Increased Activated Caspase-3 Expression in Testicular Germ Cells of Varicocele-Induced Rats

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OBJECTIVES: In mammalian cells, caspase-3 is considered to be a major executioner protease because it is essential for apoptotic cell death. We investigated the testicular germ cell apoptosis of rats with experimentally induced left varicocele by detecting activated caspase-3 expression.

MATERIALS AND METHODS: An experimental left varicocele (ELV) was created by partial ligation of the left renal vein in 24 adult male Sprague-Dawley rats as the study group. An additional 16 rats that underwent a laparotomy and renal vein handling without ligation served as the sham control group. Six rats of the study group and 4 rats of the control group were sacrificed at 2, 4, 8, and 12 weeks, respectively, following varicocele creation. The testicular tissues of the varicocele and control groups were sampled for activated caspase-3 by immunoblotting and immunohistochemistry.

RESULTS: Actual numbers of rats in the varicocele group were 4, 5, 6, and 5 at 2, 4, 8, and 12 weeks, respectively, after varicocele creation. The immunoblots of activated caspase-3 in both control and varicocele groups revealed a single band. The relative intensities of activated caspase-3 at 8 and 12 weeks significantly increased in the varicocele group compared to the ipsilateral testis of the control group (p < 0.05).

CONCLUSIONS: This animal study demonstrated increased expression of activated caspase-3 in testicular germ cells at 8 and 12 weeks after varicocele creation. That means that the increase in testicular germ cells apoptosis in rats with ELV occurred gradually. This may be a factor in varicocele causing male infertility in humans. (JTUA 17:81-6, 2006)

Key words: caspase-3, testicular germ cell, induced varicocele, rat.

INTRODUCTION

Varicocele, described as abnormal tortuosity and dilatation of the gonadal veins within the spermatic cord, is a common anomaly in adolescent and adult males with an incidence of 15%-20% of the general population [1-3]. They have long been recognized as the most common cause of male infertility in adults. About 30%-40% of male infertility was reported to be associated with varicoceles [1-3].

The mechanisms by which a varicocele leads to infertility, however, remain unclear. Recent studies have suggested a close relationship between varicoceles and apoptosis: in varicocele patients, apoptosis may play an important role in the development of oligospermia [4,5]. In the human apoptotic pathway cascade, 14 caspases...
(cysteinyl aspartate-specific proteinases) have been found to date. Among them, caspase-3 is considered to be a major executioner protease because it is essential for apoptotic death in mammalian cells [5-8]. Caspases also play central roles in regulating apoptosis in the human seminiferous epithelium [6-8]. In the present study, the expressions of activated caspase-3 (active forms of 20 and 17 kDa) [9] were detected in testes to illustrate the relationship between testicular germ cell apoptosis and the presence of a varicocele in rats.

MATERIALS AND METHODS

This study included 40 adult male Sprague-Dawley rats of the same weaning age (10 weeks) and weighing ~300g. All animals were fed the same food and were maintained in a constant environment with a 12:12-hour light-dark cycle. The rats were assigned to 2 groups. Group 1 rats (sham control, n = 16) underwent a laparotomy except that the ligatures were only placed in position and not tied. Group 2 rats (study group, n = 24) underwent surgery to induce a varicocele. Then 6 rats of the study group and 4 rats of the control group were sacrificed at 2, 4, 8, and 12 weeks following varicocele creation. Testicular tissues of rats in which a varicocele had successfully been induced in the varicocele group and all rats in the control group were sampled for activated caspase-3 by immunoblotting and immunohistochemistry.

Technique of experimentally induce left varicocele (ELV)

Each animal was anesthetized with an intraperitoneal injection of sodium phenobarbital (50 mg/kg) [10,11]. An abdominal midline incision was made. The left renal vein, inferior vena cava, and left spermatic vein were identified and a clamp was passed behind the left renal vein just distal to the spermatic vein insertion [12,13]. A 4-0 silk ligature was loosely placed around the left renal vein at this site, and a rigid hydrophilic guide wire of 0.64 mm in diameter was placed on the left renal vein [12]. The ligature was tied around the vein over the top of the guide wire. The guide wire was then withdrawn, and the vein was allowed to expand to the limits of the ligature, which caused the vein diameter to decrease to approximately 1/2 of its original diameter. The renal and spermatic veins in each animal immediately dilated. The midline incision was closed in 2 layers with 4-0 silk sutures.

Activated caspase-3 immunoblotting

Testicular tissues from the control and varicocele groups were sampled at sacrifice and stored at -80 °C before use. An activated caspase-3 (Asp 175) (SA1) rabbit monoclonal antibody (Cell Signaling Technology, Ipswich, MA) was used as the primary antibody in the present study. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Each frozen sample was mixed with 0.5 ml of lysis reagent (Promega, Madison, WI) and 51 of proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 ml aprotenin), then homogenized on ice. Aliquots of 100g of the homogenate and molecular weight standard (Bio-Rad, Hercules, CA) were heated to 100°C for 5 minutes and fractionated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 15% polyacrylamide gels. The separated proteins were then transferred to PVDF membranes (Millipore, Chelmsford, MA) by electoblotting at 100 V for 40 minutes. After incubation for 2 hours in PBST buffer containing 5% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 2 hours in primary antibody diluted in 1% BSA and 0.05% sodium azide in PBST (1:1000 dilution), washed in PBST, and reacted for 2 hours with secondary antibody (1:10,000 dilution). Blots were developed after incubation with a BCIP/NBT kit (Invitrogen, Carlsbad, CA). -Tubulin (Santa Cruz Biotechnology, sc-8035, Santa Cruz, CA) was used as an internal control. Immunoblots were photographed and imported as TIFF files into the ID image analysis software package (Kodak Digital Science, 1998). The results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

Immunohistochemistry for activated caspase-3

The testes were fixed in Bouin's solution and embedded in paraffin. Then sections (4 μm) of testicular tissues from both the control and varicocele groups were deparaffinized and stained with hematoxylin and eosin (H&E). For immunohistochemistry of activated caspase-3, deparaffinized sections were dehydrated and then immersed in 10^-4 M sodium citrate buffer (pH 6.0). Sections were then heated in a microwave oven at 60 °C for 10 minutes. Endogenous peroxidase was inactivated by incubating sections with 3% hydrogen peroxide, and nonspecific reactions were blocked by incubating sections in a solution containing 5% normal horse serum and 1% normal goat serum. Then sections were incubated with the primary antibody overnight at 4 °C. Activated caspase-3 expression was assessed using a peroxidase-conjugated rabbit monoclonal antibody IgG (Cell Signaling Technology, Ipswich, MA) (dilution 1:200). After 3 rinses with phosphate-buffered saline,
the sections were incubated with a commercial kit (PicTure™, Zymed, South San Francisco, CA) for visualization of the immunoreaction. Finally, they were rinsed with distilled water and counterstained with hematoxylin. Negative control experiments, in which goat serum was used instead of the primary antibody, were conducted (data not shown) to confirm the positive results. Then all sections were observed using an Olympus BX50 light microscope and photographed with a Nikon CP5000 digital camera.

Statistical analysis
Data were analyzed using Student’s t-test (SPSS software, Chicago, IL) with p < 0.05 being considered to significantly differ from the control group at the same sampling time.

RESULTS
The numbers of rats in which a varicocele was successfully induced in the varicocele group (total 24 rats) were 4, 5, 6, and 5 (20 rats with a successful ELV) at 2, 4, 8, and 12 weeks, respectively, after surgery (varicocele creation). The immunoblotting of activated caspase-3 in both the control and varicocele groups revealed a single band (Fig. 1). The relative intensity of activated caspase-3 in rat testis of the varicocele group was significantly higher than that of the control group (p < 0.05) at 8 and 12 weeks after varicocele creation (Table 1, Fig. 2). The intensity of activated caspase-3 expression in testicular germ cells of rats with varicocele was predominant in spermatogonia according to immunohistochemical (IHC) staining (Fig. 3).

DISCUSSION
Varicocele is the most common etiologic factor

<table>
<thead>
<tr>
<th>Group</th>
<th>Control group (no. of rats)</th>
<th>Varicocele group (no. of rats)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>62.44±14.84 (4)</td>
<td>58.49±6.50 (4)</td>
</tr>
<tr>
<td>4</td>
<td>77.37±12.30 (5)</td>
<td>80.31±13.42 (4)</td>
</tr>
<tr>
<td>8</td>
<td>83.40±9.86 (6)</td>
<td>132.55±20.36* (4)</td>
</tr>
<tr>
<td>12</td>
<td>67.62±13.90 (5)</td>
<td>207.92±44.92* (4)</td>
</tr>
</tbody>
</table>

Values of activated caspase-3 expression were normalized relative to α-tubulin expression. *p < 0.05 associated with male infertility [1-3]. However, despite data being obtained from animal models and human studies, the pathophysiology remains unclear. The importance of understanding the mechanisms of germ cell death has become evident during recent years as there is still a need for superior treatment modalities for male infertility. New studies on testicular tissues of men with varicocele have demonstrated increased apoptosis among developing germ cells, which may be the cause of oligospermia [4-8].

Caspase-dependent apoptosis is a well-characterized mechanism for removing senescent, defective, or unneeded cells [5-8], and caspase-3 appears to be the main executioner within the apoptotic cascade in mammalian cells. In general, caspases play major roles in the pathogenesis of a multiplicity of andrological disorders such as impaired spermatogenesis, decreased sperm motility, and increased levels of sperm DNA.
fragmentation [5-7]. Barqawi et al. confirmed that normal Spraque-Dawley rats demonstrate low levels of germ cell apoptosis. Conversely, rats that underwent experimental varicocele creation showed significantly increased levels of germ cell apoptosis in the ipsilateral testis 14 days following varicocele creation [11].

In this study, gradually higher activated caspase-3 expression was found in the left testis of the varicocele group than in the control group at 8 and 12 weeks after varicocele creation, although spermatogenic cells showed some degree of apoptosis according to IHC staining in the control group. A trend was revealed of increasing apoptosis with a longer duration of the varicocele [14]. Saleh et al. showed that infertile men with varicoceles had significantly greater DNA damage in their spermatozoa than did normal men [14]. Regular apoptosis of spermatogenic cells is required to maintain proper testicular homeostasis, but increased cell death may result in defective spermatogenesis leading to infertility [15].

What is the etiology of the increased testicular cell apoptosis in patients with varicocele? Researchers have hypothesized that these factors increase the volume of blood in the testis and result in venous stasis, which means decreased testicular oxygen levels [10]. Chakraborty et al. documented blood stagnation with the microcirculatory vessels of the testes in patients with varicocele, which could cause local hypoxic or ischemic
shock to the cells [10]. Hypoxic stress could increase testicular cell apoptosis and may also be a factor in varicocele-caused male infertility.

CONCLUSIONS

This animal study demonstrates that activated caspase-3 expression in testicular germ cells of rats with experimentally induced left varicocele (ELV) was significantly higher than in the control group at 8 and 12 weeks after varicocele creation. That means that the increase in testicular germ cell apoptosis in rats with ELV occurs gradually. This may be a factor in varicocele causing male infertility in humans.

REFERENCES

以實驗誘導左側精索靜脈曲張的老鼠發現活化的 Caspase-3 在其睾丸的表現量增加

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目的：在哺乳類的細胞，caspase-3 被認爲是執行細胞凋亡的主要蛋白酶。於是我們探討活化的 caspase-3 在睾丸生殖細胞的表現，藉此瞭解左側精索靜脈曲張的老鼠其睾丸生殖細胞凋亡的情形。

材料與方法：以 24 隻 Sprague-Dawley 成鼠做左側部分腎靜脈的結紮維實驗組，另外 16 隻進行假手術當對照組，分別在術後的二、四、八及十二週取下兩組的左側睾丸進行活化的 caspase-3 蛋白分析和免疫組織化學染色。

結果：精索靜脈曲張的老鼠（實驗組）在術後的第八週及第十二週活化的 caspase-3 的表現量均大於對照組，且有統計意義（p < 0.05）。

結論：本次的動物實驗證實活化的 caspase-3 在睾丸的生殖細胞表現量，尤其在第八週及第十二週後實驗組明顯大於對照組；表示生殖細胞凋亡增加，這可能也是人類精索靜脈曲張病人造成不孕的原因之一。

鍵語：活化的 caspase-3，生殖細胞，實驗誘導精索靜脈曲張，老鼠。