Cadmium Accumulation and Metallothionein Overexpression in Internal Spermatic Vein of Patients With Varicocele

Shaw-Yeu Jeng, Su-Mei Wu, and Jane-Dar Lee

OBJECTIVES
To determine the possible molecular mechanism for the thickened wall in the internal spermatic vein (ISV) of patients with varicocele, we examined the cadmium (Cd) content and metallothionein (MT) expression in these diseased vessels. Previous studies have shown that Cd might play a role in the etiology of varicocele-associated infertility. MT, a metal-binding protein, protects against cell apoptosis during hypoxia.

METHODS
The study group consisted of 20 patients with grade 3 left varicocele. The control group consisted of 15 volunteers with left-sided indirect inguinal hernia. Through a left inguinal incision, a 1-cm section of the ISV was resected from each patient to measure the Cd and MT levels. The results were analyzed using Student’s t test.

RESULTS
The Cd content in the ISV was 59.84 ± 5.7 ng/g in the control group and 192.1 ± 24.2 ng/g in the varicocele group. The relative intensity of the MT band was 40.52 ± 3.74 in the control group and 78.26 ± 5.61 in the varicocele group. MT expression was greater in the varicocele group than in the control group, and its deposition in the vascular endothelial layer was predominant using immunohistochemistry staining and confocal laser scanning.

CONCLUSIONS
The results of the present study have demonstrated a greater accumulation of Cd in the ISV of the varicocele group than in the control group. The high Cd content and hypoxic conditions would induce overexpression of MT in the diseased vessels to protect the vascular cells from apoptosis. This might be a mechanism for the thickened wall of the ISV in patients with varicocele.

V aricocele is characterized by the engorgement and dilation of the pampiniform plexus above the testis. Although it is found in 15%-20% of men, with the left side the most commonly affected,1 the investigation of these diseased vessels is still very rare. Recently, we reported that hypoxic stress occurred in the internal spermatic vein (ISV) of patients with varicocele. However, it did not increase vascular cell death or vascular wall atrophy.2 Conversely, vascular tortuosity and a thickened ISV wall were found in the diseased vessels.2 What happened in the ISV of patients with varicocele molecularly? Benoff et al.3 reported that cadmium (Cd) might play a role in the etiology of varicocele-associated infertility. Cd is a toxic metal that can induce germ cell apoptosis and cause male infertility in humans.4,5

Metallothionein (MT)—a low-molecular-weight, heavy metal-binding protein—is rich in cysteine. MT reportedly has physiologic functions, including the ability to detoxify metals, combat oxidative stress, regulate essential biometals such as zinc and copper,6,8 and protect cells against apoptosis during hypoxic conditions.9 Therefore, we measured the expression of MT and the Cd content in the ISV of patients with varicocele. Our results provide new explanations for the engorgement and dilation of the diseased vessels. To our knowledge, this is the first study of the Cd content and MT expression in the ISV of humans.

MATERIAL AND METHODS
Patients and Tissue Samples
The samples were collected from May 2006 to October 2007 from 35 young, nonsmoking patients without exposure to Cd in the soil or through agricultural activities. The study group consisted of 20 patients aged 20-25 years with grade 3 left varicocele who had undergone surgery because of scrotal pain after evaluation for varicocele by physical examination and color flow Doppler ultrasonography.10-11 Varicocele was graded according to Dubin and Amelar in 1970, as follows: grade 1, varicocele palpable only during the Valsalva maneuver; grade 2,
varicocele palpable with the patient in the standing position; and grade 3, varicocele detectable by visual scrutiny alone. To prevent interobserver bias, all physical examinations were performed by 1 physician. The control group consisted of 15 volunteers, aged 20–25 years, with indirect left-side inguinal hernia, for whom the possibility of varicocele was ruled out by physical examination and color flow Doppler ultrasonography (ISV diameter <2 mm).

All patients underwent left inguinal surgical incision. A 1-cm section of the ISV was resected and stored at −80°C for the measurement of Cd and MT expression by immunoblotting, immunohistochemical staining, and confocal laser scanning. All specimens were removed only after the patients had provided written informed consent. The institutional review board of our hospital approved this investigation.

Cd Measurements
The method used to measure Cd in the tissues was detailed in our previous study, with modifications.

Antibodies
Four primary antibodies were used in the present study: (a) MT—2 mouse monoclonal antibodies for immunoblotting (M3009-10, dilution 1:4000, U.S. Biological, Swampscott, MA) and for immunohistochemistry (MS-1175, dilution 1:200, Thermo Fisher, Fremont, CA) and immunofluorescence staining (MS-1175, dilution 1:4000, U.S. Biological, Swampscott, MA) and for immunoblotting. The method used for MT extraction and immunoblotting was a modified version of that used in a previous study. The sections were rinsed with phosphate-buffered saline. Endogenous peroxidase was inactivated with the commercial kit. Negative control experiments, in which phosphate-buffered saline was used instead of the primary antibody, were conducted to confirm the positive results for MT. Finally, the sections were counterstained with hematoxylin (catalog no. 1.05175.0500, Merck, Darmstadt, Germany) and rinsed with tap water. The sections were observed by using a light microscope (model BX50, Olympus, Tokyo, Japan), and the micrographs were reviewed.

Immunoblotting for MT
The method used for MT extraction and immunoblotting was a modification of the method described in the manufacturer’s manual and used in our previous study. The antibodies of MT and β-actin had a molecular weight of about 54 and 44 kDa, respectively. The blots were cut into upper and lower portions at feasible sites for incubation, incubated at 4°C overnight with the diluted primary antibodies, and then incubated with the diluted secondary antibody for 1 hour. Finally, the immunoreactive bands were analyzed using MCID software, version 7.0, revision 1.0 (Imaging Research, St. Catharines, Ontario, Canada). The results were converted to numeric values (normalized relative to β-actin expression) to compare the relative protein abundance of the immunoreactive bands.

Immunohistochemical Analysis of MT
The deparaffinized ISV sections (4 μm) were rinsed with phosphate-buffered saline. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide. The sections were stained with primary antibody before being analyzed with the commercial kit. Negative control experiments, in which phosphate-buffered saline was used instead of the primary antibody, were conducted to confirm the positive results for MT. Finally, the sections were counterstained with hematoxylin (catalog no. 1.05175.0500, Merck, Darmstadt, Germany) and rinsed with tap water. The sections were observed by using a light microscope (model BX50, Olympus, Tokyo, Japan), and the micrographs were reviewed.

Immunofluorescent Double Staining and Confocal Laser Scanning Microscopy
The method used for staining and microscopy was a modified version of that used in a previous study. The sections were incubated at 4°C overnight with the diluted primary antibodies (HIF-1α or MT) and then exposed to the respective secondary antibodies for 1 hour. Finally, the sections were covered by a slip with mounting solution (Zymed) before being viewed by confocal laser scanning microscopy.

To determine and compare the localization of specific proteins, immunofluorescence-stained sections were examined using a Zeiss LSM 510 inverted laser scanning microscope (Hamburg, Germany) equipped with an argon laser (488 and 543 nm) for excitation. The immunofluorescence images of HIF-1α and MT were obtained with the Alexa Fluor 488/546 filter set (BP505-530 for 488 and LP 560 for 546) controlled by the

Table 1. Cadmium content and metallothionein expression in internal spermatic vein of both groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Cadmium Content (ng/g)</th>
<th>Relative Level of MT (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>59.84 ± 5.7</td>
<td>40.52 ± 3.74</td>
</tr>
<tr>
<td>Varicocele</td>
<td>192.1 ± 24.2*</td>
<td>78.26 ± 5.61†</td>
</tr>
</tbody>
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MT = metallothionein.

Data presented as mean ± SE.

* P < .001, significantly different from control group (t test).
† P < .01, significantly different from control group (t test).
software (Zeiss LSM Image Browser, version 3.5.0.223, Hamburg, Germany). The filter set was used to separate and transmit the emission wavelengths of Alexa-488 and Alexa-546-conjugated antibodies to different photomultipliers. The micrographs taken from each photomultiplier were subsequently merged so the different-colored labels could be simultaneously visualized.

Statistical Analysis
The data were analyzed using Student’s t test, and \( P < .05 \) indicated a significant difference from the control group.

RESULTS
As shown in Table 1, Cd accumulation in the ISV was greater in the varicocele group (192.1 ± 24.2 ng/g) than in the control group (59.84 ± 5.7 ng/g; \( P < .001 \)). The immunoblots of MT showed a single band in all patients. The relative intensity was 40.52 ± 3.74 and 78.26 ± 5.61 for the control and varicocele groups (\( P < .01 \)), respectively (Fig. 1). Immunohistochemical analysis of MT in the ISV sections showed stronger staining in the vascular cytoplasm of the varicocele group than in the control group (Fig. 2).

MT expression was greater in the varicocele group than in the control group, and its deposition in the vascular endothelial layer was predominant on confocal laser scanning. In contrast, HIF-1α deposition was observed in the smooth muscle layer (Fig. 3).

COMMENT
Cd, a toxic metal, is 1 of the major toxicants in the reproductive organs of humans.\(^4,5\) Cd can induce germ cell apoptosis and cause male infertility.\(^3,5\) Benoff et al.\(^5\) reported elevated apoptosis and Cd accumulation in the bilateral testes of infertile men with left varicocele. It is able to cross the blood-testes barrier and cause seminiferous endothelium damage. Cd itself can alter the permeability of the testicular vascular endothelium and produce edema, allowing the testes to accumulate Cd more rapidly.\(^3,5,18\) Testicular damage appears to be progressive and is generally observed on both sides, even in cases of unilateral varicocele.\(^18,19\) In our previous study, HIF-1α expression in the ISV was sevenfold greater in patients with varicocele than in the control group.\(^2\) This finding indicates that hypoxic stress occurred in the diseased vessels of the patients with varicocele. Hypoxia can increase endothelial permeability in some tissues and vessels.\(^20-22\) In addition, the vascular endothelium is a primary target of Cd toxicity.\(^1,23,24\) Therefore, hypoxic stress and Cd itself contributed to more accumulation of Cd in the ISV of the varicocele group. In the present study, we observed a 3.2-fold greater content of Cd in the diseased vessels of the varicocele group than in the control group. According to a previous study, 30%-40% of men evaluated for infertility were reported to have varicocele.\(^1\) The hypoxia led to increased Cd accumulation, which might contribute to the male infertility in patients with varicocele.
Of interest was the significantly greater expression of MT in the ISV of the varicocele group, and recent studies have shown that MT was upregulated during hypoxic conditions to protect cells from apoptosis.\textsuperscript{9,25,26} Given the function of MT, a Cd-binding protein,\textsuperscript{27} it is also induced during acute stress and is a free radical scavenger that protects cells from oxidative damage during stress.\textsuperscript{28-30} Similarly, the HIF-1\textsuperscript{1}/H9251 protein functions as a coactivator of MT-I gene transcription by interacting with metal-responsive transcription factor-1 to reduce the production of hypoxia-induced reactive oxygen species.\textsuperscript{9,26} In the present study, HIF-1\textsuperscript{1}/H9251 protein deposition was observed in the smooth muscle layer on confocal laser scanning microscopy. MT expression in the ISV was greater (approximately twofold) in the varicocele group than in the control group. The Cd content was also greater in the varicocele group. The high Cd content and hypoxic conditions would induce overexpression of MT in the diseased vessels to protect the vascular cells from apoptosis and might contribute to the ISV thickening in patients with varicocele.

**CONCLUSIONS**

Cd accumulation in the ISV was greater in the varicocele group than in the control group. The high Cd content and hypoxic conditions would induce overexpression of MT in the diseased vessels to protect the vascular cells from apoptosis. This might be a mechanism for the thickened wall of the ISV in patients with varicocele. These findings might be helpful in clarifying the pathophysiology of varicocele formation and recurrence.

**References**

2. Lee JD, Jeng SY, Lee TH. Increased expression of hypoxia-inducible factor-1\textsuperscript{1} in the internal spermatic vein of patients with varicocele. J Urol. 2006;175:1045-1048.

**Figure 3.** Immunofluorescent double staining and confocal laser scanning microscopy for metallothionein (red) and hypoxia-inducible factor-1\textsuperscript{1} (green). (A) Representative internal spermatic vein section from varicocele group after metallothionein (red) and hypoxia-inducible factor-1\textsuperscript{1} (green) staining. (B) Representative internal spermatic vein section from control group after metallothionein (red) and hypoxia-inducible factor-1\textsuperscript{1} (green) staining. ×400. Red arrow indicates endothelium; green arrow, smooth muscle layer.


