NOTE *Anatomy*

Expression of CD44 Adhesion Molecule in Rat Testis with Ischemia/Reperfusion Injury

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ABSTRACT. The expression pattern of CD44 was studied in the rat testis following ischemia/reperfusion (I/R) injury to elucidate the possible role of the CD44 adhesion molecule in acute experimental testicular torsion. Western blot analysis showed that CD44 expression began to increase significantly 24 hr after reperfusion, compared with the normal control; the increased expression persisted until 96 hr after I/R. Immunohistochemistry showed that, in the normal testis, CD44 was constitutively expressed mainly in ED2-positive resident macrophages in the interstitial space. After I/R, the majority of inflammatory cells in the interstitial space surrounding the damaged tubules were ED1-positive macrophages that were CD44-positive. These findings suggest that the significant increase in CD44 expression that occurs during the delayed phase after reperfusion originates from infiltrating macrophages possibly in anticipation of the migration and adhesion of additional macrophages into the affected testis.

KEY WORDS: CD44, macrophage, testicular torsion.

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Testicular torsion has been implicated in testicular injury and infertility. The main pathology of testicular torsion is ischemia and reperfusion (I/R) injury of the testis [6]. Reperfusion is one of the most important factors in further injury [1]. I/R injuries cause the infiltration of abundant inflammatory cells into the interstitial space surrounding damaged tubules during the delayed phase after reperfusion. This suggests that, during the delayed phase of reperfusion, increased numbers of hematogenous macrophages stimulate the generation of nitric oxide (NO) and oxidative stress, which results in induced germ cell death (especially necrosis) [4, 11, 13]. The inflammatory response contributes to testicular I/R injury and is a potential cause of infertility.

The testis is an immunologically privileged site. However, little is known about the factors that regulate the formation of immune responses, in particular, the infiltration of hematogenous inflammatory cells that affect germ cell death elicited by I/R in the testis. In this study, we focused on the expression of cell adhesion molecules, which may be involved in the infiltration of inflammatory cells following testicular injury. Recent studies have reported the expression of cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), lectin, and tenascin, and their roles in the pathophysiological signs of inflammation in the rat testis [15].

The CD44 adhesion molecule, which is a hyaluronate receptor, is in a family of glycoproteins that are expressed on the cell surface [2, 10]. This molecule is widely expressed in embryonic and normal adult tissues and serves as an adhesion molecule in cell-cell and cell-substrate interactions that mediate processes such as cell migration during organogenesis and wound repair [3]. However, little is known about the expression of CD44 in the testis following I/R injury.

Based on pathological findings in the testis following I/R injury [11, 13, 14], CD44 might be an important adhesion molecule in the processes of acute testicular I/R-induced inflammation. The aim of this study was to examine the expression and localization of CD44 in the testis following I/R in order to investigate the possible role of CD44 in the course of I/R-induced injury to the testis in a rat model.

Sprague-Dawley rats were purchased from the Daehan Biolink Co. (Chungbuk, Korea) and were bred in our animal facility. A total of 30, 8- to 9-week-old male rats were used in the experiments. All experiments were carried out in accordance with the National Research *Council's Guide for the Care and Use of Laboratory Animals*.

The rats were anesthetized for surgery with chloral hydrate (Fluka, Buchs, Switzerland) (375 mg/kg body weight, intraperitoneal injection). All surgical procedures were performed as mentioned in our previous studies [11, 13, 14]. Briefly, the left scrotum was incised, and the left spermatic cord was rotated 720° clockwise to minimize individual variation in blood flow. After 1.5 hr, the torsion (*i.e.*, ischemia) was relieved (*i.e.*, reperfusion), and the testis was returned to the scrotum. Tissue samples were removed at 6, 12, 24, and 96 hr after reperfusion, immersed in 10% neutral buffered formalin, and processed for paraffin embedding for immunohistochemistry or were stored at –80°C for later biochemical analysis.

Frozen tissues were homogenized, and lysed in a buffer containing 40 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% Nonidet P-40 (polyoxyethylene [9] p-t-octyl phenol) supplemented with the protease inhibitors leupeptin (0.5 μg/m*l*), phenylmethylsulfonyl fluoride (1 mM), and aprotinin (5 μg/m*l*). Equal amounts of protein were electrophoresed

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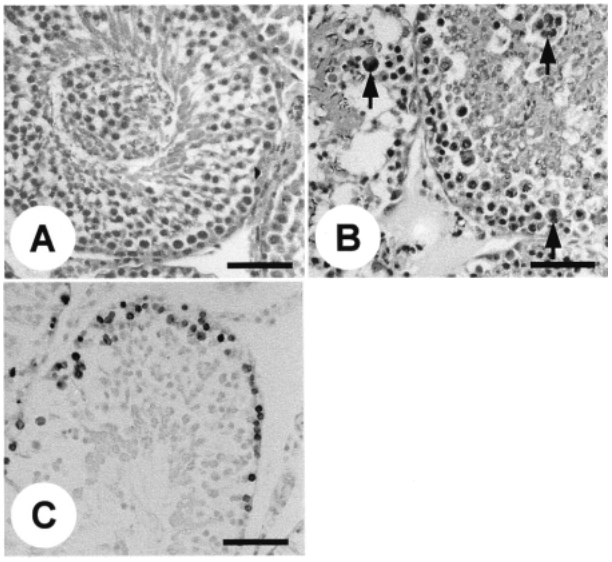

Fig. 1. Histopathological findings in normal control testis (A) and I/R rat testis 24 hr after the repair of torsion (B). The formation of multinucleated germ cells is seen in severely damaged tubules (arrows). Hematoxylin and eosin staining. (C) TUNEL staining of the rat testis 24 hr after reperfusion following ischemia. The apoptosis was localized mainly to spermatogonia and was rarely noted in primary spermatocytes at the basement membrane of the seminiferous epithelium (C). Counterstained with hematoxylin. Scale bars=50 μm.

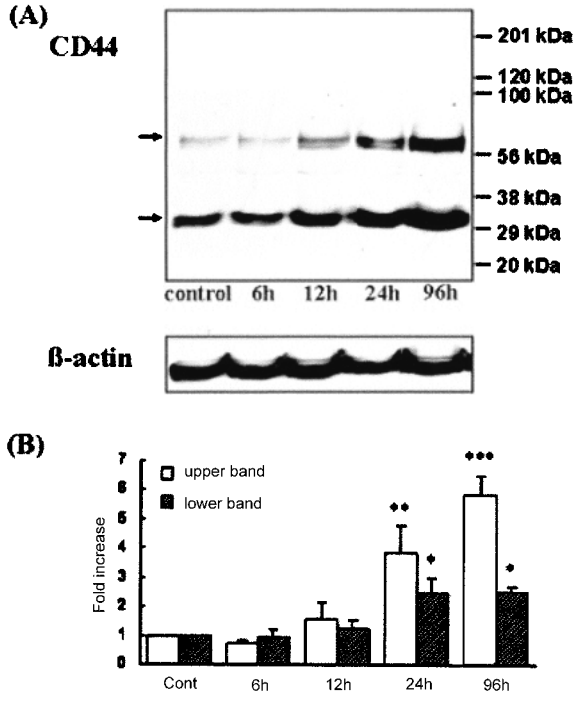

Fig. 2. Immunoblots of CD44 in normal control rat testes and rat testes at 6, 12, 24, and 96 hr after reperfusion following 1.5 hr of experimental testicular torsion. A) Representative Western blots of CD44 and beta actin in the testis with I/R. B) The amount of protein was quantified using a densitometer. The value is the fold increase relative to the density of the normal control after normalization to beta actin. The relative expression of CD44 was calculated, and the value for the normal control was arbitrarily defined as 1. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ versus normal control.

under denaturing conditions in 10% SDS-PAGE, and electrotransferred onto nitrocellulose membranes. Blotting with monoclonal anti-rat CD44 (OX49; Pharmingen, San Diego, CA) was performed, with slight modifications, as described in our previous study [12]. Subsequently, the membranes were reprobed with monoclonal antibody to beta actin (Sigma, St. Louis, MO). The density of each band was measured using a scanning densitometer (GS-700, Bio-Rad Laboratories, Hercules, CA) and analyzed using Molecular Analyst software (Bio-Rad). The ratios of CD44 to beta actin were compared using one-way analysis of variance (ANOVA), followed by a Student-Newman-Keuls *post hoc* test. In all cases, values of $p<0.05$ were considered statistically significant.

Immunohistochemical staining was performed in the manner of our previous study [12]. In brief, 5-μm sections of paraffin-embedded testis were deparaffinized, and the samples were allowed to react with primary antibodies, including monoclonal anti-rat CD44 (OX49, 1:2000; Pharmingen). To identify monocyte-like and resident macrophages, mouse monoclonal anti-rat monocyte-like macrophage (ED1, Serotec, London, U.K.) and resident macrophage (ED2, Serotec) antibodies were applied, respectively [5]. To identify T cells in the affected testes, the frozen sections were air-dried and fixed in 4% paraformaldehyde buffered with 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 20 min. The sections were allowed to react with mouse anti-T cell receptor ab (R73, Blackthorn, Bicester, Bucks, UK) antibody. The immunoreactions were visualized using avidin-biotin peroxidase complexes (Elite kit; Vector, Burlingame, CA), and the peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector).

DNA fragmentation was detected using *in situ* nick end-labeling (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling, TUNEL), performed according to the manufacturer's instructions (ApopTag® *In Situ* Apoptosis Detection Kit, Intergen, Purchase, NY).

The testicular structure of the normal control rats was normal and included germ cells, Sertoli cells, and interstitial cells (Fig. 1A). I/R injury caused the formation of multinucleated germ cells in the seminiferous tubules (Fig. 1B, arrows) and the appearance of necrotic tubules 24 hr after reperfusion, with frequent remnants of severely damaged germ cells; however, many tubules contained normal germ cells (Fig. 1, B and C). Germ cell necrosis was first noted 24 hr after reperfusion; subsequently, many tubules were damaged, and considerable necrotic debris was observed (data not shown). In addition, the number of TUNEL-positive apoptotic cells increased until reaching a significant peak 24

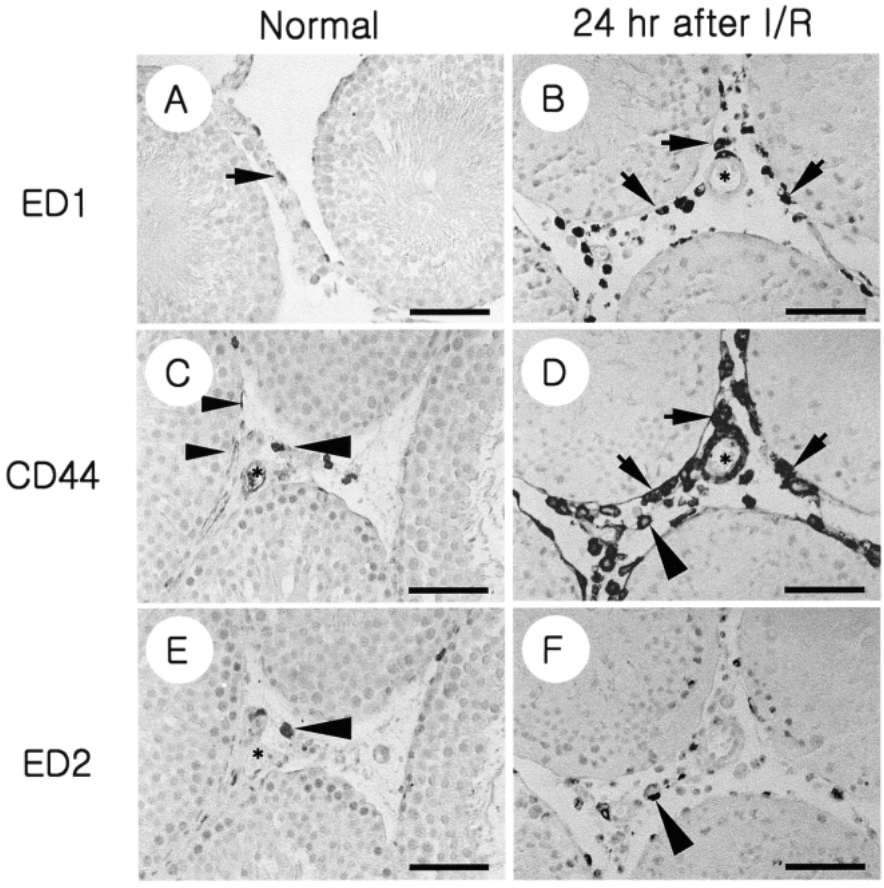

Fig. 3. Immunohistochemical detection of CD44 in the testes of normal control (A, C and E) and testicular torsion rats (B, D and F). In the normal control, CD44 is constitutively detected in some round cells (C, large arrowhead) and vessels (C, asterisk) in the interstitial space and some myoid cells (C, small arrowheads). In adjacent sections, the CD44 immunoreaction is localized in the ED2-positive resident macrophages (C and E, large arrowhead). At 24 hr after relieving the torsion, CD44 immunoreactivity is obviously increased in most of the infiltrating inflammatory cells (D). In adjacent sections, the CD44 immunoreaction is localized mainly in ED1-positive monocyte-like macrophages surrounding the severely damaged tubules (B and D, arrows) as well as ED2-positive resident macrophages (D and F, large arrowhead). Counterstained with hematoxylin. Scale bars=50 μm.

hr after I/R (Fig. 1C). However, after 48 hr of reperfusion, the number of TUNEL-positive cells declined (data not shown). This pattern paralleled that seen in our previous studies [11, 13, 14].

Western blot analysis showed two major bands of reactivity for CD44 (Fig. 2A). The testicular torsion resulted in an increased intensity of the 2 bands, one between 56 and 100 kDa and the other between 29 and 38 kDa (Fig. 2A), which is in agreement with observations in previous studies [9, 12]. As shown in Fig. 2, the expression of CD44 immunoreactivity was detected at low levels in the testes of normal controls, was significantly enhanced 24 hr after reperfusion (fold increase as mean ± S.E. for upper band: 3.85 ± 0.91, $p<0.01$ vs. normal controls; lower band: 2.49 ± 0.5, $p<0.05$; n=3) and remained elevated 96 hr after reperfusion (upper band: 5.83 ± 0.64, $p<0.001$; lower band: 2.5 ± 0.1, $p<0.05$; n=3).

In the normal control rats, CD44 immunoreactivity was constitutively detected in not only some myoid cells (small arrowheads) and vessels (asterisk), but also in some round cells of the interstitial space (Fig. 3C). The expression coincided mainly with ED2-positive resident macrophages in an adjacent section (Fig. 3C and E, large arrowhead). After the I/R injury, many inflammatory cells in the interstitial space, particularly those surrounding severely damaged seminiferous tubules, were positive for CD44 at 24 hr after reperfusion (Fig. 3D), and this pattern persisted 96 hr after the reperfusion (data not shown). The CD44 expression coincided with most of the ED1-positive monocyte-like macrophages (Fig. 3B) which are rare in normal testis (Fig. 3A), and ED2-positive resident macrophages (Fig. 3F) in adjacent sections.

To confirm the involvement of hematogenous T cells in the inflammation following testicular ischemia, we immunostained the lesions with a T cell-specific marker, R73 (monoclonal anti-TCR$\alpha\beta$). R73-positive cells were occasionally observed in the interstitial space of damaged tubules (data not shown).

This is the first study to examine the expression of CD44 in the testis following I/R injury. In this study, we found that the expression of CD44 significantly increased and that an abundant number of inflammatory cells infiltrated into the interstitial space surrounding damaged seminiferous tubules during the delayed phase after I/R.

CD44 is a transmembrane glycoprotein that is known to be involved in endothelial cell recognition, leukocyte trafficking, and the regulation of cytokine gene expression in inflammatory disease [7, 16]. However, the regulatory mechanism at the cellular level that mediates the pathogenesis of testicular I/R injury remains unclear. Recently, the expression of CD44 was studied in testis with experimental autoimmune orchitis, and the results suggested that CD44 is involved in the homing of lymphomonocytes into the rat testis with autoimmune inflammation [8]. In this study, we found that the increased pattern of CD44 expression mirrored the increase in inflammatory cell infiltration (mainly macrophages) in the testis of rats with I/R, which suggests that CD44 plays an important role in the infiltration of macrophages into I/R-injured testes. Previously, we reported that germ cell death was significantly increased in the rat testis 24 hr after reperfusion and gradually increased thereafter [13]. This phenomenon corresponds to the significant increase in CD44 expression observed in this study. Furthermore, most of the CD44-positive macrophages that were adjacent to dead or dying germ cells, which were seen near the basement membrane of the seminiferous epithelium (mainly spermatogonia), had infiltrated into the interstitial space surrounding severely damaged tubules. We recently found [11] that the expression of nitric oxide synthase was increased and localized in infiltrated inflammatory cells (mainly macrophages) of the interstitial space in the testis following I/R. Moreover, the inflammatory cells activated to express more CD44 after I/R injury may be able to adhere to the parenchyma of target organs more efficiently. Consequently, it is suggested that nitric oxide arising from most of the CD44-positive macrophages affects germ cell death in testicular I/R.

In conclusion, a significant increase in CD44 expression originates from infiltrating macrophages after I/R injury, possibly in anticipation of the migration and adhesion of macrophages into the affected testis.

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