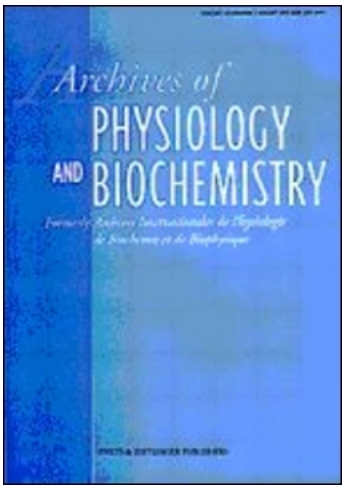

Archives Of Physiology And Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713817673

Biology of peripheral blood cells in obstructive sleep apnea - the tip of the iceberg

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Online Publication Date: 01 October 2008

To cite this Article Lavie, Lena, Dyugovskaya, Larissa and Polyakov, Andrey(2008)'Biology of peripheral blood cells in obstructive sleep apnea - the tip of the iceberg',Archives Of Physiology And Biochemistry,114:4,244 — 254
To link to this Article: DOI: 10.1080/13813450802306701
URL: http://dx.doi.org/10.1080/13813450802306701

Archives of Physiology and Biochemistry, October 2008; 114(4): 244–254

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REVIEW ARTICLE

Biology of peripheral blood cells in obstructive sleep apnea – the tip of the iceberg

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Abstract

Obstructive sleep apnea (OSA), a highly prevalent breathing disorder in sleep, characterized by intermittent and recurrent pauses in respiration, has emerged as an independent risk factor for cardiovascular morbidity and mortality. Accumulated evidence implicates Leukocyte-endothelial cell activation and adhesion as critical components that induce inflammation and injury to the vasculature resulting in the development of cardiovascular complications. Similar cellular interactions were described in conditions of ischemia/reperfusion, and various components of the metabolic syndrome as hypercholesterolemia and hypertension. The hallmark of sleep apnea – the multiple cycles of hypoxia/reoxygenation – promote oxidative stress and inflammation. These facilitate increased interactions of blood cells with endothelial cells, resulting in endothelial cell injury and dysfunction. Such events can promote atherosclerosis and the development of cardiovascular morbidities in OSA. However, inter-individual differences in response to intermittent hypoxia and activation of anti-inflammatory cytokine profiles in T lymphocytes can serve as protective mechanisms.

Key words: *Obstructive sleep apnea, inflammation, leukocytes, platelets, erythrocytes, endothelial cells.*

Introduction

Sleep medicine has witnessed an unprecedented growth in the last two decades due to the growing awareness of obstructive sleep apnea (OSA) and its profound impact on patients' quality of life and health (Tachibana *et al.*, 2005). OSA is a prevalent syndrome characterized by recurrent pauses in respiration, which result in cyclic decreases in blood oxygen saturation and sleep fragmentation. At least 4% of men and 2% of women are affected by OSA and display at least five disordered breathing events in each hour of sleep in the form of apneas or hypopneas (Punjabi, 2008; Young *et al.*, 1993). Apneas are complete cessations of breathing for at least 10 sec duration, while hypopneas are partial cessations for the same duration [defined as apnea-hypopnea-index (AHI) denoting the number of breathing arrests per hour of sleep]. These intermittent breathing arrests during sleep which are the hallmark of OSA syndrome are considered analogous to hypoxia/reperfusion injury. However, several characteristic symptoms such as excessive daytime sleepiness, chronic fatigue or neurocognitive decline are an integral part as well (Young *et al.*, 1993). Specifically, the syndrome is associated with the male gender, middle age, central obesity, smoking, sedentary life-style, and a post-menopausal status in women (Young *et al.*, 2002b). However, the most dramatic impact is on the cardiovascular system (Lavie *et al.*, 2000; Smith *et al.*, 2002), particularly in relatively young populations of 50 years or younger (Lavie *et al.*, 2005). In recent years OSA was also shown to be associated with many risk factors of the metabolic syndrome such as hyperlipidemia, insulin resistance, hypertension and obesity. These may constitute additional risk factors for cardiovascular morbidity in OSA by acting synergistically with the apnoeic events to increase cardiovascular risk (Newman *et al.*, 2001; McArdle *et al.*, 2007; Punjabi *et al.*, 2002; Reichmuth *et al.*, 2005; Lam & Ip, 2007; Meslier *et al.*, 2003; Peppard *et al.*, 2000; Young *et al.*, 2002a; Basta & Vgontzas 2007).

The association between OSA and cardiovascular morbidity has been attributed to several mechanisms as increased activation of the sympathetic nervous

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Received for publication 24 March 2008. Accepted 13 May 2008.

ISSN 1381-3455 print/ISSN 1744-4160 online © 2008 Informa UK Ltd.
DOI: 10.1080/13813450802306701

system, swings in intrathoracic pressure, altered blood coagulability, and, more recently to oxidative stress and inflammation (Carleson *et al.*, 1993; Shamsuzzaman *et al.*, 2003; Lavie, 2003). Of these, oxidative stress and inflammatory responses, which are fundamental mechanisms underlying atherosclerosis, are by far the most attractive proposed. Oxidative stress and subsequently inflammation and the acceleration of atherogenic processes are uniquely triggered by the apnea-related intermittent hypoxia (Lavie, 2003; Suzuki *et al.*, 2006). Moreover, these mechanisms are also integral parts of obesity, hypertension, and type 2 diabetes (Hotamisligil, 2006; Rudich *et al.*, 1998; Lassègue & Griendling, 2004; Stokes & Granger, 2005; Meigs *et al.*, 2007; Furukawa *et al.*, 2004). These involve activation of complex pathways and cellular interactions which culminate in endothelial dysfunction as a prelude to atherosclerosis.

The current review outlines the major epidemiological studies pointing to increased cardiovascular morbidity and associated risk factors which cluster with sleep apnea. The second part summarizes the current literature on phenotypic and functional changes and the inflammatory responses of various blood cells. The interactions of leukocytes/endothelial cells and the possible cellular mechanisms which are initiated by the breathing arrests during sleep are described, as well as their possible involvement in the development of cardiovascular morbidity via endothelial cell injury and dysfunction.

OSA and cardiovascular morbidity

The evidence supporting the association of OSA with cardiovascular morbidity is strong. At the time of diagnosis, at least 50% of the sleep apnea patients are hypertensive and 10–15% have cardiovascular disease including myocardial infarction, and stroke (Lavie *et al.*, 2000; Smith *et al.*, 2002). On the other hand, breathing disorders in sleep are prevalent among non-selected patients with cardiovascular diseases (Peker *et al.*, 1999; Mooe *et al.*, 1996). Cross-sectional prospective and population based studies support the association between cardiovascular morbidity and breathing disorders in sleep demonstrating that this association is independent of major possible confounding cardiovascular risk factors (Peppard *et al.*, 2000; Nieto *et al.*, 2000; Bixler *et al.*, 2000; Young *et al.*, 1997). Several studies also demonstrated that OSA is an independent risk factor for cardiovascular mortality (Lavie, 2008; Lavie *et al.*, 1995; Yaggi *et al.*, 2005), and that effective treatment of the syndrome significantly decreased mortality (Marti *et al.*, 2002; Marin *et al.*, 2005).

The evidence supporting a causal relationship between sleep apnea and hypertension is particularly strong. It is based on large epidemiological studies (Lavie *et al.*, 2000; Nieto *et al.*, 2000; Bixler *et al.*, 2000; Young *et al.*, 1997) and prospective studies (Peppard *et al.*, 2000). Furthermore, treatment with nasal continuous positive airway pressure (nCPAP) that ameliorated the apneas also lowered blood pressure (see a recent review in Bazzano *et al.*, 2007). These data on OSA as an independent risk factor for cardiovascular morbidity and the evidence on the mortality risk in these patients are summarized in recent reviews (McNicholas *et al.*, 2007; Lavie, 2007).

The obesity epidemic which emerged in the last two decades as one of the major cardiovascular risk factors is also associated with OSA. In many epidemiological, cross-sectional, clinic-based and population-based studies visceral obesity, in particular, was positively correlated with cardiovascular disease in these patients (Young *et al.*, 2002a; Basta & Vgontzas 2007). Notably, between 60% and 90% of OSA patients are obese (Anstead & Phillips, 1999). Similarly to OSA, obesity is associated with the male gender, post menopausal status in women, cardiovascular morbidity, hypertension, stroke, insulin resistance and type 2 diabetes (Kopelman, 2000; Wisse *et al.*, 2007). Although the nature of this association in OSA is not clear, the severity of OSA was shown to be aggravated by gaining weight, and improvement was noted under massive weight reduction by a controlled diet (Smith *et al.*, 1985) or by surgical means (Charuzi *et al.*, 1985).

Similarly, the prevalence of dyslipidemia, insulin resistance, glucose tolerance and type 2 diabetes, which constitute major risk factors for cardiovascular morbidity, were also found to be high among patients with OSA. These data on the associations of OSA with these cardiovascular risk factors are summarized in several reviews and are beyond of the scope of the current paper (Young *et al.*, 2002a; McNicholas *et al.*, 2007). All in all, there is sufficient data to suggest that cardiovascular morbidities and the associated metabolic syndrome risk factors are prevalent among patients with OSA.

Endothelial dysfunction and atherosclerosis in OSA

A large body of evidence implicates endothelial dysfunction in various conditions including the metabolic syndrome and sleep apnea. While in the normal state the endothelium regulates the vascular tone and interactions between the vessel wall and circulating substances and blood cells, endothelial dysfunction promotes an activated state. As a result, the anti-coagulant and anti-inflammatory endothelium acquires a pro-thrombotic and pro-inflammatory phenotype which initiates atherosclerosis (Libby, 2002; Davignon & Ganz, 2004). In endothelial dysfunction, the vasodilatation of the blood vessels is compromised due to decreased nitric oxide (NO) bioavailability. The impact of NO relies on its vasodilatory and protective properties which limit leukocyte recruitment and leukocyte expression of

adhesion molecules, and prevent the proliferation of vascular smooth muscle cells and aggregation/adhesion of platelets. Thus, it protects from the development of atherosclerosis. Exposure to oxidative stress, to inflammatory mediators or hypercholesterolemia, all of which promote endothelial cell activation and endothelial dysfunction, impair NO bioavailability (Libby, 2002; Davignon & Ganz, 2004; Lavie, 2004). In patients with OSA, several studies have demonstrated diminished NO bioavailability that was restored after nCPAP treatment (Schulz *et al.*, 2000b; Ip *et al.*, 2000; Lavie *et al.*, 2003). Moreover, several measures that represent early signs of atherosclerosis were shown to be elevated in OSA including increased intima-media thickness that was also severity dependent (Drager *et al.*, 2007; Minoguchi *et al.*, 2005; Drager *et al.*, 2005), arterial plaque formation (Kaynak *et al.*, 2003), calcified artery atheromas (Freidlander *et al.*, 1998), and higher pulse wave velocity (Nagahama *et al.*, 2004; Drager *et al.*, 2005). Thus, cardiovascular morbidity in sleep apnea is foreseeable. Moreover, the impact of OSA on endothelial dysfunction is further demonstrated by showing that such early signs of atherosclerosis can be improved after 3 or 4 months of treatment with nCPAP or dental device (Drager *et al.*, 2007; Itzhaki *et al.*, 2007).

Cellular and molecular mechanisms in OSA

Inflammatory pathway activation

Reactive oxygen species (ROS) and a state of oxidative stress are considered potent activators of inflammatory pathways. One of the more studied pathways of inflammatory cell activation is the upregulation of the transcription factor nuclear factor (NF) κB, which is largely affected by oxidative stress. The activation of NFκB induces increased expression of adhesion molecules and inflammatory cytokines in many cell types including leukocytes and endothelial cells (Lavie, 2008). Its up-regulation was demonstrated in polymorphonuclear leukocytes (PMNs) and monocytes of patients with OSA (Htoo *et al.*, 2006; Greenberg *et al.*, 2006; Yamauch *et al.*, 2006). Similar findings were also documented in a tissue culture model utilizing HeLa cells that were exposed to intermittent hypoxia *in vitro* (Ryan *et al.*, 2005). Moreover, intermittent hypoxia in vitro was shown to activate the NFκB in an I kappa B kinase (IKK) dependent manner, at least in part, via activation of p38 mitogen activated protein kinase (MAPK) (Ryan *et al.*, 2007). Thus, the ROS molecules produced in response to intermittent hypoxia initiate a cascade of inflammatory pathways resulting in over-expression of adhesion molecules and pro-inflammatory cytokines. These adhesion molecules facilitate the recruitment and accumulation of leukocytes and platelets on the endothelial cells lining the vasculature and promote PMNs / monocyte / lymphocyte / platelets / endothelial cells interactions. Such cellular interactions between blood cells and endothelial cells may result in injury to the endothelium (Lavie, 2008; Lavie, 2003).

Blood cell activation and expression of adhesion molecules

In the normal state circulating leukocytes and endothelial cells express basal levels of adhesion molecules. However, upon encounter with a variety of stimuli or insults including inflammation, infections, hypercholesterolemia, cytokines, hypoxia/re-oxygenation and sleep apnea, their expression is up-regulated. The expression of adhesion molecules is a highly regulated and sequential process and occurs in both endothelial cells and leukocytes. Up-regulated expression of adhesion molecules augments the interactions between these cell types and promotes the adherence of leukocytes to the vascular endothelium. Initially increased expression of selectins (L-selectins in leukocytes, E-selectins in endothelial cells and P-selectins in platelets and endothelial cells) facilitates weak binding of the leukocytes to endothelial cells. A firm binding is mediated by the integrins which also mediate transmigration into the interstitial layer through the endothelial cell layer (Libby, 2002; Panés & Granger, 1998). The interactions between endothelial cells and various leukocyte subpopulations of patients with OSA, including monocytes, polymorphonuclear leukocytes (PMNs), and numerous cytotoxic T cells expressing CD8, CD4, and $\gamma\delta$ molecules were rigorously investigated in our laboratory (Dyugovskaya *et al.*, 2002; Dyugovskaya *et al.*, 2005a; Dyugovskaya *et al.*, 2003; Dyugovskaya *et al.*, 2005b; Lavie *et al.*, 2005; Dyugovskaya *et al.*, 2008). A summary of the main phenotypic and functional changes of these cells in OSA is depicted in Table I.

Polymorphonuclear leukocytes (PMNs). The PMNs best known for their classical role as professional phagocytes are the most abundant of the leukocyte sub-populations, representing approximately 60% of all circulating leukocytes. They are short lived (up to 24 h in the blood stream) terminally differentiated cells that continuously undergo cell death by apoptosis. The constitutive apoptotic program controls and limits their life span and by that protects surrounding cells and tissues from their injurious compounds, i.e. ROS molecules, bactericidal proteins, lytic enzymes and leukotriens, which participate in inflammatory responses against invading micro-organisms, foreign particles or cellular debris. Another key feature of PMNs biology is their ability to express growth factors, inflammatory cytokines and chemokines, cell surface receptors, and adhesion molecules. Moreover, as they are the first to be recruited to inflammatory sites it is conceivable that they actively contribute to the sequential recruitment

Table I. The distribution of the main leukocyte adhesion molecules, markers, adhesion indices and reactive oxygen species (±SD) in fresh whole blood cells.

	Control	OSA	Cited by
PMNs			
CD15 (MFI)	1048 ± 370	1484 ± 470*	Dyugovskaya *et al.* (2008)
CD62L (MFI)	48.3 ± 14.7	112.3 ± 22.0*	Unpublished data
CD11b (MFI)	121.0 ± 64.0	139.0 ± 62.0 (ns)	Unpublished data
CD11c (%)	29.3 ± 26.8	27.0 ± 22.4 (ns)	Dyugovskaya *et al.* (2008)
Low-CD16 (%)	2.8 ± 0.9	1.0 ± 0.6*	Dyugovskaya *et al.* (2008)
Adhesion index	3.72 ± 0.74	3.85 ± 0.45 (ns)	Dyugovskaya *et al.* (2002)
ROS (MFI)	1079 ± 355	1482 ± 358*	Dyugovskaya *et al.* (2002)
Superoxide (nmoles/5×10^6 cells)	3.7 ± 0.5	9.3 ± 1.5*	Schulz *et al.* (2000a)
Monocytes			
CD15 (%)	1.4 ± 0.9	12.4 ± 7.4*	Dyugovskaya *et al.* (2002); Lavie *et al.*, (2005)
CD11c (%)	25.5 ± 12.5	54.8 ± 19.6*	Dyugovskaya *et al.* (2002)
Adhesion index	5.20 ± 1.80	9.80 ± 0.49*	Dyugovskaya *et al.* (2002)
ROS (MFI)	716 ± 198	962 ± 252*	Dyugovskaya *et al.* (2002)
Lymphocytes			
$\gamma\delta$ T cells/CD62L (%)	49.6 ± 11	65.0 ± 11.7*	Dyugovskaya *et al.* (2003)
$\gamma\delta$ T cells/adhesion index	3.60 ± 0.90	6.10 ± 0.90*	Dyugovskaya *et al.* (2003)
CD8+/CD40L+ (%)	3.4 ± 2.5	14.4 ± 10.5*	Dyugovskaya *et al.* (2005b)
CD8+/CD62L+ (%)	47.1 ± 14.8	44.0 ± 14.0 (ns)	Dyugovskaya *et al.* (2005b)
CD8+/adhesion index	3.54 ± 1.40	3.90 ± 0.90 (ns)	Unpublished data
CD4+/$CD28^{null}$ (%)	6.8 ± 1.3	19.9 ± 5.0*	Dyugovskaya *et al.* (2005b)
CD4+/CD62L+ (%)	75.3 ± 13.6	79.1 ± 7.9 (ns)	Dyugovskaya *et al.* (2005b)
CD4+/adhesion index	3.47 ± 1.4	3.90 ± 2.40 (ns)	Unpublished data
ROS (MFI) – all lymphocytes	209 ± 54	299 ± 79*	Unpublished data

*Statistically significant, with $p < 0.05$ at least; ns, statistically not significant; MFI, mean fluorescence intensity, denoting intensity of expression; %, representing the percentage of cells from the population which express a specific molecule; ROS, reactive oxygen species. Adhesion index was determined on human umbilical vein endothelial cells (HUVECs) in culture.

of different leukocyte populations to inflammatory sites similarly to conditions characterized by ischemia and reperfusion (McDonald, 2004). Interestingly, PMNs were shown to infiltrate eroded or ruptured plaques obtained from patients with acute coronary syndromes (Naruko *et al.*, 2002; Zidar *et al.*, 2005) and to participate in the pathogenesis of lethal myocardial reperfusion (Vinten-Johansen, 2004). Their involvement in amplifying cardiovascular morbidity was further established by depletion experiments which resulted in reduced myocardial infarct size and a protected myocardium (Jolly *et al.*, 1986; Kin *et al.*, 2006).

Investigating PMNs' life span, functions and adhesive properties in patients with OSA revealed increased production of ROS molecules and attenuation by nCPAP (Schulz *et al.*, 2000a; Dyugovskaya *et al.*, 2002). Increased expression of selectins (of the family of adhesion molecules which mediate capture and tethering) CD62 and the CD15 (which is a carbohydrate complex on selectins) of PMNs was noted in OSA as well (Table I). The up-regulated CD15 expression in OSA was also severity dependent as attested by the number of apnoeic events. However, no differences where noted between OSA and controls up to 15 apnea-hypopnea events per hour. Treatment with nCPAP effectively lowered the expression of CD15 (Dyugovskaya *et al.*, 2008). Interestingly, the expression of CD11b (Vishnevsky *et al.*, unpublished observations) or CD11c, the β-subunits of the integrins (and counter receptors for ICAM-1 on endothelial cells responsible for firm adhesion) was unaffected (Table I). This is not surprising given that adhesion of OSA PMNs to endothelial cells in vitro was also unaffected (Dyugovskaya *et al.*, 2002). The fact that selectins but not integrins of OSA patients PMNs were up-regulated implies that the interactions involving binding and tethering with endothelial cells are increased but not the firm adhesion. In addition PMN apoptosis, a fundamental injury-limiting mechanism and a key event in the control of inflammation, was also suppressed in OSA PMNs. Such suppressed apoptosis may imply that the PMNs/endothelial cells interactions initiated by the selectins could be exacerbated and by that amplify the destructive potential of PMNs towards the endothelium. It should be noted that the apneic events (AHI) but not sleep fragmentation were identified as an independent predictor of the percentage of apoptotic PMNs. Moreover, similar data were obtained in PMNs from healthy individuals exposed to experimental intermittent hypoxia in vitro (Dyugovskaya *et al.*, 2008). Thus, together with increased expression of selectins, suppressed PMNs apoptosis may promote endothelial injury in OSA.

Monocytes. Like PMNs, monocytes are best known for their classical role as professional phagocytes. However, unlike PMNs, monocytes are long lived and their initiation, participation in progression and persistence of atherosclerosis, is well established

(Libby, 2002, 2007). In inflammatory conditions they express adhesion molecules and release large quantities of ROS and inflammatory cytokines. Indeed ROS production by OSA monocytes, was increased as compared to controls (Table I). Apart from ROS, monocytes of patients with OSA were shown to express increased CD15 and CD11c adhesion molecules. Moreover, their increased expression of CD15 was dependent on the severity of the syndrome (Lavie *et al.*, 2005). Also, treatment of monocytes from healthy individuals with hypoxia *in vitro*, resulted in up-regulated CD15 expression. Unlike in PMNs, the CD11c integrin of OSA monocytes was also elevated, while treatment with nCPAP attenuated the levels of both CD15 and CD11c. Accordingly, increased adhesion of OSA monocytes was noted towards endothelial cells of venous (HUVEC) and arterial origin (HCAEC). By utilizing antibodies that neutralize selectins (anti-CD62) and integrins (anti-CD54) adhesion of monocytes to endothelial cells was attenuated to values comparable to controls (Dyugovskaya *et al.*, 2002).The involvement of monocytes in atherogenesis in OSA was further implicated by the observation that lipid uptake was increased in human macrophages that were treated with experimental intermittent hypoxia in vitro (Lattimore *et al.*, 2005).

T lymphocytes. The participation of various lymphocyte subpopulations was primarily implicated in atherogenesis through cytokine secretion and antibody production. Lymphocytes were shown to be prevalent in atherosclerotic lesions and to modulate atherosclerotic responses (Vanderlaan & Reardon, 2005; Song *et al.*, 2001). Natural killer (NK) lymphocytes, CD8+, CD4+, and $\gamma\delta$ T cells were all implicated in atherosclerotic sequelae, which further add to the complexity of atherosclerosis. Numerous T lymphocyte subpopulations were investigated in patients with OSA. Basically, all T cells studied in OSA (CD8+, CD4+, and $\gamma\delta$ T cells) express an activated and a cytotoxic phenotype. Assessment of $\gamma\delta$ T cells phenotype, and function revealed that expression of CD62L selectins was increased as compared to controls (Table I). Also adhesion to endothelial cells and cytotoxicity towards endothelial cells were higher in OSA. The higher avidity and cytotoxicity of OSA $\gamma\delta$ T cells were mainly attributed of the pro-inflammatory cytokine tumor-necrosis-factor-α (TNF-α). This since utilizing antibodies which neutralize TNF-α, abolished the cytotoxicity against endothelial cells. Cytotoxic receptors as CD56 did not seem to participate in this process in $\gamma\delta$ T cells since elimination of the CD56 bearing cells did not alter the cytotoxicity against endothelial cells (Dyugovskaya *et al.*, 2003).

Unlike in $\gamma\delta$ T cells, adhesion of CD4+ and CD8+ T cells to endothelial cells was unaffected by OSA (Table I). However, cytotoxicity towards endothelial cells was increased in both CD8+ and CD4+ T cells of OSA patients. The killing abilities of CD8+ T lymphocytes were found to be apnea-hyponea-index (AHI) severity dependent. Yet, each subpopulation employed different killing mechanisms to damage endothelial cells (Dyugovskaya *et al.*, 2005a; Dyugovskaya *et al.*, 2005b; Dyugovskaya *et al.*, 2003). While endothelial cell killing by $\gamma\delta$+ T lymphocytes was primarily mediated by TNF-α, CD8+ and CD4+ T lymphocytes utilized various other mechanisms. For instance, CD8+ T lymphocytes expressed more then three-fold of the CD40 ligand (CD40L) which is an important T cell activation marker and implicated in inflammatory cell activation in atherogenesis (Marx *et al.*, 2003). Additionally, OSA CD8+ T cells expressed higher amounts of the CD56 natural killer receptors and higher perforin levels which account for their higher cytotoxicity. Depletion of CD8+ T cells co-expressing CD56+ receptors greatly attenuated the cytotoxicity towards endothelial cells (Dyugovskaya *et al.*, 2005a). The CD4+ T cells of OSA patients contained about three-fold higher amounts of the subpopulation CD4+/CD^{28null} (Table I). These CD4+/CD^{28null} T cells are known to induce killing of endothelial cells (Liuzzo *et al.*, 2000). Yet it should be noted that the strongest cytotoxicity against endothelial cells was expressed by OSA $\gamma\delta$ T cells, CD8+ cytotoxicity was somewhat lower and that of CD4+ T cells was the lowest.

Platelets. Platelets are best known for their functions in maintaining vascular homeostasis by clot formation and wound healing. Under physiological conditions platelets circulate in a quiescent state protected from activation by inhibitory mediators released from intact endothelial cells, including NO. However, in response to endothelial dysfunction or when encountering vascular damage or under oxidative stress, platelets rapidly undergo activation, followed by interactions with monocytes and PMNs and increased adhesion and aggregation in the vessel wall (Zarbock *et al.*, 2007). This implicates their involvement in atherosclerosis. Similarly to circulating leukocytes, also platelets have been shown to acquire an activated and a pro-thrombotic phenotype in response to hypoxia/reoxygenation (Gavins *et al.*, 2007). Platelets from patients with OSA expressed increased activation and aggregability *in vitro*. The percentage of platelets expressing P-selectin (CD62P) was higher (Bokinsky *et al.*, 1995; Geiser *et al.*, 2002), mainly in the severe group of patients (Eisensehr *et al.*, 1998) and was effectively lowered by treatment with nCPAP (Hui *et al.*, 2004). In addition increases in hematocrit, blood viscosity, and fibrinogen, in patients with OSA could further affect hypercoagulability (Hoffstein *et al.*, 1994; Nobili *et al.*, 2000). Also, in an ongoing study in our lab, we found that platelets of patients with OSA form higher amounts of platelets/monocytes aggregates as

compared to their age, sex, and body mass index (BMI) matched controls (Vishnevsky *et al.*, unpublished observations). Owing to the fact that platelets were shown to play a key role in ischemic cardiovascular diseases, their altered activation state and hyper-aggregability may contribute to increased cardiovascular morbidity in OSA as well.

Collectively, the higher expression of adhesion molecules on platelets and leukocytes, the higher avidity and the ability to strongly attach to endothelial cells in culture conditions, the stronger cytotoxicity of T cells against endothelial cells, the delayed apoptosis expressed by PMNs, the higher ROS generated by monocytes and PMNs, and the higher aggregability of platelets are all markers of activation of the various blood cells investigated and can serve as markers of the possible ongoing processes that may damage the endothelium and initiate atherogenesis in patients with OSA.

Erythrocytes. The red blood cells (RBCs) constitute the major cell type in the circulation. Their primary role is the transport of oxygen to all tissues and organs. Under normal blood flow their adherence to endothelial cells is non-significant and their deformability facilitates tissue perfusion. Under hypoxic/ischemic conditions RBCs are capable of inducing and participating in inflammatory responses (Madjdpour *et al.*, 2003). However, which mechanisms are involved or whether they contribute to inflammation is unclear. Nonetheless, ROS molecules and redox sensitive transcription factors were proposed as major determinants. RBCs' adhesiveness and aggregation were shown to be elevated in cardiovascular risk factors as hypertension (Kesler *et al.*, 2006), atherosclerosis (Rotstein *et al.*, 2002) and obesity (Samocha-Bonet *et al.*, 2004). More recently, OSA was found to be associated with increased RBC aggregation/adhesion, which was correlated with an increase in the inflammatory marker CRP (Peled *et al.*, 2008). Being the major component in the circulation – the effects of hypoxia or intermittent hypoxia on RBCs functions and adhesive properties should be considered. This in particularly is important in view of the fact that RBC aggregation/adhesion is also affected by various cardiovascular risk factors.

Activated endothelial cells. As with leukocytes and platelets, endothelial cell activation results in up-regulated expression of adhesion molecules and pro-inflammatory cytokines and promotes cellular interactions. In their non-activated state endothelial cells resist adhesion with leukocytes, platelets and RBCs. However, activation or injury by various factors as hypercholesterolemia, obesity, hypertension and hypoxia/re-oxygenation triggers the expression of adhesion molecules, which mediate these interactions (Gavins *et al.*, 2007; Packard & Libby, 2008). Until recently most findings on endothelial cell activation in OSA relied on indirect evidence. For instance, the presence of soluble variants of adhesion molecules in the circulation of patients with OSA which originated from endothelial cells is indicative of their involvement. These variant adhesion molecules are shed when endothelial cells undergo activation. Several well established such molecules were identified including E-selectin and P-selectin which is stored in intracellular granules and released from activated platelets and endothelial cells (Zamarrón-Sanz *et al.*, 2006; Minoguchi *et al.*, 2007). Likewise, the intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were identified in the plasma of patients with OSA (Zamarrón-Sanz *et al.*, 2006; Ohga *et al.*, 1999; Ursavaş *et al.*, 2007; Chin *et al.*, 2000). These circulating adhesion molecules are regarded as markers of active atherosclerotic diseases, and as predictors of future cardiovascular disease (Lavie, 2005). Additionally, soluble P-selectin (sP-selectin) levels reported to be higher in OSA patients were also negatively correlated with PMN apoptosis. This indicates a possible involvement of P-selectin in the inhibition of PMN apoptosis as observed in OSA, which may exacerbate PMNs functions to injure the endothelium (Dyugovskaya *et al.*, 2008). Of note, activation of endothelial cells in vivo was recently confirmed in OSA by harvesting endothelial cells obtained from veins. In that study, the authors demonstrated that OSA directly affects the vascular endothelium by promoting oxidative stress and inflammation while decreasing NO bioavailability and repair capacity (Jelic *et al.*, 2008).

Pro-inflammatory cytokines

Pro-inflammatory cytokines, like adhesion molecules, are affected by the redox state of the cells in which they are synthesized and actively participate and modulate inflammatory responses. Various inflammatory cytokines are synthesized and released by inflammatory cells. These multi-purpose molecules regulate both the innate and adaptive immune system. Cytokines regulate macrophage activation via expression of scavenger receptors and secretion of metalloproteinases, modulate the proliferation of smooth muscle cells, the production of nitric oxide and apoptosis, and stimulate the activation of endothelial cells. All of which are steps in the progression of atherosclerosis. The main cytokines investigated in OSA include: Tumor necrosis factor-α (TNF-α) that affects the initiation and progression of cardiovascular pathology (von der Thüsen *et al.*, 2003; Ridker *et al.*, 2000), interleukin-6 (IL-6), interleukin-8 (IL-8) and the anti-inflammatory cytokine IL-10 which were all shown to be affected by OSA. Evidently, once the inflammatory response is initiated, these cytokines can in turn activate NFκB and by that can further exaggerate inflammation. In patients with OSA, increases in pro-inflammatory cytokines levels were primarily found in the circulation (Vgontzas *et al.*, 2000; Constantinidis

et al., 2008). But elevated levels in monocytes (Golan-Shany *et al.*, unpublished observations) and in various cytotoxic T lymphocytes were also observed (Dyugovskaya *et al.*, 2005a; Dyugovskaya *et al.*, 2003). Specifically, in $\gamma\delta$+ T lymphocytes the pro-inflammatory TNF-α was increased and the anti-inflammatory IL-10 was decreased whereas the opposite was noted in controls (Figure 1). Also, the expression of IL-8, a pro-inflammatory cytokine with strong chemoatractant and activating properties for PMNs was shown to increase in $\gamma\delta$ T cells of patients with OSA (Figure 1). This clearly attests to a pro-inflammatory state in these cells. In CD8+ T cells both TNF-α and IL-10 were increased. The percentage of CD8+ cells expressing TNF-α was increased by four-fold whereas an incremental increase of 1.3-fold was noted for IL-10 (Figure 2). By contrast, in CD4+ T cells the percentage of cells expressing TNF-α was unaffected by OSA, but the expression of IL-10 was increased by 4.9-fold compared with control (Figure 2). Altered cytokine balance can result in activated T cells and can lead to their differentiation into effector cells with tissue damaging potential or with abilities to diminish inflammation. Depending on the cytokines produced upon such activation, T cells can be defined as type 1 – those that secrete IL-2 (and IFN-γ), and type 2 – those that secrete IL-4 (but also IL-5, IL-6 and IL-10). Based on the ratios of IL-2 and IL-4, CD4+ and CD8+ T cells of patients with OSA had increased percentage of IL-4 expressing cells than IL-2. This suggests a type 2-dominant cytokine expression, as demonstrated in Figure 2. This shift towards a type 2-dominance may represent a compensatory response of the CD8+ and CD4+ T cells to protect from other inflammatory cells or exacerbated pro-inflammatory responses. As both IL-10 and IL-4 have been shown to possess anti-inflammatory properties they can moderate inflammation (Libby, 2007). It should be stressed, however, that unlike the data presented here on cytokine expression of specific inflammatory/immune cells, the data on cytokines levels in OSA patients thus far were mostly acquired from serum or plasma. Such data represent the overall pool of the cytokines released from various inflammatory cells and adipocytes and therefore cannot delineate a specific inflammatory/anti-inflammatory response or an on-going process as described here for the cytokines investigated in the various cells. The cytokines released by adipocytes, in particular, can pose a problem. Since the fat tissue represents a major source for cytokines/adipokines, obese subjects express higher levels of inflammatory cytokines and low grade inflammation. Such data that were demonstrated in obese and overweight patients undergoing

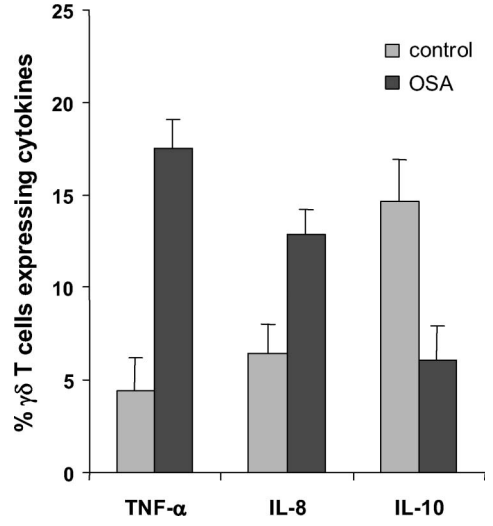

Figure 1. Percentage of $\gamma\delta$ T cells from sleep apnea patients and controls expressing the cytokines TNF-α, IL-8, and IL-10. Data adapted from Dyugovskaya *et al.* (2003).

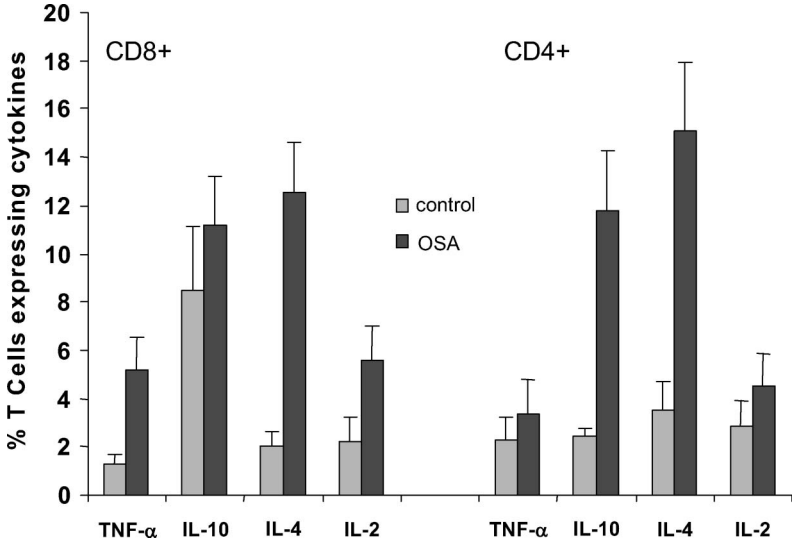

Figure 2. Percentage of CD8+ and CD4+ T cells from sleep apnea patients and controls expressing the cytokines TNF-α, IL-10, IL-4, and IL-2. Data adapted from Dyugovskaya *et al.* (2005b).

surgical treatment (Constantinidis *et al.*, 2008) clearly attest to the need to separate the obesity component from the apneic events.

Conclusion and perspectives

Inflammatory cell activation is by now recognized as a fundamental mechanism in the pathophysiology of cardiovascular morbidity. Moreover, it is also involved in hypertension, hypercholesterolemia, glucose metabolism and obesity, which are all well established cardiovascular risk factors. In recent years the participation of inflammatory cell activation was recognized as an integral part of OSA pathophysiology as well. As specified before, intermittent hypoxia resulting from the apneas has profound effects on redox systems and initiates oxidative stress and inflammation via activation of NFκB and downstream inflammatory/immune pathways. Consequently, up-regulated expression of adhesion molecules, inflammatory cytokines and ROS molecules, are evident in various leukocyte subpopulations and platelets of patients with OSA. These induce increased avidity and cytotoxicity towards endothelial cells. Increased erythrocyte aggregation was also noted in OSA, and should be considered as an important target of research. All in all, these cellular interactions in the vasculature promote endothelial dysfunction and early signs of atherosclerosis.

In view of the fact that many of these cellular events described are OSA severity dependent, it could be expected that all or most patients with OSA, in particularly those with severe OSA would suffer from various cardiovascular morbidities. This, however, is not the case. Even though cardiovascular morbidity and mortality in OSA is higher than in the general population, some OSA patients are free of cardiovascular morbidities suggesting the possible involvement of as yet unidentified protective mechanisms. Previously, we proposed that mechanisms as ischemic preconditioning inducing angiogenesis of newly formed heart collaterals may serve to protect some OSA patients (Lavie & Lavie, 2006). We also demonstrated large inter-individual differences in the angiogenic response to hypoxia that were correlated with arterial collateral formation in the heart. These may help to explain the inter-individual differences in cardiovascular morbidity (Schultz *et al.*, 1999). Here, we propose that CD8+ and even more so CD4+ T cells can participate in such protective mechanisms via shift to type 2 cytokine secretion and dominance, and by that moderate inflammation. Thus, there is a need to elucidate many basic unanswered questions for a better understanding of the sequence of interactions among all blood cells and endothelial cells, and of the cytokines produced by each cell type and the overall cytokine balance. Clarifying which additional adhesion molecules participate, what is the sequence of interactions among inflammatory cells and endothelial cells, do the leukocytes transmigrate to atherosclerotic plaques after activation, or, which of the transcription factors or transduction mechanisms participate? These are only a few examples, and a better understanding of these mechanisms can help in the development of new treatment modalities to prevent cardiovascular morbidity in OSA.

Acknowledgements

This study was supported in part by a grant from the Binational US-Israel foundation (BSF) grant no. 1006695.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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