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Interleukin-8 (IL-8) May Contribute to the Activation of Neutrophils in Patients with Peripheral Arterial Occlusive Disease (PAOD)

G. Kirk*[1], P. Hickman[1], M. McLaren[1], P. A. Stonebridge[2] and J. J. F. Belch[1]

University [1]Department of Medicine and [2]Department of Surgery, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

Objectives: *to investigate the levels of interleukin-8 (IL-8) in patients with peripheral arterial occlusive disease (PAOD) and healthy control subjects both before and after an acute exercise test.*
Materials and methods: *twenty-six patients with intermittent claudication and 22 matched healthy control subjects each had IL-8 levels measured before and after a standard acute treadmill-exercise test. Subjects walked for 10 min or until stopped by claudication pain. Serum IL-8 levels were measured before exercise was commenced and 1, 5 and 10 min after exercise was stopped.*
Results: *patients with PAOD had statistically significantly higher levels of IL-8 than healthy control subjects, before and after an acute exercise test ($p<0.00001$, Mann–Whitney). Ratios of the change of IL-8 levels post-exercise showed a statistically significant difference at the post-5-min time point ($p=0.005$), showing a difference in the change of IL-8 levels at this time point between the patient group and control group.*
Conclusions: *The increased levels and the failure of the cytokine levels to fall by the same extent after exercise in the patient group may be due to a combination of increased neutrophil activation, reduced blood flow and increased cytokine production during ischaemia–reperfusion, which is not observed in the healthy controls.*

Key Words: Peripheral arterial occlusive disease; Interleukin-8; Neutrophils; Exercise.

Introduction

Peripheral arterial occlusive disease (PAOD) is a major cause of morbidity and mortality in the Western world with 5% of males over 50 years of age developing intermittent claudication.[1] Claudication itself does not cause death, but the mortality of claudicants is approximately three times that of age- and sex-matched individuals. Approximately half the excess mortality is due to myocardial infarction.[1] Treatment for short-distance claudication or critical limb ischaemia is normally restricted to reconstructive surgery or angioplasty.[2] There is reluctance amongst vascular surgeons to offer surgery to claudicants, as the risks may outweigh the benefits. Only one-third of patients with claudication are thought to be suitable for angioplasty. Patients with mild claudication (i.e. those who are able to walk 200 m free of pain) are given the advice "stop smoking and keep walking".[3] It was Erb in 1898 who first suggested walking as a therapy for intermittent claudication. Today, it is still unclear by what mechanism exercise is responsible for any improvement, although several factors have been proposed including improved blood flow, increased functional capillary density, and altered muscle metabolism.[4]

The monocyte-derived macrophage has been implicated in many areas of atherosclerotic plaque development. The cell can release cytokines which are chemotactic towards other cells. One of these cytokines is interleukin-8 (IL-8). IL-8 is a member of a superfamily of pro-inflammatory cytokines known as chemokines.[5] IL-8 can regulate neutrophil endothelial interaction.

The atherosclerotic plaque leads to narrowing of the arteries and can lead to ischaemia on exercise which is followed by reperfusion at rest. Reperfusion is necessary to recover ischaemic tissue, but neutrophils which are adherent to the endothelium may not be removed after reperfusion, and continue, by means of utilising the cell-adhesion molecules (CAMs) and cytokines, to migrate through the vessel wall, resulting in the damage of the endothelium and local tissue.[6] IL-8 has been shown to increase the surface expression of CAMs and, therefore, can promote increased neutrophil adhesion to the endothelium.[7]

The aim of this study was to investigate the levels

* Please address all correspondence to: G. Kirk, University Department of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

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Table 1. Demographic details of subjects.

	Patients with PAOD	Controls
n	26	22
Sex		
M:F	19:7	16:6
Age (years)	66	58
Median (range)	(53–83)	(38–69)
Smoking		
Y:N:Ex	10:4:12	7:7:8
ABPI ratio	0.69	1.20
Median (range)	(0.31–0.93)	(1.00–1.90)

of IL-8 in patients with PAOD and healthy controls both before and after an acute exercise test.

Materials and Methods

Patients

Twenty-six patients with intermittent claudication and 22 healthy volunteers took part in the study. Demographic details are shown in Table 1. The patients with claudication had their disease proven by segmental Doppler pressure and/or angiography. All patients with claudication had an ankle–brachial blood pressure index (ABPI) of less than 0.95. All patients with peripheral arterial occlusive disease (PAOD) had a measured pain-free walking distance, on a treadmill at 3.2 km/h with a 10° slope, of between 50 m and 200 m. All the control subjects had an ABPI of <1.0 and gave no history of cardiovascular disease. No patients had taken any vasoactive drugs for their claudication for at least 14 days prior to the start of the study. The study was approved by the local ethics committee, and written informed consent was obtained from all volunteers.

Blood samples

All samples were taken at the same time of day to avoid influences from circadian variations known to occur in CAMs and other activation markers.[8–10] Subjects were all asked to remain supine for 1 h and during this time had a 16-gauge intravenous (i.v.) cannula inserted with a three-way tap into an antecubital-fossa vein. This was used to obtain blood samples before and after exercise. Between sampling the i.v. cannula was flushed with 2 ml of sterile heparinised saline (5 U/ml), which does not affect IL-8 levels. Five ml of blood were taken and discarded before the test blood samples were taken. After the 1-h rest period the subjects were required to stand on the treadmill and 10 ml of blood were removed and collected into a tube containing clotting beads for serum preparation. This was the pre-exercise blood sample. All subjects were then exercised on a treadmill, as above, for a maximum of 10 min or until stopped by claudication pain. Three further 5-ml samples of blood were removed at 1 min, 5 min and 10 min after the exercise was stopped. All samples were placed in a water bath at 37 °C and left to clot for 1 h. The serum was separated, aliquoted and stored at −70 °C until assayed. Serum IL-8 concentrations were measured by an ELISA (R&D Systems, U.K.). All four samples from each individual were measured on the same assay. The detection limit of the assay was 6.75 pg/ml and the interassay coefficient variation CV was 12%.

Statistics

As the data had a skewed distribution and a few outliers, the non-parametrical Mann–Whitney *U*-test was used to compare the levels of IL-8 from the two subject populations at each blood-sample time point. Repeated measures analysis of variance (repeated MANOVA) was applied to the levels of IL-8 for all the sampling time points to see if the acute exercise test had a significant overall effect on IL-8 levels. Due to the large variation in the range of IL-8 levels the ratio of change of IL-8 levels post-exercise compared with baseline was calculated for all post-exercise time points in both groups. These ratios were modelled by a normal distribution and unpaired *t*-tests were used at each of the three post-exercise time points to compare the two subject groups. A p value of <0.05 was taken to be statistically significant.

Results

All patients and controls were well matched with no statistical significant differences in age or sex distribution. The control group had slightly more non-smokers than the patients group but, with both groups having a similar proportion of current and ex-smokers, there was no relationship between smoking status and IL-8 levels (Table 1). There also appears to be no relationship between walking distances or requirement for surgery and baseline IL-8 levels.

The patients with PAOD had statistically significantly higher levels of IL-8 at all four time points (Table 2, $p<0.00001$, Mann–Whitney). The acute exercise test significantly affected the overall levels of

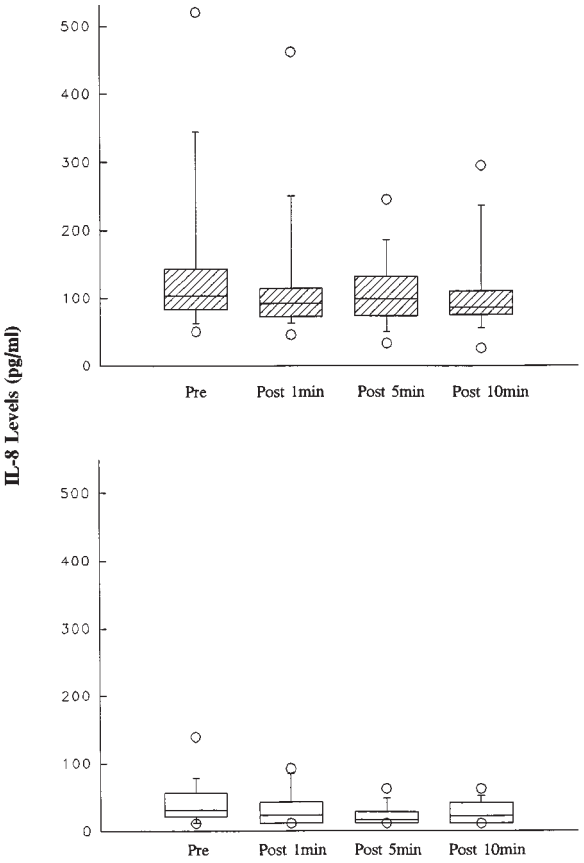

Fig. 1. Whisker box plots showing IL-8 levels (pg/ml) in patients with PAOD (hatched) and healthy controls (open) before and after an exercise test.

IL-8 in both the control group ($p<0.0001$) and the patient group ($p=0.03$) using MANOVA. The levels of IL-8 decreased after exercise in all subjects at all three time points when compared with baseline. At post-1 min and post-10 min the ratios of change did not statistically change between the two groups $p=0.25$ and $p=0.13$, respectively. Whereas the ratios of change were statistically significantly different at the post-5 min time point ($p=0.005$, unpaired t-test), showing a difference in the change of IL-8 levels at this time point between the patient group and control group (Table 2).

Discussion

The role of the neutrophils in the inflammatory response to ischaemia is well recognised.[11] During exercise the patients with claudication suffer from acute ischaemia and, on restoration of blood flow, reperfusion can lead to the emigration of neutrophils from the circulation into tissue.

In this study, we have shown for the first time that patients with PAOD have significantly higher levels of IL-8 both before and after an exercise test, which probably reflects an increase in neutrophil activation.[12,13]

The chemokine, IL-8, has been shown to enhance superoxide production and may even prime neutrophils for enhanced superoxide production in response to other stimuli.[14] Human neutrophils express receptors for IL-8 that are comparable in number and affinity to those for C5a or FMLP.[15]

It has also been shown, *in vitro*, that reactive oxygen metabolites have been shown to upregulate IL-8 production, whereas free-radical scavengers decreased local production of IL-8.[16] We have previously shown that patients with PAOD had elevated levels of free-radical production and attenuation in free-radical scavenging systems after exercise when compared with levels from exercised healthy controls.[17] We have also shown that sE-selectin, an endothelial activation marker which binds to neutrophils and monocytes, is elevated in patients with severe PAOD.[18]

IL-8 not only acts as a neutrophil attractant, but also has a neutrophil activator. It may be involved in the pathogenesis of atherosclerosis because it is chemotactic for smooth-muscle cells[19] and it downregulates L-selectin and induces β_2-integrin expression.[20]

We also show that acute exercise has less effects on IL-8 levels when investigating PAOD compared with healthy controls. The cytokine levels were significantly lowered in the healthy control subjects at all three time points after the exercise test. This may represent an attenuation in neutrophil adhesion during exercise, and perhaps an increase in blood flow and an elevation of oxygen levels reaching the exercising tissues in the healthy controls.

At the post-5-min time point the ratio of change between the two subject groups is statistically significant. If we look at the median levels of IL-8 at post-5-min we can see that in the control group the median level is almost half the pre-exercise level (53% change), whereas in the patients' group it is only very slightly decreased (5% change).

Shear stress has been shown, *in vitro*, to stimulate the increase in the amount of cytokines released from endothelial cells.[21] In the healthy control subjects, during the acute exercise test, a decrease in shear stress may therefore attenuate or even abolish this cytokine release into the circulation. Perhaps, as a consequence of increased blood vessel diameter the greater volume of red blood cells present in the dilated vessel may act as a binding site for IL-8,[5] thereby mopping up the cytokine. The slower marginal blood flow may increase the binding of the IL-8 and proteoglycans or antibodies[22] and therefore lower the circulating detected

Table 2. IL-8 levels (pg/ml) before and after an exercise test and ratio of change after exercise in patients with PAOD and healthy control subjects.

	n	Median (pg/ml)	Range (pg/ml)	Ratio of change after exercise Mean (range)
Patients with PAOD				
Pre-	26	103.9	13.7–887.5	—
Post-1 min	26	92.3	21.6–843.0	0.94 (0.54–2.30)
Post-5 min	26	99.0	11.5–401.3	0.86 (0.31–1.43)
Post-10 min	26	86.2	11.5–398.6	0.86 (0.25–1.56)
Controls				
Pre-	22	34.0*	11.5–190.7	—
Post-1 min	22	25.3*	11.5–96.5	0.82 (0.31–1.52)
Post-5 min	22	18.0*	11.5–64.0	0.63 (0.20–1.00)**
Post-10 min	22	21.7*	11.5–75.6	0.71 (0.20–1.25)

* $p<0.00001$ Mann–Whitney *U*-test; ** $p=0.005$, unpaired *t*-test (patients vs. controls).

levels. The decreasing levels of IL-8 in the healthy subjects may also indicate that moderate exercise may be beneficial.

However, in the patients with claudication the levels of IL-8 do not fall to the same extent. The binding of IL-8 to the red blood cells prevent it from being biologically active, and therefore unable to stimulate neutrophils.[5] It therefore acts as a clearance mechanism.

The difference observed between the two subject populations may be a consequence of the availability of the red blood cells and the binding of the cytokine to its receptor present on the red blood cells.[5] However, the patients with intermittent claudication may have a reduced red blood cell perfusion through the atherosclerotic blood vessels, due either to the atherosclerotic plaque itself or because erythrocytes in patients with PAOD are less deformable.[23,24] Therefore, at the site of ischaemia where larger activated neutrophils may block the blood vessel,[11] fewer red blood cells are available for binding.

A further possible explanation for IL-8 levels failing to fall by the same extent in the patient population is that free radicals increase IL-8 on reperfusion, thus attenuating any fall produced by RBC-binding. The difference observed in the claudicants may therefore be a combination of reduced blood flow and an increase in cytokine production during ischaemia–reperfusion.

There is no doubt that there is a very large variation in serum levels of IL-8 throughout our patient group. We ourselves considered issues that could have caused this. There appears to be no relationship to smoking habit or ex-smoking habit, nor to walking distance or requirement for surgery. We have tried to clarify this by putting the IL-8 level in Table 1 for both smokers and non-smokers. Whilst we do accept that there is a large variation, the fact that statistically significant changes occur after exercise perhaps indicates the strength of this change rather than the weakness.

Despite these apparently detrimental effects of acute exercise in patients with PAOD, regular exercise has been shown to be beneficial. Exercise training in claudicants results in decreased blood viscosity and an increase in red blood cell deformability.[25] Therefore, long-term exercise may improve the encounter of IL-8 and red blood cells resulting in a fall in circulating IL-8 levels, with decreased neutrophil activation. This neutrophil activation may be a crucial step in atherosclerosis progression.

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