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Pigment epithelium derived factor (PEDF) expression in the male tract of Wistar rats

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ABSTRACT

Pigment epithelium derived factor (PEDF) expression has been described in many organs as showing neurotrophic, anti-angiogenic, anti-apoptotic, anti-inflammatory, anti-oxidant and pro-cell survival properties. However, references to its activity in the male reproductive system are scarce. We aimed to characterize the expression of PEDF in the male reproductive tract of Wistar rats by using RT-PCR, western blot and immunostaining and also evaluate the effect of flutamide in PEDF expression. We found that PEDF is expressed in the epididymis, prostate and seminal vesicles in Wistar rats, but notably not in the testes. Under the effect of flutamide PEDF expression decreased, recovering by suppressing the antiandrogen. The epididymis is an essential organ in sperm maturation-storages. The role of PEDF in this physiological process has not been fully elucidated yet, but considering that in other systems PEDF has anti-apoptotic, anti-oxidants and pro-cell survival properties, its expression along the epididymis could play a role in the protection of spermatozoa while they are stored.

1. Introduction

Pigment epithelium-derived factor (PEDF) is a secretory glycoprotein member of the serine protease inhibitors family (serpins), but does not exhibit inhibitory activity [1]. This protein was originally isolated from the conditioned medium of human retinal pigment epithelium cells [2]. There many functions attributed to PEDF: neurotrophic actions inducing transformation of Y79 retinoblastoma cells into differentiated neuronal [3]. PEDF also exhibit anti-angiogenic properties antagonizing the effects of vascular endothelial growth factor VEGF [2,4]. Other works reported PEDF anti-inflammatory properties in diabetic nephropathy [5] and anti-oxidant actions [6].

Studies assessing the role of PEDF, specifically in relation to reproductive function, have focused mainly on the female tract. PEDF expression and secretion in human and murine granulosa cells were described as a hormone-dependent process. In these cells, anti-angiogenic properties were observed by antagonizing the VEGF expression in an autocrine and paracrine mode [7,8]. The exposure of granulosa cells to an oxidizing agent such as hydrogen peroxide (H_2O_2) in a dose-dependent manner decreased cell viability and expression levels of PEDF [9]. Apoptosis induced by H_2O_2 was attenuated in these cells by treatment with recombinant PEDF (rPEDF). In the female reproductive tract, PEDF levels were regulated by oestrogens, progesterone and hCG in granulosa cells [8].

PEDF related findings are very scarce in the male reproductive system. Recent studies focused on prostate cancer shown PEDF expression with a negative correlation regarding prostate cancer incidence in human patients [10]. Our group in 2010 described the rat sperm conjugation inside the epididymal lumen [11]. This sperm conjugate, only found in the caudal epididymis, is formed by a dozen sperm bound by their heads and free flagella. On analyzing this phenomenon, we described the presence of PEDF in caudal luminal fluid and their role in mature sperm conjugation [11,12]. So

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far these are the only references we have found regarding the presence of PEDF in the murine epididymis.

Numerous studies have established the strong regulation of male reproductive system by androgens [13,14]. The anti-androgenic agent flutamide has been extensively used in rats to assay the effect of androgens deprivation [15–17]. This background allows us to consider the use of this anti-androgenic agent as a suitable means of study to evaluate the influence of androgens on the expression of PEDF in the male reproductive tract.

The aims of this study were to analyse the expression of PEDF in the male reproductive tract of Wistar rats, both in control animals and in those subjected to androgen deprivation.

2. Materials and methods

Unless otherwise stated, all chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Flutamide (alpha-alpha-alpha−trifluro-2-methyl-4′-nitro-*m*-propionatoluidide) was obtained from Gador Laboratory (Argentina). Immunohistochemistry reagents were purchased from Vector Laboratories Oligonucleotides primers were provided by Invitrogen (Waltham, Massachusetts, USA). Molecular biology reagents were purchased from Promega (Madison, WI, USA) and Invitrogen -Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

2.1. Experimental design

Adult male Wistar rats were obtained from the animal facility at the Institute of Histology and Embryology, Mendoza (IHEM). Animals were maintained under controlled conditions and permitted *ad libitum* access to water and standard lab chow. All animals in this study were maintained in accordance with Guiding Principles in the Care and Use of Animals of the US National Institute of Health. The procedures performed were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL http://fcm.uncuyo.edu.ar/cicual). Endorsement N°. 34/2014. Animals were randomly separated into three groups: control (CONT); flutamide group (FLU) and post flutamide (POSTFLU) of 10 animals each. Flutamide was administered to FLU and POSTFLU animals during fifteen days. The FLU group was euthanized using a CO_2 chamber. Immediately the tissues were removed and weighed; samples were fixed and processed for immunohistochemistry, subjected to protein or RNA extraction, according to routine procedures. RNA samples were kept at −80 °C until processing. The remaining groups (POSTFLU and CONT) were maintained for an additional period of 30 days without flutamide administration to assess the recovery of androgenic function. After 30 days, animals were euthanized. Organs were isolated, and immediately processed for immunohistochemistry and extraction of proteins or RNA.

2.2. Flutamide administration

A protocol adapted from Ref. [18] was applied. Briefly, flutamide was administered via intra peritoneal injection. The dose used was 50 mg/kg body weight/day solubilized in 300 μl of the vehicle (1, 2-propanediol) for 15 days uninterrupted. Control animals were injected with 300 μl of the vehicle.

2.3. Body and organ weights

Body weights of both, animals CONT; FLU and POSTFLU were monitored daily. The obtained values were tabulated and averaged. The weight of each of the analysed organs was expressed as a percentage of body weight.

2.4. Serum testosterone levels

Blood was obtained by cardiac puncture from control animals (CONT, N = 4), flutamide treated (FLU, N = 4) and flutamide and recovered (POSTFLU, N = 4). A clot was allowed to form at room temperature and the serum was separated. Samples were analysed by chemiluminescence (one step competition assay, IBL, Hamburg, Germany) according to the manufacturer.

2.5. Immunohistochemistry

Samples were processed by a routine histological technique. Briefly, isolated organs were fixed by immersion in 10% w/v neutral buffered formalin, dehydrated through a series of graded ethanol and paraffin-embedded. Sections were then deparaffinized and heated in sodium citrate 0.001 M for antigen retrieval. Then, sections were stabilized by immersion in glycine 300 mM at room temperature. Endogenous peroxidase was blocked, washed with PBST (Phosphate buffered saline Sigma Cat. P-4417- plus 1% v/v Tween 20) and incubated in blocking solution, 60 min at room temperature. A monoclonal antibody against PEDF (MAB1059 Merck Millipore, Temecula, CA, USA) was used with a polyvalent biotinylated secondary antibody from a Vectastain Kit R.T.U. (Vecta, Burlingame, CA, USA). Later, the extravidin-peroxidase system from the same kit was applied. The reaction was visualized using amino benzidine (peroxidase substrate, Vecta). The preparations were observed and photographed with a Nikon 80i microscope camera and software features Element NIS 3.2 F.

2.6. Western blot analysis

Tissue samples were lysed with 1 ml 1X Sample buffer [19]. Protein concentration was determined by BCA (Bicinchoninic Acid assay, Sigma BCA-1). The samples, 20 μg per lane, were fractionated by SDS-PAGE using 10% acrylamide gels. Proteins were transferred to a nitrocellulose membrane (Amersham protran 0.45 NC) and non-specific binding's sites blocked by incubation with 3% Teleostean fish gelatine. Blots were incubated with Anti-PEDF antibody (MAB1059) 1:1000 or anti-Tubulin B 1:1500 (MP 08A203068) for loading control at 4 °C O.N. Conjugated anti-rabbit IgG (Life technologies 656140) or Biotin conjugated anti-mouse IgG (Sigma B 7404) were used as secondary's antibody (1:10000). Horseradish peroxidase-conjugated extravidine (Sigma) was added (1:750), with a period of incubation of 1 h, at room temperature. After adequate washing detection was accomplished with an enhanced chemiluminescence system (ECL; Amersham Biosciences) and images were obtained using an ImageQuant LAS 4000 series system.

2.7. Relative semi quantitative RT-PCR for PEDF

Total RNA was extracted from individual samples from the three groups of animals under study using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA samples were solubilized in 25 μl UltraPure DNase/RNase-free distilled water (ThermoFisher Scientific) for reverse transcription - polymerase chain reaction (RT-PCR). Concentration and purity of the samples were spectroscopically determined from the absorbance of diluted samples (1:100) at 260/280 nm. Ten micrograms of total RNA were used for retrotranscription with 200 units M-MLV enzyme reverse transcriptase (Invitrogen). Twenty microlitres of the reaction mixture were added following the manufacturer's instructions.

Semi quantitative Multiplex PCR was then performed using the retrotranscription products obtained from the three groups of animals under study. Three pairs of primers designed using Primer3 software (Simgene.com) were used for the amplification by