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Induction of the Anti-Inflammatory Cytokines Interleukins (IL)-10 and Interleukins (IL)-13 in Atherosclerotic Patients

إنتاج السيتوكينات المُضَادة للالتهابات الانترليوكين -10 والانترليوكين -13 في مَرضي تَصَلُّب الشرايين

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ABSTRACT: Interleukin (IL)-10 and IL-13 are cytokines with pleiotropic effects, mainly anti-inflammatory. Little information regarding their role in atherosclerosis is known. Thus, more data are needed to elucidate the link between IL-10 and IL-13 and risk for atherosclerosis. The present work explored the induction of these anti-inflammatory cytokines in atherosclerotic patients at gene and protein levels. In addition, the effects of the chlamydial antigen HSP60 from Chlamydia pneumoniae (C. pneumoniae) and LPS on the induction of IL-10 and IL-13 were examined. Intracellular detection of cytokine mRNA and protein level was assessed by in situ hybridization and immunohistochemistry respectively. The results of these experiments showed significantly high levels of mRNA expressed and protein produced IL-10 and IL-13 in patients compared to healthy controls. Cells stimulated with CHSP60 did reveal neither high mRNA expression nor protein production of both cytokines when comparing healthy subjects versus patients. However, CHSP60 induced more mRNA and protein levels in both healthy subjects and patients when compared to non stimulated cells. Similar results were also depicted when LPS from C. pneumoniae and E. coli were used. Thus, this study demonstrated the induction of the anti-inflammatory cytokine IL-10 and IL-13 at both mRNA and protein levels and that bacterial infection does not promote the production of disease beneficial anti-inflammatory cytokines and thus contribute to the disease by providing an opportunity for disease promoting inflammatory responses to take action..

Keywords: *Atheroma, inflammation, cytokine, mRNA, bacteria.*

المستخلص: الانترليوكين -10 (IL-10) والانترليوكين -13 (IL-13) من السيتوكينات ذات التأثيرات المتعددة خاصة كمضادات للالتهابات. دور هذه السيتوكينات في مرض تصلب الشرايين غير معروف.. وعليه فقد أصبح من المهم إن كان لها دور يتعلق بمسببات هذا المرض. الدراسة الحالية استكشفت إنتاج مضادات الالتهابات هذي على مستوى التخليق وعلى المستوى البروتيني لدى مرضى تصلب الشرايين. وبالإضافة إلى ذلك فقد تم دراسة تأثيرات بروتينات الصدمة الحرارية [Heat shock proteins (hsps)] و الليبولي سكرايد LPS الخاص ببكتيريا كلاميديا بنيمونيات (Chlamydia pneumoniae) في إنتاج هذه المضادات. نتائج هذه التجربة توضح أعلى مستوى التخليق التلقائي والبروتيني لهذه السيتوكينات (IL-10, LL-13)

للمرضى مقارنة مع الأشخاص الغير مصابين. في حالة تعريض الخلايا لـ chlamydial antigen لم يكن هناك فرق واضح في مستويات إنتاج هذه المضادات عند مقارنة المجموعتين لا على مستوى التخليق أو المستوى البروتيني بالرغم من الزيادة الواضحة في الإنتاج التخليقي والبروتيني لدى المرضى والأشخاص العاديين. نفس النتائج قد تم التوصل إليها عند استخدام الليبولي سكرايد LPS الخاص ببكتيريا كلاميديا بنيمونيات وبكتيريا الإيكولاي (E. coli). وعليه، فإن هذه الدراسة توضح إنتاج السيتوكينات المضادة للالتهابات (IL-10, LL-13) على مستوى التخليق وعلى المستوى البروتيني وأن البكتيريا لا تحفز إنتاج هذه السيتوكينات المضادة للإلتهابات وذات الدور الحميد مما يساعد على تطور المرض بإتاحة الفرصة لإنتاج السيتوكينات المساعدة في حدوث الالتهابات.

كلمات مدخلية : *تصلب الشرايين، التهاب، سيتوكين، مخلق تلقائي، بكتيريا .*

INTRODUCTION

Atherosclerosis is a pathological process that takes place in the major arteries and is the underlying cause of heart attacks, stroke and peripheral artery disease (Libby, 2001). It is no longer considered a disorder of lipid accumulation, but a disease process characterized by the dynamic interaction between endothelial dysfunction, subendothelial inflammation and the 'wound healing response' of the vascular smooth muscle cells. (Mahmoud, *et al.* 2007). The principal cell types of the artery wall the endothelial cell, the smooth muscle cell and the monocyte/macrophage are major players in the events involved in initiation and evolution of the atherosclerotic plaque (Severs and Robenek, 1992). Other important participants are platelets and lymphocytes, which have modulating influences on smooth muscle cell, endothelial cell and macrophage behavior. The earliest detectable lesions, called fatty streaks, contain macrophage foam cells that are derived from recruited monocytes. More-advanced atherosclerotic lesions, called fibro-fatty plaques, are the result of continued monocyte recruitment and smooth muscle cell migration and proliferation. Variable numbers of $CD4^+$ T cells are found in atherosclerotic lesions, and cytokines secreted by T helper 1 (Th1) - or Th2-type cells can have a profound influence on macrophage gene expression within atherosclerotic plaques (Mach, *et al.* 1999)

A number of risk factors for atherogenesis, including infectious agents, have been shown to exert their influence via inflammatory mechanisms. There is growing evidence that *Chlamydia pneumoniae* (*C. pneumoniae*) may be involved in the pathogenesis of atherosclerosis, as several studies have demonstrated the presence of the organism in atherosclerotic lesions (Jhn, *et al.* 1999). *Chlamydia pneumoniae* can initiate and propagate inflammation in ways that could contribute to atherosclerosis. Infected leukocytes may serve to disseminate an infection from the lung to other susceptible tissues including arteries (Moazed, *et al.* 1997), and (Boman and Gaydos, 2000). *C. pneumoniae* also may influence atheroma biology by modulating macrophage-lipoprotein interactions. Infected macrophages ingest excess low-density lipoprotein to become cholesteryl ester-laden foam cells, the hallmark of early lesions in atherosclerosis (Moazed, *et al.* 1997), and (Kalayoglu and Byme, 1998 a, and b). In addition, *C. pneumoniae* induces monocytes to oxidize lipoproteins, converting them to highly atherogenic forms (Kalayoglu, *et al.* 1999). *C. pneumoniae*-induced foam cell formation is mediated chiefly by lipopolysaccharide, whereas lipoprotein oxidation occurs mainly by CHSP60 (Kalayoglu, *et al.* 2000). Recent work demonstrated that the chlamydial antigen HSP60-specific regulatory T cell have inhibitory activity *in vitro* and prevent the development of plaques *in vivo* on atherosclerosis (Yang, *et al.* 2006).

Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Cytokines can be inflammatory as IL-1, IL-6, TNF-α, IFN-γ and IL-18, but some cytokines are predominantly inhibitory as for example IL-10 and IL-13, which inhibit inflammatory cytokine production by macrophages. IL-10 and IL-13 are cytokines with pleiotropic properties. Limited biochemical and clinical evidence suggest a link between

IL-10 and IL-13 and thus, more data are needed to clarify the relationship between IL-10 and IL-13 and risk for atherosclerosis.

MATERIALS AND METHODS

Study Participants:

Patients presented to Bahrain Defense Force (BDF) Hospital with coronary artery disease undergoing angiograms (n = 15) of both sexes, age >35 year were included in the study, after the purpose of the study and its implications are fully explained to them. Control subjects comprised healthy controls (n = 15). Characteristics and clinical data of patients and controls are included in Table 1. All subjects (patients and controls) filled and signed a consent form, indicating their acceptance to participate in the study. Venous blood samples (15 ml) were collected in EDTA tubes after angiogram was done and confirmed pathologic changes, and were stored at room temperature pending lymphocyte isolation.

Table 1. Characteristics of Patients and controls.

CHARACTERISTICS	PATIENTS ((N=15	CONTROLS ((N=15
Age (years, range)	(54.3 (30-72)	49.9 (37-70)
Gender (n, %)	6 Female (35.71%) 9 Male (64.29%)	6 Female (35.71%) 9 Male (64.29%)
Hypertension (%)	84.4	0
Diabetes (%)	59.4	0
Hyperlipidemia (%)	65.9	0
Glucose (3.4-6.1mmol/l)	7.6	4.6
Triglyceride (0.11-2.15 mmol/l)	1.8	0.9
Cholesterol (3.88-6.47 mmol/l)	5.4	3.8
LDL-chol. (<3.4mmol/l)	3.8	1.8
HDL-chol. (F= 0.91-1.168 mmol/l) (M=0.912.07 mmol/l)	1.17	1.0
WBC (4.4-11 ×10^3/μl)	8.72	6.98
Platelets count (150-450 ×10^6/μl)	250.8	230.9

Lymphocytes isolation:

Immediately after obtaining the blood samples lymphocytes were isolated from peripheral blood cells by overlaying carefully the diluted blood on Ficoll and centrifugation at 3200 rpm for 20 min at room temperature, followed by discarding the plasma layer and collection of the buffy coat layer. Peripheral blood lymphocytes were then washed for 3 times with 1X (Phosphate Buffered Saline (PBS) pH 7.2.

Cell cultures:

Lymphocytes were maintained in RPMI 1640 culture medium (ICN, Biomedicals, Inc.) supplemented with 5% fetal bovine serum, 1M HEPES buffer, 50μmole Mercabto ethanol, 2ml of 200 mM L-glutamine (in 500 ml medium), and 5IU/5μg Penicillin Streptomycin (ICN, Biomedicals, Inc.). The cells were transferred to adhesion slides (BioRad Lab, Munich, Germany) and were stimulated by 5pg chlamydial and *E. coli* LPS or 5ng chlamydial HSP60 (Gift from Dr. M. Majeed, Division of Medical Microbiology, Linkping University, Sweden). The cells were then incubated for overnight at (37°C-in 5% Co2). Some negative control cells were left without stimulation. The positive control cells were stimulated by 5μg of phytohemagglutinin (PHA) (Sigma, Germany).

Detection of cytokine mRNA expression by in situ hybridization

In situ hybridization was performed as previously described (Bakhiet, *et al.* 2006). Briefly, 200 μl aliquots of suspensions containing $4x10^5$ mononuclear cells (MNC) were plated on round-bottomed microtiter plates (Nunc) in triplicate. 10 μl aliquots of 5pg chlamydial LPS or 5ng chlamydial HSP60 or *E. coli* LPS or PHA were added into appropriate wells. After culture for 24 h, the cells were washed, counted and applied onto restricted areas of electronically charged glass slides (ProbeOn slides; Fisher Scientific, Pittsburgh, PA). Synthetic oligonucleotide probes (Scandinavian Gene Synthesis AB, Köping, Sweden) were labeled using ^{35}S deoxyadenosine-5′-α-(thio)-triphosphate with terminal deoxynucleotidyl transferase

(Amersham). To increase the sensitivity of the method, a mixture of four different probes was employed for each cytokine. The oligonucleotide sequences were obtained from GenBank using MacVector software. Cells were hybridized with 10^6 cpm of labeled probe per 100 μl of hybridization mixture. After emulsion autoradiography, development and fixation, the coded slides were examined by dark field microscopy for positive cells containing more than 15 grains per cell in a star-like distribution. The intracellular distribution of the grains was always checked by light microscopy and expressed as numbers per 10^4 MNC. In many positive cells, the grains were so heavily accumulated within and around the cells that it was not possible to count every single grain. In cells judged negative, the numbers of grains were mostly 0 - 2 per cell, and the grains were scattered randomly over the cells and not distributed in a star-like fashion. There were therefore no difficulties in differentiating between positive and negative cells. Variation between duplicates was <10%. A control probe used in parallel with the cytokine probe on cells from each individual revealed no positive cells.

Intracellular detection of IL-10 and IL-13 by immunohistochemistry

Cells fixation was performed in methanol for 5min. Slides were incubated with avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) to block endogenous biotin or biotin-binding proteins. Cells were permeabilized with PBS containing 0.1% saponin to allow internalization of the cytokine-specific antibodies. A total of 30 μl cytokine specific monoclonal antibody (mAb) (mouse anti-human IL-10 and mouse anti-human IL-13; R&D Systems, Oxon, UK) diluted in PBS-saponin to a final concentration of 5μl/ml was added and allowed to incubate for overnight at 4°C followed by several washes in PBS. Non-specific staining by the second-step biotinylated goat antibody caused by Fc-interactions was prevented by a subsequent incubation with 1% normal goat serum (DAKO, Patts, Glostrup, Denmark) dissolved in PBS-saponin for 30 min at room temperature. The biotin-conjugated secondary antibodies were then added. The cells were incubated with an avidin-biotin horse-radish peroxidase complex (VECTASAIN, Vector Laboratories) for 30 min in the dark at room temperature. A color reaction was developed by 3- diminnobenzidine tetrahydrochloride (DAB) (Vector Laboratories) and stopped after 2-10 min by washes in PBS. The cells were counterstained with hematoxylin and the slides were left to dry before mounting in DPX (Fluka, Switzerland). The immunocytochemically stained cells were examined in a Leica RXM microscope (Leica, Wetzlar, Germany) equipped with a 3CDD color camera (Sony, Tokyo, Japan). Counting of cytokine producing cells was performed manually using X100 objective. The frequency of cytokine expressing cells was assessed by examination of at least 10^4 cells.

Statistical analysis:

Student's unpaired *t*-test was used to measure statistical significance between two groups. In all tests $p< 0.05$ was taken as the level of significance. (*=p<0.05, **=p>0.005, ***=p>0.0005).

RESULTS

The results of this study showed significantly high levels of expressed IL-10 and IL-13 at mRNA level in patients compared to healthy controls (p<0.0005). However, cells stimulated with CHSP60 did not show significant differences between patients and healthy control for both cytokines at both mRNA and protein levels. PHA was used as control, but there were no significant differences between IL-10 and IL-13 mRNA levels induced in the cells after stimulation with PHA between patients and healthy controls. However, PHA stimulation was much higher in both patients and healthy controls compared to non-stimulated cells. Another important control was exposure of the cell from patients and controls to chlamydial and *E. coli* LPS and the data did not show significant statistical differences between the patients and healthy control subjects (Figure 1A, Figure 2A and Figure 3A-C).

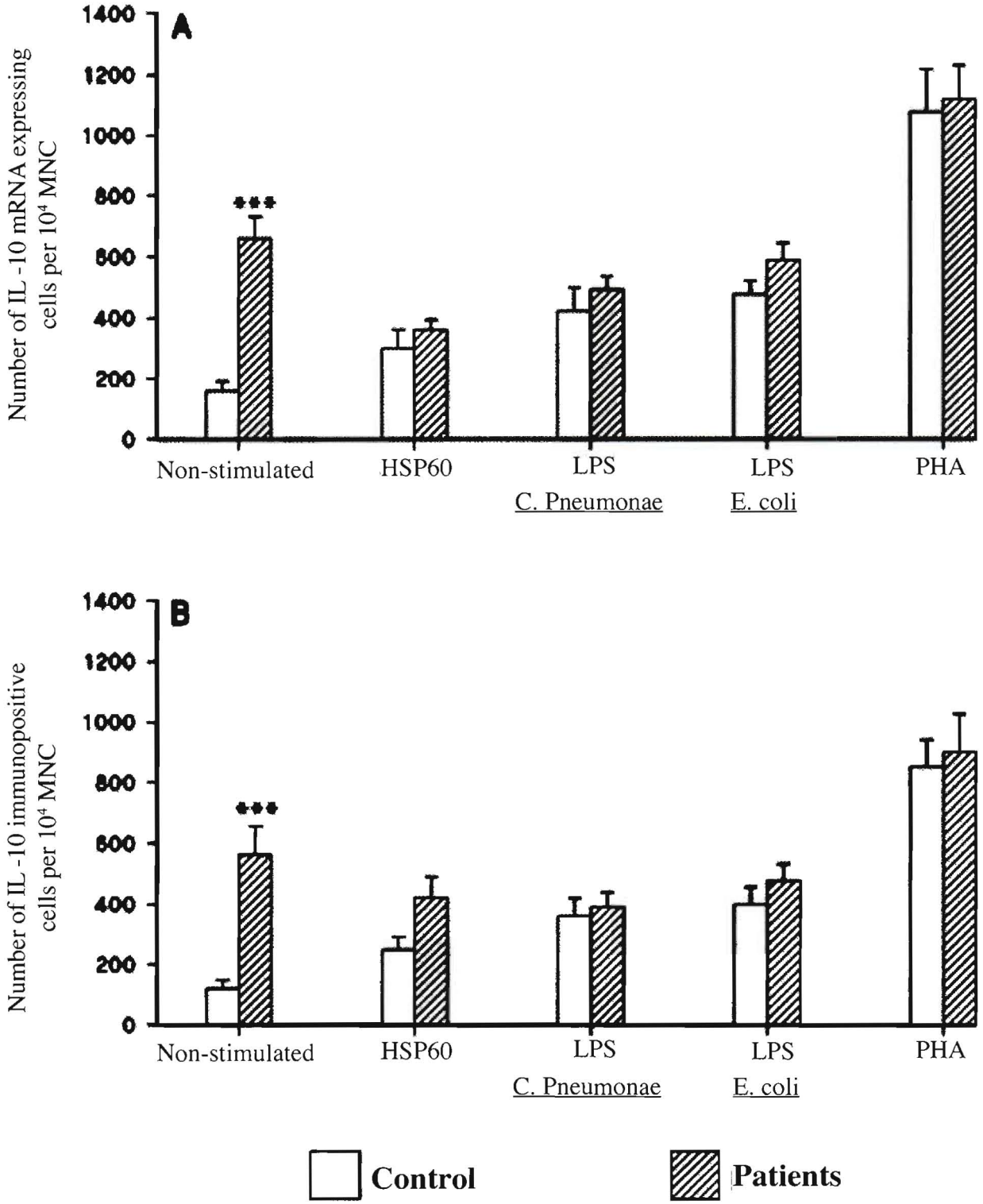

Fig.1. (A) Numbers of mRNA expressing cells for IL-10 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means ±SD are shown. (B) Numbers of immunopositive cells for IL-10 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means ±SD are shown.

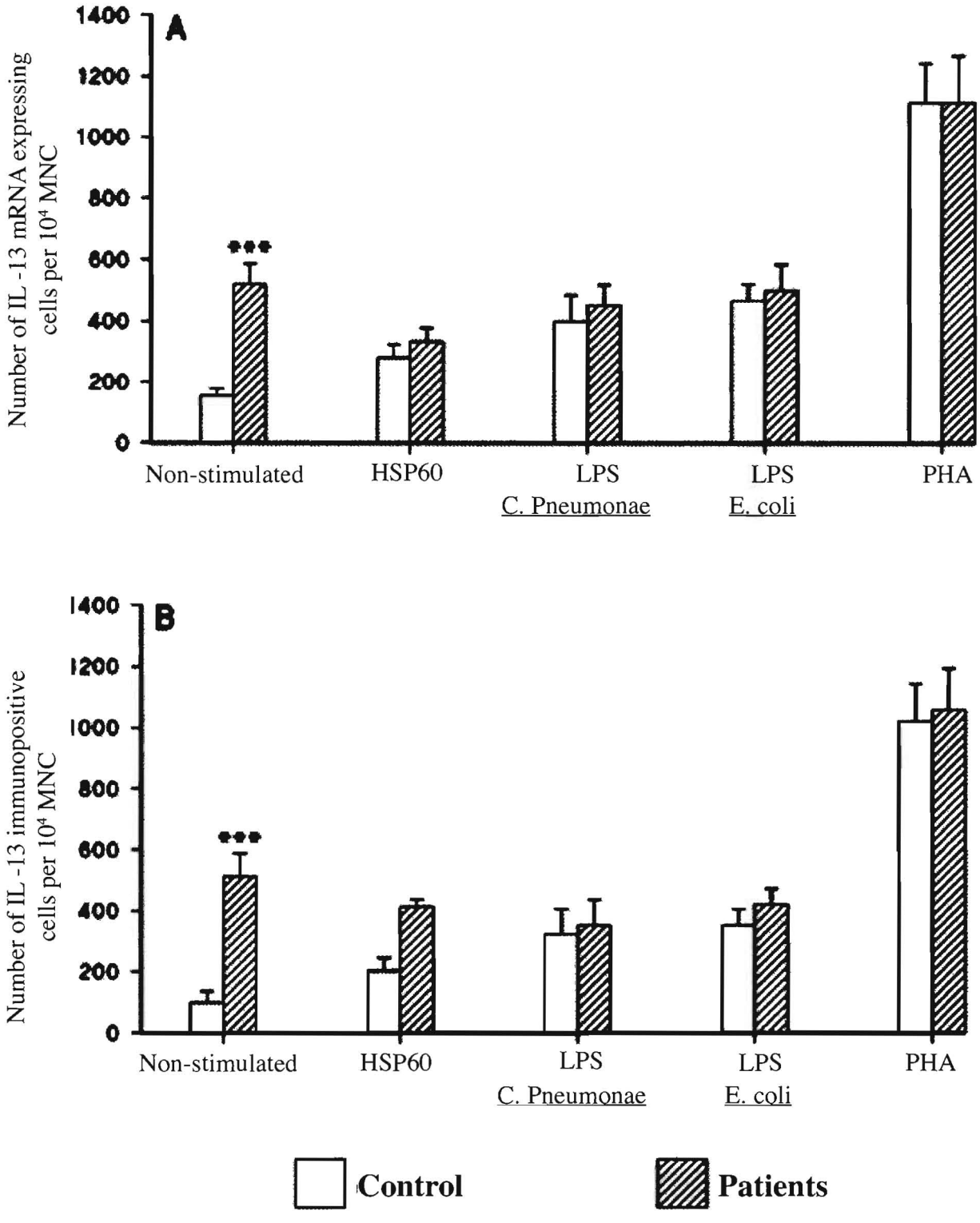

Fig. 2. (A) Numbers of mRNA expressing cells for IL-13 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means ±SD are shown. (B) Numbers of immunopositive cells for IL-13 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means ±SD are shown. (***=p>0.0005).

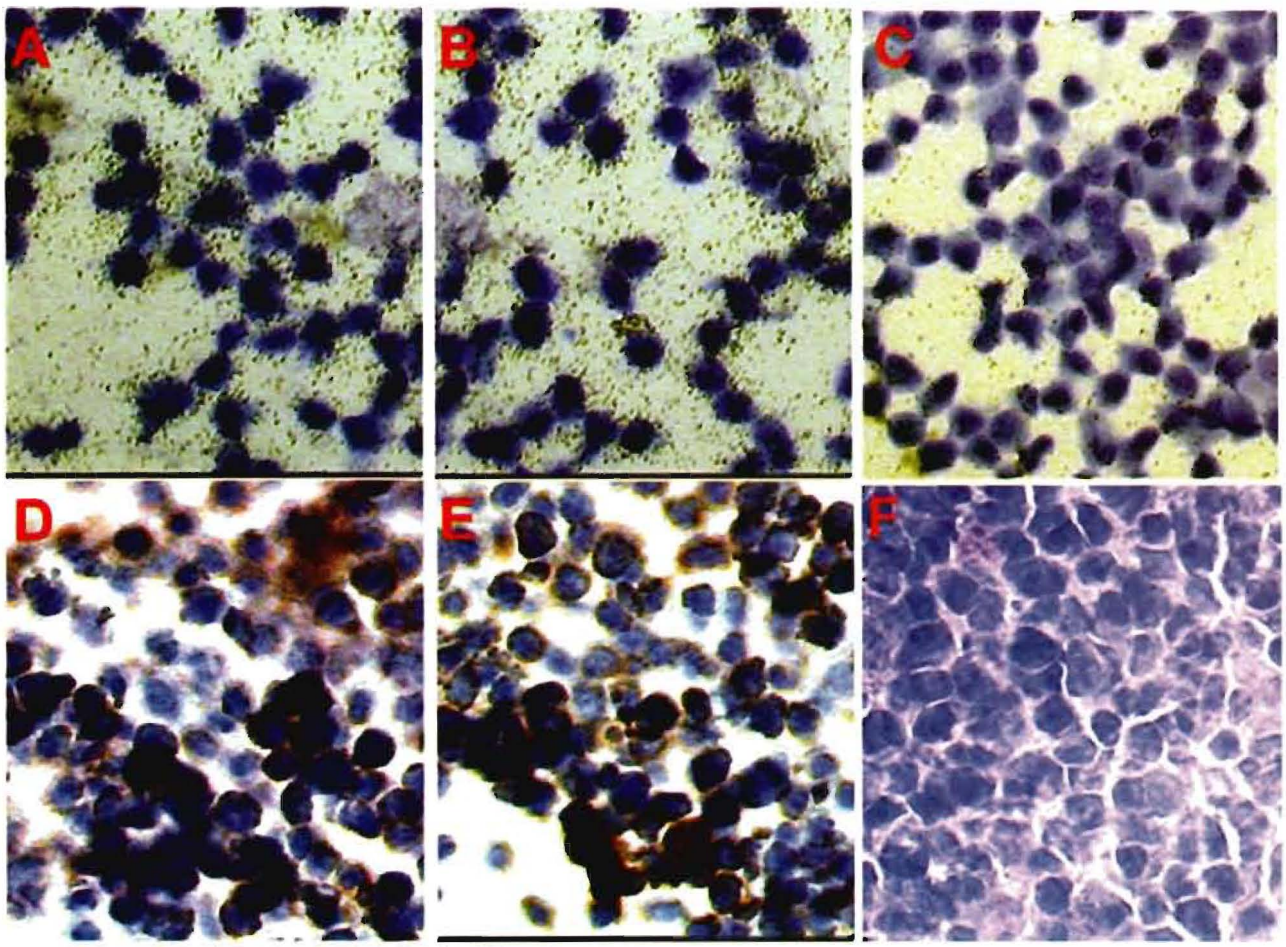

Fig. 3. (A) Cells expressing mRNA for the cytokine IL-10 produced by human mononuclear cells culture on slide. (B) Cells expressing mRNA for the cytokine IL-13 produced by human mononuclear cells culture on slide. (C) Sense control. (D) Intercellular immunohistochemistry staining an IL-10 produced by human mononuclear cells culture on slide. (E) Intercellular immunohistochemistry staining an IL-13 produced by human mononuclear cells culture on slide. (F) Non-stimulated cells (negative control). The photo represents healthy subject stained for IL-10. (*The photographs were taken in bright field microscopy (X200). (***=p>0.0005).*

To ensure that the mRNA expression has resulted in actual protein production generated during the immunopathogenetic events of the disease, the levels of IL-10 and IL-13 protein were measured by immunohistochemistry. As for mRNA levels for both cytokines, the results of this study showed significantly high levels of expressed IL-10 and IL-13 at protein level in patients compared to healthy controls ($p<0.0005$). Also, and as the results of mRNA, cells stimulated with CHSP60 did not show significant differences between patients and healthy control for both cytokines at protein levels, but higher significance was noted when IL-10 and IL-13 production from CHSP60 stimulated cells were compared to non-stimulated cells ($p<0.005$). PHA was used as control, but there were no significant differences between IL-10 and IL-13 protein levels induced in the cells after stimulation with PHA between patients and healthy controls. However, PHA stimulation for both cytokines protein was much higher in patients and healthy controls compared to non-stimulated cells. Also, exposure of the cell from patients and controls to chlamydial and *E. coli* LPS did not show significant statistical differences between the patients and healthy control subjects (see Figures, 1B, 2B and 3 C-E).

Exposure of cells to chlamydial and *E. coli* LPS showed significantly differences in both mRNA expression and protein levels for IL-10 and IL-13 compared with non-simulated cells ($p<0.005$) (see Figures 1 and 2).

DISCUSION

The present work explored the induction of potential anti-inflammatory cytokines in atherosclerotic patients. In addition, the effects of the chlamydial antigen HSP60 and LPS on the specific induction of these mediators were examined. The measurement of inflammatory mediators was conducted because atherosclerosis is considered as an unusual form of chronic inflammation occurring within the artery wall (Ross, 1999). Also, recent data demonstrated that not only pro-inflammatory processes contribute to atherosclerosis, but also anti-inflammatory cytokines may play an important role (Trompet, *et al.* 2007).

The data showed upregulation of the anti-inflammatory cytokines IL-10 and IL-13 at both gene and protein levels suggesting *de novo* synthesis of these mediators. Inflammatory cytokines such as IL-18 were proven to play a major role in atherosclerotic plaque destabilization leading to acute ischemic syndromes (Mallat, *et al.* 2001). Thus, the induction of the anti-inflammatory cytokines IL-10 and IL-13 and their *in vivo* upregulation can be explained by internal regulation to establish a balance between cytokines mediating inflammation as IL-18 and those required to slow down the immune response. IL-10 was suggested to play a protective role in atherosclerosis and its deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice (Caligiuri, *et al.* 2003). Also, effects of T-helper-lymphocyte Th1 and Th2-type cytokines on the ability of human monocytes to oxidize LDL, one of the pathological processes believed to occur in atherosclerosis were studied by (Folcik, *et al.* 1997). The ability of opsonized zymosan (ZOP)-activated human monocytes to oxidize LDL in a 24-hour period was significantly enhanced by pretreatment of the monocytes with the Th2 cytokines, IL-4, or IL-13 compared with untreated monocytes. In contrast, IFN-γ, a Th1 cytokine, inhibited LDL oxidation by activated monocytes. Treatment with IFN-γ also prevented the IL-4- and IL-13-mediated enhancement of LDL oxidation by ZOP-activated monocytes. Untreated or cytokine-treated unactivated monocytes did not oxidize LDL suggesting that the progression of atherosclerosis includes events that are immunologically mediated (Folcik, *et al.* 1997). Factors as diabetes, hypertension and hyperlipidemia are usually associated with endothelial dysfunction and several cytokines are considered to be independent risk factors for cerebrovascular and coronary artery disease. Thus, one important point to be considered here is that the majority of patients included in the study had risk factors for atherosclerosis as diabetes, hypertension and hyperlipidemia. These factors may also influence the recorded induction of the measured cytokines in atherosclerotic patients since control subjects were normal for theses disorders.

The role of anti-inflammatory cytokines in atherosclerosis is not clear and such cytokines may have dualistic effects on the disease events. They may play a beneficial role based on their anti-inflammatory activity, but their level must be strictly controlled. This was considered in view of recent data showed that elevated IL-10 concentration is associated with an increased risk for future cardiovascular events in post-menopausal women with established coronary atherosclerosis (Lakoski, *et al.* (In press) Also, genetic variation in the IL-10 gene promoter was found to be a risk of coronary and cerebrovascular event and that not only pro-inflammatory processes contribute to atherosclerosis, but that also anti-inflammatory cytokines may play an important role (Trompet, *et al.* 2007).

Interestingly, and in this context, the chlamydial antigen HSP60 did not induce neither IL-10 nor IL-13 expression at both mRNA and protein levels. This suggests that the infection with Chlamydia may contribute to atherosclerosis by failing to stimulate cytokines to the level needed for protection from atherosclerosis. However, excessive induction of such cytokines may on the other hand constitute a risk factor (Lakoski, *et al.* (In press).

In conclusion, this work demonstrates the production of the anti-inflammatory cytokines IL-10 and IL-13 that might be involved in the resolution of disease process. Challenges with chlamydial antigens did not stimulate further production of those cytokines suggesting

that chlamydial infection contribute to the development of the disease by inability to generate host beneficial pro-inflammatory cytokines.

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