Neutrophil Response to Replantation of Large Human Extremities

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Abstract

Background: Polymorphonuclear leukocytes (PMN) play a pivotal pathogenic role in ischemia/reperfusion injury of various tissues. The aim of the present study was to investigate the effect of replantation of large extremities on the function of circulating PMN in human patients.

Patients and Methods: PMN were isolated from whole blood up to 90 min after vessel repair and reperfusion. PMN superoxide anion production was measured by a cytochrome C reduction assay. Ten patients with amputations of the leg (n = 2), lower leg (n = 5), upper arm (n = 2), forearm (n = 1), and three subtotal amputations of the lower leg with severe vessel damage were enrolled.

Results: In four of six reamputated patients, total time of limb ischemia exceeded 5 h and PMN superoxide production was substantially increased at 60 min after reperfusion. With successful replantation, time of ischemia was < 5 h and PMN superoxide production did not further increase during reperfusion.

Conclusion: The neutrophil response to replantation of large extremities is associated with the time of ischemia which may be involved in multiorgan dysfunction syndrome observed in some of these patients.

Key Words
Reperfusion · Skeletal muscle · Replantation · Neutrophil · Superoxide

Introduction

Emergency replantation of large human extremities is a challenging clinical task and demands high standards in surgical technique and postoperative management. Moreover, decision-making for both replantation and reamputation exclusively depends on experience of the responsible surgeons and is influenced by a variety of individual factors such as duration of ischemia, additional injuries, patient age, and preexisting disease [1]. In some cases, an overwhelming systemic inflammatory response with impeding multiple organ failure jeopardizes survival of the patient and forces the surgeon to reamputate the limb [2].

Several groups have developed clinical scoring systems, including the “Mangled Extremity Severity Score” (MESS) or the “Mangled Extremity Syndrome Index” (MESI), to provide objective decision criteria for replantation of large extremities and to prevent life-threatening complications [3, 4]. Furthermore, criteria were developed to help determine when to undertake replantation in patients who have sustained multiple trauma [5]. In most cases, these algorithms allow assessment of the severity of injury for individual patients. However, so far, pathophysiology of both ischemia/reperfusion and tissue injury plays an inferior role in the clinical decision process. Although many experimental studies have been performed to elucidate the relevant mechanisms and inflammatory cascades of postischemic tissue injury [6], to our knowledge, no studies have dealt with the neutrophil response after replantation in human patients.

Current concepts of ischemia/reperfusion injury to skeletal muscle characterize the early period of reperfusion by both the release of humoral mediators (e.g., complement, kinins, coagulations factors) and parallel activation of local cell systems (endothelium, macrophages, fibroblasts). The local release of potent inflammatory mediators (e.g., cytokines, arachidonic acid derivatives, xanthine oxidase) and the expression...
of adhesion molecules trigger a secondary cellular inflammatory reaction [7]. Leukocyte-endothelial interactions represent a key element of postischemic endothelial cell and tissue injury. Leukocyte adhesion seems to be a precondition for intravascular release of aggressive leukocyte enzymes or oxygen radicals and allows interstitial infiltration by primed and activated polymorphonuclear leukocytes (PMN) [8].

Determination of PMN superoxide production is appropriate to describe the function of circulating neutrophils and allows interpretation of their function in vivo [9]. In previous studies of tourniquet ischemia of the upper extremity in human patients, we demonstrated a significant early activation of circulating leukocytes during their passage of the reperfused limb [10]. With these findings in mind, we developed the hypothesis that neutrophils may also play a pathogenic role during the inflammatory response following replantation of large human extremities.

**Patients and Methods**

Patients with amputations or posttraumatic ischemia of large extremities admitted to the Department of Trauma Surgery at the University of Saarland, Germany, were enrolled. The decision for replantation was individually based on the trauma surgeon’s evaluation of the condition of the amputated limb, time of ischemia, concomitant injuries, and overall clinical condition of the patient. Since cell studies had to be performed immediately, not all patients replanted by our trauma institution could be enrolled in the study. Following primary stabilization and diagnostic procedures in the emergency room, patients underwent immediate surgical treatment. The clinical course of the patients was documented until discharge. Patients entered the study with their informed consent or that of the next of kin, and in compliance with the institution’s requirements for studies in humans. Patients were part of a study of severely traumatized patients approved by the local ethics committee.

**Isolation of Circulating Neutrophils**

Arterial heparinized whole blood samples (10 ml) were taken before release of the arterial anastomosis and then 30, 60, and 90 min during limb reperfusion, as performed in our previous study of tourniquet ischemia of upper extremities [10]. PMN were isolated immediately using a Percoll® density gradient (Pharmacia, Uppsala, Sweden) as described by Hjorth et al. [11]. Briefly, an isotonic (final osmolarity: 284.5 mOsm) Percoll® gradient was prepared in a 12-ml polystyrol tube by layering Percoll® with lower density (D = 1.077 g/ml) on Percoll® with higher density (D = 1.090 g/ml). 4 ml blood was centrifuged at 350 g for 5 min and plasma was frozen by liquid nitrogen and stored at −78 °C for later biochemical evaluation. The cell sediment was resuspended with 4 ml phosphate-buffered saline (PBS; 10 mM phosphoric acid, 138 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4, Sigma Chemie, Deisenhofen, Germany), then layered on top of the Percoll® gradient and ultimately centrifuged at 400 g for 20 min at room temperature. PMN were recovered from the layer between the low- and high-density gradient, mixed with PBS, and centrifuged at 350 g for 5 min at 4 °C. Contaminating erythrocytes were removed by hypotonic lysis of the granulocyte sediment. Purity and viability of PMN, as tested by trypan blue exclusion technique, were >95%.

**PMN Superoxide Production**

PMN superoxide production was measured by a continuous SOD-inhibitable ferricytochrome C reduction assay using a dual beam spectrophotometer (Perkin Elmer, Lambda 16, Überlingen, Germany) [12, 13]. Briefly, isolated PMN were suspended in PBS (10⁷ cells/ml), 100 ml of the cell suspension containing minimal essential medium (MEM Dulbecco for chemiluminescence, 1.0 g/l D-glucose, 50 µM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, HEPES; Biochrom KG, Berlin, Germany) and 50 ml cytochrome C (50 nmol; cytochrome C type III from horse heart) were stimulated with formyl-methionyl-leucyl-phenylalanine (fMLP, final concentration 0.5 × 10⁻⁷ M). The reference cuvette contained superoxide dismutase (250 U, SOD from bovine erythrocytes, activity: 2,500–7,000 U/mg protein). The rate of superoxide production was determined by the slope of the linear part of the curve. Ferricytochrome C reduction was continuously read at 550 nm over 10 min. The nanomolar extinction coefficient of 0.0211 for the reduction of ferricytochrome C was used to calculate superoxide anion production [14]. Superoxide anion production was expressed as nmol/min/10⁶ PMN. All assays were performed in duplicate. Normal value of PMN superoxide production derived from 19 healthy persons was 1.36 ± 0.1 nmol/min/10⁶ PMN.

**Results**

Ten patients with traumatic ischemia of large extremities were enrolled: upper leg amputation (n = 2), lower...