskolin. It is well known that pregnancy hormones like P4 and E2 control gene expression in placental cells [8]. It is likely that the anti-protease and anti-microbial actions of SLPI will be involved in key reproductive events such as fertilization, implantation and pregnancy.

P4 is also required for maintenance of the pregnancy by stimulating and maintaining uterine functions, necessary for early embryonic development, implantation, placentation and fetal development. After the luteo-placental shift in steroidogenesis (at around the 7th or 8th week of human gestation), the placenta becomes the principal source for production of P4 and the synthesis in P4 increases rapidly throughout the course of pregnancy.

Cyclical expression of SLPI and elafin, particularly in endometrium, suggests that their expression may also be influenced by steroid hormones such as P4 [9]. There are relatively few studies examining this aspect, although it has been reported that SLPI expression is upregulated by P4 treatment of cervical explants [10]. There is new evidence showing that SLPI is a P4-regulated gene and that the hormone is likely to regulate its expression in female reproductive tract tissues [11]. Serum P4 concentrations are in the nanomolar range during the secretory phase of the menstrual cycle and are in the micromolar range in the fetus-placental unit during pregnancy. Considering this it was felt important to check the effect of addition of different concentrations of P4. Furthermore, it has been reported that 90% of P4 is metabolized within 24 h in cell culture experiments [11-13].

SLPI gene is shown to be P4 regulated in Ishikawa endometrial epithelial cells and its presence has been demonstrated in this cell type in humans and non-human primates [11,14]. Velarde et al. [15] have demonstrated that BTEB1 (basic transcription element binding protein-1) as a functional P4 receptor-A and P4 receptor -B interacting partner in the P4 mediated transcriptional activation of SLPI gene in Ishikawa endometrial epithelial cells [15]. These reports as well as the availability of the specific inhibitors to block the action of P4 and E2 prompted us to examine the role of steroids in regulation of SLPI in BeWo cells.

Results of our studies which involved the quantitation of the mRNA, clearly established that SLPI is regulated by P4 in BeWo cells and the action of P4 is specific, as the effect of up-regulation by P4 was inhibited by RU486. The above studies clearly suggest that the effect of P4 on SLPI expression is mediated via the nuclear P4 receptor since this action is effectively antagonized in the presence of the anti-gestogen, RU486. Similar studies on T47D breast epithelial cell line showed an up-regulation of SLPI gene and protein expression on addition of P4 and RU486 and other anti-gestogens were able to inhibit this increase in SLPI expression [11]. In our studies, addition of E2 even at high concentration did not have any effect on the expression of SLPI in BeWo cells. This was again emphasized by the fact that aromatase inhibitors fadrazole could not inhibit the upregulation of expression induced by P4. These studies with BeWo cells as well as studies on non-human primates [14] and the present study using human choriocarcinoma cells clearly demonstrate SLPI is a P4 regulated gene.

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