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Comparison of Apoptotic Gene Expression Profiles Between Peyronie's Disease Plaque and Tunica Albuginea*

Porównanie profili ekspresji genów apoptotycznych płytki włóknistej występującej w chorobie Peyroniego i osłonki białawej

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The fibrotic plaques of Peyronie's disease and other localized fibrotic conditions have been considered to be the result of an abnormal wound healing process. The potential role of regulatory disorders of apoptosis in abnormal wound healing may also play a role in the development of Peyronie's disease.
Objectives. To examine the phenomenon of apoptosis in Peyronie's disease, authors quantified differential levels of gene expression of apoptotic proteins, Fas, Fas Ligand, Bcl-2, p53, Caspase 3 and 8 in Peyronie's plaque and tunica albuginea.
Material and Methods. Eight patients with Peyronie's disease undergoing surgical correction of the curvature had biopsy specimens taken from both the Peyronie's plaque and normal tunica albuginea. Messenger RNA expression of the apoptotic proteins in the plaque and normal tunica was measured by reverse transcriptase PCR.
Results. Apoptotic gene expression was lower than the housekeeping gene`s in half of the tunica albuginea samples and two thirds of the plaque samples. Overall mRNA expressions in the plaque were not significantly different from the normal tunica albuginea.
Conclusions. The fibrotic plaques of Peyronie's disease and other localized fibrotic conditions have been considered to be the result of an abnormal wound healing process. The potential role of regulatory disorders of apoptosis in abnormal wound healing may also play a role in the development of Peyronie's disease. In this study, the lower expression of apoptotic genes may cause the persistence of collagen producing cells which were up-regulated for unknown reasons and consequently result in plaque formation. Similar expression levels of apoptotic genes in both tunica albuginea and Peyronie's plaques may be due to the generalized physiopathologic alterations in tunica albuginea that lead to plaque formation at a vulnerable region subjected to recurrent traumas (**Adv Clin Exp Med 2012, 21, 5, 607–614**).

Key words: apoptosis, fibrosis, Peyronie's disease, messenger RNA, caspase.

Streszczenie

Wprowadzenie. Płytka włóknista występująca w chorobie Peyroniego i inne zaburzenia zwłókniające miejscowo zostały uznane za wynik nieprawidłowego procesu gojenia ran. Regulacja zaburzeń apoptozy w nieprawidłowym gojeniu się ran może również odgrywać rolę w rozwoju choroby Peyroniego.

*This study was supported by a research fund of Istanbul University Project no. p597.

Cel pracy. W celu zbadania zjawiska apoptozy w chorobie Peyroniego autorzy ilościowo porównali stężenie ekspresji genów apoptotycznych białek Fas, FasL, Bcl-2, p53, kaspazy 3 i 8 w płytce włóknistej w chorobie Peyroniego i osłonce białawej.
Materiał i metody. Od ośmiu pacjentów z chorobą Peyroniego, u których przeprowadzono chirurgiczną korektę krzywizny prącia, pobrano wycinki zarówno płytki Peyroniego, jak i prawidłowej osłonki białawej. Ekspresję przekaźnikowego RNA białek apoptotycznych płytki i prawidłowej osłonki mierzono za pomocą metody *PCR* z odwrotną transkryptazą.
Wyniki. Ekspresja genów apoptotycznych była mniejsza niż genów metabolizmu podstawowego w połowie próbek osłonki białawej i dwóch trzecich próbek płytki. Ogólna ekspresja mRNA na płytce nie różni się istotnie od ekspresji na osłonce białawej.
Wnioski. Płytka włóknista występująca w chorobie Peyroniego i inne zaburzenia miejscowo zwłókniające zostały uznane za wynik nieprawidłowego procesu gojenia ran. Regulacja zaburzeń apoptozy w nieprawidłowym gojeniu się ran może również odgrywać rolę w rozwoju choroby Peyroniego. W tym badaniu mniejsza ekspresja genów apoptozy mogła spowodować przetrwanie komórek wydzielających kolagen, których liczba zwiększała się z nieznanych powodów, a tym samym spowodowała tworzenie się płytki. Przyczyną podobnego stężenia ekspresji genów apoptotycznych zarówno białawej osłonki, jak i płytki w chorobie Peyroniego może być ogólna patofizjologiczna zmiana osłonki białawej, która prowadzi do tworzenia płytki we wrażliwej części ciała podatnej na nawracające urazy (**Adv Clin Exp Med 2012, 21, 5, 607–614**).

Słowa kluczowe: apoptoza, zwłóknienie, choroba Peyroniego, przekaźnikowe RNA, kaspaza.

Peyronie's disease (PD) is a localized connective tissue disorder leading to the formation of fibrous plaques in tunica albuginea (TA) and erectile tissue. These plaques contain excessive amounts of collagen and fibroelastic proliferation with a swelling of the extracellular matrix that leads to different degrees of curvature and narrowing as well as penile pain and erectile dysfunction [1]

The etiology of PD is poorly understood. The favorite hypothesis is penile trauma. The bending stresses happening in the coitus are believed to result in delamination of the tunical fibers and consequently in microhemorrhages, acute, and subsequently chronic inflammation and finally scar formation [2]. The fibrous plaque of PD is assumed to develop from an inelastic scar tissue, the result of an abnormal healing process, resembling its counterparts in the other parts of the body [3]. The development of fibrotic plaque involves both the proliferation of myofibroblasts from the fibroblast population normally present in penile TA and stimulation of collagen synthesis [4]. The role of myofibroblasts in wound healing is to approximate the edges of the wound and then they are removed by apoptosis. Their prolonged existence is associated with abnormal wound healing and scar formation [5–7].

Apoptosis is a physiological phenomenon that balances proliferation and cell death. The dysregulation of apoptotic cell death is thought to play an important role in the abnormal wound healing process. There are too many genes to differentiate the inducing and inhibiting ones in the apoptotic process.

Alterations in the expression of some selected genes associated with the apoptotic mechanism in the formation of Peyronie plaques were noted in some studies [8–10].

In this preliminary study, the gene expressions of proteins associated with apoptosis (Fas, Fas Ligand, Bcl-2, p53, Caspase 3 and 8) were compared in Peyronie plaque and healthy TA.

Material and Methods

Patients

Eight patients (mean age: 49.3 ± 12.3 years) were included in the study. The curvatures had been stable for at least 1 year. The degree of the curvature was calculated after combined intracavernous injection of papaverine (60 mg) and phentolamine (1 mg) and sexual stimulation (CIS) by the same physician (Ateş Kadıoğlu). None of the patients had diabetes mellitus or hypertension. Erectile dysfunction was seen in 2 patients and managed with oral medication and penile prosthesis implantation. The international index of erectile function (IIEF) scores of these patients were 7 and 8.

After a circumcising incision, the penis was degloved. With an artificial erection, the location and severity of the curvature was established. Buck's fascia along with the dorsal neurovascular bundle were mobilized off of the underlying tunica albuginea with a lateral approach. The plaque was incised at the maximal erectile curvature during an artificial erection. The plaque incision was carried around to the lateral corporal body and to release tension with an inward pointing V on either end. A venous patch was placed into the tunical defect. Artificial erection was performed after the graft was closed in a watertight fashion. Any remaining curvature seen after the venous patch was corrected with a Nesbitt procedure.

Ethic committee approval was received and conforms to the provisions of the Declaration of Helsinki.

Tissue Harvesting, Storage and RNA Isolation

Excisional biopsies of the Peyronie's plaque and normal TA were performed during surgery. After excision, tissues were fresh frozen in liquid nitrogen and immediately transferred to cryovials preserved at –80°C.

The tissues removed were pulverized into a fine powder under liquid nitrogen by using mortar and pestle.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was extracted from the frozen tissue using an RNeasy fibrous tissue kit (Qiagen, Maryland, USA). cDNA was synthesized from 1 μg of total RNA using random hexamers [11].

Validation of Relative Gene Expression by Quantitative Fluorescent PCR

PCR was carried out with LightCycler, a rapid thermal cycling instrument by Roche (Roche Diagnostics GmbH, Germany) in capillary glass tubes. Fas, FasL, Bcl-2, Caspase 3, Caspase 8, p53 and beta2-microglobulin (housekeeping gene) products were generated by separate PCR reactions using primers. For each reaction, a 2 μl of cDNA sample was used. Also a 10 μl PCR reaction mix including 3mM $MgCl_2$ 5μM of each primer and 1 μl master mix (LightCycler FastStart DNA Master SYBR Green 1 Roche, Mannheim, Germany) were used with and without 10 pmol of the primers. PCR amplifications were performed using cDNA specific primers that anneal to sequences in exons on both sides of an intron in order to exclude amplification of contaminating genomic DNA. Different sets of primers were tested to optimize the cDNA amplification so that the Fas, FasL, Bcl-2, Caspase 3, Caspase 8, p53 and beta 2-microglobulin were amplified under the same PCR conditions.

The amplification program consisted of 1 cycle at 95°C with a 60-second hold, followed by 45 cycles at 95°C with a 10-second hold, annealing temperature at 55°C with a 10-second hold and 72°C with a 20-second hold. Amplification was followed by melting curve analysis using the program run for one cycle at 95°C with a 0-second hold, 65°C with a 10 second hold and 95°C with a 0-second hold in the step acquisition mode.

Each PCR run included 6 points of the standard curve (5-fold serial dilutions of human blood cDNA), a non-template control with water and unknown cDNA samples.

The concentration of each gene was determined on the basis of a kinetic approach using the LightCycler software. PCR data was analyzed using the LightCycler Software (version 3.4). The

Table 1. Demographic characteristics of the patients with Peyronie's disease who underwent surgical interventions

Tabela 1. Charakterystyka demograficzna pacjentów z chorobą Peyroniego poddanych zabiegom chirurgicznym

Patient No. (Nr pacjenta)	Age – years (Wiek – lata)	Side, angle of the curvature (Strona, kąt krzywizny)	Comorbidity (Choroby ogólnoustrojowe)	ED	Surgery (Operacje chirurgiczne)	Duration of the curvature – years (Czas występowania krzywizny – lata)
1	54	D 70	–	–	IVP	1
2	46	R 50	–	–	IVP	2
3	61	V 80	–	+	IVP	2
4	54	D 90	–	–	IVP+Plic	3
5	42	L 30, V 20	–	–	IVP+Plic	3
6	63	R 90, D 45	–	+	Prost+RFG	3
7	51	L 50	–	–	IVP+Nesbitt	2
8	24	L 30, V 10	–	–	IVP+Plic	7

D: Dorsal, V: Ventral, L: Left, R: Right, Comorbidities: Hypertension, Diabetes mellitus, ED: Erectile Dysfunction, IVP: Incisional venous patch, Plic: Plication, Prost: Prosthesis, RFG: Rectus fascia graft.

D: grzbietowy, V: brzuszny, L: lewy, R: prawy. Choroby współistniejące: nadciśnienie tętnicze, cukrzyca, ED: zaburzenia erekcji, IVP: pooperacyjna łata żylna, Plic: fałd, Prost: proteza, RFG: przeszczep powięzi.

Table 2. Comparative gene expressions in tunica albuginea (TA) and Peyronie's plaque (PP). Cas3: Caspase 3, Cas8: Caspase 8

Tabela 2. Porównanie ekspresji genów w białawej osłonce (TA) i płytce włóknistej Peyroniego (PP). Cas3: kaspaza 3, Cas8: kaspaza 8

Patient #	Tissue	Fas	FasL	Bcl-2	p53	Cas3	Cas8
1	TA	0.01	1.88	1.66	1.87	1.39	1.33
	PP	0.00	11.51	33.04	113.73	36.06	0.16
	PP/TA	0.00	6.12	19.94	60.96	25.91	0.12
2	TA	0.00	11.47	4.40	3.08	4.02	0.25
	PP	0.00	12.63	4.02	8.79	506.36	4.72
	PP/TA	0.00	1.10	0.91	2.86	126.08	18.56
3	TA	0.00	0.00	5.35	0.92	0.52	1.42
	PP	0.00	0.06	2.00	0.59	0.17	0.07
	PP/TA	0.00		0.37	0.63	0.33	0.05
4	TA	0.12	53.47	11.52	5.38	11.19	1.13
	PP	0.00	0.15	20.24	0.84	0.00	0.90
	PP/TA	0.00	0.00	1.76	0.16	0.00	0.79
5	TA	0.00	0.16	2.60	0.84	0.16	0.90
	PP	0.00	1.00	4.09	1.00	0.30	0.30
	PP/TA	0.00	6.19	1.57	1.19	1.84	0.33
6	TA	0.00	8.41	3.91	1.80	0.00	1.82
	PP	0.00	10.00	1.83	1.00	0.00	3.45
	PP/TA	0.00	1.19	0.47	0.55	0.00	1.90
7	TA	0.18	0.00	4.88	1.48	0.45	0.74
	PP	0.00	0.00	0.07	0.16	0.03	0.12
	PP/TA	0.00	0.00	0.01	0.11	0.06	0.16
8	TA	0.00	45.88	13.55	0.13	0.13	2.04
	PP	0.00	0.00	0.10	0.23	0.02	0.06
	PP/TA	0.00	0.00	0.00	1.74	0.13	0.00

values for each PCR product were normalized against beta2-microglobulin mRNA to compare their expressions in normal TA and Peyronie's plaque. Quantitative PCR assays were conducted in duplicate for each sample and a mean value was used to calculate mRNA levels.

Statistical Analysis

A Mann-Whitney-U test was performed to determine the difference between gene expressions in TA and Peyronie's plaque. Statistical calculations were performed using PASW version 18 (SPSS Inc., Chicago, IL, USA). The null hypothesis was rejected if p was < 0.05.

Results

Apoptotic Gene Expressions in Tunica Albuginea

Fas expression was observed in the TA biopsy specimens of 3 patients. The FasL gene was not apparently expressed in 2 patients. The median value for FasL expression was found to be 5.14. Expressions of Bcl-2, p53 and Caspase 8 genes were detected in all patients with corresponding median values of 4.63, 1.64, and 1.23, respectively. The Caspase 3 gene was expressed in all but one patient with a median value of 0.48 (Tables 2 and 3).

Table 3. Expressions of apoptotic genes in Peyronie's plaque (PP), and tunica albuginea (TA). Cas3: Caspase 3, Cas8: Caspase 8

Tabela 3. Ekspresja genów apoptotycznych białawej osłonki (TA) i płytki włóknistej Peyroniego (PP). Cas3: kaspaza 3, Cas8: kaspaza 8

	N	Min	Max	Mean	SD	Median	P
Fas (TA)	8	0	0.1800	0.0382	0.0709	0	0.234
Fas (PP)	8	0	0	0	0	0	
FasL (TA)	8	0	53.4662	15.1582	21.8160	5.1425	0.574
FasL (PP)	8	0	12.6326	4.4187	5.8175	0.5738	
Bcl-2 (TA)	8	1.6570	13.5548	5.9829	4.2517	4.6391	0.442
Bcl-2 (PP)	8	0.0725	33.0384	8.1718	11.9946	3.0086	
p53 (TA)	8	0.1323	5.3761	1.9371	1.6405	1.6413	0.574
p53 (PP)	8	0.1570	113.7304	15.7911	39.6775	0.9180	
Cas 3 (TA)	8	0	11.1917	2.2330	3.8535	0.4855	0.505
Cas 3 (PP)	8	0	506.3645	67.8663	177.6266	0.0975	
Cas 8 (TA)	8	0.2543	2.0351	1.2027	0.5791	1.2306	0.234
Cas 8(PP)	8	0.0604	4.7202	1.2220	1.8186	0.2307	

Apoptotic Gene Expressions in Peyronie's Plaque

The Fas gene was not expressed in any of the 8 Peyronie's plaques. FasL was expressed in all but 2 patients, with a median value of 0.57. The Bcl-2 gene was expressed in all patients with a median value of 3. In one patient the expression of the p53 gene in his Peyronie's plaque was increased approximately 62 fold. This level of increased p53 expression (median: 0.91) was not detected in the remainder of the patients. Although Caspase 3 was expressed at an extremely higher level in one patient (126 fold), when considering all the patients with Peyronie's plaque, its expression remained at a relatively lower level. In 2 patients its expression was not observed (median value: 0.09). Caspase 8 gene was expressed in all patients with a median value of 0.23 (Table 2 and 3).

An increase of more than 1.5 fold was detected in 10 and 13 out of 40 apoptotic genes in Peyronie's plaque and TA relatively in comparison. However 17 genes were similarly expressed in both tissues.

As for gene expressions in the plaque, 3 of 10 genes which were expressed in higher amounts were detected in patients #1 and 2, while in patient #5, two genes and in patients #6 and 8, one gene was expressed more than 1.5-fold

Comparative Evaluation of Apoptotic Gene Expressions in Tunica Albuginea, and Peyronie's Plaque

A statistically significant difference was not detected between the expression of the above-mentioned genes in Peyronie's plaque and TA (Table 3).

Discussion

Similarities between the pathophysiologic features of Peyronie's disease and abnormal wound healing have suggested the possible role of mechanisms of abnormal wound healing in the development of Peyronie's plaque [2].

During normal wound healing, scar tissue forms as a result of a decrease in the cellularity of hypercellular granulation tissue. A decrease in cellularity due to apoptosis has been thought to have a major role during the transition between granulation tissue and scar [12]. Fas, Fas ligand, caspase cascade and the p53 and BCL2 gene families play important roles in the genetic regulation of apoptosis.

Although similarities between the pathophysiological mechanisms of Peyronie's disease and abnormal wound healing have been established, there are only a limited number of studies evaluating the apoptotic processes in Peyronie's disease. Still, a limited number of studies have investigated

expressions of apoptotic genes in Peyronie's disease.

Present findings have revealed that the Fas receptor gene involved in the first step of extrinsic apoptosis is not expressed (or expressed in scarce amounts) in TA or Peyronie's plaque. Increased Fas gene expression induces proliferation, and its decreased levels trigger apoptosis of fibroblasts [13, 14]. In these studies, Fas ligand levels have not been investigated.

In this study, Fas ligand was expressed in relatively higher amounts in TA when compared with its receptor (Fas). Although not to the extent seen in TA, expression of Fas ligand also increased in Peyronie's plaque relative to its receptor. In a study performed on mice, higher rates of Fas, FasL and apoptosis were detected in the infarct region during the acute post-myocardial infarction (MI) period (1st week). In mice devoid of Fas receptors, apoptosis could not be detected during the post-MI period despite the presence of FasL genes [15]. Despite higher levels of Fas L expression in tumor cells of various types of cancers, in many individuals these tumors have been demonstrated to be resistant to apoptosis, and protect themselves from the counter-effects of the immune system by inducing apoptosis in immune cells [16–21]. In light of the above-mentioned information, the data authors have obtained suggests that in the absence of Fas receptor, Fas L is not able to induce apoptosis which might lead to plaque formation.

With its proapoptotic, antiapoptotic members, the Bcl-2 gene family has an important role in the regulation of intrinsic apoptotic processes. Bcl-2 is an antiapoptotic member of the gene family. In Bcl-2 expression analyses conducted in cultures of myofibroblasts, recruited from hypertrophic scar and normal wound healing sites, relatively higher rates of expression have been found in the hypertrophic scar tissue [22]. In 19 of 20 keloid tissues where analysis of Bcl-2 was conducted, higher expression of the Bcl-2 gene was detected in keloid tissues [23]. Like the studies mentioned above, in this study increased levels of antiapoptotic Bcl-2 gene expression in normal TA and plaque tissues were detected.

The p53 protein detects DNA lesions such as nucleotide mismatches, fractures of DNA spirals and DNA lesions induced by chemotherapy. It also suppresses cell cycles and induces intrinsic apoptosis leading to cellular death. p53 is the primary mediator of intrinsic apoptosis. In malignant cases where the p53 gene is lacking, genomic instability and inhibition of apoptosis have been noted [24]. Aberrant p53 functions might lead to cellular proliferation and cell immortality resulting in the development of malignity. Under appropriate conditions, deterioration in the functionality of p53 might lead to cellular proliferation, and non-malignant fibromatosis [25]. Dysfunctional p53 genes and increases in the expression of Bcl-2 have been associated with higher rates of cellular proliferation and decreased cell death rates [26]. Similar to the results of studies about keloids, in this study, expressions of p53 in Peyronie's plaques were found to be decreased while those of the antiapoptotic Bcl-2 gene were increased, suggesting enhanced cellular proliferation. Higher levels of p53 gene expression were detected in 5 patients' TA. However no significant difference between the expressions of p53 in plaques and TA were observed.

Serine proteases called caspases constitute the last phase of the cell death signal in apoptosis. Caspases play key roles in the inhibition and induction of apoptosis. Activation of caspase is associated with the induction and its inhibition is linked with the arrest of apoptosis.

In the molecular assessment of apoptosis, the evaluation of caspase 3, which constitutes the final step in the intrinsic and extrinsic pathways, is extremely important. Activation of caspase 3 is the uniting point of the intrinsic and extrinsic apoptosis pathways. Following induction of apoptosis in normal and keloidal fibroblasts with staurosporine, caspase 3 activation was detected only in normal fibroblasts [27]. In this study, caspase 3 expression was detected at a lower rate in the normal TA of 5 patients and plaque formation of 6 patients, which suggests the general presence of apoptotic inhibition in the tunica albuginea of Peyronie's patients.

Caspase 8 is the first activated caspase in extrinsic apoptosis and plays a role in the activation of caspase 3. When compared with expressions in the TA, the expression of caspase 8 in Peyronie's plaque was found to be decreased in all but 2 cases. As supported by expressions of Fas and Fas ligand, the extrinsic apoptotic activity in Peyronie's plaque seems to be at minimal level.

While the role of the inhibition of apoptosis in the pathophysiology of an abnormal wound healing process is almost completely acknowledged, results supporting the possible role of the inhibition of apoptosis could not be obtained in all studies related to apoptotic gene expression [28]. In this study, contrary to expectations, gene synthesizing proteins involved in the apoptotic cascade were expressed at higher levels than anticipated. These results refute the belief that apoptosis is a simple biochemical cascade, and suggest that this cascade is under the influence of hormonal, paracrine and autocrine factors [29]. Another reason for monitoring some gene expressions in favor of apoptosis might be related to surgical intervention which

was performed after maturation of the plaque or retrieval of biopsy material during alleviation of the influence of paracrinal and hormonal factors. In their biomolecular study of apoptosis activation in PD, Loreto et al. also found extrinsic apoptosis pathway induced, which could underpin plaque stabilization and the halting of fibrosis progression in maturated plaque.

In 2 patients the authors detected that 6 of 10 apoptotic genes were expressed at higher rates (> 1.5 fold) in Peyronie's plaque relative to normal tunica albuginea. In these 2 patients, a pathologic process specific to the plaque region rather than an apoptotic defect in the entire tunica albuginea might be considered. However when the whole patient population was taken into consideration similar gene expressions in both plaque and tunica albuginea suggest a disorder affecting the entire tunica albuginea rather than a localized condition.

Similar expressions in Peyronie's plaque and tunica albuginea suggest that Peyronie's plaque is not a localized disease of tunica albuginea, rather it develops from the vulnerable regions of TA exposed to deleterious factor(s), trauma being the prominent one.

In spite of the numerous studies emphasizing the association between Peyronie's disease and abnormal wound healing, only a small number of studies have addressed the role of apoptosis inhibition in this process. In conclusion, Peyronie's disease might be the result of defective apoptosis affecting tunica albuginea. For definite conclusions, detailed investigations with larger patient populations should be performed.

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