Role of leucocytes in damage to the vascular endothelium during ischaemia-reperfusion injury

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ABSTRACT

During this investigation, a model of tourniquet-induced forearm ischaemia-reperfusion injury is employed to investigate the role of leucocytes in damage to the vascular endothelium during ischaemia-reperfusion injury. Leucocyte entrapment is investigated by measuring the concentration of leucocytes in venous blood leaving the arm. Neutrophil and monocyte leucocyte subpopulations are isolated by density gradient centrifugation techniques. Cell surface expression of CD11b and the intracellular production of hydrogen peroxide are measured via flow cytometry. Plasma concentrations of elastase and von Willebrand factor (vWF) are measured using enzyme-linked immunosorbemt assay (ELISA) techniques. During ischaemia-reperfusion, there was an increase in CD11b cell surface expression on neutrophils ($P=0.040$) and monocytes ($P=0.049$), and a decrease in peripheral blood leucocytes ($P=0.019$). There was an increase in the intracellular production of hydrogen peroxide by leucocyte subpopulations ($P=0.027$ [neutrophils], $P=0.091$ [monocytes]) and in the plasma elastase concentration ($P=0.05$). There was also a trend to increasing plasma concentration of vWF ($P=0.0562$), which was measured as a marker of endothelial damage. Ischaemia-reperfusion results in increased adhesiveness, entrapment and activation of leucocytes. Even following a mild ischaemic insult, this leucocyte response was followed immediately by evidence of endothelial damage. These results may have important implications for understanding the development of chronic diseases that involve mild ischaemic episodes.

KEY WORDS: Endothelium, vascular.
Ischemia.
Leukocytes.
Reperfusion.

Introduction

Leucocytes are white blood cells that help to fight foreign material such as bacteria and viruses. They play a critical role in the development of ischaemia-reperfusion injury and other clinically important inflammatory conditions. Leucocytes are involved intrinsically in cell–cell adhesion interactions with the vascular endothelium.[1–4]

Ischaemia is defined as an inadequate local blood supply to a part of the body. Ischaemia causes tissue injury and when blood flow is re-established, during reperfusion, further injury to the host tissue can occur.[5] Ischaemia-reperfusion injury occurs in diseases such as atherosclerosis, myocardial infarction, stroke and peripheral vascular disease, and during surgical procedures that involve the application of a tourniquet.[6–9] This justifies further investigation of the mechanisms of leucocyte involvement during ischaemia-reperfusion injury.

The mechanism of leucocyte adhesion to the endothelium involves expression of specific adhesion molecules on the surface of leucocytes and of the endothelium. Specific adhesion molecules that mediate adhesive interactions include CD11b/CD18 on neutrophils and monocytes. These bind to their corresponding ligands (e.g., ICAM-1) to facilitate leucocyte–endothelial cell interactions.[10–12]

Interactions between leucocytes and the vascular endothelium are essential for normal haemostasis. Leucocytes adhere to the vascular endothelium, causing entrapment at the site of ischaemia and reperfusion. This entrapment has two effects: first, cells are concentrated in tissues where they can cause damage and inflammation; and second, decreased blood flow through the tissue increases the degree and length of the ischaemic episode.[13–15]

The precise mechanism by which ischaemia and reperfusion cause tissue damage is unclear. Ischaemia-reperfusion injury causes activation of neutrophils and monocytes. This results in the release of cytotoxic molecules, which could damage the vascular endothelium and surrounding tissues. Examples of these include the release of the proteolytic enzyme elastase and of reactive oxygen intermediates (ROIs) such as hydrogen peroxide, by activated phagocytic leucocytes. These may contribute to diseases such as atherosclerosis, myocardial infarction, stroke and peripheral vascular disease, and may cause host cell damage following surgery involving the application of a tourniquet.[16,17]

Previous studies have used animal and human models of ischaemia-reperfusion injury.[18–21] Leucocytes are involved intrinsically in the inflammatory reaction and thus deserve research attention. This investigation involves a human study that provides a good model of ischaemia-reperfusion injury, enabling the assessment of the role leucocytes in this process.

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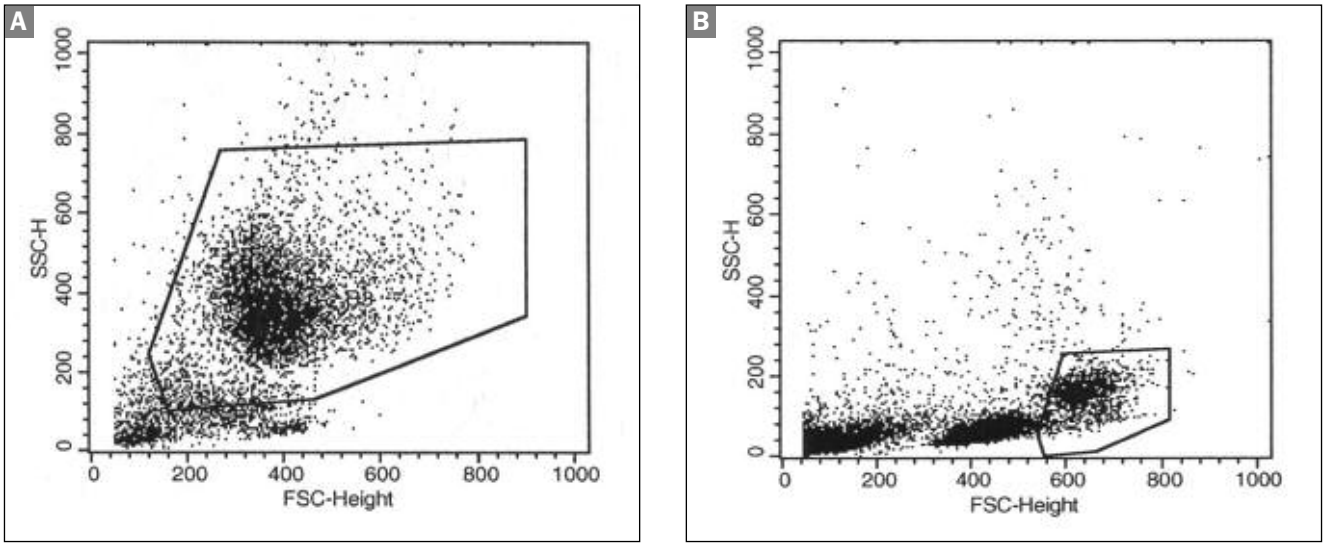
Fig. 1. Gating of leucocyte subpopulations during flow cytometric analysis. Gates were adjusted so that the percentage of cells analysed were identical to those identified using Coulter MicroDiff.[18] Lymphocytes, red blood cells and debris were excluded from defined gates. Leucocyte subpopulations were selected by assignment of gates normally associated with (A) neutrophils and (B) monocytes.



The main aim of this investigation is to determine the role of leucocytes in damage to the vascular endothelium during ischaemia-reperfusion injury. Furthermore, it tests the hypothesis that the mechanisms of leucocyte adhesion, entrapment and activation during tourniquet-induced forearm ischaemia and reperfusion lead to the subsequent damage to the vascular endothelium.

Materials and methods

Subjects

Ethical approval for this study was received from the local research ethics committee. Twenty healthy volunteers (10 male and 10 female) were recruited after informed consent. The test subjects were aged between 22 and 48 years (mean: 28 years), and all except four were non-smokers. None had a history of cardiovascular disease.

Blood collection and cell counting

Blood samples were collected by venepuncture of the antecubital vein into Vacutainers containing dipotassium EDTA. Following venepuncture, red cell, platelet and partial differential white cell counts were performed using a Coulter MicoDiff automated cell counter.

Tourniquet-induced forearm ischaemia-reperfusion injury

Initially, a venous blood sample was taken from the contralateral arm of each subject, which provided baseline measurements. A sphygmomanometer was placed around the upper experimental arm and inflated to approximately 20 mm Hg above systolic blood pressure.[22–24] A further blood sample was taken from the experimental arm via the antecubital vein after 10 min of ischaemia. The tourniquet was then removed to allow full reperfusion of the arm, and further blood samples were collected after 5 min and 15 min reperfusion.

Cell suspensions

Purified neutrophils and mononuclear cell suspensions were prepared by density gradient sedimentation on Ficoll Hypaque solutions, as described by Lennie *et al*.[25] Following isolation, cells were resuspended in phosphate-buffered saline (PBS) supplemented with dipotassium EDTA (1 mg/mL) to yield a final cell count of 2 x 10^6 cells/mL.

Cell surface expression of CD11b

The monoclonal antibodies used were murine IgG1 isotype control and murine IgG1 anti-human CD11b (clone 44). Both were purified immunoglobulin/fluorescein isothiocyanate (Ig/FITC) conjugates (Oxford Biotechnologies, UK). Following isolation of leucocyte subpopulations and adjustment to concentrations (2 x 10^6 cells/mL), 10 µL monoclonal antibody (0.1 mg/mL) was added to 100 µL cell suspension and incubated at room temperature for 30 min prior to assay analysis using flow cytometry of gated neutrophils and monocytes (Fig. 1).

Intracellular hydrogen peroxide production

Intracellular hydrogen peroxide (H_2O_2) production was assessed by adapting a technique described previously by Bass *et al*.[26] The assay is based on the oxidation by H_2O_2 of non-fluorescent 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) to stable and fluorescent dichlorofluorescein. Production of H_2O_2 was assessed in cells using a fixed volume of 0.5 mL cell suspension (2 x 10^6 cells/mL) mixed with 0.5 mL DCFH-DA (20 µmol/L) in PBS. Cells were incubated in the dark at 37°C for 30 min before immediate measurement using flow cytometry of gated neutrophils and monocytes (Fig. 1).

Plasma concentration of leucocyte elastase and von Willebrand factor

Blood samples were centrifuged at 1500 x*g* for 10 min within 4 h of collection. Plasma was removed and stored at –80°C. Plasma neutrophil elastase concentration was measured by a sandwich-type ELISA using sheep anti-human neutrophil elastase and peroxidase-conjugated sheep anti-human α1-antitrypsin (The Binding Site, UK), as described previously.[27] Plasma vWF concentration was measured as

Fig. 2. Effect of tourniquet-induced forearm ischaemia-reperfusion injury on CD11b cell surface expression of neutrophils and monocytes. Points represent mean±SD (P=0.040 [neutrophils], P=0.049 [monocytes], determined by ANOVA; n=20).

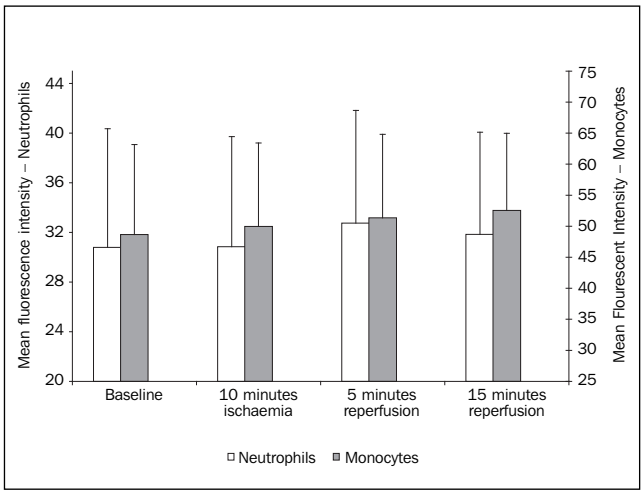


Fig. 3. Effect of tourniquet-induced forearm ischaemia-reperfusion injury on leucocyte concentration. The points represent mean±SD (P=0.019, determined by ANOVA; n=20).

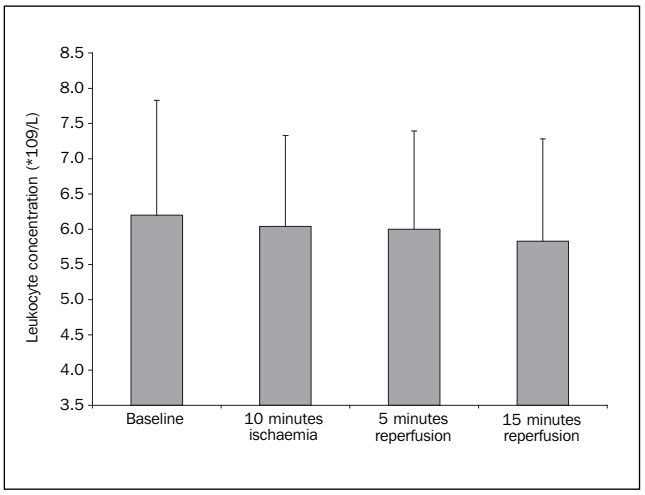


Fig. 4. Effect of tourniquet-induced forearm ischaemia-reperfusion injury on intracellular H202 production by neutrophils and monocytes. Points represent mean±SD (P=0.027 [neutrophils], P=0.091 [monocytes], determined by ANOVA; n=20).

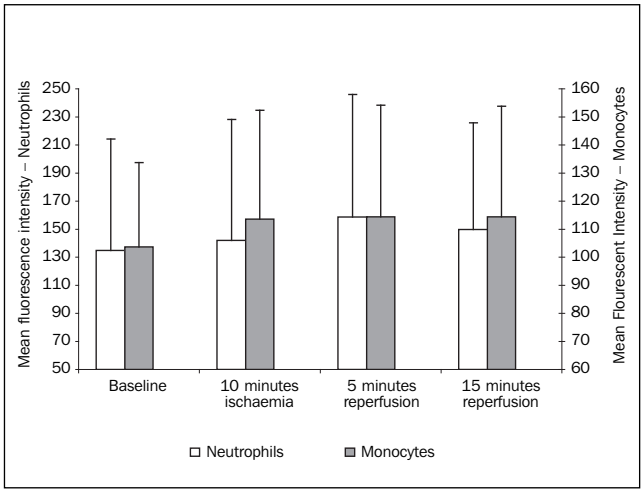
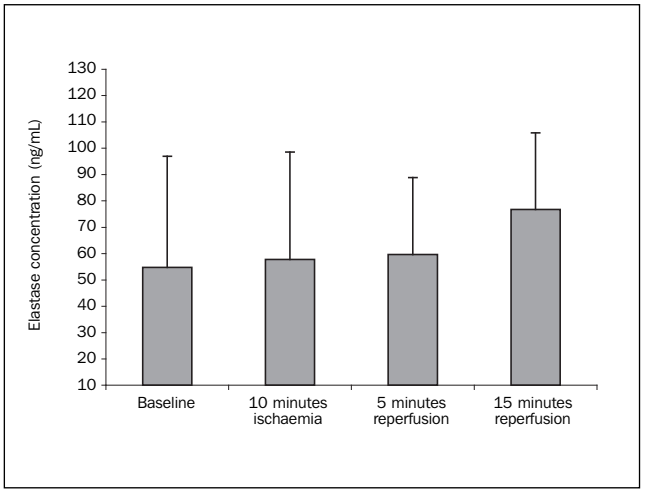


Fig. 5. Effect of tourniquet-induced forearm ischaemia-reperfusion injury on plasma α_1-antitrypsin leucocyte elastase complex concentration. Points represent mean±SD (P=0.05 determined by ANOVA; n=20).



described previously by a sandwich-type ELISA technique using rabbit anti-human vWF and rabbit anti-human vWF peroxidase conjugate (Dako, UK).[16,28]

Statistical analysis

Results were presented as mean ± standard deviation (SD). Changes in the measured parameters during ischaemia and reperfusion were determined by repeated measures analysis of variance (ANOVA). Statistical significance was set at $P \leq 0.05$.

Results

During tourniquet-induced forearm ischaemia-reperfusion injury there was a significant increase in CD11b cell surface expression, represented by mean fluorescence intensity (MFI) for neutrophils and monocytes (Fig. 2). During 10 min of ischaemia and 5 min reperfusion there was an increase in CD11b cell surface expression on neutrophils. Following 15 minutes reperfusion, neutrophil CD11b cell surface expression decreased towards baseline levels (P=0.040). Monocyte CD11b cell surface expression showed a steady increase during ischaemia and reperfusion (P=0.049), with overall CD11b cell surface expression consistently higher than that seen on neutrophils. Total leucocyte concentration decreased significantly (P=0.019), with levels changing from 6.20 ± 1.62 x 10^9/L at baseline to 5.83 ± 1.45 x 10^9/L after 15 min reperfusion (Fig. 3).

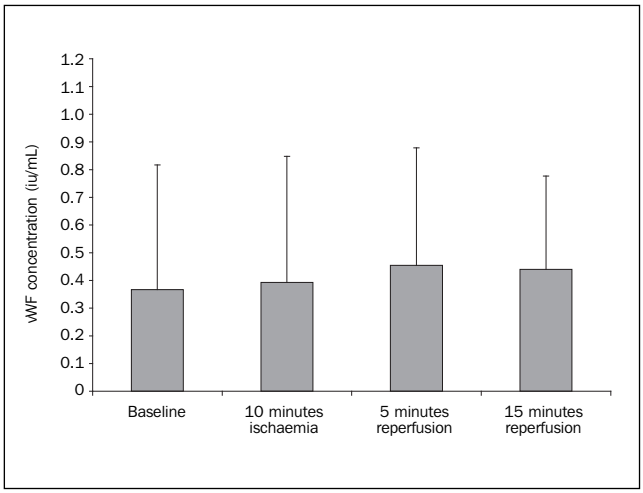
Tourniquet-induced forearm ischaemia-reperfusion injury caused an increase in intracellular H_2O_2 (represented by MFI) in neutrophils and monocytes (Fig. 4). The intracellular H_2O_2 production in neutrophils increased following ischaemia and 5 min reperfusion, with a decrease in intracellular H_2O_2 production towards baseline levels following 15 min reperfusion (P=0.027). Intracellular H_2O_2

production by monocytes increased following 10 min ischaemia and showed no change up to 15 min reperfusion (P=0.091).

Leucocyte elastase concentrations increased following ischaemia-reperfusion injury (Fig. 5), from baseline during 10 min ischaemia and 15 min reperfusion, with the most noticeable change in concentration evident between 5 min and 15 min reperfusion (P=0.05).

The vWF concentration increased during ischaemia and early reperfusion (Fig. 6), from baseline during 10 min ischaemia and 5 min reperfusion, with concentration decreasing towards baseline levels following 15 min reperfusion (P=0.0562).

Fig. 6. Effect of tourniquet-induced forearm ischaemia-reperfusion injury on plasma vWF concentration. Points represent mean±SD (P =0.0562, determined by ANOVA; n=20).



Discussion

Investigations, *in vivo*, of the role of leucocytes in damage to the vascular endothelium during ischaemia-reperfusion injury provided some interesting results. Leucocytes showed increasing entrapment during ischaemia-reperfusion and this was supported by an increase in CD11b cell surface expression on neutrophils and monocytes.

CD11b is expressed on leucocytes and binds to adhesion molecules such as ICAM-1 expressed on the surface of vascular endothelium. This increased CD11b expression may play a central role as the mechanism by which leucocyte entrapment in the microcirculation occurs. Evidence of this is supported by the detection of sensitised circulating phagocytes. This suggests that the vascular endothelium may be exposed to considerable expression of adhesion molecules, which facilitates leucocyte entrapment.

During forearm ischaemia-reperfusion, leucocyte levels in whole blood decreased. A possible explanation for this could be increased entrapment of leucocytes in the microcirculation. The entrapment results in the leucocytes adhering to the vascular endothelium, which leads to cell activation. Evidence of leucocyte activation was supported by the increase in intracellular H_2O_2 production by neutrophils and monocytes, and by the increase in plasma elastase concentration.

Extracellular liberation of elastase can be expected to occur simultaneously with the release of proteolytic enzymes such as cathepsin G and lysozyme. Under pro-inflammatory conditions, the action of these enzymes, together with other bioactive agents such as ROIs, could cause considerable damage to host tissue.

There is increasing evidence of endothelial dysfunction and damage during ischaemia-reperfusion injury. This investigation demonstrated that vWF is an established marker of endothelial damage,[28] as reflected by the increase in vWF concentration during ischaemia and early reperfusion. Although further increase in vWF concentration was not seen following 15 min reperfusion, the results may not negate the concept of increasing leucocyte activity, as the experiments were conducted over a relatively short period of reperfusion (15 min), and it is during reperfusion that most host cell damage is though to occur.[5] However, the results suggest increasing liberation of vWF from the endothelium into the plasma, and may indicate that damage to the vascular endothelium occurs during tourniquet-induced forearm ischaemia-reperfusion injury.

Owing to the binding and bridging function of vWF, high levels of circulating vWF may contribute to the development of pathological states in which ischaemia-reperfusion injury is an underlying process. These include atherosclerosis, myocardial infarction, stroke and peripheral vascular disease. Evidence of increasing endothelial damage measured during the present study provides an opportunity to investigate other markers of endothelial damage to support these findings.

Ischaemia-reperfusion injury is an aspect of many clinically important conditions. Leucocytes involved in the inflammatory response (i.e., neutrophils and monocytes) have been shown in previous studies to play a central role in precipitating and exacerbating ischaemia-reperfusion injury. However, an important aspect of the present study was to provide a better understanding of the mechanism by which these cells are involved in this process.

Using a human model of mild ischaemia-reperfusion injury, rapid accumulation of effector leucocytes in the microvasculature was detected. Neutrophils and monocytes analysed *ex vivo* were shown to express elevated levels of cell-surface CD11b and intracellular ROIs, indicating their high activation state following ischaemia-reperfusion. Importantly, progressively increased circulating levels of leucocyte-derived proteolytic elastase was detected during ischaemia-reperfusion injury. This trend was reflected by increased levels of circulating vWF, a marker of endothelial damage.

These studies reveal that during even very brief periods of ischaemia, inflammatory leucocytes are rapidly activated, accumulate in the vasculature and release bioactive molecules extracellularly. Even after only mild ischaemic insult, this leucocyte response is followed by evidence of endothelial damage. These results may have important implications for understanding the development of chronic diseases that involve mild ischaemic episodes. □

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