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BLOCKADE OF P-SELECTIN REDUCES NEUTROPHIL INFILTRATION INTO THE ISCHEMIA-REPERFUSION INDUCED MURINE TESTIS

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Germ cell apoptosis after ischemia-reperfusion (IR) of the testis is dependent on neutrophil recruitment to the testis. Intravascular adhesion molecules like the P- and E- selectins play an important role in this recruitment. The purpose of this study was to inhibit neutrophil recruitment to the IR- induced testis by using a function-blocking monoclonal anti-mouse P-selectin antibody.

Adult mice were subjected to a 2 h period of testicular torsion (ischemia) followed by detorsion (reperfusion). Ten minutes after the onset of reperfusion mice received either the function-blocking monoclonal P-selectin antibody (FBMAB group) or the isotype-matched control antibody (IMCA group). Separate groups of mice underwent a sham operation (SO group) or received 500 ng of TNFα (IF group) to induce inflammation.

Mice were sacrificed 24 hr after reperfusion and testiscular interstitial cells were isolated and analyzed for the presence of neutrophils by means of flow cytometry.

The function-blocking monoclonal P-selectin antibody reduced neutrophil recruitment to the IR-induced testis significantly. Therefore, blocking P-selectin may be therapeutically beneficial to protect postischemic testis.

Key words: antibodies, neutrophils, P-selectin, testis, torsion

INTRODUCTION

Ischemia-reperfusion (IR) of the testis results in germ cellspecific apoptosis and is dependent on neutrophil recruitment, which requires expression of selectins on the luminal surface of the testicular vascular endothelium (Lysiak *et al.*, 2000; Turner *et al.*, 1997). The selectin family of adhesion molecules, i.e. the P- E- and L selectins, mediate the initial attachment of leukocytes to the venular endothelial cells before their firm adhesion and diapedesis at the sites of tissue injury and inflammation. L-selectin is expressed on all leukocytes, P-selectin is expressed on platelets and endothelial cells, and E-selectin is found exclusively

on endothelial cells (Tedder *et al.*, 1995). P-selectin participates in leukocyte capture and rolling on the venular endothelial surface upon inflammatory stimuli (Ley *et al.*, 1995) and is transported to the endothelial cell surface within minutes of injury (Jones *et al.*, 1999). P-selectin can persist in a synthesis-dependent manner for hours after ischaemia–reperfusion injury (Chukwuemeka *et al.*, 2005). In the clinical setting ischemia-reperfusion of the testis results from torsion of the spermatic cord (Lysiak *et al.*, 2003). The resultant germ cell-specific apoptosis after testicular IR is dependent on neutrophil recruitment to the testis and E-selectin appears to be the key cell adhesion molecule on the testicular endothelial cells responsible for mediating neutrophil recruitment (Lysiak *et al.*, 2001). E-selectin participates in both processess and allows for the slow rolling of neutrophils to endothelial cells (Kunkel and Ley, 1996). E-selectin expression on the testicular vascular endothelium is up-regulated following IR or after treatment with tumor necrosis factor α (Weller *et al.*, 1992) or interleukin 1β (Keelan *et al.*, 1994). Expression is maximal 3-8 h after stimulation and gradually decreases within 12-24 h (Weller *et al.*, 1992).

Previous studies have demonstrated that blocking E-and P-selectins in IR-induced tissue injuries (Singbartl and Ley, 2000; Singbartl *et al.*, 2000) and inflammation (Gotoh *et al.*, 2004; Homeister *et al.*, 1998) reduces neutrophil migration to the tissues. Therefore, blockade of P- and E-selectins function may have therapeutic benefits. Blocking P- selectin was able to reduce neutrophil recruitment to IR-induced organs as heart (lefer *et al.*, 1996), liver (Singh *et al.*, 1998), kidneys (Singbartl *et al.*, 2000) and brain (Huang *et al.*, 2000), but it has not been investigated in IR-injury of testis.

The purpose of the present study was to examine the inhibitory effect of a function-blocking monoclonal antibody against mouse P-selectin on the recruitment of neutrophils into the IR- induced testis.

MATERIALS AND METHODS

Experimental testicular torsion

The Animal Research Committee at the University of Virginia approved the study protocol. Adult male C57BL/6 mice were anesthetized with an intraperitoneal injection of ketamine (Ford Dodge, Iowa; Ketaset) and xylazine (Burns Vet supply. Westbury, NY) mixture (respectively, 60 mg/kg and 5 mg/kg) and unilateral testicular torsion was performed (n=12), as described by Lysiak *et al.* (2001). A low midline incision was performed and the testis was exposed through the incision, the gubernaculum was incised, and the testis was freed from the epididymo-testicular membrane. The testis was rotated 720^{o} for 2 h (ischemia), during which time it remained in the abdomen with a closed incision. After 2 h the incision was reopened, the testis was counter rotated to the natural position (reperfusion) and the testis was reinserted into the scrotum and the incision was closed. Ten minutes after onset of reperfusion, 6 mice received either 100 μg of a function-blocking monoclonal P-selectin antibody intravenously via the retro-orbital sinus (FBMAB group; Clone: Rb40.34 produced at the University

of Virginia lymphocyte Culture Center, Charlottesville VA, described in reference Bosse *et al.*, 1994) or 100 μg of isotype-matched control antibody (IMCA group; Sigma, I4131, IgG from rat serum). Separate groups of mice (n=6 each) underwent a sham-operation (SO group) or received 500 ng of TNFα (IF group) to induce inflammation. Intratesticular injection of TNFα was performed as described by Lysiak *et al.* (2003). Briefly, mice were anesthetized with a mixture of ketamine and xylazine (60 mg/kg and 5 mg/kg, respectively), and the testis was exteriorized through a low midline laparatomy, 500 ng of TNFα in a 10 μL volume was injected with a glass micropipette sharpened to a 50 μm tip. After the injection the testis was reinserted into the scrotum and the incision sutured. Mice in the sham-operated group (SO group) were subjected to the same surgical procedure as the FBMAB and IMCA groups except that on completion of the torsion maneuver the testis was immediately counter rotated. All mice were sacrificed 24 h after reperfusion or intratesticular injection and the testis were removed for flow cytometry study in order to determine neutrophil content.

Isolation of testicular interstitial cells

For isolation of interstitial cells (both inflammatory cells and testis-resident cells as germ cells/Leydig cells) the testis was decapsulated and placed in 3 mL of RPMI1640 (Dulbecco's) containing 100 U/mL Collagenase Type 2 (Worthington Biochemical Corp.) and 0.1 M DnaseI (Sigma; type IV). This was subsequently incubated for 10 min. in a 34^{o}C water bath while shaking. After incubation, 40 mL of 0.1 M EDTA in HBBS was added and none-interstitial contents (containing seminiferous tubules) were allowed to settle by incubation on ice for 3 minutes. The supernatant (containing interstitial testicular cells) was collected and washed once in PBS. Isolated interstitial cells were counted by trypan blue exclusion and further processed for flow cytometric analysis.

Flow Cytometric Analysis

Isolated testicular interstitial cells were resuspended in 50 μg/mL, Fc-block (anti-CD16/32; clone G412, BD Pharmingen) in PBS + 0.5% BSA and incubated for 15 minutes at 4^{o}C in order to block Fc-receptor binding of antibodies used for staining of cells. Subsequently, the cells were transferred to a 96-wells round-bottomed plate. Surface staining for leukocyte-specific antigens and identification of neutrophils was then performed by using a combination of the following antibodies; Gr-1-PE (clone RB6-8C5; BDPharmingen), CD45-APC (clone 30-F11, BDPharmingen), CD11b-APCAlexaFluo750 (Clone M1/70; Ebioscience) and F4/80-biotin (clone BM8; Caltag Lab.). After 20 minutes incubation at 4^{o}C, the cells were washed twice with PBS + 0.5% BSA and subsequently incubated with Streptavidine-FITC (BDPharmingen) for 20 minutes at 4^{o}C. After three washes the cells were resuspended in 100 μL PBS + 1%BSA+0.1M EDTA plus 5 μL of the viability dye 7-AAD (BDPharmingen). Samples were acquired on a FACScan upgraded with a blue laser by Cytek (Freemont, CA, USA) to allow 5 color-analysis. Data compensation and analysis was performed by using the Flowjo-software (Treestar) as follows: only live cells (7-AAD-negative) were included and

subsequently infiltrating leucocytes were identified as CD45-positive cells. Neutrophils were identified as CD45-positive cells expressing high levels of Gr-1 and simultaneously expressing CD11b (Mac-1) and negative for the macrophage-marker F4/80.

RESULTS

To determine if a function-blocking monoclonal P-selectin antibody was able to modify neutrophil recruitment to the IR-induced testis, we performed flow cytometric analysis of isolated testicular cells 24 h after reperfusion. In order to identify the neutrophils we used a specific combination of fluorchrome-conjugated monoclonal antibodies that have been reported to properly identify neutrophils in the mouse (Lagasse *et al.*, 1996). Briefly, this includes Gr-1 (which is a cell surface molecule expressed highly on neutrophils and weakly on monocytes / macrophages and dendritic cells), CD11b (which is a cell surface molecule expressed on neutrophils and monocytes/macro-phages and dendritic cells) and F4/80 (not expressed on neutrophils, but expressed on monocytes / macrophages). In addition neutrophils have a high granular content compared to monocytes / macrophages and dendritic cells. This property can be identified by looking at the SSC of the cells with a flow cytometer (granular cells having a high SSC).

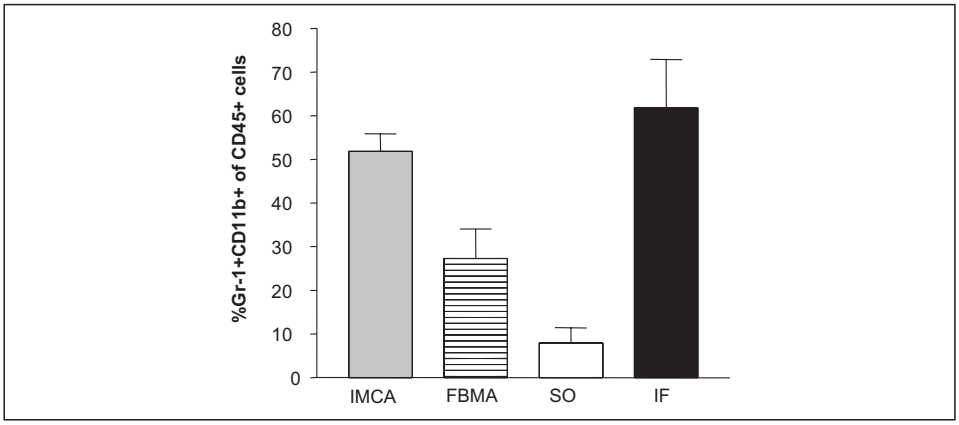

Figure 1. The percentage of neutrophils in the testicular cells

Figure 1 presents the % of neutrophil (Gr-1- and CD11b-positive and F4/80-negative) leucocytes (CD45-positive cells) in live interstitial testicular cells isolated from mice that were sham-operated (SO) as compared to IR-testis given isotype control (IMCA) or a function-blocking monoclonal antibody to P-selectin (FBMAB). The administration of FBMA reduces the % of neutrophils in the isolated interstitial cells significantly ($p<0.05$). As a positive control mice were injected with TNFα (IF) which shows the detection of neutrophil recruitment in isolated interstitial cells.

Flow cytometric analysis of infiltrating leucocytes (CD45-positive cells) showed that intratesticular TNFα injection induces inflammation and caused a significant neutrophil recruitment to the testis as compared to the sham-operated group 60 ± 12 and 7 ± 3%, respectively (Figure 1).

Most importantly, the CD45-positive cells present in the cells isolated from the testis showed a significant reduction in the percentage of neutrophils in the testis of the FBMAB group as compared to the IMCA group (figure 1; 26 ± 4 vs. 52 ± 10% Gr-1+CD11b+ of total leucocytes; P = 0.05).

DISCUSSION

Neutrophil recruitment to the affected organs is one of the hallmarks of IR injury (Singbartl and Ley, 2000; Huang *et al.*, 2000) and selectin-targeted therapeutic agents are proven to be effective in blocking many of the pathological effects resulting from leukocyte entry into the sites of inflammation (Tedder *et al.*, 1995).

The aim of this study was to inhibit neutrophil infiltration into the IR-induced testis with a function-blocking monoclonal P-selectin antibody. In this study we performed the flow cytometric analysis for identifying neutrophil recruitment to the testis. We demonstrated that the percentage of neutrophils in the infiltrating leucocytes of IR-induced testis in FBMAB and IMCA groups were 26% and 52%, respectively. This demonstrates that a function-blocking monoclonal P-selectin antibody is able to reduce neutrophil infiltration by 50% in the testis of FBMAB group compared with IMCA group.

Previous studies have demonstrated that blocking P-selectin protects from IR-induced acute organ failure and reduces neutrophil accumulation into the postischemic tissues (Singbartl *et al.*, 2000; Lefer *et al.*, 1996; Goussev *et al.*, 1998; Singh *et al.*, 1998). Singbartl *et al.* (2000) have reported that blocking P-selectin with a function-blocking monoclonal P-selectin antibody dramatically reduced renal myeloperoxidase activity in post ischemic kidneys and protected from the development of renal failure even after the onset of reperfusion in mice, indicating that attenuation of neutrophil infiltration can be therapeutically beneficial.

In this study we are the first to describe the inhibitory effect of function-blocking monoclonal P-selectin antibody in the IR-induced testis and the present data are consistent with the results of other investigators who demonstrated that blockade of P-selectin reduces neutrophil migration to IR-induced organs (Singbartl *et al.*, 2000; Lefer *et al.*, 1996; Goussev *et al.*, 1998; Singh *et al.*, 1998).

In conclusion, blockage of P-selectin even after the onset of reperfusion with function-blocking monoclonal P-selectin antibody reduces neutrophil recruitment to the IR-induced murine testis. Blockade of P- selectin may be therapeutically beneficial to protect postischemic testis.

Further studies should be done to identify the blockade effects of anti E-selectin antibody alone and a combination of anti E- and P-selectin antibody on the migration of neutrophils into the IR-induced testis.

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BLOKADA P – SELEKTINA REDUKUJE INFILTRACIJU NEUTROFILIMA U SEMENICIMA MIŠEVA POSLE ISHEMIJE I REPERFUZIJE

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SADRŽAJ

Apoptoza specifična za germinativne ćelije, posle ishemije i reperfuzije semenika, zavisi od mobilizacije neutrofilnih granulocita koji onda naseljavaju ovaj organ. Važnu ulogu, pri tome, imaju intravaskularni adhezivni molekuli u koje spadaju P- i E- selektini. Cilj naših ogleda je bio da se ispita inhibicija ovog procesa upotrebom monoklonskih anti-mišijih antitela protiv P-selektina.

Odrasli miševi su bili podvrgnuti torziji funikulusa tokom dva sata, čime je izazivana ishemija, a zatim su funikulusi oslobađani, čime je postignuta reperfuzija. Deset minuta nakon reperfuzije, životinjama su aplikovana anti-mišija antitela protiv P-selektina ili izotipska kontrolna antitela. Posebne grupe miševa su bile podvrgnute "sham" operaciji ili su primale 500 ng TNFα radi izazivanja inflamacije. Miševi su žrtvovani 24 sata posle reperfuzije i prisustvo neutrofilnih granulocita je utvrđivano protočnom citometrijom u grupi izolovanih intersticijalnih ćelija.

Primenjena anti-mišija antitela protiv P-selektina su značajno smanjivala naseljavanje neutrofilnih granulocita u tkiva ovog organa pa prema tome mogu imati terapijsku primenu u sanaciji posledica ishemije testisa.