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The Testosterone Production and the Regulation of the Expression of Genes of Several Proliferation Factors in Older Men

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Abstract

Objective: **The likelihood of the development of oncological pathology increases significantly among men over 40 years of age; from this period men have less testosterone circulating in their blood. The reduction of the testosterone level leads to increased mitotic activity, a breakdown in the regulation of the cell cycle, and inhibiting the beginning of apoptosis. The objective of this research work was to study the cause and effect that link between the development of partial androgen deficiency of aging men (PADAM) and the change in expression of genes of several factors that show proliferative activity.**

Patients and Methods: **In this study, 12 patients with age ranged from 56 to 72 years, with partial androgen deficiency were analyzed. Patients were given 40 mg of andriol (testosterone undecanoate) once each morning. All patients were put into one group, for which the research results were compared before and one month after the beginning of androgen-replacement therapy.**

Results: **One month after the beginning of androgen-replacement therapy, the patients showed a decreased expression of bFGF genes (on average by 4.9 times), ER (on average by 11.5 times), bcl-2 (on average by 4.5 times) and an increased expression of the insulin gene (on average by 26 times) as compared with the original values before the study began.**

Conclusion: **Normalization of testosterone production by using androgen-replacement therapy leads to a decrease in proliferative activity, the restoration of regulation of the cell cycle, and a reduction in insulin resistance among men of older age groups.**

Key words: **Testosterone, estrogen receptor, main fibroblast growth factor, epidermal growth factor, insulin, insulin receptor, bcl-2, PSA**

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Introduction

The likelihood of the development of oncological pathology increases significantly after 40 years of age in men.[1] From this period men show a reduction in the amount of testosterone that circulates in their blood. This phenomenon is called partial androgen deficiency of aging men (PADAM).[2]

A decrease in the number of cell-producers of testosterone (Leydig cells) leads to an inadequate response to luteinizing hormone (LH) incretion. Under a mechanism of inverse feedback, the levels of LH and, secondarily, follicle stimulating hormone (FSH) increase,[3] thereby disrupting the regulation mechanisms in the system of the gonads-hypophysis-hypothalamus.[4] Meanwhile the activity of 5α-reductase and aromatase increases.[5,6] The reduction in the testosterone level leads to an increase in mitotic activity, thereby hindering the onset of apoptosis.[6]

An increase in proliferative activity together with insulin resistance is a partial result of the expression of metabolic syndrome. The latter reflects the series of compensatory-adaptive reactions, which take place among men of older age group for the most part due to the decrease in these men's testosterone level.[6]

The given factors have a strong influence on the development of cancer of the prostate, bladder, large intestine, and several other organs.[7,8]

The goal of this research work is to study the cause and effect links between the development of PADAM and the change in expression of genes of several factors that show proliferative activity.

Patients and Methods

This study analyzed 12 patients with partial age-related androgen deficiency. Their ages ranged from 56 to 72. In order to be included in this study the patients had to have fit several criteria, including being male, being older than 40 years of age, and having a decreased level of common (<10.4 nmol/L) and/or free testosterone (<110.0 pmol/L) in the blood serum[3] (Table 1). Patients with infectious diseases of the lower urinal tracts, varicocele, or prostate cancer were excluded from the study. In order to check for these diseases, patients were given rectal analysis and transrectal ultrasound scanning of the prostate gland, while prostate-specific antigen (PSA) which was supposed to be no higher than 4 ng/ml was measured in their blood serum. Patients with improper liver function, in other words, whose values of activity of alanine aminotranspherase (ALT) was greater than 26 ME/L, or aspartate aminotranspherase (AST) higher than 25 ME/L and common bilirubin higher than 21 mkmol/L were also excluded from the study; as were those with serum creatinine higher than 0.11 mmol/L and those who had medical treatment over the last three months using antiandrogens or finasterid, trauma of the central nervous system, epilepsy, or punctures of the brain with amnesia.

Patients were given 40 mg of andriol (undecanoate of testosterone) once a day in the morning. All patients made up one group. The research results of this group were compared before and one month after androgen-replacement therapy.

Hormonal research and reading PSA levels

The levels of common and free testosterone in the blood serum, as well as the level of PSA were measured using an immuno-fermenting method. In order to take these measurements, blood was taken from the veins on an empty stomach at a fixed time (08.00-10.00).[9,10]

The level of common testosterone was measured using test-kits made by the DPS Company (USA), while free testosterone was measured using test-kits made by

Table 1 Levels of common and free testosterone and PSA before and one month after the beginning of androgen-replacement therapy

	Common testosterone (nmol/L)	Free testosterone (pmol/L)	PSA (ng/ml)
μ ± σ before	17.1 ± 5.9	31.0 ± 16.3	1.7 ± 1.2
μ ± σ one month after	19.8 ± 5.9	39.3 ± 16.4	1.6 ± 1.4

N = 12; PSA: prostate-specific antigen; μ ± σ: average values and standard deviations

Diagnostic Systems Laboratories Inc. (USA), and prostate-specific antigen was measured using kits made by DPC (USA).

The sensitivity of the method used for reading levels and the coefficients of variation were, for common testosterone 0.2 nmol/L and 8%, for free testosterone 0.1 pmol/L and 5.4%, and for PSA 0.01 ng/ml and 8%.

Evaluation of the level of expression of specific genes

Evaluation of the level of expression of proteins, as m-DNA was accumulated, was done using a method of half-quantity reverse transcription of the polymerase chain reaction. Separation of total cytoplasmatic ribonucleic acid from blood cells was done using a modification of the Georgiev-Manteyev method.[11,12]

After drawing blood, the anticoagulant EDTA was added to the preparations to make up to 25 mM. The preparations were then frozen and kept at a temperature of -20 ˚C. After thawing the preparations, the red blood cells were broken down using osmotic shock, for which two volumes of 0.07 M NaCl were added to the preparations. Red blood cells were stained, while leukocytes were settled down using a centrifuge for 10 minutes at 2000 turns per minute at a temperature of 4 ˚C. The remaining hemoglobin was taken away while the sediment was washed using 0.14 M NaCl, followed by centrifuging as described above. The leukocytes resulted from this process were re-suspended in 0.14 M NaCl. An equal amount of phenol was then added (pH 6.0) and the mixture was incubated for 15 minutes at room temperature while constantly stirring. The water phase was taken away using a centrifuge at 4000 turns per minute over a period of 10 minutes, and then extracted with an equal amount of phenol (pH 8.0) in the presence of **B** 0.5% SDS. The phases were separated using a centrifuge at 12,000 revolutions per minute over the course of 5 minutes. The water phase was deproteinized three times in a series using a mixture of phenol-chloroform (1:1), while the remaining traces of phenol were taken away using an extract of chloroform.

The resulting ribonucleic acid was broken down by adding three volumes of ethanol and 0.1 volume of 3M of a solution of acetate natria, pH 5.2/0.1M of acetate magina/1 mM of EDTA followed by incubation at a temperature of -20 ˚C over the course of 12 hours. Ribonucleic acid was then collected using a centrifuge (12000 revolutions per minute for 10 minutes at a temperature of + 4 ˚C), and the resulting sediment was washed using 80% ethanol, then dried, and dissolved in deionized water that didn't have any ribonucleic acidase.

The concentration of ribonucleic acid was spectrophotometrically evaluated for its optical density at a wavelength of 260 nm. After further processing using DNA-ase, the lack of genome DNA in the samples was confirmed by the negative results of PCR with primers to the fragment of the gene β-actin. The preparations were balanced in terms of their content of ribonucleic acid, and were used in order to stop the reaction of reverse transcription. The synthesis of k-DNA was done with the help of reverse transcriptase ImProm II (Promega, USA), using random gexanucleotide primers under conditions recommended by the producer.

The expression of specific genes was studied using the PCR method, employing amplification of fragments of genes Bcl-2, estrogen receptor 1 (ESR1), insulin receptor (INR), main growth factor of fibroblasts (bFGF), epidermal growth factor (EGF) and β-actin (Table 2) on a matrix received from k-DNA.

All PCR reactions were done under identical conditions, and repeatability was ensured by using a commercial mixture for PCR called PCR Master Mix (Promega, USA), which included all necessary components of the reaction. The reactions were made in a volume of 20 microliters, and the reactionary mixture included 10 microliters of 2X PCR Master Mix, 1 microliter of 10 mM solution of primers, and 1 microliter of k-DNA. The temperatures of the annealing, which corresponded to the primers used, are shown in Table 2. The temperature profile of the typical PCR reaction included prior melting at 93 ˚C for 2 minutes; 40 cycles under the conditions of 92 ˚C for 30 seconds, annealing for 30 seconds at an according temperature, elongation for 30 seconds at a temperature of 72 ˚C; and a final elongation for 10 minutes at a temperature of 72 ˚C. In order to have a positive control for amplification, the researchers used a preparation of human DNA, and the size of amplicon was estimated using markers from the molecular mass.

In order to evaluate the accumulation of the PCR products, these products were fractioned in 6% PAAG, then stained with bomide etide. Their image was then digitalized and their density was measured using a program made by Total Lab v.2.01.

Table 2 Characteristics of primers used in this research work

	Sequence of primers	Index in the gene bank of the gene-target	Temperature of the annealing	Size of amplicon (p.o.)	Source
Bcl-2 gene	BCL2F: 5'-GTGGAGGAGCTCTTCAGGGA-3' BCL2R: 5'-AGGCACCCAGGGTGATGCAA-3'	NM_000633, GI:4557354	57 ˚C	304	J. Oral Pathol Med, (1999) 29, 63-69
ESR1, the gene of the estrogen receptor 1	ER-F: 5'-TGAACCGTCCGCAGCTCAAGATC-3' ER-R:5'-GTCTGACCGTAGACCTGCGCGTTG-3'	NM_000125, GI:4503602	65 ˚C	151	Mol. Endocrinol, (2005), 19, 1740-1751
INR, gene of insulin receptor	INR-F: 5'-ACTGACCTCATGCGCATGTGCTGG-3' INR-R: 5'-GCCCGTTTTTCTTGCCTCCGTTCAT-3'	NM:M10051, GI:186439	65 ˚C	319	Arch Gynecol Obstet (2004) 270: 170-173
bFGF, main fibroblast growth factor gene	bFGF-F: 5'-CCCGACGGCCGAGTTGAC-3' bFGF-R: 5'-CACATTTAGAAGCCAGTAATCT	NM:M27968, GI:182562	52 ˚C	157	Experimental Cell Research (2004) 299 (2), 286-293
EGF, epidermal growth factor gene	EGF-F: 5'-CAGGTAATGGAGCGAAGCTTTCAT-3' EGF-R: 5'-GAGTTAAATGCCTACACTGTATCT-3'	NM 001963, GI:6031163	52 ˚C	624	Journal of Pediatric Surgery (2003), 38 (11), 1656-1660
β-actin gene	Actin-F 5'-GCCGAGCGGGAAATCGTGCGT -3' Actin-R 3'-GCCACCTGCTACCTCCCCGGC-5'	NM:AK223055, GI:62897670	70 ˚C	506	Graciously offered by Dr. M.Tarunina

Table 3 Values of the expression of the genes IR, ER, bFGF, EGF, bcl-2 before and one month after the beginning of androgen-replacement therapy

	IR	ER	bFGF	EGF	bcl 2 gene
$\mu \pm \sigma$ before	0.01 ± 0.01	0.73 ± 0.82	0.49 ± 0.67	0.23 ± 0.31	0.27 ± 0.31
$\mu \pm \sigma$ after 1 month	0.26 ± 0.36	0.14 ± 0.25	0.10 ± 0.19	0.02 ± 0.03	0.06 ± 0.07
$\bar{d}$	-0.25	0.59	0.39	0.21	0.21
$s_{\bar{d}}$	0.11	0.22	0.18	0.09	0.10
t	-2.24	2.72	2.22	2.29	2.14

N = 12, IR : insulin receptors; ER : estrogen receptors; bFGF : main fibroblast growth factor; EGF : epidermal growth factor;
$\mu \pm \sigma$: average values and standard deviations; $\bar{d}$: average values of the changes in parameters; $s_{\bar{d}}$: standard errors; t : Student criteria

The estimation of the total quantity of k-DNA received as a result for each sample was done on the accumulation of products of PCR with primers to the constituted expressed gene (housekeeping gene) β-actin. The expression of genes was evaluated half-qualitatively for the accumulation of specific M-ribonucleic acid and were calculated as the relation of optical densities of the products PCR, amplified on k-DNA matrixes of the genes being studied, to optical densities of products of PCR with primers to the gene for β-actin.

Statistical analysis

The results of the study were analyzed using a method of dispersion analysis of repeated measurements. The significance of the differences in the changes between the results received before and one month after the beginning of androgen-replacement therapy was done using paired Student criteria. All data in this text and in all tables are given as average values and standard deviations ($M \pm \sigma$). The average values of the changes in parameters ($\bar{d}$) are also given, as are their standard errors ($s_{\bar{d}}$) and the values of the Student criteria (t).[13]

Results

One month after the beginning of androgen-replacement therapy, patients showed a decrease in the expression of the genes main fibroblast growth factor (bFGF), estrogen receptors (ER), epidermal growth factor (EGF) and bcl-2, and an increased

expression of the gene insulin receptors (IR) as compared to their original values (Table 3).

Discussion

Due to the inter-dependence of neurohumoral regulatory processes,[3] inadequate testosterone production as a result of a reduction in the number of cell-producers of testosterone (Leidig cells) leads not only to an increase in the formation of LH, but has an influence on the entire endocrinal regulation system as well. An entire series of genetically determined compensatory-adaptive reactions takes place, and these reactions have an effect at endocrinal, paracrinal, and autocrinal levels. These changes are combined with an increase or a decrease in the expression of the according genes.

The development of compensatory reactions is determined by the physiological role of testosterone. The latter takes part in cell growth and cell differentiation processes.[7,14] PADAM leads to a break down of the natural cycle of those cells which have androgen receptors. The transition of the androgen-independent pool of transitory-proliferation cells into the androgen-dependent pool of transitional cells,[7] which requires the presence of a physiologically necessary level of testosterone in order to continue development, is accompanied by a breakdown in the process of the differentiation of these cells. The inadequate production of testosterone makes it harder for cells with androgen receptors (AR) to pass through the testosterone-dependent cell development stage, and impairs programmed cell death. The given processes are followed by malignant growth.[15]

The series of compensatory reactions which forms is aimed at making up for the inadequacy of the mitogenic action of testosterone through the means of an increase in aromatase and 5α-reductase activity, and increased synthesis of a series of cell growth factors, as well as higher levels of endocrinal activators of mitosis (somatrope hormone, insulin) and vitamin D.[6,16]

An increase in aromatase and 5α-reductase activity is conditioned by the physiological value of estrogens and 5α-dihydrotestosterone. Estrogens induce intensive mitogenesis in tissues which contain specific receptors.[17] 5α-Dihydrotestosterone and testosterone, which connect with one and the same inner-cell receptor,[3] stimulate proliferation of activity of those cells having androgen receptors.[18]

The receptor apparatus which accepts the signal, together with cells, tissues and organs that make secretions form a unified interdependent system.[3] Activation of receptors on the cell membrane increases the effect of the according regulating factor.[14] The expression of AR and ER is added by increased 5α-reductase and aromatase activity. This increase is observed when the testosterone level goes down.[15] The fact that this reaction is typical is proven by the significantly higher level of the expression of the ER gene among patients with PADAM as compared to the analogous value one month after the beginning of androgen-replacement therapy ($p < 0.05$).

A decrease in the production of testosterone among men with PADAM and, correspondingly, a reduction in testosterone mitogenic activity, are compensated for by an increase in the synthesis of peptide growth factors, such as bFGF and several others.[6] The main growth factor of fibroblasts (bFGF) has the most expressed stimulatory effect on proliferation of the epithelial cells. In terms of its mitogenic activity, bFGF is stronger than EGF and other cell growth factors.[7]

A decrease in the quantity of insulin receptors (insulin resistance) leads to a reactionary increase in the level of insulin. At this point insulin-independent sugar diabetes (type 2 diabetes) starts to develop. Insulin increases mitotic cell activity.[3] From this point of view, insulin resistance can be seen as a mechanism for increasing the insulin level, and correspondingly, for increasing its mitogenic activity. An increased insulin level is characteristic of the stage of tumorous growth promotion.[8,19] A low level of the expression of the gene for insulin receptors among patients with PADAM, as compared to the same value a month after the beginning of androgen-replacement therapy ($p < 0.05$), testifies to the fact that the number of insulin receptors is reduced when testosterone production in men of older age groups goes down.

The fact that there is a reduction in the expression of ER, bFGF, EGF, and bcl-2 genes, as well as an increase in the expression of the gene for insulin receptors, is entirely due to the fact that a testosterone preparation was taken by patients. This demonstrates PADAM's leading role at increasing the risk of carcinogenesis among men of older age groups.

The increase in the level of expression of these genes in men with PADAM is accompanied by an increase in the level of estrogens, bFGF, EGF, and insulin in the blood plasma This increase stimulates proliferative activity.[6,15] An increase in the level of mitogenic factors in the blood plasma puts both androgen-dependent and androgen-independent cells of the organism under equal conditions.

Insulin, main fibroblast growth factor (bFGF), and epidermal growth factor (EGF) stimulate extragonadal production of testosterone by other tissues, for which such a function is not common under normal conditions (Pechersky et al., 2005). Thus, among the majority of patients studied, even despite the fact that these patients belonged to older age groups, when making a primary analysis, normal levels of common testosterone were found (Table 2).

Extragonadal testosterone production is directed at compensation for partial age-related androgen deficiency. However, the compensatory incretion of hormones by cells and tissues for which endocrinal functions are not typical is not regulated,[3,14,20] and is inadequate. This is proven by the signs of inadequate testosterone regulation observed among patients with PADAM in the form of atrophy of androgen-dependent tissues and the expression of AR in these tissues.15 Thus despite additional extragonadal testosterone production, there is no inverse development of compensatory reactions, as conditioned by PADAM, including an increase in proliferative activity.

Lymphocytes and macrophages can synthesize testosterone from androstenedione, as well as demonstrate aromatase activity, thereby transforming androgens into estrogens. The androgen dependency of the reaction of cell immunity is confirmed by the presence of AR in lymphocytes and macrophages. There is an increased expression of AR, ER, PR, bcl-2 and p53 of lymphocytes and macrophages of the tumor infiltrate and peritumorous tissues among patients with PADAM.[15] Lymphocyte and macrophage infiltration adds to the overall compensatory reaction in certain places. This compensatory reaction is aimed at increasing mitotic activity.

The participation of lymphocytes and macrophages in the process of steroidogenesis (analogous to the majority of other cells in the organism) testifies not only to the close interaction between the immune and endocrinal systems,[21] but also to the potential participation of the majority of cells in the body in mechanisms for regulation.

Each of the eukaryotic cells (with minor exceptions) is a carrier of the individual's entire genetic information. While the genome is almost identical for all cells in the organism, their proteometabolism are determined by inner and outer physiological factors. The fact that cells are included in compensatory reactions means that this process is capable of modulating their metabolism, which is proven by the hormonal activity of a significant number of non-endocrine cells in the organism. For example, firstly extragonadal testosterone production is developed among patients with PADAM,[15] secondly cancerous cells of the prostate gland synthesize analogues of hypophysial hormones,[22-24] thirdly there is an extragonadal production of estrogens by fatty and several other types of tissues taking place among women[21] in the menopause period, and lastly myocytes of the wall of arterioles (which are transformed into epithelioid cells), and mesangiocytes of the kidneys over a long period of ischemia begin to produce rheniums.[20] Apparently, the situational manifestation of compensatory hormonal activity by the majority of cells and tissues including tumorous cells forms a diffusive endocrinal system (APUD-system).[15]

The strongest expression of extragonadal testosterone production among men with andropause is observed when there is a significant increase in mitotic activity of cells when they go through malignant transformation. The malignant transformation of cells is the most strongly expressed form of the manifestation of compensatory changes as PADAM develops,[15] which determines its physiological role.

Among patients with PADAM who were given androgen-replacement therapy, we regularly observed an increased expression of the antiapoptotic gene bcl-2 ($p < 0.05$) one month after therapy as compared to the expression before therapy. This allows us to speak of an increased risk of blastomatose transformation.

When the production of testosterone is decreased in the male body, androgen-dependent cells turn out to be in the worst situation: an increase in the level of promoter factors of carcinogenesis in the blood plasma is accompanied by a breakdown in the development of cells during the androgen-dependent stage.[15] Likewise, cases of prostate cancer make up a significant part of all the oncological diseases among older-aged men.[25]

For this same reason, among men with prostate cancer, primary analysis usually reveals androgen-dependent tumors[26] which are formed from cells of which tumorous transformation began at the androgen-dependent stage. An increase in proliferative activity, together with insulin resistance, is a partial manifestation of metabolic syndrome, (X-syndrome), the development of which among men is due, to a significant extent, to a decrease in the production of testosterone.[6]

The given changes represent a series of compensatory-adaptive reactions which develop among men with PADAM, and have a systematic character. Apparently, the male organism has formed standard genetically-determined variants of functioning of the endocrinal and paracrinal-autocrinal regulation systems over the course of evolution. These variations match both normal conditions and the majority of pathological states a man can have, one of which is a reduction in the production of testosterone.

The absence of an adequate incretion of testosterone by Leidig cells in response to LH activity is accompanied by an expression in bFGF, EGF, and bcl-2 genes, as well as by a decrease in expression of the insulin gene. When partial age-related androgen deficiency is corrected, however, the expression of these genes is reversed. This suggests that the changes in gene expression which take place due to PADAM are not permanent.

The change in the pattern of gene expression which takes place during the process of phylogenesis depends on the level to which testosterone production has deviated from physiological norms. The new variations of the functioning of the endocrinal and paracrinal-autocrinal regulation systems, when testosterone production is made, are through a mechanism of negative feedback.

The necessary level of testosterone is formed in the body as a response to LH incretion when androgen-replacement therapy in patients with PADAM is made properly. Autocrine-paracrine interaction is also normalized, thanks to the obstruction-free passage of androgen-dependent cells through the testosterone-dependent cell development stage. When testosterone regulation is rehabilitated, there is no longer any need for compensatory-adaptive reactions to take place in the body, as happens in men with PADAM. One can observe a decrease in the expression of the ER, bFGF, EGF, and bcl-2 genes, and an increase in the expression of the IR gene ($p < 0.05$).

Conclusion

The recovery of testosterone production helps make a decrease in proliferative activity and the rehabilitation of regulation of the cell cycle, as well as to reduce insulin resistance among older men. These changes suggest that metabolic syndrome is possibly reversed (X-syndrome), which represents to a significant degree a phylogenetically-formed response to a decrease in testosterone production. Taking this into consideration, attempts to change the expression of individual genes without taking into effect the systematic nature and succession of the changes that take place seem hardly promising.

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