ORIGINAL ARTICLE

Increased expression of hypoxia-inducible factor-1α and connective tissue growth factor accompanied by fibrosis in the rat testis of varicocele

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Abstract

Objective: We investigated hypoxia inducible factor-1α (HIF-1α), connective tissue growth factor (CTGF) expression and fibrosis in the testis of rats with surgically induced varicocele.

Materials and methods: A total of 47 adult male Sprague-Dawley rats were arranged in 3 groups, namely group 1 (varicocele operation 4 weeks ago, n = 10; sham operation 4 weeks ago, n = 5), group 2 (8 weeks, n = 11; n = 5), and group 3 (12 weeks, n = 11; n = 5). The rats in every group underwent bilateral orchiectomy 4, 8, and 12 weeks after the operations, respectively. HIF-1α and CTGF expression of both testes in group 3 were studied by real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Fibrotic change was assessed by quantitative image analysis.

Results: HIF-1α mRNA expression in testes tissues in varicocele operation and sham controls showed no significant differences in RT-PCR. However, CTGF mRNA expressions in left testes were found to be significantly different between varicocele operation and sham controls. HIF-1α staining was present in both testes of all specimens and CTGF staining was present in 10 left and 8 right testes of 11 specimens. However HIF-1α and CTGF staining were absent in control group. There were significant fibrotic changes of both testes in groups 2 and 3. There were significant differences in fibrotic change along the durations of surgical varicocele.

Conclusions: This study reveals that experimental varicocele in the rat is associated with HIF-1α and CTGF expression and it is accompanied by fibrotic change in the testis.

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Introduction

Varicoceles, defined as tortuous and abnormally dilated veins of the spermatic cord, are present in 15% of the normal male population and about 40% of men with infertility. The most common findings in the histopathology are hyperplasia of Leydig cells, a lower number of spermatogonia per tubule, stoppage of spermatogenesis, and sloughing of the germinal epithelium. Additionally, it has been described that myofibroblasts are transformed into fibroblasts, causing peritubular and perivascular fibrosis in adult males with varicocele. These changes are bilateral even in patients with unilateral varicocele. The pathophysiological mechanisms proposed for varicocele include venous stasis, increased testicular pressure and temperature, reflux of adrenal metabolites, and endocrine disorder at the level of the testes. However, there are controversies in these theories. Therefore, there is a need for further research to fully understand the pathophysiology of varicocele.

It has recently been shown that hypoxia is a contributing factor to the pathophysiology of varicocele. Moreover, an association between the HIF-1α and the CTGF in the hypoxic state has been proven by showing an upregulated expression of the CTGF via activation of the HIF-1α in dermal fibroblasts or epithelial tubular cells of mouse kidney. Based on these results, it seems reasonable to postulate that the HIF-1α and CTGF play a role in the pathological process of varicocele. Hence we try to investigate the expression of HIF-1α, CTGF, and histological changes including fibrosis in testes of rats with surgically induced varicocele to reveal the pathophysiology of varicocele.

Materials and methods

Animals

51 male Sprague-Dawley rats between 12 and 13 months of age were included in the study. All the animals were fed equal amounts of the same food and kept in a constant environment with a light–dark cycle of 12h:12h. The rats were assigned to one of three groups: group 1 (4 weeks post-surgery of varicocele, n = 10; 4 weeks after simulated surgery), group 2 (8 weeks, n = 11; n = 5), and group 3 (12 weeks, n = 11; n = 5). Four rats were excluded because of death after surgery. Two of them in group 1, one in group 2, and one in group 3.

Technique of experimental varicocele

Each animal was anesthetized with an intramuscular injection of zolazepam hydrochloride (10 mg/kg) and xylazine (7 mg/kg). An abdominal incision was made in the midline. The abdominal content was displaced to the right to visualize the left kidney, the left adrenal vein, the left renal vein, and the left spermatic vein at its junction with the left renal vein. The outer diameter of the left internal spermatic vein was measured at the level of the ilioinguinal vein through a metal micrometer to assess the development of varicocele. Using a careful blunt dissection, the tissue attached to the left vein was removed in a medial position to the insertion of the left spermatic vein and the left adrenal vein. At this point, a silk was tied 6 zeros around the 0.035-in. guidewire (Terumo Guide Wire, Terumo, Frankfurt, Germany), which...
was cut about 4 cm and placed in the left renal vein. The
guidewire was then withdrawn and there was a reduction of
about half the diameter of the left renal vein. The dis-
tal part of the renal vein in each animal was immediately
dilated.

The rats in the control group underwent the same opera-
tion, except that only the ligatures were placed in position,
but they were not tied. After closing the incision in the mid-
line in two layers with 3-0 silk sutures, the rat was put into
the cage and fed as previously. The experimental protocol
was approved by the Committee of Care and Use of Animals
and was in accordance with the Declaration of Helsinki and
the guidelines of the International Association for the Study
of Pain.

Histopathology

The rats of groups 1, 2, and 3 underwent bilateral orchi-
tomy 4, 8, and 12 weeks after the respective operations.
The rats of the control groups were also put down in the same
intervals. We confirmed the dilation of the left internal sper-
matic vein measuring its external diameter at the level of
the ilioilumbal vein before being fixed in Bouin’s solution
and embedded in paraffin, while the rest was stored in a
cryotube and immersed in liquid nitrogen only for HIF-1α
staining. Histological sections of both testes of each animal
were prepared and marked with hematoxylin–eosin for his-
tological examination. The diameters of the seminiferous
tubes were measured in 25 tubules using the Leica IM-50
software (Leica Imaging Systems, Cambridge, England) using
an optical microscope (BX-50; Olympus, Tokyo, Japan) with
a target of magnification 10×.

Real-time reverse transcription polymerase chain
reaction

The real-time reverse transcription polymerase chain
reaction (RT-PCR) was analyzed in 22 samples of groups
3 and 10. The total RNA was extracted from the testes
using a Trizol kit (Invitrogen Corp., Carlsbad, CA, U.S.A.).
The reverse transcription was performed using 1.5 μg total
RNA in 20 μl using a reverse transcription kit (Invitrogen
Corp., Carlsbad, CA, USA). For the real-time RT-PCR
assays, a master mix of the following components at the
indicated concentrations was prepared: 2.5 μl of each
primer (9 μM), 2.5 μl probe (2.5 μM), 2.5 μl water and
12.5 μl TaqMan PCR 2× master mix (Perkin-Elmer Applied
Biosystems, Lincoln, CA, USA). The nucleotide sequences
of the primers were: 5′-CCCCAGATTCAGATCCAGACA-
GAG-3′ and 5′-CATGCATGTCCATTGTCCG-3′ for
HIF-1α, 5′-CTAAGACCTTGGATGAGG-3′ and 5′-
CTCAAGATGTCATTGTCCG-3′ for CTGF. Five microlitre
mixture was added for reverse transcription as a template
of PCR. We performed a relative and quantitative real-time
RT-PCR using the reactants mentioned and a sequence
detection system ABI Prism 7000 (Perkin-Elmer Applied
Biosystems, Lincoln, CA, USA). We used the following pro-
cedure: after the initial activation of uracil-N-glycosylase
at 50 °C for 2 min, AmpliTaq Gold was activated at 95 °C
for 10 min. The PCR consisted of 45 cycles of amplification
(denaturation at 95 °C for 15 s, tempered at 60 °C for 1 min,
and extension at 60 °C for 1 min). During the PCR amplifi-
cation, the amount of amplified product was monitored by
continuous measurement of fluorescence. The expressions
of HIF-1α and CTGF were normalized to GAPDH expression
(VIC/MGB probe, limited primer) as follows: the number of
cycles in which the transcription of HIF-1α or CTGF genes
(threshold cycle, Ct) was detected was normalized against
a GAPDH Ct, which is called a deltaCt. The expression of
HIF-1α or CTGF relating to the reference was expressed as
2 deltaCt, where deltaCt represents the differences
between the deltaCt values of the test and reference
groups. The intra-assay coefficients of variation Ct were
below 11.0% for HIF-1α, below 6.48% for CTGF, and below
16.7% for GAPDH.

Immunohistochemistry for hypoxia inducible
factor-1α and connective tissue growth factor

The left and right testes of the experimental and control
group in group 3 were analyzed by immunohistochemistry
using the indirect immunoperoxidase method with sec-
ondary antibody conjugated with dextran polymer labeled
with peroxidase (Envision™; DAKO, Carpinteria, CA, USA).11
The expression of HIF-1α was measured using mouse polyclonal
IgG antibodies (1:25, sc-10790; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The CTGF expression
was measured using goat polyclonal IgG antibodies (1:25,
sc-14939; Santa Cruz Biotechnology). In the control plates,
the primary antibody was replaced with normal serum. In
each section, we evaluated the immunohistochemical staining
of HIF-1α and CTGF as absent or present, as described
in previous studies.7,12 We compared the staining group fre-
frequencies.

Quantitative assessment of testicular fibrosis

The sections of groups 1, 2, and 3 fixed in Bouin’s solu-
tion were stained according to Masson’s trichrome method
to detect testicular fibrosis, as previously described.13 We
performed the quantitative analysis of fibrosis as follows:
each section was analyzed under the microscope described
above with a 20× objective. 5 fields were analyzed in each
section. In order to achieve a proper assessment, all the chosen
fields included all the structures, such as seminiferous
tubules, interstitium, and vessel. The fibrosis was quantified
using the Leica Qwin software (Leica Imaging Systems),
calculating the areas stained blue divided by the total area of
the field and multiplied by 100.

Statistical analysis

We used the SPSS version 13.0 for Windows (SPSS Inc.,
Chicago, IL, USA) for the statistical analysis. We used the
Mann-Whitney test to detect the differences between the
varicocele and control group. The Kruskal–Wallis test was
used to detect the differences between groups 1, 2, and 3
(the durations of varicocele). All the values were expressed
Table 1 Diameters of the seminiferous tubules.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Mean diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Group 1</td>
<td>10</td>
<td>272.2 ± 20.5(^a)</td>
</tr>
<tr>
<td>Control 1</td>
<td>5</td>
<td>294.4 ± 9.1</td>
</tr>
<tr>
<td>Group 2</td>
<td>11</td>
<td>245.1 ± 39.2(^a)</td>
</tr>
<tr>
<td>Control 2</td>
<td>5</td>
<td>275.3 ± 15.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>11</td>
<td>230.1 ± 39.2(^b)</td>
</tr>
<tr>
<td>Control 3</td>
<td>5</td>
<td>275.0 ± 20.3</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05, in comparison with the corresponding control group.
\(^b\) p < 0.01, in comparison with the corresponding control group.

as mean standard deviation. We considered a p value < 0.05 as significant.

Results

Assessment of the varicocele

All the rats (47) undergoing partial ligation of the left renal vein and included in this experiment showed an objective dilation of the left internal spermatic vein when they were put down. No animal in the control group showed dilation of the left internal spermatic vein.

Body weight

At the time of sacrifice, the mean weights of group 1, control 1, group 2, control 2, group 3, and control 3 were 499.0 ± 39.3; 514.2 ± 28.1; 571.1 ± 32.5; 580.1 ± 61.8; 660.2 ± 35.9, and 655.4 ± 68.33 g, respectively. There were no significant differences (p > 0.05) between the varicocele groups, the control groups, or between groups 1, 2, and 3.

Histopathology

Testicular weight

The mean weight of the left testes in group 1, control 1, group 2, control 2, group 3, and control 3 were 1.62 ± 0.13; 1.59 ± 0.15; 1.61 ± 0.29; 1.69 ± 0.09; 1.49 ± 0.49; and 1.73 ± 0.19 g, respectively. The mean weights of the right testes in group 1, control 1, group 2, control 2, group 3, and control 3 were 1.61 ± 0.17; 1.58 ± 0.16; 1.69 ± 0.11; 1.71 ± 0.09; 1.60 ± 0.28; and 1.74 ± 0.19 g, respectively. There were no significant differences between varicocele and control groups or between the durations of varicocele.

Diameters of seminiferous tubules

Mean values are shown in Table 1. The mean diameters of the left tubules in groups 1, 2, and 3 were significantly lower (p < 0.05) than those of the corresponding control groups. The mean diameters of the right tubules in groups 2 and 3 were significantly lower than those of the corresponding control groups. There were significant differences in both testes according to the duration of the varicocele (p = 0.01 and 0.04 for left and right testes, respectively).

Histological changes

The sections of the left and right testes of the control groups showed no significant histological abnormalities. The main histological changes in the varicocele groups were reduced spermatogenesis, desquamation of germ cells, maturation stoppage, increased congestion, and perivascular fibrosis. Among 11 samples, there were severe histological changes in 4 left testes and two right ones in group 2 and 7 left testes and 5 right ones in group 3. However, there were no severe changes in the testes in group 1.

mRNA expression of hypoxia inducible factor-1α and connective tissue growth factor in testicular tissue

The RT-PCR quantitative analysis of HIF-1α in both testes in group 3 revealed no significant differences in mRNA expression (Fig. 1). However, the quantitative analysis of RT-PCR in group 3 revealed a significant difference in the mRNA expression of CTGF in the left testes between the operated and the control group; but it did not reveal significant differences in the mRNA expression of CTGF in the right testes (p = 0.024 and 0.095 respectively, Fig. 2).
Immunohistochemistry of hypoxia inducible factor-1α and connective tissue growth factor

There was staining of HIF-1α in both testes of all the specimens in group 3, whereas there was not in the control group. The HIF-1α expression was detected in the nucleus of the germ cells, the cytoplasm, and the vascular endothelium (Fig. 3). There was CRGF staining in 10 left testes and 8 right ones of 11 specimens in group 3. However, there was not in the control group. The CTGF expression was detected only in the interstitium, this being the most prominent staining in the cytoplasm of the fibroblasts. Additionally, it was detected mainly in the perivascular area of group 3, while it was not detected in the control group (Fig. 4).

Quantitative assessment of testicular fibrosis

The fibrosis was significantly higher in groups 2 and 3 than in the corresponding controls (Table 2). Much of this increase could be attributed to increased interstitial fibrosis, which was more than three times higher in group 3 than in controls. Moreover, the fibrosis increased significantly in the left and right testes with the varicocele duration ($p=0.01$ and $<0.01$ for left and right testes, respectively).

Discussion

It is known that the etiology of varicocele is multifunctional, from anatomical variations to endocrine dysfunction. Among the causes, the association between hypoxia and varicocele has been investigated for several decades. Researchers have managed the hypothesis that poor venous return, which increases blood volume in the testes and results in venous stasis, leads to a decrease in testicular oxygen levels. Turner et al. showed that the bilateral change in testicular function induced by experimental left varicocele was not related to the testicular blood gas concentrations. However, Ozturk et al. found a significant increase in biochemical indicators of tissue hypoxia in the unilateral experimental varicocele in rats and found that chemical sympathectomy can prevent this increase. Moreover, recent studies have shown an overexpression of HIF-1α in the testis of rats with experimental varicocele and the internal spermatic vein of the male.

The HIF-1 is a master regulator of the transcription factors of the family of hypoxia inducible factors, and it consists of α and β subunits. It is known that the HIF-1 activity is determined primarily by the HIF-1α. Under hypoxia, the HIF-1 binds to hypoxia responsive genes to recover oxygen homeostasis activating hypoxia sensitive genes such as erythropoietin, vascular endothelial growth factor, β3 transforming growth factor, and insulin-like growth factor.

The CTGF is a cellular matrix protein belonging to the cysteine-rich family overexpressed in 61/nephroblastoma, and it is involved in the proliferation, adhesion, chemotaxis, and matrix production in a variety of cell types such as fibroblasts, mesangial, endothelial cells, and chondrocytes. The CTGF expression increases during wound repair, inflammation, fibrotic diseases, tumor growth, and angiogenesis. The expression of CTGF has also been associated positively with the degree of fibrosis and, indeed, the protein excretion of CTGF has been identified in the urine samples of patients.
with renal disease as an effective indicator of renal function, where high values of CTGF protein reflect increased kidney damage.\textsuperscript{17,18} Since the CTGF appears to be a critical factor in the development of fibrosis in other organs, we decided to investigate its potential role in the pathophysiology of varicocele associated with HIF-1α.

In the present study, we found that experimental varicocele caused a significant decrease in the diameter of the seminiferous tubules and increased testicular fibrosis compared with the control group, and that changes worsened as the varicocele lasted longer. Additionally, we showed overexpression of HIF-1α and CTGF in the testes of rats with varicocele, detected by immunohistochemistry. These results are consistent with recent research on HIF-1α and CTGF in other organs.\textsuperscript{9,10} Wiener et al. reported mRNA of HIF-1α in organs of humans, rats, and mice in both normoxic and hypoxic conditions.\textsuperscript{19} It has also been shown that the mRNA and the CTGF proteins increase in response to hypoxia in line with MDCA231 human breast cancer.\textsuperscript{20} It has even been shown that localized hypoxia may contribute to the development of fibrotic regions within the kidney, and that by means of pVHL inactivation, a significant increase in mRNA expression of CTGF with subsequent activation of HIF-1 in murine kidneys was achieved.\textsuperscript{9} Therefore, these results suggest that upregulated by HIF-1α CTGF induced in hypoxia might increase testicular fibrosis.

The relation between fibrosis and loss of testicular spermatids in one study suggested that fibrosis could be something associated with the process that causes spermatogenic damage, or that it could exacerbate this process by interfering with the function of the seminiferous tubules.\textsuperscript{13} Anyway, it was concluded that testicular fibrosis worsened with the vasectomy obstructive interval and that it was associated with adverse outcomes of fertility after vasostomy.

In this study, we also found that longer durations of varicocele exacerbated testicular fibrosis. Moreover, based on the findings of the recent reports mentioned above, increased expression of HIF-1α and CTGF could be the cause of this change after the varicocele operation.

This study shows that experimental varicocele in rats is associated with HIF-1α and CTGF expression accompanied by histological abnormalities and fibrotic changes in the testes. As far as we know, this is the first study that shows that there is CTGF expression in combination with fibrosis in the testicular tissue in the context of varicocele. These results suggest that the pathophysiological changes related with fibrosis are associated with testicular dysfunction when there is varicocele.

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**Conflict of interest**

The authors declare that they have no conflict of interest.
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