Specific expression pattern of tissue cytokines analyzed through the Surface
Acoustic Wave technique is associated with age-related spontaneous benign
prostatic hyperplasia in rats

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ABSTRACT

The aim of the study reported herein was to evaluate the suitability of the Surface Acoustic Wave (SAW) technique as a possible diagnostic tool in benign prostatic hyperplasia (BPH). Moreover, for the first time, the BPH model was a totally physiological using naturally aged rats with spontaneous, age-related BPH instead of the pharmacologically induced models usually used. Eighteen male Wistar rats were distributed according to their age: 6 weeks (young), 12 weeks (adult) and 12 months (old) old. Prostate gland was removed and analyzed by mini-arrays, Western blotting (WB) and SAW techniques. Mini-arrays indicated that there were significant differences in the expression of 29/34 inflammation-related cytokines. WB was carried out to confirm the results after selection of 4 cytokines from which one showed no changes, namely PDGF-AA, and the other three, which significantly increase in older animals, were CD86, β-NGF and VEGF. Notwithstanding, WB of old rats yielded confusing results due to an anomalous migration of proteins, dismissing this technique as an useful tool in these animals. Accurate results in old rats were uniquely obtained by using the SAW technique. Thus, SAW analysis showed that there were not differences among groups in the amount of PDGF-AA. On the contrary, SAW analysis showed that amounts of CD86, β-NGF and VEGF in old rats were 2.0, 1.9 and 5.7-fold higher than that from young ones, respectively. These results indicate that SAW is a highly accurate technique for determining changes in the cytokines expression in BPH.

1. Introduction

Age-related benign prostatic hyperplasia (BPH) is a very common disease in elderly males in mammals [1,2]. One of the most common features of BPH is the presence of an inflammatory process that can be acute, chronic active or chronic inactive [3–6]. The prevalence of inflammation in BPH is very high, ranging from 70% to 100% depending on the study [7–9]. The clinical importance of this BPH-associated inflammatory process is relevant, since some of the observed clinical signs in BPH are a consequence of the enlargement of the prostate gland induced by the inflammation process [10]. Thus, analyzing the BPH-linked inflammation is important in order to improve both the diagnosis and the prognosis of the process. Additionally, the adequate diagnosis of BPH is relevant taking into consideration that this process is commonly lined with the first steps in the instauration of prostate tumor processes with much worst prognosis. Inflammation is a complex process mediated by pro-inflammatory cytokines, chemokines, prostanoids and growth factors [see 11 for a review]. The etiology of prostatic inflammation is not completely elucidated. At this time, the current data seems to indicate that the onset of prostatic inflammation is launched under a multifactorial etiological basis that involves factors such as infection [12,13], systemic inflammation, components from both the environment and the diet, oxidative stress, urine reflux and systemic steroids [14]. Despite this, according to the literature, only ageing and androgenic imbalance, either concomitantly or not, has been definitely related to BPH development [15–17].

From a broad point of view, a close relationship between the development of cancer, whether derived from a previous hyperplastic process or not, and chronic inflammation has long been recognized [18–21]. Thus, like BPH cells, tumor ones produce cytokines, chemokines and growth factors, such as IL-1β, IL-3, IL-6, IL-11, IL-23, and...
TNF-α [22,23]. These mediators attract leukocytes, such as neutrophils, macrophages, eosinophils, mast cells and lymphocytes [19]. The attracted leukocytes in turn release several mediators, such as cytokines, cytotoxic mediators and mediators of cell death [24,25].

Finally, the factors released from leukocytes then act on a broad phenomena linked not only to the inflammatory process, but also to carcinogenesis, such as cell proliferation and migration, tissue remodeling, metabolism and genomic integrity. [26]. In this manner, cytokines play an important role in carcinogenesis and subsequent development of the tumor process as they are involved in apoptosis, angiogenesis and cell adhesion and transformation [27,28].

Prostatic diseases can be diagnosed and differentiated among them using a wide variety of diagnostic tools. In this sense, the most common diagnostic tool for detecting early prostate cancer in men is the determination of serum prostate-specific antigen (PSA) levels. However, PSA can be increased not only due to presence of prostatic cancer, but also due to BPH or chronic prostatitis [36]. In addition, PSA levels can also be altered due to the handling of samples, laboratory procedures and standardizations [37], levels of serum androgens and ejaculations [38,39]. Thus, results are not always completely reliable. Another important diagnostic tool for prostate alterations is biopsy. However, prostatic biopsy also gives negative results up to two-thirds of patients with prostatic alterations [36]. Therefore, new and more precise diagnostic tools are needed to reach accurate diagnosis in prostatic pathologies. These difficulties in diagnosis are also accompanied with troubles in finding an optimal laboratory model for the study of BPH and/or prostatic tumors. Thus, these laboratory models have been obtained basically from two sources. The first source is male rats and mice in which the BPH has been induced through the administration of inducing substances like formalin, testosterone, sulpiride or even specific microbial strains in order to induce prostate inflammation as a previous step to the establishment of BPH [29–33]. The second source is specific cell lines, such as LNCaP cell line [34] and PC3 cell line [35]. Both sources, although reliable, do not strictly follow a physiological pattern for the BPH onset, limiting thus their practical use in the precise knowledge of BPH.

Taking all of this information into account, the aim of this manuscript was focused on two aspects. The former was to explore the possibility of finding feasible techniques that could improve the diagnosis of prostatic alterations, in order to avoid troubles linked to the currently utilized diagnostic tools, and the latter was to explore the possibility of utilizing ageing rats as a feasible model for the study of BPH.

In this sense, the search of a feasible technique that could be applicable to the diagnostic of prostate alterations was centered on the Surface Acoustic Wave (SAW) technique. In fact, the SAW technique has been already applied to optimize diagnosis and even to develop potential new therapeutically approaches in processes such Alzheimer’s disease, Parkinson’s disease and epidermal growth factor-linked tumors [40–44]. The main reason for the application of the SAW technique in diagnosis/prognosis is centered in the fact that SAW is a very powerful tool for label-free detection of a broad range of numerous analytes, from small molecules, peptides, proteins and lipid membranes to whole cells and cell cultures. SAW biosensor is a chip-based bioaffinity system to study several affinity interactions (antibody-protein, protein-protein, ligand-protein, etc). The biosensor is based on the conversion of a high-sensitivity detection. Furthermore, interactions on the gold-coated chip surface can be observed at near-physiologic conditions [45].

Regarding on the second aim of the study, the experimental model used rats divided in three separate groups according to their age. The levels of several inflammatory cytokines from prostate samples of male Wistar rats of 6 weeks (young), 12 weeks (adult) and 12 months (old) were firstly analyzed by mini-array technique. It is important to point out that this is the first study that reports results from rats that present spontaneous, age-related BPH, in contrast of previous reported works that BPH was pharmacologically induced. Therefore, this age-related model reflects in a more accurate manner the initial processes leading to the onset of BPH. After an accurate analysis of the mini-arrays results, the next step was to focus further analyses on 4 cytokines that showed separate levels in age-related BPH, namely platelet-derived growth factor AA (PDGF-AA), cluster of differentiation 86 (CD86), nerve growth factor β (β-NGF) and vascular endothelial growth factor (VEGF).

PDGF-AA has been described to play a relevant role in prostate tumorigenesis as well as cancer progression. Nevertheless, regarding to the literature, it has not been associated with BPH [46,47]. CD86, which is also known as B-lymphocyte activation antigen B7-2, is a type I membrane protein member of the immunoglobulin superfamily. CD86 is the ligand for two different proteins on the T cell surface, CD28 (for autoregulation and intercellular association) and CTLA-4 (for attenuation of regulation and cellular disassociation) [48]. The co-stimulatory signal through CD86 to its counter receptor CTLA-4 on T cells has been shown to play an important role in the induction of T-cell-mediated immunity against tumors [49]. NGF is a growth factor that is essential for the development and maintenance of the nervous system, which is also involved in angiogenesis, facilitating tumor genesis of breast, oral and prostate cancers [50]. VEGF is a potent growth factor and angiogenic cytokine that stimulates proliferation and survival of endothelial cells, as well as promotes angiogenesis and vascular permeability [51]. VEGF is implicated in the induction of tumor metastasis and intraocular neo-vascular syndromes [52,53].

Before the SAW analysis, mini-arrays results for these cytokines were validated through a Western blot analysis, which confirm the specificity of the obtained results. Despite this, Western blot results of samples from old rats show technical interferences that preclude the utilization of this technique for an accurate detection of cytokines in these animals, emphasizing the usefulness of the SAW technique for this purpose. Results indicate that SAW can be a useful and practical tool for an early diagnosis of BPH through the highly sensitive and specific detection of changes in prostate protein expression.

2. Material and methods

2.1. Animals and sample collection

Eighteen Wistar male rats were included in the present study. The animals were distributed into three different groups according to their age. The first group was formed by 6 young males 6 week-old and weighing from 178.66 g to 199.42 g. The second group was that of 6 adult males 12 week-old and weighing from 336.6 g to 416.8 g. Finally, the third, old rats groups, included 6 rats 12 month-old, weighing from 612.3 to 745.0 g.

An intraperitoneal injection of a mixture (3:1; v: v) of ketamine, 100 mg/Kg rat body mass (Imalgene®, Merial Laboratorios S.A., Barcelona, Spain), and xylacine, 10 mg/Kg rat body mass (Rompun®, Bayer Hispania S.L., Barcelona, Spain), was used to euthanize animals. Prostate glands were immediately surgically removed by medial laparotomy. Half of each prostate gland was fixed with 10% paraformaldehyde, while the other half was snap frozen in liquid nitrogen and kept frozen at −80 °C until analyses were performed. The experimental protocol was approved by the Ethical Committee of the University of Barcelona (DAAM-8153). All the experiments were performed in accordance with the guidelines and regulations established by the University of Barcelona.

2.2. Experimental design

Paraformaldehyde samples were used to perform the histological study of the prostate glands with the aim of stabilizing the degree of benign prostatic hyperplasia. Additionally, frozen samples were used to
analyze the expression of 34 cytokines on the prostate glands by mini-arrays analysis. Once the expression of the different cytokines was analyzed and compared among the three groups of animals, four proteins were selected for further analysis based on the results obtained through the miniarray analysis. The first protein was PDGF-AA, which did not show variations among experimental groups. The second and third proteins, CD86 and β-NGF, showed significant (P < 0.05) variations in the old rats group when compared with the other experimental ones. Finally, the fourth selected protein was VEGF, which showed significant (P < 0.05) variations among all experimental groups. The proteins were subsequently analyzed through Western blotting (WB) and Surface Acoustic Wave (SAW) techniques for quantification purposes.

2.3. Histology

After 24 h of 10% (w: v) paraformaldehyde fixation, prostate samples were embedded in paraffin and 5 µm thick sections were obtained. Paraffin sections were then stained with Harris’ haematoxylin-eosin and analyzed under a light microscope.

2.4. Mini-arrays analysis

Thirty-four cytokines were evaluated with a commercial protein mini-array system kit (RayBio® C-Series Rat Cytokine Antibody Array C2, RayBiotech Inc., Norcross, GA, USA). The analyzed cytokines are listed in Fig. 1. For this purpose, prostate gland samples were thawed in 1 mL of the ice-cold extraction buffer included in the kit and immediately homogenized by using an Ultra-Turma™ T25 basic homogenizer (IKA®-WERKE, Staufen, Germany). Samples were subsequently centrifuged at 13000 × g for 20 min in a 4 °C chamber and supernatants were kept. Total protein content of the supernatants was determined by the Bradford method using a commercial kit (Bio-Rad Protein Assay Dye Reagent; Bio-Rad Laboratories Headquarters, Hercules, CA, USA). Once total protein content was determined, samples were diluted in Tris-Bru®er included in the mini-array kit to a

2 mL of HRP-conjugated streptavidin for 2 h. Subsequently, membranes were subjected to a final washing step with both, Washing Buffer I and Washing Buffer II as previously described. All incubations described herein were performed at room temperature. Finally, membranes were incubated with the commercial detection buffer provided by the manufacturer for 2 min and exposed to X-ray film. The resultant X-ray films from cytokines were scanned and the intensity of the expression analyzed using an image analyzer (Multi Gauge 2.0, Fujifilm Europe, Düsseldorf, Germany). This software provided a numerical expression of intensity in arbitrary units for every single analyzed spot. An equation of expression was calculated from the mean numeric expression of the positive and the blank spots (Fig. 2) in order to quantify the intensity of every cytokine spot. In order to clarify the possible statistical differences among the evaluated groups, the percentage obtained for adult rats was transformed into 100%, while young and old rats values were transformed into their corresponding percentage by multiplying by the factor used to transform the adult value into 100%.

2.5. Western blotting analysis

Western blotting analysis was performed according to Sirois and Dore [55]. Briefly, after determining protein concentration by the Bradford technique, proteins were separated by SDS-PAGE electrophoresis in 10% (w: v) acrylamide gels and transferred to nitrocellulose membranes. The efficacy of transference was evaluated by 0.1% Poncet® A dye (w:v). Membranes were then incubated with 5% (w: v) BSA diluted in Tris-Buffered Saline (TBS) solution (25 mM Tris-HCl, pH 7.4, 0.14 mM NaCl) during 1 h at room temperature to block non-specific binding. Afterwards, membranes were probed against PDGF-AA (R&D Systems; Abingdon, UK), B7-2/CD86, β-NGF and VEGF (Novus Biologicals, Littleton CO, USA) antibodies at a 1:1000 concentration. Antibody binding was visualized by chemiluminescence (Western blotting luminol reagent, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) according to the manufacturer’s instructions and exposed to X-ray film.

Once cytokines expression was evaluated, membranes were exposed to a stripping buffer (0.2 M glycine, 0.05 mM Tween 20, pH 2.2) to remove primary antibodies for PDGF-AA, CD86, β-NGF and VEGF and their corresponding secondary antibody. Then, the membranes were blocked again and exposed to α-tubulin antibody (ABR Affinity BioReagents, CU, USA), which was used as an internal standard to verify that the same amount of protein was loaded for every sample. Finally, X-ray films from every cytokine and their corresponding α-tubulin were scanned and the intensity of the expression analyzed using an image analyzer (Multi Gauge 2.0, Fujifilm Europe, Düsseldorf, Germany).

2.6. Surface Acoustic Wave technique

Bioaffinity analyses of prostate homogenates were performed with a Sam5® Blue SAW biosensor (Nanotemper, Munich, Germany). This device consists of a biosensor unit, an auto-sampler and a microchip module with a gold layer sensing surface on a quartz chip. The chip surface was coated with dextran and functionalized with a flat 16-mercaptohexadecanoic acid self-assembled monolayer (COOH-SAM), as previously described [56].

Antibody against studied cytokines was immobilized on the SAM by carboxyl-group activation with 200 mM (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) [57]. A solution of 500 nM of Anti-cytokine antibody (300 µL) was immobilized on the SAM, followed by capping of unreacted carboxyl groups with 1 M ethanolamine, pH 8.5. Dilutions of homogenate samples were injected and affinity binding performed at a flow rate of 15 µL/min. Furthermore, to avoid the aggregation of proteins as well as their nonspecific interactions with the biosensor chip devices, 0.005% (v:v) Tween20 and 3 mM EDTA was required in the running PBS buffer for bioaffinity binding analysis [58,59]. Thus, all affinity binding
experiment were performed in PBS buffer (pH 7.5) with 0.005% (v: v) Tween 20 and 3 mM EDTA at room temperature. At the end of each measurement cycle (which is an injection of an homogenate sample for measurement of cytokine affinity interaction with its respectively immobilized antibody), the sensor chip surface was washed by a 30–40 µL injection of 0.5% (w: v) sodium dodecyl sulfate (SDS) in the running buffer at 15 µL/min, to remove the tightly bound proteins from the surface, and the baseline of the sensorgram returned to the same level to that before the injection of the homogenate sample. Binding curves were analyzed using the Origin Pro v8.5.1 SR2 software (OriginLab, Northampton, USA) and the integrated FitMaster V 4.2 (Nanotemper, Munich, Germany).

2.7. Statistical analyses

Statistical analyses were performed using the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Data were compared through one-way analysis of variance (ANOVA) to establish the differences among the three groups included in the study. The minimal level of significance was set at $P < 0.05$.

3. Results

3.1. Histology

All of the analyzed rats pertaining to the young animal group (6 weeks old) showed a normal histological architecture of the prostatic tissue with no sign of prostatic hyperplasia (Figs. 3A1, 3A2). Otherwise, all of the adult rats group (12 weeks old) showed slight benign prostatic hyperplasia characterized by the presence of irregular glands, poorly delimited prostatic lobes and slight inflammatory infiltration (Figs. 3B1, 3B2). Finally, all animals included in the old rats group (12 months old) showed moderate to severe benign prostatic hyperplasia characterized by the presence of atrophied prostatic glands, proliferative epithelium, hyperchromatic cells and severe inflammatory infiltrate (Figs. 3C1, 3C2).

3.2. Mini-arrays

The mini-array allowed analyzing the expression of a total of 34 inflammation-related cytokines. Of the 34 cytokines analyzed, 29 showed statistically significant ($P < 0.05$) differences. Only Fas Ligand, IL-13, MMP-8, PDGF-AA and RAGE showed no statistical differences among the evaluated groups (Fig. 1). Furthermore, 25 out of 29 cytokines that showed any significant difference also showed a significant ($P < 0.05$) increase in the old rats group when compared with the other two groups. Likewise, these 25 cytokines did not show any significant difference when comparing young and adult rats groups (Table 1). Finally, the other 4 cytokines out from the 29 ones that showed significant differences yielded significant ($P < 0.05$) differences among the three evaluated groups (young, adult and old rats).

These 4 cytokines were CINC-3, MCP-1, TIMP-1 and VEGF (Table 1). Furthermore, the highest amount observed for this 4 cytokines were obtained in the old rats group, whereas the young animals group showed significantly ($P < 0.05$) lower values of all 4 proteins when compared with both the adult rats group and the old animals one (Table 1).

3.3. Western blotting

No significant differences were observed in PDGF-AA expression when comparing young and adult rats. In addition, the increase of the VEGF intensity band in adult rats when compared with the young ones (Fig. 4) was much more evident than that observed in case of both CD86 and β-NGF (data not shown). Regarding to old rats, Western blotting yielded no clear bands, but a greatly diffuse spot on the X-ray film that precluded any possible analysis of bands in these animals.

3.4. Surface Acoustic Wave technique

On one hand, in the chip functionalized with Anti-rPDGF-AA, any interaction was observed with the diluted 1/1000 samples showing that the concentration of PDGF-AA protein was too low. However, when samples were diluted 1/100 in PBS and injected to the biosensor, association and dissociation curves were clearly detected indicating that the concentration of the targeted protein was high enough. No significant differences in bioaffinity were observed among diluted 1/100 young, adult and old rat prostate samples (Fig. 5A).

On the other hand, statistically significant differences ($P < 0.05$) in CD86, β-NGF and VEGF concentration in young, adult and old rat prostate homogenates were detected (Fig. 5B). To relatively quantify the amounts of cytokines present in the three groups of animals, the maximum value of phase for each sample was taken and statistical treatment was performed. Following this analysis, the concentration of CD86 and β-NGF in prostate homogenates of adult rats were 1.3 and 1.4-fold upper than that in young rats, whereas their concentrations in old rats were 2.0 and 1.9-fold greater than that in adult rats, respectively (Figs. 5C and 5D). Finally, the VEGF concentration in adult rat prostate samples was 2.5-fold higher than that in young rat prostate samples. However, the VEGF concentration in old rat prostate homogenates was more than 5.7-fold higher than that observed in young rat homogenates (Fig. 5E).

4. Discussion

In the present study, the suitability of the SAW technique as a diagnostic tool for BPH taking as a basis the BPH-linked changes in prostate expression of specific cytokines is brought to light for the first time. In fact, the previous mini-arrays analysis already showed the BPH-linked cytokines expression alteration of prostate parenchyma, since of the 34 analyzed cytokines, 29 showed some statistically significant difference among the different groups, with old rats showed...
Table 1

Cytokines quantification, expressed in percentage, analyzed by mini-arrays technique.

| Cytokine         | Young rats (n = 6) | Adult rats (n = 6) | Old rats (n = 6) |
|------------------|--------------------|-------------------|-----------------|
| Fas Ligand       | 111.59 ± 28.46     | 100.00 ± 7.44     | 68.47 ± 21.51   |
| IL-13            | 102.33 ± 34.75     | 100.00 ± 19.19    | 103.87 ± 43.90  |
| MMP – 8          | ND                 | 100.00 ± 103.40   | 67.56 ± 24.93   |
| PDGF-AA          | 73.38 ± 7.35       | 100.00 ± 27.96    | 115.67 ± 12.80  |
| RAGE             | 77.62 ± 13.07      | 100.00 ± 42.46    | 125.84 ± 13.45  |
| Activin A        | 128.4 ± 33.76      | 100.00 ± 46.0     | 3386.3 ± 255.4  |
| Agrin            | 89.5 ± 6.71        | 100.00 ± 14.5     | 1292.5 ± 9.0    |
| CD86             | 97.0 ± 20.7        | 100.00 ± 7.6      | 3186 ± 50.4     |
| β-NGF            | 82.8 ± 20.4        | 100.00 ± 7.1      | 674.2 ± 45.1    |
| CINC – 1         | 95.0 ± 13.0        | 100.00 ± 5.3      | 165.8 ± 22.7    |
| CINC – 2a        | 98.7 ± 11.6        | 100.00 ± 9.7      | 231.7 ± 29.4    |
| CNTF             | N. D.              | 100.00 ± 100.1    | 975.5 ± 121.7   |
| Fractalkine      | N. D.              | 100.00 ± 10.2     | 1098.5 ± 142.6  |
| GM-CSF           | 105.5 ± 184.5      | 100.00 ± 71.3     | 9912.0 ± 1947.3 |
| ICAM – 1         | 230.0 ± 105.4      | 100.00 ± 50.7     | 1544.9 ± 238.7  |
| INF-γ            | 172.8 ± 52.0       | 100.00 ± 44.3     | 1190.2 ± 136.8  |
| IL-α             | 110.2 ± 17.0       | 100.00 ± 9.3      | 194.5 ± 10.8    |
| IL-β             | N. D.              | 100.00 ± 79.1     | 5094.0 ± 802.4  |
| IL – 1 R6        | N. D.              | 100.00 ± 73.1     | 3270.5 ± 312.1  |
| IL – 2           | 41.7 ± 36.4        | 100.00 ± 74.0     | 1285.0 ± 76.0   |
| IL-4             | N. D.              | 100.00 ± 69.6     | 1086.9 ± 120.0  |
| IL – 6           | N. D.              | 100.00 ± 81.0     | 4706.9 ± 610.6  |
| IL – 10          | N. D.              | 100.00 ± 102.8    | 3244.2 ± 641.9  |
| Leptin           | 58.3 ± 35.1        | 100.00 ± 40.8     | 691.6 ± 94.3    |
| LIX               | 70.7 ± 11.0        | 100.00 ± 33.8     | 554.9 ± 50.0    |
| L-selectin       | N. D.              | 100.00 ± 45.4     | 2273.1 ± 244.6  |
| MIP – 3α         | 37.7 ± 15.1        | 100.00 ± 61.7     | 831.7 ± 122.2   |
| Prolactin R      | 107.6 ± 33.6       | 100.00 ± 25.9     | 297.1 ± 99.3    |
| Thymus           | 100.5 ± 5.5        | 100.00 ± 23.7     | 172.0 ± 13.8    |

Results are expressed as mean ± s.e.m. Minimal level of significance was set at P < 0.005. Differences among the groups are expressed with different superscripts letters. N.D.: Not detected.

Fig. 3. Prostate gland histology. A1) Prostate gland from a young rat, 200 µm magnification. A2) Prostate gland from a young rat. 100 µm magnification. Note the absence of inflammatory response and tissue hyperplasia. B1) Prostate gland from an adult rat, 200 µm magnification. Note the presence of irregular glands indicating the presence of BPH. B2) Prostate gland from an adult rat. 50 µm magnification. Note the presence of poorly delimited prostatic lobes and inflammatory infiltration, indicative of light benign prostatic hyperplasia. C1) Prostate gland from an old rat. 200 µm magnification. Note the presence of atrophied prostatic glands with proliferative epithelium and hyperchromatic cells. C2) Prostate gland from an old rat. 50 µm magnification. In addition to the glandular atrophy and the proliferative epithelium, inflammatory infiltrate can be also observed, indicating a moderate to severe benign prostatic hyperplasia.

Fig. 4. Western blotting for PDGF-AA, CD86, β-NGF and VEGF. No significant difference between young and adult rats in PDGF-AA expression were observed. In the case of CD86 and β-NGF, their expression in between young and adult rats had slightly differences, while adult rats showed a significant higher expression than young rats for VEGF.

significantly higher values in all cases (Table 1). Taking into account the close relationship between inflammation and the increase of cytokines expression, our results would logically indicate that inflammatory changes linked to age in the prostatic parenchyma are related with the presence of cytokines in the prostatic parenchyma. These results are in agreement with previous studies which have observed that the BPH-associated prostatic inflammation is characterized by an elevated presence of T cells as well as several cytokines from the interleukin (IL) family [60–72], IFN-γ [71,73] and TGF-β [67,71,74–78]. However, it is noteworthy that all of these previous studies focused on in vivo induced BPH in rats have been performed in pharmacologically induced-BPH [see 79–81 for some examples]. This will surely induce important differences in the specific expression of cytokines when comparing with the results showed in this manuscript, since the inflammatory mechanisms launched here are strictly related with aging. This implies that, in case of this manuscript, the induction of a prostate inflammatory response was not acute as was that induced by drugs as in previous studies, [79–81]. In this way, taking into account the great differences in both the timing and the aggressiveness of the inflammatory induction between spontaneous aging BPH model and pharmacologically-induced BPH one, it is reasonable to detect differences in the specific expression of cytokines in both models. Moreover, the slow, chronic inflammatory response associated with aging is a
much more physiological mechanism to induce BPH, and probably closer to the spontaneous process. Thus, we would recommend an aging-based rat model to study the mechanism/s involved in the launching of BPH, since this model could more precisely emulate the mechanisms involved in the spontaneous process, increasing the accuracy in the interpretation of results. In this sense, the information yielded by our results indicate that the older is the animal, the more severe are the changes in the prostatic tissue and also the expression of pro-inflammatory cytokines would have a more prominent prospective value in order to understand the progressivity of the inflammatory process linked to BPH than results obtained through other, non-spontaneous but artificially induced, in vivo experimental models. Taking this into account, our results showed that only Fas-Ligand, IL-13, MMP-8, PDGF-AA and RAGE showed no statistical difference in their expression among the three evaluated groups. On the other hand, only CINC-3, MCP-1, MIP-3α, TIMP-1 and VEGF showed significant

**Fig. 5**. Quantifications of cytokine’s amounts in rat prostate homogenate samples by SAW technique. Average of SAW sensograms of PDGF-AA (A), CD86 (B), β-NGF (C) and VEGF (D) (Left) and their concentrations in young, adult and old rat prostates (Right). The PDGF-AA concentration in the three evaluated groups of rats is not significantly different, while the CD86 and β-NGF concentrations among these groups are statistical different. Besides, the VEGF concentration in young, adult and old rat prostates is clearly and significantly different. A relatively quantification of cytokine concentration could be done after a statistical treatment of data. The phase's values at 20 s before the end of an injection period are plotted as a bar graphic. Data are expressed as mean ± standard deviation values.
differences among young, adult and old male rats. However, CD86 and β-NFG did not present significant differences between young and adult rats, but an important statistical difference between adult and old rats.

As detailed above, according to the results obtained with the mini-arrays, four cytokines were selected for further analyses. On the one hand, PDGF-AA was selected as a negative control because no significant difference among the three groups was observed. Due to the fact that CD86, β-NFG and VEGF showed statistically significant differences among at least two evaluated groups of rats and its previously described specific relationship with prostatic pathologies, they were selected as positive controls. Both PDGF-AA and VEGF are involved in the process of vasculogenesis and angiogenesis [82,83], which are closely related with both inflammation [84,85] and prostate alterations like BPH and carcinoma [86–91], although the exact role of both cytokines is not well known. This is highlighted in the case of PDGF-AA, which, according to the literature, has not been associated with BPH. However, since none of the old individuals included in the study was diagnosed with prostatic carcinoma we cannot affirm or exclude a possible role of PDGF-AA in the tumorigenesis process of rat prostate. On the contrary, the strong neovascularizing action of VEGF has been widely related with prostatic pathologies [92–94], emphasizing thus the importance of our results. Additionally, in the case of CD86 and β-NFG both cytokines were reported to participate in carcinogenesis [49,50]. These results are in concordance with the results obtained in the mini-array analysis, where a significant increase of CD86, β-NFG and, specially, VEGF expression has been detected in old individuals.

The results obtained in this manuscript clearly indicate that Western blotting was not appropriate for the analyses of prostatic tissue even to confirm the results observed through the mini-array technique. This is evident in samples from old rats, which showed no clear band, regardless the antibody analyzed, but a dispersed and diffuse spot that made the quantitative analyses impossible. A possible explanation might be the changes in the extracelluler matrix (ECM) of the prostate gland. The ECM of the prostate gland is composed by a network of glycoproteins, glycosaminoglycans and proteoglycans [92]. It is known that changes in the ECM in some tissues, such as the prostate gland, increase the risk of disease with age [93,94]. Several glycosaminoglycans [95,96], as well as glycoproteins [97], are increased in prostatic pathologies, including age-linked hyperplasia. Thus, the high concentration of these substances in the prostate gland might turn Western blotting into a non-suitable technique for analyzing prostatic samples from old rats. Obviously, a deeper analysis to confirm that the alteration in the ECM components alters the efficacy of the Western blotting technique would be needed.

In contrast with Western blotting, the SAW technique yield suitable, reproducible and sensitive results when analyzing the expression of the selected cytokines. This great improvement is closely related by the mechanisms in which is based this technique. Therefore, some of the applied techniques.

M. D.-L.: Responsible of performing SAW analysis. Additionally, she contributed to the analysis an interpretation of data, drafting the article and final approval of the submitted version.

S.B.: Main responsible for the coordination of SAW analysis and the interpretation of data obtained after the combination of the results for all of the applied techniques.

J.E.R.-G.: Responsible for the conception and design of the study. Likewise, he collaborated in the interpretation of data, drafting and revising the manuscript and final approval of the submission.

J.M.F.N.: Together with J.E.R.-G., responsible for the conception and design of the study. Likewise, he collaborated in the interpretation of data, drafting and revising the manuscript and final approval of the submission.

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