ORIGINAL ARTICLE

L-Selectin and Leukocyte Function in Skeletal Muscle Reperfusion Injury

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Hypothesis: Treatment with anti–L-selectin monoclonal antibody will reduce venular neutrophil-endothelial rolling (flux and velocity) and adhesion associated with ischemia reperfusion injury in rat skeletal muscle.

Design: Prospective, randomized experimental trials.

Setting: Basic science research laboratory.

Materials: Male Wistar rats weighing 109 ± 5 g (mean ± SEM).

Interventions: Gracilis pedicle muscle flaps were elevated and microcirculation was observed by intravital microscopy. Two groups were evaluated: (1) the control group, which received 4 hours of global ischemia, and (2) the experimental group, which received 4 hours of global ischemia, plus treatment with anti–L-selectin monoclonal antibody 30 minutes before reperfusion.

Main Outcome Measures: The number of rolling and adherent leukocytes in postcapillary venules were counted in the 2 groups at baseline and at 1 through 5, 10, 15, 20, 30, 45, and 60 minutes of reperfusion.

Results: Treatment with the monoclonal antibody to L-selectin significantly reduced the number of rolling leukocytes (flux) at 2 through 5, 20, 30, 45, and 60 minutes of reperfusion compared with controls ($P<.05$). Use of the monoclonal antibody significantly reduced the number of adherent neutrophils at 5, 10, 15, 20, 30, 45, and 60 minutes of reperfusion ($P<.05$). There was no significant difference in leukocyte velocity.

Conclusion: L-Selectin plays a significant role in leukocyte rolling and adherence to venular endothelium in rat skeletal muscle ischemia reperfusion injury.

Arch Surg. 1999;134:1079-1081

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ADVANCEMENTS in microsurgical techniques have enabled surgeons to perform free tissue transfer and replantation of traumatically amputated limbs and digits. These procedures necessitate periods of global ischemia. Reperfusion injury of skeletal muscle continues to be a limiting factor in successful outcomes when these procedures are associated with prolonged periods of ischemia.

Experimental evidence[1-3] strongly suggests that tissue damage secondary to ischemia reperfusion (IR) injury is largely mediated by activated neutrophils that accumulate in reperfused tissues. Neutrophil adhesion is mediated by adhesion molecules on neutrophil and vascular endothelium. Circulating neutrophils express at least 2 classes of cell surface glycoprotein adhesion molecules that are required for margination and diapedesis. Neutrophil rolling is mediated by the selectin family of adhesion molecules.[4,5] Firm adherence to vascular endothelium is mediated by leukocyte integrins (β_2 subfamily).[6] Neutrophil rolling seems to be the initial step required for adhesion and is initiated by the binding of L-selectin to its counterreceptor on the endothelium.[7]

The purpose of this experiment was to determine the effect of treatment with L-selectin on postcapillary venular neutrophil rolling and adhesion function associated with IR injury in rat skeletal muscle. Data are reported as mean ± SEMs.

RESULTS

Administration of monoclonal antibody to L-selectin significantly reduced the rolling leukocyte flux at 2 through 5, 20, 30, 45, and 60 minutes of reperfusion after global ischemia ($P = .01$, post hoc $P<.05$) (**Figure 1**) and significantly reduced the number of adherent neutrophils at 5, 10, 15, 20, 30, 45, and 60 minutes of reper-

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MATERIALS AND METHODS

RAT GRACILIS MUSCLE MICROCIRCULATION MODEL

Sixteen male Wistar rats (109 ± 5 g) were anesthetized (50 mg/kg) and supplemented throughout the experiment (10 mg/kg) with intraperitoneal sodium pentobarbital. The right thigh musculature and femoral vasculature were exposed, and the gracilis muscle was dissected free on its vascular pedicle using standard microsurgical techniques. Each rat was then positioned on its right side on a specially constructed insulated microscope stage. The muscle flap was placed in phosphate-buffered saline solution (pH 7.4) on a raised glass pedestal. The gracilis muscle microcirculation was then transilluminated and projected onto a high-resolution video screen at ×832 magnification. The pedestal was sealed with a silicone plug during ischemia and between data collection times to prevent dehydration and environmental gas exchange with the exposed surface of the muscle.

After a stabilization period to assess the presence of normal directional flow, the number of rolling and adherent neutrophils visible in postcapillary venules were counted as baseline measures. Results of previous studies[8] show this preparation to be stable during an 8-hour period, with sham-operated (gracilis flap raised, observation only) (n = 6, 17 venules) animals showing no significant difference in the number of rolling and adherent leukocytes from baseline (1.7 ± 0.6 rolling and 1.2 ± 0.5 adherent neutrophils) through 6 hours of observation. A microvascular clamp was placed across the pedicle artery and vein to initiate global ischemia. The animals were randomly assigned to (1) the control group (n = 8), which received 4 hours of global ischemia, or (2) the experimental group (n = 8), which received 4 hours of global ischemia and treatment with monoclonal antibody to L-selectin (purified hamster anti-rat CD62L; Pharmingen, San Diego, Calif). The monoclonal antibody (2.17 mg/kg) was infused (0.097 mL/min) into the contralateral femoral vein 30 minutes before reperfusion of the ischemic tissue. After 4 hours of global ischemia, the vascular clamp was removed, reestablishing blood flow to the muscle. The number of rolling and adherent leukocytes were counted at 1 through 5, 10, 15, 20, 30, 45, and 60 minutes of reperfusion. Data were collected after 60 seconds of observation within a fixed 100-µm venule segment. Leukocyte flux (the number of cells rolling past a fixed point per minute), velocity (in micrometers per second), and number of adherent neutrophils were measured in the experimental group, and the values were compared with those in the control group at the same times.

All activities involving animals were conducted in accordance with all applicable provisions of the Animal Welfare Act, the US Government Principles Regarding the Care and Use of Animals (Public Health Service Policy on Humane Care and Use of Laboratory Animals), and the University of Nevada Animal Welfare Assurance. All experiments were reviewed and approved by the University of Nevada Laboratory Animal Care and Use Committee, Las Vegas.

STATISTICAL ANALYSIS

Analysis of variance and the Duncan multiple range test were used to determine significant differences between groups ($P \leq .05$).

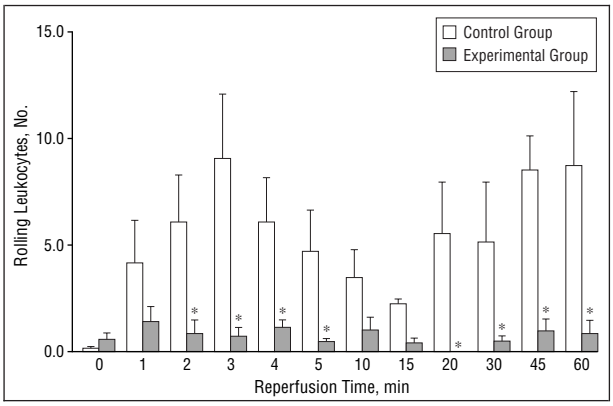

Figure 1. *Rolling leukocyte flux (mean ± SEM) in the control group, which received 4 hours of global ischemia, and the experimental group, which received 4 hours of global ischemia and treatment with monoclonal antibody to L-selectin, at baseline and at 1 through 5, 10, 15, 20, 30, 45, and 60 minutes of reperfusion. Asterisk indicates* P<*.05 vs control at same time.*

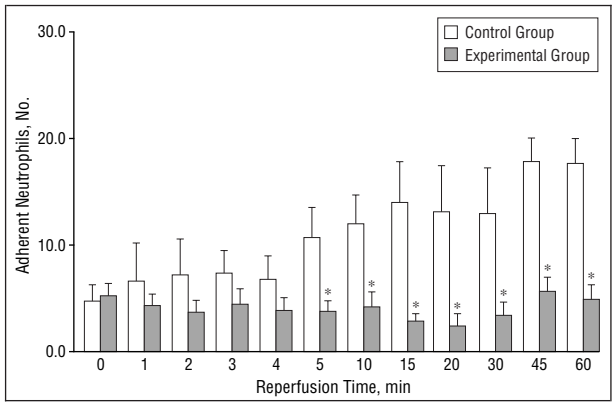

Figure 2. *Number of adherent neutrophils (mean ± SEM) in the control group, which received 4 hours of global ischemia, and the experimental group, which received 4 hours of global ischemia and treatment with monoclonal antibody to L-selectin, at baseline and at 1 through 5, 10, 15, 20, 30, 45, and 60 minutes of reperfusion. Asterisk indicates* P<*.05 vs control at same time.*

fusion compared with controls ($P = .004$, post hoc $P < .05$) (**Figure 2**). There was no difference in leukocyte velocity between groups.

COMMENT

In recent years, a significant amount of experimental work has been aimed at understanding the basic mechanisms of reperfusion injury. This area of research has direct clinical application to the field of plastic surgery, in which extremity and digit replantation often follows periods of prolonged global ischemia. Only through a detailed understanding of the mechanisms involved in IR injury can therapeutic measures be initiated to reduce or modify the injury.

A significant amount of evidence suggests that activated neutrophils play an important role in tissue in-

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jury associated with IR. Results of experiments[9,10] in pigs and dogs demonstrate the infiltration of neutrophils into skeletal muscle that has been subjected to IR injury. It has also been shown that altering the adherent properties of neutrophils improves the survival of tissues subjected to ischemia. Monoclonal antibodies to neutrophil adhesion molecules have been demonstrated[2,11-14] to ameliorate the ill effects of IR injury in several different animal models.

Circulating neutrophils express at least 2 types of cell surface glycoprotein adhesion molecules—β_2 integrins (CD11b/CD18 and CD11a/CD18) and L-selectin.[6,7] Experimental evidence[15] suggests that both L-selectin and β_2 integrin expression are required for neutrophil aggregation. In vitro data[15] suggest that, before a neutrophil can firmly adhere to the endothelium, rolling must occur. Rolling is initiated by L-selectin and its endothelial ligand under sheer stress.[16] It has been suggested[17] that L-selectin not only initiates neutrophil rolling but may also act as a signal transducer for β_2 integrin–dependent adhesion. Up-regulation of β_2 integrin is not required for initial adherence to the endothelium but seems to be required for sustained adhesion.[18,19]

It is now believed that L-selectin modulates the first step of neutrophil adherence. Monoclonal antibodies to L-selectin have been demonstrated to inhibit adherence of neutrophils in mesenteric venules subjected to IR injury.[7] Because the predominant type of selectin varies, depending on the vascular bed under investigation, we chose to examine the role of L-selectin in mediating IR injury in rat skeletal muscle.

In our experimental model, rolling leukocyte flux was significantly reduced by treatment with monoclonal antibody to L-selectin administered before reperfusion of the ischemic tissue. These results suggest that L-selectin modulates the initial step in neutrophil adherence in rat skeletal muscle. The antibody-treated group also demonstrated a decrease in number of adherent neutrophils, supporting the theory that rolling is required before adhesion can occur. There was no difference in mean leukocyte velocity between the experimental and control groups. The question of whether hemodynamic factors can affect leukocyte flux has been addressed by Lindbom et al,[20] who demonstrated that leukocyte rolling, total flux rates, and mean leukocyte rolling velocity remained constant among vessels with different flow rates, indicating a dependency on the selectin rather than on circulatory hemodynamic factors.[20]

In summary, IR injury is a complex and multifactorial phenomenon. Although L-selectin is only one factor, it seems to play an important role in the neutrophil-endothelial interactions associated with IR injury.

Abstract presented at the 12th Annual Meeting of the American Society of Reconstructive Microsurgery, Boca Raton, Fla, January 13, 1997.

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