Overexpression of Metallothionein in the Endothelium of Varicocele Veins—A Study Using Confocal Laser Scanning Microscopy

Chin-Cheng Yi1, Wen-Kai Yang1,2, Jane-Dar Lee1,3*
1Division of Urology, Department of Surgery, Armed Forces Taichung General Hospital, Taichung, Taiwan
2Department of Life Sciences, National Chung-Hsing University, Taichung, Taiwan
3Central Taiwan University of Science and Technology, Taichung, Taiwan

Objective: Metallothionein (MT), a metal-binding protein, protects cells against apoptosis under hypoxia. Hypoxic stress causes pathophysiologic changes in the internal spermatic vein (ISV) of patients with varicocele. The use of confocal microscopy and fluorescent compounds permits visualization of the vascular wall organization and molecular changes that may occur. We studied MT expression and distribution in the ISV using confocal laser scanning microscopy.

Materials and Methods: The study group consisted of 20 patients with grade 3 left varicocele. The control group consisted of 10 volunteers with left indirect inguinal hernia. Through a left inguinal incision, a 1-cm section of the ISV was resected from each patient to detect MT expression by immunohistochemical stain and confocal microscopy.

Results: Immunohistochemical analysis of MT in the ISV sections showed stronger staining in the varicocele group than in the control group. The use of confocal microscopy provided a detailed vascular wall structure, and also revealed that the MT protein was distributed significantly in the endothelium.

Conclusion: The hypoxic conditions induced the overexpression of MT, which is deposited in the endothelial layer of varicocele veins, as determined by confocal microscopy. MT has the function to protect vascular cells from apoptosis, and this may be a mechanism for the thickened wall of the ISV that is present in varicocele patients.

*Corresponding author. Division of Urology, Department of Surgery, Taichung Armed Forces General Hospital, No. 348, Section 2, Chung-San Road, Taiping, Taichung, Taiwan. E-mail: jane.dar@yahoo.com.tw

1. Introduction

In 1957, Minsky created the first confocal microscope.1 In recent years, technological improvements have contributed greatly to the development of modern digital imaging methods, laser technology, and the availability of fluorescent probes. The use of confocal microscopy allows an integrated view of the interrelationships of the different elements of the vascular wall, which is made possible by the use of fluorescence.2 This technique is useful not only for imaging the vascular wall structure, but also for the visualization and quantification by the intensity of fluorescence.2

Varicocele is characterized by the engorgement and dilatation of the pampiniform plexus above the testis. Despite the fact that varicocele is found in 15–20% of men,3 investigation of these diseased vessels is still very rare. Recently, we reported that hypoxic stress occurred
Metallothionein in the endothelium of varicocele veins

in the internal spermatic vein (ISV) of patients with varicocele. However, the hypoxic stress did not result in increased vascular cell death or vascular wall atrophy. Conversely, dilated and thickened walls were found in the varicocele veins. Thus, it is possible that dysregulation of apoptosis occurs in these diseased vessels.

Metallothionein (MT) is a heavy metal-binding protein with a low molecular weight. This protein has important physiologic functions, including the ability to detoxify metals, to combat oxidative stress, to regulate essential biophysical functions, including the ability to detoxify metals such as zinc and copper, and to protect cells against apoptosis under hypoxic conditions. To the best of our knowledge, this is the first report that investigated the expression and distribution of MT in the varicocele veins of humans. Here, we show that MT plays a preventative role in the diseased vessels through the utilization of confocal microscopy.

2. Materials and Methods

2.1. Patients and venous samples

This study included 30 nonsmoking young patients who were recruited between May 2006 and October 2007. The study group consisted of 20 patients aged 20–25 years with grade 3 left varicocele, who had prior complaints of scrotal pain, and following an evaluation for varicocele by physical examination and color-flow Doppler sonography, received surgery. Varicocele was graded according to Dubin and Amelar in 1970. To prevent interobserver bias, all of the physical examinations were performed by a single physician. The control group consisted of 10 volunteers aged 20–25 years with an indirect left inguinal hernia; the possibility of varicocele in these patients was ruled out by physical examination and color-flow Doppler sonography (ISV diameter, <2 mm).

All of the patients underwent a left inguinal surgical incision, and a 1-cm section of the ISV was resected and stored at –80°C for the detection of MT expression by immunohistochemical staining and confocal laser scanning. All of the specimens were removed only after written informed consent was obtained from the patients. This investigation was approved by the institute ethics committee of Taichung Armed Forces General Hospital.

2.2. MT antibody

Metallothionein mouse monoclonal antibody (MS-1175; Thermo Fisher Scientific, Fremont, CA, USA) was used for immunohistochemical staining and confocal laser scanning. All of the specimens were removed only after written informed consent was obtained from the patients. This investigation was approved by the institute ethics committee of Taichung Armed Forces General Hospital.

2.3. Immunohistochemical staining for metallothionein

The method used here was modified from a previous study. The deparaffinized ISV sections (4 μm) were rinsed with phosphate-buffered saline. In order to inactivate the endogenous peroxidase, the ISV sections were incubated with 3% hydrogen peroxide. The sections were stained with primary antibody prior to analysis 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 under a light microscope (model BX50; Olympus, Tokyo, Japan), and the micrographs were reviewed.

2.4. IF staining and confocal laser scanning microscopy

Staining and microscopy were performed according to previously published methods, with some modifications. The sections were incubated at 4°C overnight with the diluted primary antibody, 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 using confocal laser scanning microscopy.

In order to determine and compare the localization of specific proteins, IF-stained sections were examined with a Zeiss LSM 510 inverted laser scanning microscope (Carl Zeiss, Hamburg, Germany) equipped with an argon laser (543 nm) for excitation. The IF images of MT were obtained with the Alexa Fluor 546 filter set (LP 560 filter for 546-nm excitation), controlled by the software (Zeiss LSM Image Browser version 3.5.0.223; Carl Zeiss). The filter set was used to separate and transmit the emission wavelengths of the Alexa Fluor 546 conjugated antibodies to different photomultipliers. The micrographs taken from each photomultiplier were subsequently merged so that the different-colored labels could be visualized simultaneously.

3. Results

Immunohistochemical analysis of MT in the ISV sections showed stronger MT-staining in the vascular cytoplasm of the varicocele group than in the control group (Figure 1). The distribution of MT (red color, Figure 2) in whole vascular layers and in the endothelial layer was predominant, as observed by confocal laser scanning (Figure 2). The use of confocal microscopy revealed a detailed...
vascular wall structure and distribution of the MT protein in the different layers, far above the power of traditional light microscopes.

4. Discussion

Confocal microscopy has many advantages over the traditional microscope, including the ability to look deeply into intact tissue and inside cells with less resulting photo-damage, and the ability to reconstruct three-dimensional images; this is a great benefit to the biological sciences. The major application for confocal microscopy in the field of biomedical research is for the imaging of either live or fixed tissues that have been labeled with one or more fluorescent probes. The results of microscopic immunohistochemical stain showed the protein expression of whole section. However, the use of confocal microscopy can produce optical sections throughout relatively thick tissues without the need for cutting thin slices, and thus provide identification of the different layers of vessels by differences in fluorescent intensity.

We have previously reported that hypoxia-inducible factor 1α expression in the ISV of varicocele patients was significantly higher (i.e., sevenfold) than in the control group. Previous studies have reported that MT is upregulated as a result of hypoxia in the kidney, colorectal carcinoma, and human prostate cancer cells. Interestingly, there was a significantly greater expression of MT in the endothelium of ISV in the varicocele group, as demonstrated by confocal microscopy. Recently, many studies have shown that MT is upregulated under hypoxic conditions to protect cells from apoptosis. MT, a metal-binding protein, is also induced during acute stress, and is a free-radical scavenger that protects cells from oxidative damage during stress. Similarly, the hypoxic factor is a coactivator of the metallothionein-I gene and reduces the production of hypoxia-induced reactive oxygen species. In the present study, MT expression in the ISV was higher in the varicocele group than in the control group. The hypoxic conditions might induce an overexpression of MT in the endothelium of the ISV in order to protect vascular cells from apoptosis, and may play a role in the dilated and thickened wall of ISV of varicocele patients. The use of confocal microscopy can provide a better analysis of the vascular structure and molecular changes in both physiologic and pathologic situations, greatly assisting biomedical studies.
In conclusion, our data revealed that there was a higher expression of MT in the varicocele group than in the control group. The hypoxic conditions might induce overexpression of MT in the endothelium of ISV of the varicocele group, as observed by confocal microscopy. MT has the function to protect vascular cells from apoptosis. This may be a mechanism behind the generation of the thickened wall of the ISV in varicocele patients.

Acknowledgments

This study was supported by a grant from Taichung Armed Forces General Hospital, Taichung, Taiwan.

References


