Induction of interleukin-1β and interleukin-6 gene expression in hypoperfused skeletal muscle of patients with peripheral arterial disease

Marco Testa, Ermenegildo De Ruvo, AnnaRita Russo, Franco Citterio*, Francesco Serino**, Antonella Mangoni***, Maurizio C. Capogrossi***, Giovanni Sperti

*Institute of Cardiology, *Department of Surgery, Catholic University of the Sacred Heart, **Division of Vascular Surgery, ***Laboratory of Vascular Pathology, Istituto Dermopatico dell Immacolata, Rome, Italy*

Key words:
Hypoperfusion; Ischemia; Inflammation; Peripheral arterial disease; Gene expression.

Background. **A growing amount of data supports the role of inflammation in the pathophysiology of atherosclerotic diseases but the cellular source of cytokines has not been clearly identified. Cytokines could be produced by inflammatory cells, activated endothelial and smooth muscle cells, and by the tissue exposed to recurrent ischemia. Accordingly, we evaluated whether hypoperfusion induces gene expression of interleukin (IL)-1β and IL-6 in the skeletal muscle of patients with peripheral arterial disease and critical limb ischemia.**

Methods. **Skeletal muscle biopsies were obtained, during a femoral-distal bypass, from normoperfused (control) and hypoperfused skeletal muscles in 8 patients. Gene expression was assessed by semiquantitative reverse transcriptase-polymerase chain reaction, using glyceraldehyde-phosphate-deydrogenase mRNA levels as a normalization factor.**

Results. **In the hypoperfused biopsies, the level of IL-1β gene expression was significantly higher in all but 2 patients (mean upregulation > 8.8 fold, p = 0.043), and the level of IL-6 gene expression was significantly higher in all but 1 patient (mean upregulation > 23.7 fold, p = 0.031).**

Conclusions. **We report that IL-1β and IL-6 gene expression is markedly upregulated in hypoperfused skeletal muscle of patients with critical lower limb ischemia. To our knowledge this is the first report of a local activation of the inflammatory cascade at the level of hypoperfused skeletal muscle. This activation, which could worsen symptoms and tissue viability and be involved in the pathophysiology of reperfusion injury, might be considered as a therapeutic target. It remains to be investigated whether our results may also apply to coronary artery disease.**

(Ital Heart J 2000; 1 (1): 64-67)

Received November 18, 1999; revision received December 20, 1999; accepted December 22, 1999.

Address:

Dr. Marco Testa

Istituto di Cardiologia Università Cattolica del Sacro Cuore Policlinico Agostino Gemelli Largo Agostino Gemelli, 8 00168 Roma E-mail: ma.testa@agora.stm.it

Introduction

A growing amount of data supports the role of inflammation in the pathophysiology of atherosclerosis, the natural history of coronary artery disease and ischemia-reperfusion injury.

In acute coronary syndromes, high levels of proinflammatory cytokines have been demonstrated and may be associated with adverse prognosis[1,2]. Similarly, in chronic stable angina increased levels of macrophage-colony stimulating factor, interleukin (IL)-1b, and IL-6 have been shown to correlate with both the number of diseased vessels and a lower threshold for ischemia[3].

Contrasting data are available on the activation of inflammatory pathways in peripheral arterial disease[4,5].

The cellular source of cytokines in coronary artery and peripheral arterial disease has not been clearly identified. Cytokines could be produced by inflammatory cells present in the plaque, activated endothelial and smooth muscle cells, circulating blood cells, and also by the tissue exposed to recurrent ischemia[6-9].

Accordingly, we have evaluated whether hypoperfusion induces local activation of proinflammatory pathways in the skeletal muscle of patients with peripheral arterial disease. To this aim we assessed gene expression of IL-1b and IL-6 in biopsies obtained from hypoperfused skeletal muscle and control normoperfused skeletal muscle in the same patient.

IL-1b and IL-6 are two closely related cytokines, both central to the process of inflammation, and share several mechanisms of induction[6,7].

IL-1b is the prototypic multifunctional cytokine, part of a complex family that also comprises IL-1a, IL-1 receptors, a specific receptor antagonist, IL-1Ra, and an IL-1b converting enzyme. IL-1b has a promoter/enhancer region typical of inducible genes

that contains cAMP response elements, activating protein-1 site, nuclear factor kB and nuclear factor IL-6 sites[6]. Its gene expression is induced by hypoxia and ischemia, as well as by several neuroactive and inflammatory substances, in a variety of cell types including peripheral blood mononuclear cells, macrophages, endothelial and smooth muscle cells. The central role of IL-1b in the inflammatory cascade is partly due to its ability to activate the nuclear translocation of nuclear factor kB and the mitogen-activated protein kinase (MAPK) pathway[10,11]. Many of the effects elicited by IL-1b are mediated by prostaglandins and nitric oxide[10]. The proinflammatory properties of IL-1b could be relevant to the development of the atherosclerotic lesion, tissue damage after ischemia, restenosis after coronary angioplasty, and myocardial function.

IL-6 is also a multifunctional cytokine produced by and affecting several cell types[7]. IL-6 synthesis is elicited by IL-1 and tumor necrosis factor in a range of target cells, including endothelial cells[12]. Its expression is also induced by hypoxia and ischemia and this is due to enhanced transcription driven by the nuclear factor IL-6 site in the IL-6 promoter[8]. Tissue-specific mechanisms underlying this activation have been demonstrated[13].

IL-6 has proinflammatory and procoagulant properties and is one of the major inducers of acute phase hepatic proteins[14], such as C-reactive protein and fibrinogen that are elevated in unstable coronary syndromes. It may also play a role in the progression of atherosclerotic lesions by inducing the generation of oxidized LDL, by affecting platelet function and by regulating the leukocyte recruitment.

Methods

Population. Patients with peripheral arterial disease and critical limb ischemia (diagnosed according to the criteria of the Second Consensus Conference on critical limb ischemia)[15], undergoing a surgical procedure of femoral-distal bypass, were eligible for the study. Patients with other diseases limiting their walking ability (i.e. orthopedic diseases), systemic infectious or immune diseases, and malignancies were excluded from the study.

Before the intervention all patients underwent a thorough clinical evaluation and routine blood tests. An echo-Doppler study and angiography were performed in all patients.

Eight patients were recruited, 5 men and 3 women, with a mean age of 67.9 years (range 57-85 years). Seven patients suffered from diabetes, 5 were hypercholesterolemic (total cholesterol > 200 mg/dl), 5 had hypertension, 5 were former smokers and 1 a current smoker. Seven patients had ischemic lesions and 1 had pain at rest. All patients were on acetylsalicylic acid and 3 patients on low molecular weight heparin. All patients were also assuming other medications, as required by their comorbid conditions.

The study was approved by the Ethics Committee of the Catholic University of the Sacred Heart of Rome (Italy), and each patient gave informed consent.

Muscle biopsy RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). During the surgical procedure two fragments of skeletal muscle were obtained from a proximal muscle of the thigh, perfused by arteries without flow-limiting stenoses (normoperfused biopsy), and from a leg muscle, perfused by the critically stenosed superficial femoral artery about to be bypassed (hypoperfused biopsy).

The biopsies were immediately frozen in liquid nitrogen and stored at -70°C.

Total RNA was isolated using TRIzol (Gibco, Gaithersburg, MD, USA) and cDNA was synthesized using random hexamers and M-MuLV reverse transcriptase (Gibco, Gaithersburg, MD, USA). IL-1b, IL-6 and glyceraldehyde-phosphate-dehydrogenase (GAPDH) were amplified using AmpliTaq Gold DNA polymerase (Perkin-Elmer Biosystem, Foster City, CA, USA) and gene specific primers (IL-1b 5'-CAC ATG GGA TAA CGA GGC TT-3' and 5'-TCT TTC AAC ACG CAG GAC AG-3'; IL-6 5'-ATG AAC TCC TTC TCC ACA AG-3' and 5'-GAA GAG CCC TCA GGC TGG AC-3'; GAPDH 5'-CTG CAC CAC CAA CTG C-3' and 5'-CCA CCA CTG ACA CGT T-3'). Primer sets amplified across intron/exon boundaries to allow the identification of products amplified from contaminating genomic DNA (IL-1b 254 vs 1490 bp; IL-6 630 vs 4276 bp; GAPDH 278 vs 487 bp). However, in no instance was genomic DNA contamination detected.

For each gene the PCR protocol was optimized in order to maximize sensitivity while remaining in the linear phase of the amplification for the samples with the highest levels of specific mRNA. In particular we performed 35 cycles of amplification for IL-1b and IL-6 and 25 cycles for GAPDH. The PCR products were separated on ethidium bromide stained agarose gel and band intensities were measured by dedicated software (Phoretics Int., Newcastle-upon-Tyne, UK). Data are expressed in arbitrary units as the ratio of IL-1b/GAPDH and IL-6/GAPDH band intensities for the same cDNA sample.

Statistical analysis. For the statistical analysis a value just below that of the faintest band measurable on the same gel was assigned to PCR products below the limit of detection.

The significance of differences in mRNA levels between the normoperfused and the hypoperfused skeletal biopsies was assessed by two-tailed Student's t-test for paired data. To correlate the levels of gene expression for IL-1b and IL-6 the Pearson product-moment correlation coefficient was used. A probability value of $p < 0.05$ was assumed to be significant.

Results

We examined the levels of gene expression for IL-1b and IL-6 in skeletal muscle biopsies obtained, during

femoral distal bypass, from a normoperfused skeletal muscle and a hypoperfused skeletal muscle of the same lower extremity using semiquantitative RT-PCR. GAPDH mRNA levels were also determined for the same preparations of cDNA and used to normalize for any variation in cDNA quantity.

PCR products for IL-1b were detectable in 5 out of 8 normoperfused biopsies and in all hypoperfused biopsies. The level of gene expression was significantly higher in the hypoperfused biopsy in all but 2 patients with a mean upregulation > 8.8 fold ($p = 0.043$). Individual data are shown in figure 1.

PCR products for IL-6 were detectable in only 3 out of 8 normoperfused biopsies and in 7 out of 8 hypoperfused biopsies. The level of gene expression was significantly higher in the hypoperfused biopsy in all but 1 patient with a mean upregulation > 23.7 fold ($p = 0.031$). Individual data are shown in figure 2.

The levels of gene expression for IL-1b and IL-6 in the hypoperfused biopsies correlated tightly ($r = 0.965$, $p < 0.001$).

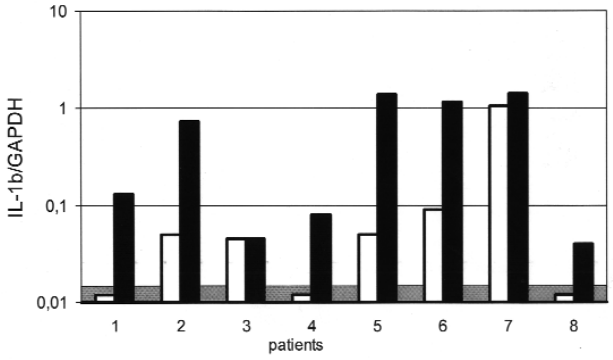

Figure 1. *IL-1β gene expression, normalized for GAPDH levels, in normoperfused biopsies (*empty bars*) and hypoperfused (*solid bars*) biopsies in individual patients numbered 1 to 8. The* bars within the gray area *represent undetectable PCR products to which a value just below the limit of detectability was assigned for statistical purposes. IL-1b/GAPDH = ratio of IL-1β/GAPDH band intensities (arbitrary units).*

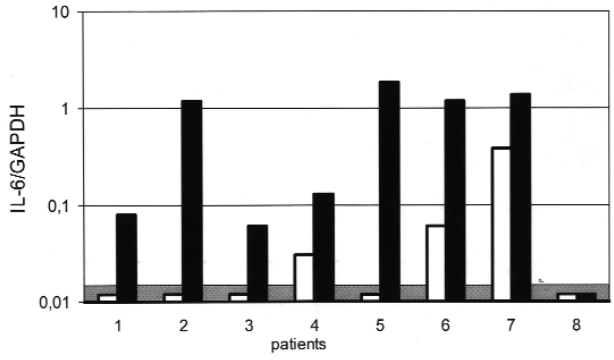

Figure 2. *IL-6 gene expression, normalized for GAPDH levels, in normoperfused biopsies (*empty bars*) and hypoperfused (*solid bars*) biopsies in individual patients numbered 1 to 8. The* bars within the gray area *represent undetectable PCR products to which a value just below the limit of detectability was assigned for statistical purposes. IL-6/GAPDH = ratio of IL-6/GAPDH band intensities (arbitrary units).*

Discussion

In investigating possible sources of inflammatory mediators in atherothrombosis, most of the attention has been focused on the arteries where atherosclerotic plaques are localized or on circulating inflammatory cells. However, the possible contribution of the ischemic tissue, and in particular its microcirculation, has not been thoroughly evaluated.

In this paper we report that IL-1b and IL-6 gene expression is markedly upregulated in hypoperfused skeletal muscle of patients with critical lower limb ischemia. While other investigators have reported on circulating markers of inflammation in patients with peripheral arterial disease[4,5], to our knowledge this is the first report of a local activation of the inflammatory cascade at the level of hypoperfused skeletal muscle.

In our study the hypoperfused skeletal muscle in one limb was compared with normoperfused skeletal muscle in the same patient. For this reason, the non-homogeneity in risk factors, associated diseases, and drug regimens among patients is of little significance for a qualitative analysis of the differences in cytokines gene expression between the normoperfused and the hypoperfused tissue. We found a several-fold upregulation of both IL-6 and IL-1b in the biopsies from hypoperfused muscles in the majority of patients. However, both the levels of gene expression in the normoperfused (control) biopsies and the extent of the upregulation were highly heterogeneous. The reasons for this heterogeneity are not apparent from our data, and may actually be linked to the above-mentioned differences among patients as well as to differences in the severity of hypoperfusion. Genetic polymorphisms might also be involved. Recently it has been shown that a polymorphism involving a guanine/cytosine substitution at -174 in the 5' flanking region of the IL-6 gene[16] affects the IL-6 response to stimuli.

We believe that our preliminary findings have several relevant pathophysiological, clinical and therapeutic implications. The molecular alterations induced by ischemia and reperfusion have extensively been investigated in the heart[17-20], while little data are available on skeletal muscle. Several cytokines, including IL-1b and IL-6, are induced during myocardial reperfusion and different molecules are thought to variably affect the processes related to ischemia and reperfusion. Prior studies on how sustained ischemia *per se* affects cytokine gene expression have been highly inconsistent[17,20-22]. Our data indicate that inflammatory pathways are already activated in chronically hypoperfused skeletal muscle. This activation, possibly due to chronic ischemia, repeated burst of ischemia and reperfusion or altered levels of shear stress, could constitute a base for further activation during therapeutic reperfusion, and consequent reperfusion injury, and might be considered as a therapeutic target. Whether blunting the chronic inflammatory activation can alter ischemia-reperfusion

injury remains to be investigated. It is also likely that different cytokines play contrasting roles in the pathophysiology of ischemia-reperfusion injury (for instance a cardioprotective role has recently been attributed to IL-6)[9] and that therapeutic intervention should be targeted to specific molecules rather than to the whole inflammatory cascade. Even when reperfusion is not a clinically available option, local inflammatory activation, which might worsen symptoms and tissue viability, could be considered as a therapeutic target. Finally inflammation might accelerate the progression of atherosclerotic lesions.

It remains to be investigated whether our results, as well as the possible pathophysiological and clinical implications, may also apply to coronary artery disease.

References

1. Biasucci LM, Vitelli A, Liuzzo G, et al. Elevated levels of interleukin-6 in unstable angina. Circulation 1996; 94: 874-7.
2. Biasucci LM, Liuzzo G, Fantuzzi G, et al. Increasing levels of interleukin (IL)-1Ra and IL-6 during the first 2 days of hospitalization in unstable angina are associated with increased risk of in-hospital coronary events. Circulation 1999; 99: 2079-84.
3. Ikonomidis I, Andreotti F, Economou E, Stefanidis C, Toutouzas P, Nihoyannopoulos P. Increased proinflammatory cytokines in patients with chronic stable angina and their reduction by aspirin. Circulation 1999; 100: 793-8.
4. Baba T, Uchimura I, Fujisawa K, et al. Production of interleukin-6 induced by hypoxia linked to peripheral arterial disease. Ann NY Acad Sci 1997; 811: 542-8.
5. Fiotti N, Giansante C, Ponte E, et al. Atherosclerosis and inflammation. Patterns of cytokine regulation in patients with peripheral arterial disease. Atherosclerosis 1999; 145: 51-60.
6. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996; 87: 2095-147.
7. Hirano T. Interleukin 6 and its receptor: ten years later. Int Rev Immunol 1998; 16: 249-84.
8. Yan SF, Tritto I, Pinsky D, et al. Induction of interleukin 6 by hypoxia in vascular cells. J Biol Chem 1995; 270: 11463-71.
9. Gwechenberger M, Mendoza LH, Youker KA, et al. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. Circulation 1999; 99: 546-51.
10. LaPointe M, Isenovic E. Interleukin-1b regulation of inducible nitric oxide synthase and cyclooxygenase-2 involves the p42/44 and p38 MAPK signaling pathways in cardiac myocytes. Hypertension 1999; 33 (Part II): 276-82.
11. Ridley SH, Sarsfield SJ, Lee JC, et al. Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase. Regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. J Immunol 1997; 158: 3165-73.
12. Beyaert R, Cuenda A, Vanden Berghe W, et al. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor. EMBO J 1996; 15: 1914-23.
13. Yan SF, Zou YS, Mendelsohn M, et al. Nuclear factor interleukin 6 motifs mediate tissue-specific gene transcription in hypoxia. J Biol Chem 1997; 272: 4287-94.
14. Morrone C, Ciliberto G, Oliviero S, et al. Recombinant interleukin 6 regulates the transcriptional activation of a set of human acute phase genes. J Biol Chem 1988; 61: 588-602.
15. European Working Group on Critical Limb Ischemia. Second European consensus document on chronic critical leg ischemia. Circulation 1991; 84 (Suppl): IV1-IV26.
16. Fishman D, Faulds G, Jeffery R, et al. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. J Clin Invest 1998; 102: 1369-76.
17. Chandrasekar B, Mitchell DH, Colston JT, Freeman GL. Regulation of CCAAT/enhancer binding protein, interleukin-6, interleukin-6 receptor, and gp130 expression during myocardial ischemia/reperfusion. Circulation 1999; 99: 427-33.
18. Chandrasekar B, Streitman JE, Colston JT, Freeman GL. Inhibition of nuclear factor kB attenuates proinflammatory cytokine and inducible nitric-oxide synthase expression in postischemic myocardium. Biochim Biophys Acta 1998; 1406: 91-106.
19. Frangogiannis NG, Youker KA, Rossen RD, et al. Cytokines and the microcirculation in ischemia and reperfusion. J Mol Cell Cardiol 1998; 30: 2567-76.
20. Kukielka GL, Wayne Smith C, Manning AM, et al. Induction of interleukin-6 synthesis in the myocardium. Potential role in postreperfusion inflammatory injury. Circulation 1995; 92: 1866-75.
21. Muraoka KI, Shimizu K, Sun X, et al. Hypoxia, but not reoxygenation, induces interleukin 6 gene expression through NF-kB activation. Transplantation 1997; 63: 466-70.
22. Herskowitz A, Choi S, Ansari AA, Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. Am J Pathol 1995; 146: 419-28.