REVIEW ARTICLE

INFLAMMATORY RESPONSE IN INFECTIVE ENDOCARDITIS

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Endocarditis remains a devastating disease with a high mortality despite timely diagnosis and treatment. The mainstays of treatment include appropriate antibiotics and when indicated, removal of the septic focus. This sounds extremely simple and belies the necessity for a sophisticated multidisciplinary approach to its treatment, the success of which depends not just on the right antibiotic at the right dosage via the right portal, but also on a profound understanding of the inflammatory and infective pathophysiology at work. This review aims at assisting both the clinician and the lab-based physician in the task.

In 1885 Osler described the clinical course of infective endocarditis (1). Until the introduction of antibiotics the course of the disease was mostly fatal. The introduction of penicillin in 1941 made the disease treatable. However the mortality was considerable, mainly due to heart failure secondary to valve destruction (2). In 1960 Kay was the first one to report surgical treatment for a valve lesion caused by infective endocarditis (3). In the USA infective endocarditis has an annual incidence of 5-7 cases per 100,000 persons (4). Despite the progress in diagnosis, antimicrobial treatment and surgical techniques, the mortality varies from 5% in uncomplicated cases to 59% in complicated cases (5), and in 2-31% etiologic diagnoses cannot be obtained (6).

The pathogenesis of infective endocarditis is not fully understood. The disease involves a complex interaction of bacteria, endocardium and blood components.

Heart failure is sometimes out of proportion to the valve incompetence. Bacteria of low virulence can induce severe disease when present in vegetations. Extracardiac manifestations such as renal lesions, arthritis, vasculitis, splenomegaly, splenic infarcts cutaneous and ocular signs cannot be explained alone by the presence of circulating bacteria (7).

Understanding the inflammatory response and the interaction between the bacterium, white blood cells, platelets and endothelial cells and the role of various cytokines may explain some of the pathophysiological findings associated with infective endocarditis. In addition, a better understanding of the inflammatory response to infective endocarditis may improve the diagnosis rate (especially in cases of culture negative endocarditis), the follow up of the activity of the disease, the treatment, and above all the survival and cure rate.

Animal studies
The vegetation
Data based on experimental endocarditis

Infected endocarditis is characterized by a combination of systemic and local findings. The central lesion is the vegetation which is an infected fibrin platelet thrombus which forms on the surface of damaged cardiac valves.

It is well recognized that the normal endothelium

Key words: infective endocarditis, blood culture negative endocarditis vegetation, inflammatory markers
is refractory to the development of infection and that pre-existing endocardial lesions are required for the establishment of experimental endocarditis (8). A widely used animal model of experimental bacterial endocarditis is a catheter-induced valve lesion followed by bacterial colonization proposed by Garrison and Freedman (9). The preliminary stage of infective endocarditis is denudation of the endothelial cell, exposure of the underlying extracellular matrix and local deposition of platelets and fibrin. This process leads to the formation of a non-bacterial thrombotic vegetation (10). In the next stage, bacteria which are in the blood stream colonize the sterile vegetation leading to the formation of an infected vegetation Subsequently, interactions between bacterial cells, platelets and white blood cells occur through the activation of an inflammatory response which leads to the growth or disappearance of the vegetation.

**Procoagulant activity following endothelial cell injury**

Fibrin is the primary constituent of the vegetation in infective endocarditis, and Tissue Factor (TF) expression is a major marker of coagulation activation on infected valves.

The initial trigger to increased procoagulant activity (PCA) is endothelial cell damage rather than bacterial infection (11). Exposure of the valve stromal cell to the blood induced PCA following interaction with lipopolysaccharides (LPS) and IL-1. Induction of tissue factor activity (TFA) by bacteria mainly depends on their ability to internalize within the endothelial cell (12)

**Bacterial colonization within the vegetation tPMP**

Once there is activation of the coagulation system and the inflammatory response, host defense mechanisms are activated against the invading bacteria. Activated platelets are capable of secreting thrombin that induces platelets microbicidal peptides production (tPMP) (13). These peptides limit the progression of the disease. *Staphylococcus aureus* strains resistant to tPMP may grow faster in the presence of tPMP than susceptible strains, and the vegetation size rate and progression rate was higher in rabbits infected by tPMP resistant strains (14-16). tPMP susceptibility of the infective organism may affect the entire course of the disease regarding both local and systemic lesions. Resistance to tPMP was more prevalent in *S. viridans* bacteria cultured from blood of patients with IE than from those cultured from the blood of bacteremic patients without IE (17). Thus tPMP may be an important host defense factor against IE due to certain bacteria.

Bacterial colonization on the vegetation is a complex process involving interaction between the infecting organism, endothelial cells, platelets and monocytes. After the initial response of the endothelium to injury and platelets to bacterial invasion, the monocytes play a major role in the pathogenesis of infective endocarditis. Monocytes augment the induction of TFA in bacterium infected endothelial cells (18-19). The mechanism of this process is partially mediated through the adhesion of the monocyte to the infected endothelial cell and by the proinflammatory cytokine IL-1 (18). After cytolysis of endothelial cells by *S. sanguis*, *S. aureus* or *S. epidermidis* TFA persists from monocytes bound to bacteria-infected endothelial cell matrix (20). Adhesions of monocytes to the vegetation via fibronectin receptors stimulate monocyte TFA (21). Later, IL-1 and TNF-alpha induce IL-10 production which down regulates monocyte TFA (22). From these data it seems that the growth of the vegetation and TFA of the monocyte result from a balance between pro- and anti-inflammatory cytokines.

Bacteria resist host defense by various mechanisms. Durac and Beeson (23) found that the majority of *S. sanguis* strains in the vegetation are phagocytosed, transported in the blood, and deposited on the vegetation in adherent monocytes. The monocyte disappears and viable bacteria persist in the vegetation. Recently it was found that monocytes infected by oral streptococci are transformed to short-lived IL-12 producing mature dendritic cells (24). These cells live in the vegetation for a few days as macrophages and can survive at the site of inflammation and kill microorganisms for a long period of time.

**Other virulence factors related to the vegetation**

Fibronectin plays a major role in bacterial adhesion to the vegetation. It was found that in some streptococcal and staphylococcal species the ability
to bind to fibronectin and to other extracellular matrix proteins on the vegetation increases the virulence of the disease (8, 25). Toy et al (26) found that some \textit{S. aureus} strains can adhere to fibronectin adhered to solid fibrin thrombi. Adherence ability of \textit{S. aureus} to fibronectin was directly related to their number on damaged heart valves and the virulence of the disease (27-28). \textit{S. mutans} colonization was directly related to sucrose-derived exopolysaccharide production (29).

Glucosyl transferases (GTFs) secreted by some strains of \textit{S. viridans} are important modulators of the inflammatory response in early stages of the disease. It was found that levels of IL-6 and tumor necrosis factor alpha secreted from endothelial cells of mice infected by \textit{S. viridans} strains were directly related to their GTFs expression (30). During the chronic stage of the disease, IL-6 production persists, but at that stage it may also originate from the monocytes (30). Another study showed that GTF was capable of inducing the expression of adhesion molecules and the release of IL-6 and plays an important role in the transition from neutrophil to monocyte recruitment in the early stages of IE (31). Aggregation substances (AS) and enterococcal binding substances (EBS) secreted by \textit{E. faecalis} are essential for the development of vegetation. Rabbits infected by strains lacking AS and EBS did not develop cardiac vegetations, whereas those infected by strains able to produce either AS or EBS developed small vegetations and those that were infected by both AS and EBS producing bacteria produced large vegetations (32).

The presence of bacteria within the vegetation depends on monocytes, platelets and endothelial function and the ability of the invading organism to resist phagocytosis by the polymorphonuclear cell (PMN). Young et al. (33) found that \textit{S. gordonii} strains that resist phagocytosis by PMN were found within the vegetation.

Infective endocarditis is characterized by vegetation formation which is the local inflammatory response to the disease. The formation of the lesion is a complex process that starts on damaged endothelial cells which induce TFA and activates platelets, leading to the formation of a sterile vegetation. Bacterial adherences to the vegetation promote a cascade of interactions between endothelial cells, platelets, PMNs, monocytes and infecting organisms. The growth of the vegetation is determined by the ability of the organism to adhere to the vegetation and to resist various host defense mechanisms, and also by the inflammatory response of the host.

\textbf{Systemic reaction}

In addition to the local reaction within the site of the endothelial injury, IE is characterized by a spectrum of systemic reactions involving the heart, distant organs such as the spleen, kidneys, skin and eyes as well as the cardiovascular system. The degree of heart and distant organ involvement might be determined in the early stages of the disease mediated by tPMP. In a rabbit experimental model Kupferwasser et al (14) showed that in tPMP resistant bacteria strains there was a higher degree of perivalvar damage, aortic regurgitation, and degree of impairment of left ventricular function as well as a higher degree of renal infarcts. AS and EBS (aggregation substance and enterococcus binding substance) secreted by \textit{E. faecalis} modulated the systemic reaction and the virulence of the disease. A rabbit model with bacteria lacking these substances did not cause clinical signs of the disease, whilst bacteria, having both substances, caused 100% lethal disease associated with cardiac and lung cell destruction, splenomegaly and minimal pericardial inflammation. Animals infected with one substance producing bacteria developed an intense pericardial reaction and a relatively low mortality rate of 16% (32). It was hypothesized that AS and EBS secreting strains produced factors that prevented pericardial inflammation, either through destruction of inflammatory cells or by preventing PMN influx into the site of infection (32). The resistance of \textit{S. gordonii} to PMN killing determines the virulence of the infection. (33).

\textbf{Clinical applications}

Infective endocarditis is a serious disease associated with significant morbidity and mortality. The diagnosis, especially in culture negative endocarditis or in cases where antimicrobial treatment had been started before diagnostic tests, might be impossible to achieve. Other cases may have a complicated course associated with severe complications such as thromboembolic events resulting in an increased mortality. Monitoring of
the disease activity might be important to prevent disease exacerbation and other complications. A better understanding of the inflammatory process may enable an earlier diagnosis, anticipation of a complicated course and a better monitoring of the disease activity which might lead to better results.

**Diagnosis**

Early diagnosis of IE is very important, as the earlier the diagnosis is made and proper treatment started, the higher the rate of survival and the lower the rate of complications. Diagnosis of blood culture negative endocarditis (BCNE) is very difficult and not always possible. BCNE occurs in 2.5-31% of IE cases (6). The Duke criteria perform poorly in these patients (34). In such cases a proper antibiotic treatment may be delayed, and inflammatory markers may contribute to the diagnosis of IE.

Rheumatoid factor (RF) is considered as a minor sign among the Duke criteria for the diagnosis of IE. Comparison has been made of C-reactive protein, sedimentation rate, rheumatoid factor (RF) and TNF levels in patients with definite IE and rejected IE (according to Duke criteria). It showed that only RF was significantly higher in definite IE patients (35). In 427 cases of Q fever endocarditis systematic RF examination enabled the diagnosis of definite endocarditis in 8 out of 427 cases (6). Other studies showed that IL6 and IL2-R levels were elevated both in patients with culture positive and culture negative endocarditis (36-37). It was proposed to include IL-6 and IL-2R as a diagnostic criterion in IE (37). TNF, CRP, WBC, IL-1 and ESR were not found to contribute to the diagnosis of IE (35-37).

From these data it seems that inflammatory markers play a secondary role in the diagnosis of IE. However in BCNE the role of inflammatory markers in the diagnosis may be more important. Inclusion of IL-6 or IL-2R as minor criteria should be further investigated.

**Pathogenesis**

Q fever endocarditis is a challenging form of IE to treat. Coxiella burnetii is an intracellular pathogen; vegetations are rare and blood cultures are often negative. This frequently leads to a delayed diagnosis and treatment which results in a poor prognosis. During the early stage of the infection it was found that monocytes and peripheral mononuclear blood cells produce higher levels of TNF alpha and IL-1 beta (38). Ongoing Q fever endocarditis is associated with a specific defective killing of C. burnetii which does not exist in uninfected patients or in patients who were recently cured of the disease (39). While survival of the pathogen within the infected monocyte is mediated by TNF (39), it was found on the other hand that TNF with interferon gamma (IFN-gamma) induces apoptosis of infected monocytes and enhances killing of the pathogen (40).

**Course**

In addition to the value of early diagnosis, it is very important to monitor the disease activity. Inaccurate monitoring of the disease activity may lead to premature completion of antimicrobial therapy which may increase the risk of disease exacerbation. On the other hand, unnecessarily prolonged treatment may expose the patient to iatrogenic complications. Measuring IL-6 and IL-2R levels in IE patients revealed that their levels are continuously decreasing during antibiotic treatment, and therefore their level can monitor the activity of the disease (36-37).

The inflammatory process can also be investigated locally. IL-8 and TNF alpha were studied in excised heart valves of IE patients. It was found that IL-8 and the greatest amount of inflammation were seen in patients with short preoperative treatment course. No such relationships were seen in TNF-alpha containing cells (41). It is concluded that IL-8 containing cells in infected heart valves could be used as a marker of disease activity.

A serious complication of IE is thromboembolism. Echocardiographic studies to correlate the size and morphology of the vegetation with thromboembolic events were controversial, therefore other possible mechanisms other than just the presence of vegetation may influence the occurrence of this complication (42). Korkmaz et al (43) found that levels of p-selectin and E-selectin were highly significant rates in patients with thromboembolic events. Kupferwasser et al. (44) found that embolic events occurred more frequently among patients with elevated antiphospholipid antibodies (APA) levels. They concluded that APA levels in
patients with IE are related to increased risk for major embolic events. Buyubasyk et al. (45) found that concentration of prothrombin fragment 1+2 (PF1+2), thrombin-antithrombin III complex (TAT), plasminogen activator inhibitor 1 (PAI-1), beta thromboglobulin (beta-TG), and platelet factor 4 (PF-4) were all higher in comparison with patients without thromboembolic complications. From these data it seems that thromboembolic complications in IE patients is not merely related to the vegetation but is rather a complication of a hypercoagulable state which results from the inflammatory process.

**Summary**

IE is a severe disease associated with significant morbidity and mortality despite the advances in antibiotic treatment and surgical techniques. Understanding the mechanisms of the inflammatory response may improve the results.

The result of the inflammatory reaction is the formation of a vegetation that is initially sterile. Later it becomes infected on an injured valve endothelium which may lead to valve destruction followed by a systemic reaction with disturbances of the coagulation system and damage to the other organs such as the kidneys, eyes, heart, and spleen.

Even though the diagnosis of IE is based on positive blood cultures of the causative organisms, in cases of BCNE or intracellular organisms the diagnosis may be extremely difficult. In other cases the diagnosis may be clinically uncertain. A further understanding of the role of serum cytokine concentrations may lead to a more accurate and rapid diagnosis. Assessment of IL-6 and IL-2r could provide new diagnostic criteria for inflammation. Assessment of the IL-8 and TNF in valves excised during the operation for valve replacement can contribute to the information regarding the disease activity. (This may be especially important during replacement of an undiagnosed infected valve for other reason than IE)

In cases of defective ability of the monocytes to kill intracellular *C. burnetii* which is mediated by TNF-alpha, probably interference with TNF activity (by using anti-TNF antibodies) may improve the monocyte ability to eliminate the infecting organism. Therapeutic strategies based on cytokine antagonists may limit the putative monocyte traffic and thus enhance the efficacy of antibiotic treatment. Further investigations on the various serum cytokines can give more information about the disease activity and may predict a complicated course of the disease.

IE patients with embolic events have increased E- and P- selectin, PF1+2, ASA, TAT, beta TG, PF4 and PAI-1 levels. Determination of these molecules may provide a marker to identify patients in high thromboembolic risk from IE, and probably anticoagulant treatment may help reduce the risk.

We hope that in the future manipulations on the inflammatory reaction will limit valve destruction and remote organ damage, will improve bacterial elimination by the host, enhance the efficacy of antibiotic therapy, will reduce the need for valve operations and reduce the morbidity and mortality associated with the disease.

**REFERENCES**


NOVEL MUTATION DETECTION OF AN INFLAMMATORY MOLECULE ELASTASE II GENE ENCODING NEUTROPHIL ELASTASE IN KOSTMANN SYNDROME

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Severe congenital neutropenia (SCN), often referred to as Kostmann syndrome, is a rare immune deficiency syndrome diagnosed at or soon after birth, characterized by maturation arrest of myeloid cells at the promyelocyte stage of hematopoiesis. In severe congenital neutropenia due to disorder of neutrophil production, patients are predisposed to recurrent bacterial infections. Recently, there have been reports of detected mutations in neutrophil elastase II (ELA2) gene in genomic DNA of severe congenital neutropenia. In this study we attempted to determine whether there is any mutation in elastase II gene encoding. Peripheral blood was collected from five patients with severe congenital neutropenia and 20 healthy individuals. Total RNA was isolated using RNA standard techniques from fresh separated cells by Polymorphoprep. RNA was analyzed by employing PCR amplification of reverse transcribed using a total of ten specific primers. We amplified five exons of ELA2 gene separately and sequenced each exon. Mutational analysis was performed by directed capillary sequencing method. We found mutations in all severe congenital neutropenia patients and no mutation in 20 healthy individuals. The most mutations were in exon 4 and no mutation was found in exons 3 and 5.

Key words: elastase two, severe congenital neutropenia, polymerase chain reaction, polymorphonuclear, reverse transcription

Severe congenital neutropenia (SCN), often referred to as Kostmann syndrome, is a rare immune deficiency syndrome diagnosed at or soon after birth. Congenital neutropenia is a severe disorder of neutrophil production that brings about lifelong problems with recurrent infections (1). Severe congenital neutropenia, often referred to as Kostmann syndrome, was first described in northern Sweden as an autosomal-recessive disorder affecting neutrophil production. Congenital neutropenia and cyclic neutropenia predispose patients to recurrent fever, pharyngeal ulcers, sinusitis, and cellulites and, less frequently, bacterimia and severe deep tissue infections (2-3). Congenital neutropenia and cyclic neutropenia occur both sporadically and as inherited disorders. Severe congenital neutropenia occurs because of inefficacious granulocyteoeisis or maturation arrest in the bone marrow. This implies that primitive myeloid progenitor cells may proliferate normally, but are lost during the differentiation process. These patients, usually children, are extremely susceptible to bacterial and fungal infections because their neutrophil counts are

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extremely low and generally do not increase with infections. These children develop life-threatening neutropenia (4).

Although neutropenia in this disorder has been attributed to impaired or ineffective neutrophil production, the molecular and cellular basis for the disease has remained largely unknown (5). It was found through positional cloning studies, that the locus for severe congenital neutropenia was mapped to chromosome 19p13.3, a region containing the genes for three neutrophil protease: azurcidin, proteinase 3, and neutrophil elastase (so called ELA2) (6). Neutrophil elastase is a potent 218 amino acid protein synthesized by CD34+ precursor cells during the initial stage in the production of the primary granules of the neutrophil (7). The cellular changes due to the mutations of neutrophil elastase in congenital and cyclic neutropenia are not yet clear (8). Recently, it has been reported that mutations in the gene for neutrophil elastase ELA2 seems to be involved in the etiology of both cyclic and severe congenital neutropenia (9). As an initial step towards understanding neutrophil elastase gene expression, the present study aims at characterizing the ElA2 mutations at RNA level and amino acid changes at protein level of the ElA2 for better understanding the role of ElA2 gene encoding in pathophysiology of severe congenital neutropenia.

MATERIALS AND METHODS

Five severe congenital neutropenia patients and twenty healthy individuals as control group took part in this study. All patients and normal subjects were studied according to the Declaration of Helsinki. Written informed consent was obtained from each individual. Patients were referred to the immunodeficiency clinic at the Children’s Medical Center Hospital affiliated to Tehran University of Medical Sciences.

Consent for these studies was obtained from the parents of the patients under the auspices of the human subjects committee of the Tehran university of medical Sciences. Diagnoses were assigned at enrollment by established criteria of the SCNIR.

The diagnosis of severe congenital neutropenia was based on blood neutrophil counts less than 500/µl obtained during the 3 months after birth: a typical pattern of recurrent fevers, chronic gingivitis and infections at irregular intervals: a bone marrow aspirate showing “maturation arrest” at the promyelocyte stage. Healthy individuals with normal blood counts served as controls. Peripheral blood samples (6 ml) were collected from the cubital vein and in tubes containing anticoagulant. Peripheral polymorphonuclear cells were isolated by polymorphoprep (Axis-Shield Poc AS, Oslo, Norway). The polymorphonuclear layer was collected and washed three times in phosphate buffer saline (PBS). The total mRNA was isolated from lymphocytes by RNA blood minikit (Roach, Germany), and the amount and purity of the RNA was determined by spectrophotometry. Elastase II mRNA expression was determined by RT-PCR. Total RNA amounting to 1500 ng was reverse transcribed into first-strand cDNA by using random hexamers and 2-5 units of multiscribe (recombinant moloney murine leukemia virus) reverse transcriptase in a final volume of 40 µl (10). Primers for exons EL1, EL2, EL3, EL4, EL5 and the whole exon as control were designed using primer express software to exclude amplification of genomic DNA and pseudo genes. 75 ng of cDNA was used for PCR amplification in a final volume of 25 µl with 1 unit of Taq DNA polymerase (Roach, Germany). PCR was carried out in a PCR machine (Techneh, USA). For each of the 5 exons and PCR reactions, negative control (water) and positive control (pooled cDNA) from several healthy blood donors (peripheral blood polymorphoneuclear) were used in parallel with every set of experiments. β-actin was used to amplify a 140 base pair fragment that served as an internal standard for cDNA preparation. β-actin primer pair was used in the PCR to normalize the concentration of cDNA. An aliquot of each amplification reaction was loaded in ethidium bromide stained in 1.5% agarose gel, and electrophoresis performed at 100v for 45 min to confirm the expected size of the amplified fragment.

Analyses of mutations were performed by capillary sequencing. Each product of Elastase II gene (Exon 1 to 5) was sequenced by DNA sequencer ABI 3700 capillary system (Applied, Biosystem, USA). Products were cleaned using the PCR purification kit (Qiagen, Germany) and were directly sequenced in both directions using the same primers. For the complete ELA2 coding region cDNA was produced by reverse transcription and it was used as a template for PCR with the specified primers.

Bidirectional sequencing was performed as above for each mutation; at least 20 healthy controls were included.

RESULTS

The aim of this study is to determine mutational change in different exons of Elastase II gene at RNA level in patients with
severe congenital neutropenia compared to healthy individuals. In order to test this hypothesis, we examined mutation detection of the different elastase II exons (ELA1-5). The experiments were performed using peripheral blood polymorphonuclear cells RNA with highly sensitive methods. The results of the present study revealed that all different Elastase II gene exons (ELA1-5) were expressed in peripheral blood polymorphonuclear cells in 20 healthy individuals. RT-PCR was then carried out on five samples from SCN patients. Fig. 1 shows agarose gel electrophoresis of RT-PCR products amplifications in SCN patients. Elastase II gene specific amplification products were different in size of exon1,2 (211bp), exon 3 (146bp), exon 4 (247bp) and exon 5 (176 bp). Moreover, we designed primers to amplify whole exon as a positive control (735bp). Elastase II gene in peripheral blood polymorphonuclear was expressed on 20 normal individuals and five SCN diseases. The results presented here provide direct evidence that human polymorphonuclear express ELA2 gene. The specificities of the obtained PCR products for the respective ELA2 fragments were confirmed by capillary sequencing analysis. The PCR products were tested by blasting against the entire human genome (NCBI-national center for biotechnology information 2006) to exclude sequencing at unwarranted sites (11).

Mutational analysis was carried out by capillary sequencing. Each product of Elastase II gene(Exon 1 to 5) was sequenced by DNA sequencer ABI 3700 capillary system. For each mutation found, at least 20 healthy controls were included. Fig. 2 presents a sample of capillary sequencing electropherogram analysis of mutation of normal allele of Elastase II exon 4 in healthy individuals. Capillary sequence analysis of mutation by electropherogram documented in the same allele of Elastase II exon 4 mutation (C to A) in SCN (Fig. 2). Mutational analyses was performed by directed capillary sequencing method. We found mutations in different exons of Elastase II gene encoding in all severe congenital neutropenia patients compared to no mutation in 20 healthy individuals.

Table I presents the results of mutational analyses in SCN by bidirectional capillary sequencing. The mutations were found in high frequency in exon 4 and 2 respectively but no mutation was found in exon 3. All patients had different heterozygous base substitution changes at RNA and at protein level (Table I). Fig. 2 is a schematic diagram showing the location of mutations in the ELA2 gene in cyclic neutropenia. It is thus most likely that a novel 50% frequency of polymorphism in exon 2 occurs (GCG 44-NCBI, GCA44-Iranian) in the Iranian population which had not been reported previously. Moreover, only one coding sequence change in the gene for neutrophil elastase was observed in 20 controls from normal individuals, and this change is presumed to represent a polymorphism, because of its appearance.

<table>
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<th>Patient</th>
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<td>4</td>
<td>TCT&gt;GTC</td>
<td>Val169 Ser</td>
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<td>2</td>
<td>1,4</td>
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<tr>
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<td>4</td>
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<td>5</td>
<td>2</td>
<td>AAT&gt;GAA</td>
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also in cyclic neutropenia. Fig. 2 shows a schematic diagram indicating the location of polymorphism in the ELA2 exon 2 in healthy and severe congenital neutropenia.

DISCUSSION

This report describes the occurrence of mutations of the gene encoding neutrophil elastase in five patients with severe congenital neutropenia in comparison to 20 healthy individuals. A previous study suggested that the pathophysiology of congenital neutropenia may be related to mutations of elastase II gene (12-13). The findings reported here suggest a primary role for mutations of neutrophil elastase in causing most cases of SCN. This protease is normally synthesized and packaged in promyelocytes at an early stage in neutrophil development (14-15). Neutrophil Elastase has many recognized and possible substrates, including coagulation proteins, growth factors, and intracellular signaling molecules (16-17). It is normally synthesized in the developing neutrophil as a proenzyme but is stored in the primary granules in its active form ready with full enzymatic activity when released from the granules, normally at sites of inflammation (18-19). In the present study, a mutation in the ELA2 gene was detected in all apparently SCN. None of the mutation was found in the 20 healthy controls. These results are compatible with those published previously showing that mutations in ELA2 is the cause of cyclic neutropenia (20). The current study is the first report which indicates that mutations occur at RNA level of ELA2 in patients with SCN. The results presented in this study confirm the high frequency of heterozygous mutations in the neutrophil elastase gene in SCN (21). Moreover, we found new mutations and 50% frequency polymorphism in SCN. These data further strengthen the argument for the role of neutrophil elastase mutations in the pathogenesis of SCN. It should be noted that the patients with clinical manifestation of the disease with ELA2 mutations could actually have autosomal recessive disease.

The pathogenicity of mutations in ELA2 remains a unknown (22). Neutrophil elastase has no known role in myelopoiesis. Interestingly, heterozygous and homozygous ELA2 knockout mice have normal neutrophil counts, and only the latter has a higher
demonstrable susceptibility to infection (16-17). Experiments in which the mutant enzyme has been transfected into cell lines (rat basophilic cells RBL-1) and murine myeloblasts (32D) have shown reduced levels of elastase activity in some patients (23). However, other investigators have reported reduced elastase activity as a more general phenomenon in congenital neutropenia (24). At present it is rather difficult to arrive at a definite conclusion that ELA2 mutation is the only factor which contributes to SCN. It appears likely that pathogenesis of this disease is due to an alteration of function in the mutant protein, allowing the enzyme to bind to a novel substrate involved in myelopoisis changing its binding to an intracellular inhibitor. An alternative explanation may be related to changes in intracellular packaging. Recent evidence demonstrates increased susceptibility to apoptosis of myloid precursors in severe congenital neutropenia (25), however, the link between this and mutant neutrophil elastase has not been established. This report describes the occurrence of mutations of the gene encoding neutrophil elastase in five patients with SCN.

Mutations in the gene for neutrophil elastase ELA2 were reported to be involved in the etiology of both congenital and cyclic neutropenia (20, 26-28). The mutations in SCN which were found in the present study mostly showed amino acid exchange at protein level. This particular mutational change has not previously been reported. This was not found in the 20 healthy donors tested, thus a polymorphic change is excluded (28). There are results reported by others which corroborate our hypothesis: (I) a small group of cyclic neutropenia patients revealed no or silent ELA2 mutations; (II) some single ELA2 mutations have been described to occur in SCN as well as in CN and (III) rarely Kostmann’s syndrome, but most probably also some of the sporadic CN cases are of autosomal recessive inheritance (27, 29-30).

This is in contrast to an autosomal dominant pattern of inheritance with heterozygous ELA2 mutations as a single cause for the disease. All ELA2 mutations which so far have been reported for CN and CYN were found to be heterozygous and the evaluated patients were characterized by one ELA2 mutation in one allele (20, 26-27, 30).

Moreover, it has been recently reported that by using a positional cloning approach and candidate gene evaluation, a recurrent homozygous germ line mutation in HAX1 in three pedigrees were identified. HAX1 encodes the mitochondrial protein HAX1, which has been assigned functions in signal transduction and cytoskeletal control. It has been suggested that HAX1 is a regulator of myeloid homeostasis and underlines the significance of genetic control of apoptosis in neutrophil development. ELA2 was also sequenced, associated with cyclic and congenital neutropenia in all affected individuals with HAX1 mutations. However, no affected individual was found with mutations in both ELA2 and HAX1. Our findings confirm by these studies that suggesting ELA2 and HAX1 define two mutually exclusive groups of individuals with SCN (31).

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SERUM AMYLOID A ACTIVATION OF INFLAMMATORY AND ADHESION MOLECULES IN HUMAN CORONARY ARTERY AND UMBILICAL VEIN ENDOTHELIAL CELLS

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Inflammation is considered to be the driving force leading to atherogenic and atherosclerotic mechanisms. Increased levels of SAA predict the risk of coronary artery disease and even mortality from cardiovascular disease in humans. Recent animal and human studies have indicated that SAA plays a causal role in atherogenesis, although it is largely unclear how this occurs. The objectives of this study are to understand the role of SAA in activating possible atherogenic inflammatory responses in human coronary artery endothelial cells (HCAEC) and to compare them with human umbilical vein endothelial cells (HUVEC). Our hypothesis is that vein and artery endothelial cells have different expression patterns and levels, leading to differential inflammatory responses. HUVEC and HCAEC were grown in order to analyze the effects of SAA on endothelial expression of pro-inflammatory cytokines, such as IL-6, chemokines, such as IL-8, and adhesion molecules (s-ICAM, s-VCAM, E-selectin) by reverse transcription-PCR and ELISAs. We compared the dose responses of SAA between HUVEC and HCAEC. SAA activated both HUVEC and HCAEC pro-inflammatory factors in a dose-dependent manner. In comparison however, HCAEC showed a strikingly greater sensitivity to SAA, with a higher level of expression of all pro-inflammatory markers at much lower concentrations of SAA, and their much greater stimulation at higher SAA concentrations. SAA also generated a dose-dependent positive feedback response on its own mRNA expression in HCAEC as compared to HUVEC. In summary, there are distinct significant differences in the levels of inflammatory markers and adhesion molecules between HUVEC and HCAEC SAA induced dose responses that could potentially account for HCAEC greater susceptibility to inflammation and atherogenesis.

Inflammation is known to be a major driving force in atherogenesis and in the initiation of coronary plaque formation (1). Cohort studies have reported that acute inflammatory parameters [such as C-reactive protein (CRP) or serum amyloid A (SAA)], cellular adhesion molecules, cytokines and chemokines are all elevated among patients at risk for future coronary occlusion (2-4). Increased SAA levels predict the risk of coronary artery disease in humans (5) and multivariate studies have identified SAA as an independent predictor of mortality in acute myocardial infarction patients (6). Recent animal and human studies indicate that SAA plays a causal role in atherogenesis (7), however it is largely unclear how the induced SAA play this role.

The endothelium is heterogeneous due to its

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Key words: serum amyloid A, endothelial cells, interleukin-6, adhesion molecules
vascular bed and tissue type origin. Different endothelial cell phenotypes exhibit differential responses to changes in their environment and their underlying molecular mechanisms and signaling (8). Thus, endothelial cells derived from the umbilical vein have specific and intrinsic expression patterns of inflammatory molecules as compared to coronary arterial endothelial cells.

Widespread expression of SAA has been reported in histologically normal human tissues (9). Endothelial cells in atherosclerotic lesions of human coronary and carotid arteries have been found to express SAA mRNA, in addition to other cells such as smooth muscle cells, macrophage-derived “foam cells,” adventitial macrophages, and adipocytes (10). SAA has also been found to be present in atherosclerotic plaques (11), and was locally increased at the site of ruptured plaques in acute myocardial infarction in comparison to CRP (12). These findings enhance the postulation that SAA is involved in atherogenesis, atherosclerosis and thrombogenesis.

Human SAA belongs to a multigene family comprising of induced SAA1 and SAA2 isoforms, jointly named acute phase SAA (A-SAA1/2), and the constitutively expressed SAA4 (C-SAA) (13). SAA3 gene is not expressed in humans (14). Multiple events can trigger the stimulation of A-SAA synthesis in the liver. The expression of SAA is most potently regulated by the pro-inflammatory cytokines IL-1, TNF-α and IL-6 and their synergistic actions. During inflammation, elevated SAA production is primarily due to its increased transcription (15) and stability of mRNA (16). Studies of the mammalian SAA gene promoter indicate involvement of NF-κB, AP-2, NF-IL-6, SAF, SP-1, YY1 and CEB/P factors in the transcriptional regulation of SAA (17), which can be cell (18) and stimuli specific (19).

A-SAA is a prominent positive acute phase reactant (20) which itself has been shown to stimulate pro-inflammatory cytokines, such as IL-1, TNF-α, IL-6, chemokines, such as IL-8, the inflammatory regulator NF-κB as well as cyclooxygenases in epithelial cells, fibroblast-like synoviocytes and neutrophils (21-23). A-SAA can induce extracellular matrix enzymes, such as matrix metalloproteinases (MMPs) (24-25), collagenase and stromelysin, which are important in repair processes after tissue injury (26), but following their prolonged production these enzymes can play degradative roles. A-SAA has potent chemoattractant activity for, among other human cell types, monocytes (27), phagocytes (28), T cells (29) and mast cells (30), the in vivo consequence of which is their recruitment to inflammatory sites. Little however is known about the role of A-SAA in activating human endothelial cells.

The objectives of this study are to investigate the role of acute phase SAA in stimulating possible atherogenic inflammatory responses in the endothelium and to compare these responses between two different human endothelial cells. We used primary human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAEC) in order to determine SAA dependent expression of the pro-inflammatory marker IL-6, chemokine IL-8 and adhesion molecules (ICAM, VCAM and E-selectin) and to find out whether A-SAA itself is expressed in these endothelial cells.

MATERIALS AND METHODS

Materials

The lyophilized recombinant human SAA (endotoxin tested) was purchased from CytoLab (Peprotech/Asia), spun down and reconstituted according to the manufacturer’s instructions. The recombinant SAA used is a hybrid of SAA1 and SAA2, corresponding to SAA1α, except for the Nt methionine and a substitution of asparagine to aspartic acid at position 60 and arginine to histidine at position 71, which are known to be present in SAA2β. Human recombinant IL-1β was obtained from Invitrogen Europe (KemoMed, SI). Aliquots of both reagents were stored at -20°C until used.

Cell culture

Human umbilical vein endothelial cells (HUVEC) passage 1 and human coronary artery endothelial cells (HCAEC) passage 3 were purchased from Cambrex BioScience, Belgium. Cells were grown under standard 5% CO₂ humidified atmosphere at 37°C in endothelium growth medium (EGM-2), supplemented with singlequots EGM-2-MV (HCAEC with 5% fetal bovine serum) and singlequots EGM-2 (HUVEC with 2% fetal bovine serum). Cells were routinely passaged with Trypsin/EDTA, grown in 6 well plates (TPP, Switzerland) and were used for experiments at low passages between 3 and 6. Two hours prior to each experiment cell cultures were
serum starved and fresh serum-free medium was delivered again immediately prior to treatments.

Measurement of human IL-6, ICAM, VCAM and E-selectin proteins
HUVEC and HCAEC supernatants were collected 24 h after starting cell culture treatments and spun for 3 min at 5,000 rpm, aliquoted and stored at -20°C. Samples were thawed and allowed to reach room temperature and, when necessary, diluted in the standard diluent buffer provided by the ELISA manufacturers. The concentrations of human released IL-6, soluble ICAM-1, soluble VCAM-1 and E-selectin were measured in duplicate using ELISA kits (BioSource, Belgium) following the manufacturer’s instructions. Statistical analysis was performed using analysis of variance, from the mean ± SD of three separate experiments with *p<0.05; **p<0.01 compared to basal levels of cell expression.

RNA isolation, reverse transcription, and polymerase chain reaction (PCR) analysis
Total RNA was extracted using RNAgents. Total RNA Isolation System (Promega, Europe) following the manufacturer’s instructions. RNA purity and amount were estimated by spectrophotometry. 1 μg of total RNA was transcribed into cDNA by Reverse Transcription System (avian myeloblastosis virus, Promega, Europe) and PCR (with PCR MasterMix, Promega, Europe) was performed for β-actin, IL-6, IL-8, IL-8R, SAA1/2, ICAM-1, VCAM-1 and E-selectin. The specific sets of primers, conditions and generated fragment sizes are listed in Table I. β-actin was used in order to compare equal loading, normalize the data and establish consistency of the PCR reactions. A negative PCR control reaction was run alongside all reactions. The PCR products were loaded onto 4% agarose E-gels (Invitrogen, Europe), electrophoresed and viewed under UV illumination.

RESULTS
SAA dose dependent stimulation of IL-6 in HUVEC and HCAEC
To determine whether and to what extent SAA increases the inflammatory response in HUVEC and HCAEC, released protein levels of IL-6 of supernatants were measured by ELISA (Fig. 1, panel A) following a 24 h period of incubation with SAA, and isolated the RNA and performed RT-PCR (Fig. 1, panels B and C).

Firstly, confluent cultures of normal human HUVEC and HCAEC were incubated with SAA in varying concentrations from 0-2000 nM. We then examined the effect of SAA increasing doses on IL-6 protein levels (Fig. 1 panel A) and on IL-6 mRNA expression (Fig. 1 panels B and C) and compared them in HUVEC and HCAEC. The results showed that HCAEC strikingly elevated both IL-6 released protein and mRNA expression following SAA stimulation. HCAEC already exhibited a significant increase of IL-6 protein following stimulation with the lowest SAA concentration of 10 nM (p<0.05) and in contrast there was no such significant increase of IL-6 found in HUVEC at this concentration. At the highest SAA concentration of 2000 nM, IL-6 released protein levels on HCAEC were 4 fold higher than on HUVEC. These significant differences were not only confirmed by the IL-6 mRNA expression levels following RT-PCR in HUVEC (Fig. 1, panel B-middle) and HCAEC (Fig. 1, panel C-middle), but

Table I. PCR primers and conditions.

<table>
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<tr>
<th>Name</th>
<th>Primer sequence (all 5' to 3')</th>
<th>T&lt;sub&gt;a&lt;/sub&gt;</th>
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<th>Fragm. size</th>
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<tr>
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<td>s: ATGAACCTCTTCTCCACAGGC</td>
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<tr>
<td>IL-6 as: GAAAGGCGCCTGAGCGGACTG</td>
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<tr>
<td>s: CAAGGAGGTCTAAGAACTT</td>
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<tr>
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<td>303 bp</td>
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<tr>
<td>E-selectin as: ACTTGAGTCACCGAGCGAGG</td>
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<td>30</td>
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Ta, temperature of annealing; Cycle #, number of cycles used in PCR; Fragm. size, fragment size
showed even greater differences (larger than 20 fold as measured by densitometry-data not shown) than was found at the protein levels. A PBS background control was always included in the experiments (lane 1, 0nM SAA), IL-1β was used (1 ng/ml) as a positive control and a PCR negative control was run with all reactions. Taken together, there were striking and significant differences on both IL-6 expression levels (mRNA and protein) between HUVEC and HCAEC in response to SAA with HCAEC giving a much higher response to all SAA concentrations.

In order to determine the effect of SAA increasing doses on chemokine expression, IL-8 mRNA levels were also compared in both HUVEC (Fig. 1, panel B-bottom) and HCAEC (Fig. 1, panel C-bottom). Similarly to IL-6 mRNA expression levels, IL-8 expression in HCAEC showed SAA dose dependency starting already at 10 nM. In HUVEC IL-8 mRNA was not shown to be expressed until SAA concentrations of 1000 nM and greater. IL-8 receptor mRNA expression however, did not correlate to increasing doses of SAA and stayed around background levels in both HUVEC and HCAEC (data not shown).

Expression of adhesion molecules (s-ICAM-1, s-VCAM-1, E-selectin) with increasing concentrations of SAA

The response of both cell types to SAA was further examined with influence on adhesion molecule expression (Fig. 1, panel B-bottom). Similarly to IL-6 ELISA of supernatants (Fig. 1, panel A) and HCAEC (Fig. 1, panel B-bottom) and HCAEC (Fig. 1, panel C-bottom).
The SAA dose dependent response of all adhesion molecules was also confirmed at the mRNA levels (at 2000 nM SAA, sICAM-1 is 1.5 fold higher on HUVEC, while IL-6 is 4 fold higher on HCAEC).

Expression of SAA mRNA levels as positive feedback to its own stimulation

In order to determine whether SAA is expressed in both HUVEC and HCAEC and whether its own expression is dose dependently induced, we performed RT-PCR. Following a 24h incubation of HUVEC and HCAEC with increasing doses of SAA resulted in a dose dependent SAA mRNA expression, but only in HCAEC (Fig. 3, panel A). SAA mRNA expression was slightly detected even at background control levels in HCAEC, but was not detected at all in HUVEC, even with the highest concentration (2000 nM) of SAA used (Fig. 3, panel B).

DISCUSSION

In the past 10 years there has been an increasing body of reports strengthening the postulation that inflammation not only provides the baseline for future atherogenetic processes leading to atherosclerosis but is also a prerequisite at different stages of plaque build-up and/or plaque rupture, the latter providing a gateway to thrombosis (1-2).
Many human vascular diseases are restricted to specific vessel types, and endothelial cells derived from these vessels can elicit completely different responses. For example, the susceptibility of arterial and vein vessels to atherosclerosis is different as is the contribution of platelets to the pathogenesis of arterial and venous thrombosis (31). There is a lack of reports describing the possible mechanisms of SAA in arterial and venous thrombosis, even though antibodies against SAA have been described, and association was found between deep vein thrombosis and anti-SAA antibodies (32). Previously our group has indicated that SAA could serve as a good predictor of progression from a non-inflammatory thrombotic condition to an inflammatory one (33).

To date there have been a limited number of reports indicating SAA expression in either human artery and/or vein endothelial cells. A-SAA protein was identified in the supernatants of primary synoviocyte cultures, and its expression co-localized with vascular endothelial cells (34). Next, the same group showed that A-SAA mRNA and its receptor FPRL1 mRNA were present in endothelial cells isolated from the synovial tissue of patients with RA and other categories of inflammatory arthritis (35). In human microvascular endothelial cells A-SAA was shown to increase adhesion molecule expression, endothelial cell tube formation and migration and induced NF-κB translocation (36).

SAA expression in endothelial cells lining blood vessels was first reported in 1994 by Meek et al. (12), which was later confirmed, and studies further expanded on human aortic smooth muscle cells (37) which were found to express both the acute and constitutive isoforms of SAA. HUVEC was not found to express any detectable levels of SAA (37), which we can also confirm with our current data (Fig. 3, panel A).

We report in this study the following novel observations: first is the exclusive SAA mRNA expression in HCAEC versus HUVEC and its own positive feedback (Fig. 3B versus Fig. 3A); second is the striking difference between IL-6 levels of HCAEC and HUVEC, with the coronary artery cells having a ~4 fold higher induction of IL-6 released protein (Fig. 1A) and a ~20 fold higher induction of IL-6 mRNA expression (Fig. 1B and C, densitometry not shown); and third is that in HUVEC the level of adhesion molecule expression is slightly greater than in HCAEC (Fig. 2A). These observations lead to the speculation that HCAEC are more responsive to A-SAA in inducing pro-inflammatory IL-6 and IL-8 than adhesion molecules in comparison to HUVEC and that different receptors and/or signaling pathways may be activated. To date there has been only one other report in literature describing SAA induced IL-6 and IL-8 protein secreted levels in HCAEC presented as a comparison to its main theme adipokine/adipocyte study, which however, did not show a dose dependent response or any confirmation.

Fig. 3. Expression of SAA with increasing concentrations of SAA. A: RT-PCR representative experiment (from three separate experiments performed) of β-actin and SAA PCR from HUVEC (concentrations of SAA: Lane 1) 0nM, 2) 10nM, 3) 100nM, 4) 500nM, 5) 1000nM, 6) 2000nM) B: RT-PCR representative experiment (from three separate experiments performed) of β-actin and SAA PCR from HCAEC (concentrations of SAA: Lane 1) 0nM, 2) 10nM, 3) 100nM, 4) 500nM, 5) 1000nM, 6) 2000nM). - negative PCR reaction, + positive IL-1β control.
at the mRNA expression levels (38).

Our current data provide an indication of greater susceptibility of coronary artery endothelial cells to inflammatory stimuli and a confirmation of the potential role which SAA plays in cardiovascular diseases.

In the future we plan to expand our studies to include a more complete study of A-SAA-receptor signaling and the inhibitory mechanisms of SAA induced proinflammatory effects in human endothelial cells.

ACKNOWLEDGEMENTS

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REFERENCES


MODULATION OF GRO-α AND TNF-α PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS TREATED WITH KILLED HELICOBACTER PYLORI

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GRO-alpha seems to play an important role in recruiting and activating neutrophils during Helicobacter pylori infection. In the present study, we examined how treatment with killed H. pylori or/and live H. pylori may differentially influence the in vitro GRO-alpha and TNF-alpha release by peripheral blood mononuclear cells (PBMC). The amounts of TNF-alpha and GRO-alpha produced by PBMC after stimulation with live H. pylori were higher than those produced after stimulation with a combination of killed and live H. pylori and the latter were higher than those produced after stimulation with killed H. pylori. In conclusion, the treatment of peripheral blood mononuclear cells with killed H. pylori down-regulates the production of GRO-alpha. Taken together, our data demonstrate that treatment with killed H. pylori could represent an innovative approach during gastric infection supported by H. pylori.

H. pylori is the most common bacterial pathogen involved in human gastrointestinal pathology. Gastric colonization by H. pylori, a minimally invasive gram-negative bacterium, is the major cause of chronic active gastritis and is often associated with both duodenal and gastric ulceration, as well as gastric carcinoma and mucosa-associated lymphoid tissue lymphoma (1-3). The local inflammation induced by H. pylori is characterized by infiltration of neutrophils, lymphocytes, plasma cells and monocytes in the gastric mucosa and by the local production of cytokines and chemokines (4-5). Among these, GRO-α, a CXC chemokine, seems to play an important role in recruiting and activating neutrophils in the gastric mucosa. In addition, TNF-α has been demonstrated to be important for the up-regulation of GRO-α production and that at high concentrations, it can by itself injure the gastric mucosa and may be responsible for severe pathology (6-7). GRO-α possesses potent neutrophil-stimulating activity by inducing chemotaxis, shape change, a rise in intracellular free calcium levels, exocytosis, and the respiratory burst in these cells (8). GRO-α was initially isolated and characterized by its growth stimulatory activity on malignant melanoma cells (9). Moreover, this peptide can regulate endothelial cell proliferation, stimulating angiogenesis (10).

In a previous study, carried out in an in vitro model (11), we demonstrated that the immunological disorders determined by H. pylori infection could be related to a shift from a Th-2 to a Th-1 type cytokine profile. In light of these results, in the present study we analyze the direct effect of H. pylori (live or...
gentamicin-killed) on human PBMC in order to evaluate the in vitro production of TNF-α and of GRO-α which seems to represent a key factor in the clinical outcome of inflammatory diseases and malignancies including gastric carcinoma.

MATERIALS AND METHODS

PBMC were obtained from healthy, *H. pylori*-seronegative donors, after centrifugation of heparinized venous blood over Ficoll-Hypaque gradient (Pharmacia, Milan, Italy) (12). PBMC were then washed twice in RPMI 1640 medium and cultured in 24 well plates (Corning, Bibby srl, Milan, Italy) at a concentration of 2x10⁶ (colony-forming unit) CFU/mL per well in RPMI 1640 medium. PBMC were cultured at 37°C in 5% CO₂ atmosphere, in RPMI 1640 (Biochrom KG Seromed, Milan, Italy) supplemented with 50 mM 2-mercaptoethanol, 1 mM pyruvate, 1 mM non-essential aminoacids, 1 mM HEPES and 5% fetal calf serum (FCS) (Biochrom KG Seromed, Milan, Italy). Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma, Milan, Italy) were found to contain <10 pg of endotoxin per mL.

Supernatants from PBMC in different experimental conditions were collected and analysed for the presence of TNF-α and GRO-α by an immunoenzymatic method: human TNF-α Quantikine immunoassay and human GRO-α Quantikine immunoassay, (all from R&D Systems, Milan, Italy); the limit of detection was respectively 4.4 pg/mL for TNF-α and 10 pg/mL for GRO-α. Monoclonal anti-human TNF-α antibody (ND₄₀) for this lot of anti-human TNF-α antibody was 0.015-0.06 µg/mL in the presence of 0.25 ng/mL of human TNF-α (R&D Systems, Milan, Italy). Monoclonal anti-human GRO-α antibody (ND₄₀) for this lot of anti-human GRO-α antibody was 1 µg/mL in the presence of 6 ng/mL of rhGRO-α (R&D Systems, Milan, Italy).

*H. pylori* isolated from the antral mucosa and associated duodenal ulcer was plated on Skirrow’s agar and incubated at 37°C in a microaerophilic environment for 5 days, harvested and diluted in sterile phosphate-buffered saline (PBS) (pH 7.2).

The concentration of bacteria was estimated by measuring the absorbance of the suspension and comparing the value to a standard curve. The standard curve was generated by measuring the absorbance of an array of serially diluted samples before quantifying the number of viable bacteria in each sample by a colony assay. After centrifugation at 2,500 x g for 15 min, bacteria were re-suspended in PBS to a range of concentrations from 10⁵ to 10⁹ CFU/mL, diluted in PBS. The motility of the organisms was confirmed by phase-contrast microscopy prior to use. For the experiments with killed bacteria, *H. pylori* was treated with gentamicin 4 mg/mL (Seromed, Milan, Italy) for 45 min at 4°C, washed, and diluted in PBS to the same concentrations as the live bacteria (13).

The combined treatment was performed by adding a suspension of gentamicin-killed bacteria (1.2 x 10⁶ killed CFU/ml) to PBMC for 20 hours, and after this period adding 1.2 x 10⁹ CFU/mL live *H. pylori* for further 24 hours. After 44 hours supernatants were harvested, centrifuged and stored at −80°C until cytokine assays.

To determine the effect of different incubation times on cell viability a colorimetric assay was used as described by Mosmann (14). The assay is based on the tetrazolium salt 3-(4,5 dimethylthiazol-2-yl)2,5 diphenyltetrazoliumbromide (MTT), (Sigma, Milan, Italy) a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, PBMC were seeded in 96-microwell plates at 2x10⁴ per well, then treated with live or killed *H. pylori* for 24 or killed + live *H. pylori* for 44 hours. MTT diluted in saline solution was added to the cells and incubated for 4 hours, then acid propan-2-ol (0.04M HCl in propan-2-ol) (Sigma, Milan, Italy) was used to solubilize the formed crystals. The plates were read with a microELISA reader using a wavelength of 570 nm. Cytotoxicity percentage was calculated as follows:

\[
\text{Cytotoxicity} = \frac{(\text{experiment OD} - \text{lysis control OD})}{(\text{cell control OD} - \text{lysis control OD})} \times 100
\]

Results are expressed as the means of five experiments + standard deviation (SD). Data were analysed by one way ANOVA and the Student-Newman-Keuls test. Differences were considered statistically significant for p value of <0.05.

RESULTS

The effects of different concentrations of live *H. pylori* on cytokine release by PBMC are reported in Fig. 1. The results demonstrate that the production of GRO-α and TNF-α was dose-dependent. In particular, a significant differential stimulation was evident at a concentration of 1x10⁹ CFU/mL. In a second series of experiments, we examined how treatment with killed *H. pylori* or/and live *H. pylori* infection may differentially influence the in vitro cytokine release by PBMC. Results reported in Fig. 2 show that infection with live *H. pylori* triggers PBMC to release marked amounts of GRO-α compared with those induced by killed *H. pylori* (1972 ± 414 pg/mL vs 642 ± 121 pg/mL; p<0.05).
The combined treatments induced a down-regulation of GRO-α production compared to live *H. pylori* (1161 ± 185 pg/mL vs 1972 ± 14 pg/mL; p<0.05). In order to verify whether these results were influenced by the incubation time and additional treatment, the cell viability was analyzed by MTT test. It was found that the different incubation times (24 and 44 hours) as well as the treatment with killed and live *H. pylori* did not significantly influence the cell viability (data not shown).

**Fig. 1.** Effects of *Helicobacter pylori* concentration on GRO-alpha and TNF-alpha production by PBMC. Results represent the means of five experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.

**Fig. 2.** Production of GRO-alpha by PBMC after treatment with live, killed and killed+live *Helicobacter pylori* in presence or not of monoclonal antibodies vs TNF-alpha. Results represent the means of 5 experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.
Furthermore, as shown in Fig. 3, similar behaviour was seen for TNF-α. In particular, whereas live *H. pylori* infection induced marked levels of TNF-α compared with killed *H. pylori* (3285 ± 92 pg/mL vs 1436 ± 186 pg/mL; p<0.05), the combined effect (killed + live) resulted in higher levels of TNF-α compared to the treatment with killed *H. pylori* (2317 ± 417 pg/mL vs 1436 ± 186 pg/mL; p<0.05), but lower when compared with live *H. pylori* (2317 ± 417 pg/mL vs 3285 ± 492 pg/mL; p<0.05).

To verify a possible correlation between TNF-α and GRO-α we added monoclonal anti-TNF-α or anti-GRO-α antibodies to PBMC in all experimental conditions. As shown in Fig. 2 the addition of monoclonal Ab-TNF-α determined a down-regulation of GRO-α production. In particular, the addition of anti-TNF-α to PBMC respectively incubated with live, killed or killed+live *H. pylori*, decreased the levels of GRO-α produced by these cells (540 ± 86 pg/mL; respectively p<0.05). /mL vs 1972 ± 414; 180 ± 27 pg/mL vs 642 ± 121; 364± 61 vs 1161 ± 185.

These results demonstrate that the strong increase in GRO-α expression in PBMC infected with live *H. pylori* was, at least in part, dependent on the presence in supernatants of TNF-α. On the contrary, as shown in Fig. 3, the addition of monoclonal antibodies anti-GRO-α did not influence the TNF-α release. This finding demonstrates that TNF-α production is not supported by GRO-α. Furthermore, as shown in Fig. 2 and 3, the combined treatment with killed+live *H. pylori* produced the same effect, though to a lesser extent, on TNF-α and GRO-α release observed with killed *H. pylori*.

**DISCUSSION**

Few studies have investigated the expression of GRO-α and its role in the outcome of the *H. pylori* infection. Such knowledge is critical to fully understanding the relationships of *H. pylori* infection with gastric carcinoma.

Evidence for *in vivo* GRO-α induction in *H. pylori* infection has been reported by Suzuki *et al.*, who demonstrated increased mucosal levels of this chemokine in infected individuals, as well as decreased levels after eradication of infection (15). It is generally agreed that PBMC are important sources of chemokines and other proinflammatory mediators. In particular, it was shown that several chemokines are an essential component of the primary innate

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**Fig. 3.** Production of TNF-alpha by PBMC after treatment with live, killed and killed+live *Helicobacter pylori* in presence or not of monoclonal antibodies vs GRO-alpha. Results represent the means of 5 experiments using PBMC of 5 different donors. Data are shown as mean ± standard deviation.
immune response to *H. pylori* infection (4, 16-17).

In this paper we report our experimental data that further characterize the chemokine response induced in PBMC in vitro treated with live or killed *H. pylori*. Our results demonstrate that treatment with live *H. pylori* triggers PBMC to release marked amounts of GRO-α compared with those induced by killed *H. pylori*. In light of the results obtained with GRO-α and in order to better understand its role during *H. pylori* infection, we made a pre-treatment of PBMC with killed *H. pylori* and after 20 hours we infected the same cells with live *H. pylori*. Our findings demonstrate that the combined treatments induced a down-regulation of GRO-α production compared to live *H. pylori*. Furthermore a similar behaviour was seen for TNF-α.

To verify a possible correlation between TNF-α and GRO-α we added monoclonal anti-TNF-α or anti-GRO-α antibodies to PBMC in all experimental conditions. The addition of monoclonal Ab-TNF-α determined a down-regulation of GRO-α production. On the contrary, the addition of monoclonal antibodies anti-GRO-α did not influence the TNF-α release. These results demonstrate that the strong increase in GRO-α expression in PBMC infected with live *H. pylori* was, at least in part, dependent on the presence in supernatants of TNF-α. On this basis, we can speculate that TNF-α induced by *H. pylori* may exert a pathogenic effect not only by itself but also by supporting GRO-α production. Our results are in agreement with those of other Authors which demonstrate that TNF-α is important for the up-regulation of GRO-α production by stimulated human endothelial cells (18).

In previous studies, we reported that treatment of peripheral blood mononuclear cells with killed *H. pylori* promoted the release of anti-inflammatory cytokines such as IL-10 and IL-4, thus subverting immunological disorders accounted for by a shift from a Th-2 to a Th-1 type cytokine profile determined by live *H. pylori* infection (11, 19).

These data indicate that treatment with killed *H. pylori* may alter the balance of cytokines in the environment of cells, preventing potentially harmful effects of high levels of TNF-α and GRO-α representative of the deleterious Th1 immune response usually associated with *H. pylori* infection. In fact, these in vitro results demonstrate that pre-treatment with killed *H. pylori* plays a protective role during *H. pylori* infection decreasing inflammatory response supported by Th-1 cytokines and chemokines.

Taken together our results suggest that treatment of PBMC with killed *H. pylori* could subvert the environment of cytokine patterns responsible for the inflammatory process and marked recruitment of monocytes and lymphocytes in gastric mucosa during in vivo *H. pylori* infection. Therefore, it could be speculated that treatment with killed *H. pylori* could represent an innovative therapeutical approach during *H. pylori* gastric infection.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


TREATMENT OF MILD TO MODERATE PLAQUE PSORIASIS WITH CALCITRIOL OINTMENT APPLIED WITH OR WITHOUT A DOSING DEVICE

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Results of topical treatments can be influenced by several factors, including accurate dosing based on the affected skin area. The aim of this open-labelled multicenter study is to evaluate if correct dosing of calcitriol ointment has an impact on the clinical response of plaque psoriasis. For this purpose, patients with plaque psoriasis eligible to be treated with calcitriol ointment were randomized to treatment with a ‘standardized dose’ method, using a dosing device (N. 100), or treatment without the device (N. 101), for 12 weeks. Regardless of the use of the dosing device, calcitriol ointment caused a significant reduction of both the severity of signs and symptoms and the extent of body surface area affected after both 4 weeks and 12 weeks. Clinical response was not significantly different between the two groups. Local adverse events occurred in 12 subjects (of whom five treated with the device) and were mild and transient in most cases.

Vitamin D3 analogues are well-established topical treatments for psoriasis and are a first-line approach to mild/moderate chronic plaque psoriasis. These drugs are thought to inhibit proliferation and promote differentiation of keratinocytes by regulating intracellular calcium concentrations. Calcitriol (1alpha,25-dihydroxyvitamin D3) is the physiological active form of vitamin D3 and is an essential hormone for calcium homeostasis. Biological activity of calcitriol occurs through the binding to nuclear receptors, which are found in several tissue types and cell lines, including keratinocytes and fibroblasts in the skin (1-2). Several studies have shown that calcitriol modulates not only keratinocyte proliferation and differentiation but also inflammation and immune response in vivo (3-8). These effects are important for the therapeutic activity of calcitriol ointment in psoriasis documented by several clinical studies (9-14).

Results of topical treatments can be conditioned by several factors, including patient adherence and accurate dosing. For example, underdosing may reduce the efficacy of topical medications, as well as overdosing may augment the risk of side effects.

In this study we evaluate whether correct dosing of calcitriol ointment influences the efficacy and tolerability outcomes in patients with mild to moderate plaque psoriasis. For this purpose, we analyse the results obtained with or without the use of a dosing device.

Key words: plaque psoriasis, calcitriol, vitamin D3 analogue, topical treatment, dosing device

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MATERIALS AND METHODS

The study was designed as an open-labelled multicenter assessment of the efficacy and tolerance of calcitriol 3 microg/g ointment (Silkis®, Galderma S.p.A., Italy) used for 12 weeks by adult patients with mild to moderate chronic plaque psoriasis, affecting less than 35% of the body surface area (BSA).

The exclusion criteria included: forms of psoriasis other than plaque-type; concomitant skin diseases; hypercalcemia or other abnormalities in calcium and phosphorus metabolism; relevant liver and kidney disorders; pregnancy and breast feeding; hypersensitivity to any ingredients of the study products (e.g., both Silkis® ointment and emollients); the use of any drugs known to affect psoriasis severity and study evaluations; unwillingness to adhere to the study procedures. Patients must not have received anti-psoriasis treatments during a fixed period prior to the start of the study defined as follows: 12 weeks for biologicals, 4 weeks for other systemic therapies and phototherapy, 2 weeks for topical medications and 1 week for emollients or keratolytics.

After oral consent, patients applied calcitriol ointment twice daily to all skin lesions for 12 weeks, with or without the aid of a dosing device. In order to avoid potential selection bias, consecutive patients were alternately randomized to one of the two treatment methods according to a 1:1 ratio. The dosing device (Silkidose®), patented by Galderma Laboratories, consisted of a graduated spatula that allowed the patients to quantify the exact amount of the ointment to be applied on the basis of the size of affected skin areas (15). So, patients who belonged to the ‘standardized-dose’ treatment regimen received the device and an instruction form reporting the exact number of doses per administration, whereas the quantity of the ointment to be applied was left to the discretion of patients in the ‘unstandardized-dose’ group.

No active anti-psoriasis treatments were permitted during the study period, with the exception of emollients (a cream containing 10% urea, Nutraplus®, or another containing 10% urea and 5% lactic acid, Nutraplus Forte®) which were used by patients on an ‘as-needed’ basis.

Evaluations were made at the baseline (W0), at week 4 (W4), and at the end of treatment (W12). At each visit, both the extent of BSA affected and the severity of symptoms and signs were evaluated. BSA affected was reported as the percentage of the total area for each of the following body sites: head, upper limbs, trunk and lower limbs. The severity of erythema, scaling and thickness was graded using a 5-score rating scale (0= absent; 1= mild; 2= moderate; 3= severe; 4= very severe). The same scale was used by patients for the assessment of symptoms (pruritus/burning).

Changes of clinical parameters in each group were analysed under a statistical point of view by the Wilcoxon matched-pairs signed-ranks test, whereas differences between the two groups at each visit were evaluated using the Mann-Whitney U test (significance for p values <0.05 in both cases).

At the end of treatment, regardless of its duration, patients and dermatologists gave their independent opinion about the efficacy of the study treatment, rating it on the basis of a 4-point scale. Patients were also asked to score the acceptability of treatment.

Details of adverse events (AEs) were obtained throughout the study period.

RESULTS

The study was conducted between October 2005 and May 2006. A total of 201 patients (109 males) aged 18 to 80 years (mean age, 42.2) with chronic plaque psoriasis were enrolled and received study treatment. Of these, 100 patients were required to use the dosing device and 101 patients entered the ‘unstandardized-dose’ treatment with calcitriol ointment. At the baseline, clinical parameters did not significantly differ between the two groups (Table I) with the exception of BSA affected of the trunk which was greater in the group of patients who did not use the dosing device as compared to the other group. No relevant inter-group differences were observed in the distribution of the gender of patients and affected sites nor in the type of emollient used throughout the study period (Table I).

Eight patients (four patients in each group) withdrew from the study after W4 and did not undergo clinical assessment at W12 visit. Three patients were lost to follow-up; reasons for discontinuation in the other cases were treatment-related AEs in four cases (two in each group) and lack of efficacy in another patient.

Independently of the use of the dosing device, calcitriol ointment caused a significant improvement of psoriasis lesions at W4 and W12 and reduced both the severity of signs and symptoms (Fig. 1), and the extent of BSA affected (Fig. 2). Clinical response after both 4 weeks and 12 weeks did not significantly differ between the two groups.

In general, calcitriol ointment was well tolerated. No serious AEs were observed. Local AEs such as irritation and pruritus occurred in 12 subjects (of
whom five treated with the device) and were mild and transient in all cases but four in whom these events were responsible for study interruption. The development of AEs did not appear to be related to either the type of emollient concomitantly used or the site of treatment, with only two subjects reporting local irritation after application to the scalp or the face.

At the end of treatment period, the dermatologists’ and patients’ global assessment of efficacy was considered positive in the majority of cases with a similar rate in the two treatment groups (Table II).

Approximately three-quarters of patients in both groups judged as good or excellent the acceptability of treatment.

DISCUSSION

The efficacy, safety and tolerability of calcitriol ointment for the treatment of plaque psoriasis has been demonstrated by several studies (8-14, 16-21), which also showed that calcitriol is well tolerated even when applied to sensitive skin areas and is less irritating than calcipotriol. Our cumulative

<table>
<thead>
<tr>
<th>Total number of patients (n)</th>
<th>CALCITRIOL WITH DOSING DEVICE</th>
<th>CALCITRIOL WITHOUT DOSING DEVICE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Male</td>
<td>52</td>
<td>57</td>
</tr>
<tr>
<td>- Female</td>
<td>48</td>
<td>44</td>
</tr>
<tr>
<td>Affected sites (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Head</td>
<td>47</td>
<td>39</td>
</tr>
<tr>
<td>- Upper limbs</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td>- Lower limbs</td>
<td>76</td>
<td>82</td>
</tr>
<tr>
<td>- Trunk</td>
<td>58</td>
<td>63</td>
</tr>
<tr>
<td>% BSA affected: mean (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Head</td>
<td>8.2 (15.1)</td>
<td>4.35 (7.03)</td>
</tr>
<tr>
<td>- Upper limbs</td>
<td>12.5 (12.4)</td>
<td>12.1 (11.4)</td>
</tr>
<tr>
<td>- Lower limbs</td>
<td>11.4 (12.15)</td>
<td>12.4 (12.3)</td>
</tr>
<tr>
<td>- Trunk</td>
<td>7.9 (11.8)*</td>
<td>12.7 (15.3)*</td>
</tr>
<tr>
<td>Severity score: mean (SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Erythema</td>
<td>2.1 (0.7)</td>
<td>2.1 (0.7)</td>
</tr>
<tr>
<td>- Scaling</td>
<td>2.5 (0.8)</td>
<td>2.4 (0.6)</td>
</tr>
<tr>
<td>- Thickness</td>
<td>2.0 (0.9)</td>
<td>1.8 (0.75)</td>
</tr>
<tr>
<td>- Symptoms (pruritus/burning)</td>
<td>1.8 (0.8)</td>
<td>1.9 (0.8)</td>
</tr>
<tr>
<td>Emollients used during the study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Nutraplus® cream</td>
<td>48</td>
<td>50</td>
</tr>
<tr>
<td>- Nutraplus Forte® cream</td>
<td>52</td>
<td>51</td>
</tr>
</tbody>
</table>

*Table I. General characteristics and baseline clinical parameters. At the baseline, no significant inter-group differences were observed in the distribution of the gender of patients and clinical parameters with the exception of BSA affected of the trunk which was greater in the group of patients who did not use the dosing device as compared to the other group (*p<0.05 – Mann-Whitney U test). The type of emollient used throughout the study period did not differ between the two groups. BSA affected and severity score are reported as mean value along with standard deviation (SD).
Eur. J. Inflamm. results confirm that calcitriol 3microg/g ointment is an effective and safe treatment for chronic plaque psoriasis, even when used in affected areas of the head (scalp and face).

The primary objective of our study was to determine whether the use of an ‘exact-dose’ treatment, achieved with the aid of a dosing device (Silkidose®), has an impact on efficacy and/or safety results.

The problem of accurate dosing is theoretically important for efficacy and tolerability of topical medications. The estimation of the adequate amount of topical treatment is individualized after the evaluation of the lesional skin areas and the subsequent calculation of the dose to be applied according to the BSA affected. There are no standardized procedures in dermatology which allow patients to exactly dose their topical treatment. One of the best known methods of dosing measurement consists in the use of fingertip units, originally created for topical therapy in children (22). However, in clinical practice, prescription of topical treatments usually disregards dosing procedures or at least takes into account only generic instructions (i.e. application of ‘small quantities’; limit of application on a pre-determined body surface or limit of consumption of a fixed amount per day or per week in accordance with the package insert of medications).

The cumulative results of our study suggest that the application of an exact dose of calcitriol ointment, achieved by the use of a graduated spatula (Silkidose®), gave no advantages compared to the conventional treatment without the dosing device.

**Fig. 1.** Change in the severity of signs and symptoms during treatment with calcitriol ointment applied with (A) or without (B) the dosing device. Variation of clinical parameters as compared to baseline in each group: p<0.01 at W4, p<0.001 at W12; differences of parameters between the two patient subgroups at each visit: p>0.05.

results confirm that calcitriol 3microg/g ointment is an effective and safe treatment for chronic plaque psoriasis, even when used in affected areas of the head (scalp and face).

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The cumulative results of our study suggest that the application of an exact dose of calcitriol ointment, achieved by the use of a graduated spatula (Silkidose®), gave no advantages compared to the conventional treatment without the dosing device.

**Fig. 2.** Change in the extent of affected skin areas during treatment with calcitriol ointment applied with (A) or without (B) the dosing device. Variation of affected BSA as compared to baseline in each group: p<0.01 at W4, p<0.001 at W12; differences of values between the two patient subgroups at each visit: p>0.05.
In fact, the efficacy and tolerability results obtained with the two treatment methods were similar. Although there are no reliable reasons for these results, some reasonable speculations can be made. The risk of underdosing is probably low with an ointment and can be counterbalanced by the intrinsic characteristic of the formulation which allows a deep percutaneous penetration and a great spreadability (23). Overdosing may be a real problem when substances with a high irritating potential or an unfavourable safety profile are applied to the skin. This is not the case of calcitriol ointment, which is well tolerated in clinical practice and in experimental conditions (9-13, 16, 18-20). Moreover, patients with a chronic skin condition like psoriasis usually have gained a lot of experience in the management of their disease with topical therapy.

However, the dosing method in our study was well accepted by patients. Another consideration derived from our results is that calcitriol ointment can be safely combined to background treatment with urea-based emollients which can in turn enhance the activity of drugs in psoriasis acting as ‘therapeutic moisturizers’ (24).

ACKNOWLEDGEMENTS

The authors thank Dr Monica Carbonara (Bari, Italy) for her support in the statistical analysis.

REFERENCES

of psoriasis vulgaris with calcipotriol/betamethasone
diproprionate combination followed by calcipotriol
and assessment of the adjuvant basic use of urea-
CARVEDILOL INCREASES ATRIAL NATRIURETIC PEPTIDE PLASMA LEVELS IN HYPERTENSIVE PATIENTS


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Twenty five patients with moderate essential hypertension were studied for 30 days in order to evaluate the medium term effects of carvedilol on atrial natriuretic peptide (ANP) levels. This drug blocks the β₁ and β₂ adrenenergic receptors as well as α₁ adrenergic receptors. In addition, it has strong antioxidative and antiproliferative properties. The drug was given orally at a dose of 12.5 mg b.i.d. Quantitative determination of human ANP was made by radioimmunoassay procedure (RIA). At the end of this clinical trial, mean plasma levels of ANP had risen during treatment by 21.18% (from 37.60 pg/ml to 45.83 pg/ml) while both systolic (SBP) and diastolic (DBP) blood pressure as well as diameters of the left cardiac cavities had decreased in a statistically significant way. The ratio ANP/SBP was also increased by 46.6% in a statistically significant way. These findings support the suggestion that the increase in plasma ANP following the administration of β-adrenergic blockers to hypertensive patients is a primary effect of beta blockade and not a mechanical one secondary to a negative inotropic action on the left ventricle and obviously contributes to the anti-hypertensive action.

Major interest has been focused upon the investigation of unconventional methods of the anti-hypertensive action of drugs used for the treatment of arterial hypertension. A strong motive for the interest in this particular subject is derived from the fact that no conventional hemodynamic effect can provide a satisfactory explanation for the chronic long-term anti-hypertensive efficacy of β-adrenergic blockers. The investigation, which was conducted by the research group of the 2nd Department of Cardiology with the cooperation of the Departments of Pharmacology and Biochemistry at the Medical School of the Aristotle University of Thessaloniki, resulted in a number of presentations and publications in Greece and abroad. According to the investigation, ANP seems to be an important mediator between the β-adrenergic blockers and the therapy of arterial hypertension. Since then, interest has been expanded to investigate other anti-hypertensive agents and their linkage to ANP and the results of such investigation have been confirmative (1-4).

Subsequently, it sounded reasonable to investigate a possible role of ANP upon the anti-hypertensive action of drugs used for the treatment of arterial hypertension. A strong motive for the interest in this particular subject is derived from the fact that no conventional hemodynamic effect can provide a satisfactory explanation for the chronic long-term anti-hypertensive efficacy of β-adrenergic blockers. The investigation, which was conducted by the research group of the 2nd Department of Cardiology with the cooperation of the Departments of Pharmacology and Biochemistry at the Medical School of the Aristotle University of Thessaloniki, resulted in a number of presentations and publications in Greece and abroad. According to the investigation, ANP seems to be an important mediator between the β-adrenergic blockers and the therapy of arterial hypertension. Since then, interest has been expanded to investigate other anti-hypertensive agents and their linkage to ANP and the results of such investigation have been confirmative (1-4).

Subsequently, it sounded reasonable to investigate a possible role of ANP upon the anti-hypertensive action

Key words: carvedilol, ANP, hypertension, β-adrenergic blockers

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This study aims to investigate the medium-term anti-hypertensive effect of carvedilol in relation to plasma ANP concentration changes in patients with moderate uncomplicated essential hypertension. Carvedilol is a racemic form made up of S(-)carvedilol and R(+)carvedilol. Carvedilol has multiple actions. R(+)carvedilol blocks the β₁ and β₂ adrenergic receptors as well as α₁ adrenergic receptors. The S(-)carvedilol has only α₁-inhibiting properties. In addition, carvedilol has strong antioxidative and antiproliferative properties (5).

**MATERIALS AND METHODS**

Twenty-five patients (11 males and 14 females) aged between 33 and 78 years (mean age 53.8 ± 11.5) with mild to moderate uncomplicated essential hypertension were enrolled in this study. All patients satisfied the criteria to be treated with β-adrenergic blocker carvedilol and had resting heart rate of ≥70/min with normal atrioventricular conduction. All patients had normal renal function, as detected by conventional biochemical tests, or by radioisotopic studies whenever it was necessary. All patients had normal left atrial and ventricular dimensions as well as systolic ventricular function as detected by the echocardiogram. There was no evidence of ischemia on the surface resting ECG. Whenever it was necessary, ischemia was ruled out with myocardial perfusion imaging (SPECT, Thallium-201). The patients were on no drug treatment before the trial or otherwise all anti-hypertensive medications were discontinued 2 weeks prior to enrollment in the trial, since they had no severe hypertension. Patients were adequately informed regarding the objectives of the trial and written consent form was signed. After a washout period of 15 days for those who were receiving anti-hypertensive medications, as well as behavioral modification for the rest, if blood pressure was still persistently higher than 150/90, drug treatment was initiated. Prior to this, an echocardiogram

**Table I. Changes in the parameters studied before and after 30 days treatment with carvedilol 12.5 mg daily (means ± SD, n: 25).**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>Units</th>
<th>Changes %</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>168.33±29.7</td>
<td>128.95±13.3</td>
<td>mmHg</td>
<td>-23.4</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>DBP</td>
<td>97.91±15.87</td>
<td>81.25±6.95</td>
<td>mmHg</td>
<td>-17.0</td>
<td>p~0.001</td>
</tr>
<tr>
<td>LVESD</td>
<td>3.34±0.62</td>
<td>3.20±0.62</td>
<td>Cm</td>
<td>-4.19</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>LVEDD</td>
<td>5.06±0.57</td>
<td>4.86±0.62</td>
<td>Cm</td>
<td>-3.95</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>LVEF</td>
<td>63.67±7.53</td>
<td>65.94±8.82</td>
<td>%</td>
<td>+3.56</td>
<td>p-0.05</td>
</tr>
<tr>
<td>LAD</td>
<td>3.658±0.47</td>
<td>3.523±0.47</td>
<td>Cm</td>
<td>-3.69</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>P-Waves</td>
<td>133.33±36.21</td>
<td>17.60±14.64</td>
<td>msec</td>
<td>-11.79</td>
<td>p-0.01</td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>37.60±16.34</td>
<td>45.83±24.54</td>
<td>pg/mL</td>
<td>+ 21.18</td>
<td>p&gt;0.1</td>
</tr>
<tr>
<td>ANP/SBP</td>
<td>0.236±0.11</td>
<td>0.346±0.20</td>
<td>%</td>
<td>+46.6</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

SBP, systolic blood pressure; DBP, diastolic blood pressure; LVESD, left ventricular end systolic diameter; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fracture; LAD, left atrial diameter; ANP, atrial natriuretic peptide (plasma concentration); WT, Wilcoxon test.
was performed on all patients. Parameters measured were the dimensions of the left ventricle and the left atrium on M-mode echocardiogram in the left parasternal location using the long axis view with the conventional method. The values shown in the Tables are mean values of three to four measurements. Subsequently, P wave duration was measured using the signal average electrocardiogram (SAECG) with the patient being in the supine position. The duration of the atrial electrogram expressed by the filtered signal in the SAECG indicates the time from the initiation till the end of repolarization of the atrial myocardium. At the end of the measurements, the patients were kept in a supine position for 30 minutes. A vein puncture was performed in a peripheral vein to determine the concentration of plasma ANP. Blood pressure from the right arm was measured using a mercury sphygmomanometer while the patient was seated.

All the patients received carvedilol of the Roche company at the dosage of 12.5 mg b.i.d. per os. Thirty days after starting treatment, the above measured parameters were measured again in the same sequence three hours after taking the morning dose. ANP was measured again in blood samples taken by vein puncture three hours after the last drug dose administration.

Parallel Control groups consisting of patients receiving anti-hypertensive drug of another category were not included in this study because drugs such as β-adrenergic blockers, ACE inhibitors and calcium entry blockers have previously been shown to increase ANP plasma levels (1-4, 6-9).

Blood samples for plasma ANP level determination were collected in pre-chilled tubes containing EDTA-2Na (1.5 mg/ml blood) and aprotinin (500KIU/ml blood), placed on ice, and immediately centrifuged at 1500 g for 10 min. at 4°C. Plasma was frozen and stored at -70°C until the assay was performed. Quantitative determination of human ANP was made by radioimmunoassay procedure (RIA) combined with an extraction step using reagents supplied by Nichols Institute Diagnostics B.V (California, USA). Determination by RIA was performed by using an ANP (125I) radioimmunoassay (RIA) system according to the manufacturer’s instructions. Intrassay and interassay variations were 3.9% and 9.7% respectively. The results obtained (mean ± SD) were expressed as pg/mL. Normal values calculated in 30 healthy male individuals, mean age 51 years (range 30-60), in our laboratory were 35.3 ± 9.5 pg/mL (10-12).

The values obtained for each parameter, before and during treatment, were expressed in the form of mean ±1 standard deviation and the statistical analysis was carried out using the Students paired t-test as well as the Wilcoxon challenge because the distribution was not always regular.

RESULTS

Results are shown in detail in Table I and Fig. 1 and 2. Mean plasma levels of ANP rose after treatment by 21.18% (from 37.60 pg/ml to 45.83 pg/ml) while both systolic blood (SBP) and diastolic blood (DBP) pressure as well as left cardiac cavities diameters were decreased in a statistically significant way. The ratio ANP/SBP was also increased by 46.6% in a statistically significant way.

DISCUSSION

From this clinical study, it was found that carvedilol efficiently reduced the systolic and diastolic blood pressure by 23% and 17% respectively (Table I). Left ventricular dimensions were reduced
and ejection fraction was increased very slightly but in a statistically significant way although the patients had no signs of cardiac failure. The message derived from the above observations is that carvedilol did not exert any negative inotropic effect.

During this trial, a statistically significant reduction by 12% of atrial electrogram duration was observed, detected by the SAECG. The main factors regulating this time are the velocity of conduction and the length of the pathway. Given the fact that β-adrenergic stimulation exerts positive dromotropic effect on the myocardium, its inhibition should have the tendency to prolong the parameter under investigation. The reduction of atrial electrogram duration reflects the shortening of the length of the pathway, that is a reduction in the dimensions of both atria. This finding is aided by the echocardiogram where there was a confirmed reduction in the size of the left atrium.

The main finding of this trial was that the levels of plasma ANP showed a tendency to increase by 21.18% during the treatment with carvedilol. What was also interesting is the fact that there was a statistically significant increase of 46.6% in the ratio of plasma ANP divided by systolic blood pressure following the reduction of arterial blood pressure (Table I, Fig. 1). Systolic blood pressure has been positively associated with the mechanical stimulus affecting ANP secretion, therefore a reduction of plasma ANP concentration was to be expected. The diverting behavior of ANP and blood pressure clearly indicated by the variation of A.N.P./S.B.P. ratio together with the reduced atrial dimensions strongly support a primary stimulation for ANP secretion and not a mechanical one secondary to a negative inotropic action on the left ventricle.

This conclusion is in accordance with our previous observations on humans with older β-adrenergic blockers (1-4) as well as those of other researchers (6-9, 13-14, 18-21). The use of such control groups would be inappropriate. The only possible method was to compare ANP plasma levels before the treatment with carvedilol and during the treatment on the same patients.

The present report comprises a proof of the theory derived from clinical trials that was suggested by C. Papadopoulos, B. Kokkas et al and first published in 1992 (22).

REFERENCES


INTRARENAL RESISTIVE INDEX IN PATIENTS WITH TYPE 2 DIABETES MELLITUS WITH AND WITHOUT MICROALBUMINURIA

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Diabetic nephropathy affects a subset of about 30% of patients with type 1 Diabetes Mellitus (DM); it also develops in a less defined percentage (20-30%) of patients with type 2, after a period of 15-20 years. It is usually divided into stages. The aim of this study is to assess the usefulness of duplex sonography with Doppler wave form analysis in the evaluation of early diabetic nephropathy, in order to detect patients at risk for irreversible renal disease. 262 patients (61 males, 201 females; age range: 48-81 years) with type 2 diabetes mellitus were studied; 100 healthy volunteers with no evidence of diabetes mellitus (74 females, 26 males; age range: 50-80 years) composed the control group. All of them underwent duplex Doppler sonography of the kidneys; a scanner with a 3.5 MHz transducer (Toshiba 270 SSA) was used, Pulsatily Index (P.I.) and Resistive Index (R.I.) of Doppler waveform were obtained at the intrarenal arteries; the average value of 3 bilateral measurements was taken. Doppler sonography was done by the same authors without knowledge of the patient group (case or control). Both indexes (P.I. and R.I.) resulted to be higher in patients with DM compared to controls in patients with microalbuminuria: P.I. = 1.49 +/- 0.34 vs. 1.07 +/- 0.06, \textit{p}< 0.05; R.I. = 0.79 +/- 0.15 vs 0.60 +/- 0.03, \textit{p}<0.05. Even if our data have to be confirmed by further studies, they suggest that duplex Doppler sonography may be a useful complementary test in the evaluation of diabetic nephropathy, especially in the early stages, in order to identify more patients at risk of developing diabetic nephropathy.

Diabetic nephropathy is the most frequent cause of chronic renal failure: in Italy and in southern Europe it accounts for more than 15-20%, while in northern Europe and in the USA for 30 %; its prevalence is 30% in type 1 diabetes 10-20 years after the onset of disease and 20-30 % in type 2 diabetes (1). Considering the greatest incidence of the latter, it is clear that most of the diabetic patients with renal failure are of type 2 . Diabetic nephropathy has various stage of development. Up to 15 years ago there were no methods capable of identifying patients with early diabetic nephropathy, at a probable reversible stage (2). Years later a correlation between hypertension and glomerular damage was detected and some indices as nephromegaly, the increased filtration rate and mycroalbuminuria were found to be predictive of evolution in overt nephropathy. Microalbuminuria was firstly considered to be a
marker of incipient diabetic nephropathy (3-4). Now it is widely believed to be a sign of early stage of disease. In the urine from normal subjects is possible to find a low amount of albumin to a variable rate at different hours of the day and according to physical activity. The threshold value is 30 mg/die (5). The appearance of microalbuminuria in a diabetic patient sometime is concomitant with the onset of hypertension that critically increases the progression of albuminuria and the decline of glomerular filtration (6). In the last 30 years, studies evaluating renal haemodynamics have dramatically changed, previously being of an invasive type until the coming of ultrasonography (7).

Color-Doppler ultrasonography has provided a haemodynamic evaluation of renal flow in a totally non-invasive manner. In the kidney, Doppler ultrasound allows an accurate visualization of vasculature. By Doppler US it is possible to obtain evaluations both of qualitative type (presence, direction and type of flow) and quantitative (resistivity and pulsatility index as measure of resistance in the arterious district) (8). In the past, alterations of renal haemodynamics have been described in the course of diabetic nephropathy at the stage of renal failure. Resistive Index (RI) resulted to be different in normal subjects compared to diabetic patients and in the latter at different creatinin levels; it was concluded that RI represents a predictive index in the advanced stage of diabetic nephropathy (9).

In diabetic patients there are alterations of arterial vasculature caused by the microvascular damage characteristic of the disease. The first study investigating these alterations was that by Platt in 1989 (10) after which many other authors investigated the microvascular alteration in diabetic nephropathy in order to detect early vascular damage in type 1 and the type 2 diabetes mellitus (11). The need of a test that could be simple, safe and repeatable, at low cost, useful in detecting diabetic nephropathy at an early stage, has motivated many investigators continue eco-Doppler study of renal flow in diabetic nephropathy (12-13).

Microalbuminuria in diabetic patients represent a marker predictive of renal damage, but recent studies point out that when microalbuminuria is present it already implies a structural damage of glomerule in the context of renal and sistemic vascular damage (14).

The pre-albuminuric stage of diabetic nephropathy can not be evaluated by the use of a laboratory test; when microalbuminuria is detectable, there are pathological lesions, even if at an early stage. In the pre-albuminuric stage there are alterations of zonal hemodynamics with increased intraglomerular pressure (15).

The aim of this study is to measure these haemodynamic alterations by echo-color-power-Doppler and to confirm the value of RI as marker of early incipient nephropathy.

MATERIALS AND METHODS

The study population consisted of 262 patients (61 males, 201 females; age range: 48-81 years) with type 2 diabetes mellitus, who had been recruited from the Diabetes Outpatient Clinic of our hospital between January 2000 and December 2005. The control group was made up of 100 healthy volunteers with no evidence of diabetes mellitus (74 females, 26 males; age range: 50-80 years). Exclusion criteria for both groups were: current use of tobacco; current or past disease of the kidneys or urinary tract (including cysts, kidney stones, solid tumors, acute or chronic inflammatory diseases); systemic disease with renal involvement; congestive heart failure; arterial hypertension; chronic liver disease; pregnancy; current use of drugs that could affect haemodynamic parameters (e.g. finasteride, nitrates, calcium antagonists, ACE inhibitors, aminophylline, theophylline, estrogen-progestin compounds).

Blood glucose (measured in venous plasma), glycosylated hemoglobin, serum creatinine (all three measured after a fast of 10 h or more), and 24 h urinary excretion of albumin (measured by radioimmunoassay) were measured in all 362 participants. In diabetic patients, three 24-h urine specimens were assayed, and the average of the three results was used for analysis; for control patients, only one 24-h specimen was assayed. Albumin excretion rates of <30 mg/day were considered normal; rates of 30-300 mg/day were classified as microalbuminuria.

Based on the results of these tests, the diabetic patients were divided into three groups: Group I included patients with normal albumin excretion and serum creatinine levels (i.e. <1.3 mg/dL); Group II patients had microalbuminuria with normal serum creatinine levels; and Group III presented microalbuminuria and serum creatinine levels of >1.3 mg/dL.

Moreover, to assess whether this subdivision into three
Table I. *Characteristics of the two study groups.*

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Group (n=262)</th>
<th>Healthy Controls (n=100)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Females (%)</td>
<td>201 (76.7%)</td>
<td>74 (74.0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age (yrs.)</td>
<td>65.5 +/- 6.25</td>
<td>64.4 +/- 6.26</td>
<td>NS</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>25.2 +/- 2.33</td>
<td>25.1 +/- 2.25</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table II. *Characteristics of the three groups of type 2 diabetic patients studied.*

<table>
<thead>
<tr>
<th></th>
<th>Group I (n=145)</th>
<th>Group II (n=45)</th>
<th>Group III (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>63.0 +/- 4.3</td>
<td>65.5 +/- 5.5</td>
<td>69.5 +/- 4.7</td>
</tr>
<tr>
<td>No. females (%)</td>
<td>107 (73.8%)</td>
<td>35 (77.8%)</td>
<td>59 (81.9%)</td>
</tr>
<tr>
<td>Years from diagnosis</td>
<td>7.4 +/- 2.5</td>
<td>12.8 +/- 3.3</td>
<td>20.1 +/- 5.3</td>
</tr>
<tr>
<td>Microalbuminuria (mg/day)</td>
<td>13.2 +/- 6.4</td>
<td>63.7 +/- 23.6</td>
<td>73.6 +/- 29.5</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.84 +/- 0.16</td>
<td>0.98 +/- 0.15</td>
<td>1.85 +/- 0.28</td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)</td>
<td>7.44 +/- 0.49</td>
<td>8.15 +/- 0.70</td>
<td>8.57 +/- 0.72</td>
</tr>
<tr>
<td>GFR estimated by Cocroft-Gault formula (ml/min)</td>
<td>F 75.5 +/- 15.5</td>
<td>F 65.5 +/- 9.5</td>
<td>F 29 +/- 5</td>
</tr>
<tr>
<td></td>
<td>M 103 +/- 24</td>
<td>M 84 +/- 16</td>
<td>M 39.5 +/- 7.5</td>
</tr>
</tbody>
</table>

*F: female patients  M: male patients*

Table III. *Resistance and Pulsatility indices in the renal arteries of type-2 diabetics and healthy controls.*

<table>
<thead>
<tr>
<th></th>
<th>Group I (n=145)</th>
<th>Group II (n=45)</th>
<th>Group III (n=72)</th>
<th>Controls (n=100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance Index (I.R.)</td>
<td>0.68 +/- 0.04</td>
<td>0.78 +/- 0.05</td>
<td>0.91 +/- 0.08</td>
<td>0.60 +/- 0.03</td>
</tr>
<tr>
<td>Pulsatility Index (I.P)</td>
<td>1.13 +/- 0.12</td>
<td>1.19 +/- 0.26</td>
<td>1.74 +/- 0.27</td>
<td>1.07 +/- 0.06</td>
</tr>
</tbody>
</table>
Fig. 1. Intrarenal eco colour Doppler in a normal subject, within normal range. Resistive Index (R.I.) = 0.63 and Pulsatily Index (P.I.) = 1.00.

Fig. 2. Intrarenal eco colour Doppler in a diabetic patient without microalbuminuria. Both indexes resulted to be increased (R.I. = 0.65 and P.I. = 1).

Fig. 3. Intrarenal eco colour Doppler in a diabetic patient with microalbuminuria and Nephropathy. Further increased R.I. and P.I. value (R.I. = 0.86 and P.I. = 1.86).
groups according to the level of serum creatinine reflected the real difference in value of glomerular filtration rate (GFR), for each patient estimation of GFR was made by the Cockroft-Gault formula (16). All participants underwent color and power Doppler studies, which were performed with a Toshiba 240SSA scanner and a 3.5-MHz convex probe. The examinations were carried out by two operators who were blinded to the subject’s group origins. After standard sonographic examination of the kidneys and urinary tract, power Doppler was used to determine the RIs and PIs of the intrarenal arteries in each kidney.

Resistive Index and Pulsatility Index were calculated according to the following formula:

\[ \text{RI} = \frac{S - D}{S}, \text{where } S \text{ is the height of the systolic peak and } D \text{ is the height of the end diastolic trough; } \frac{\text{PI}}{\text{mean of the velocity}} (17). \]

The mean of three measurements was used for analysis, as recommended by Platt (18).

SPSS software was used for statistical analysis. Preliminary testing verified that the distribution of the data was normal (KS). For this reason, parametric tests were chosen for inferential analysis of the study variables. The chi square test was used to evaluate differences between qualitative variables. Correlation between variables was assessed with the Pearson correlation coefficient. A \( p \) level of \( p <0.05 \) was considered significant.

**RESULTS**

As shown in Table I, the diabetic and control groups were not significantly different in terms of sex, age, or body-mass indices. Based on the results of enrolment lab work, the 262 diabetic patients were divided into three subgroups: Group I included 145 patients (38 males, 107 females) with normal renal function and no signs of microalbuminuria; Group II included 45 patients (35 males, 10 females) with microalbuminuria but normal renal function; and Group III contained 72 patients (13 males, 59 females) with microalbuminuria and renal insufficiency. The characteristics of these three subgroups are summarized in Table II.

According to the estimation of GFR by the Cockroft-Gault formula, the division in the three groups did not change, as reported in Table II.

The mean age of Group I patients was significantly lower than those of the other two groups \( (p<0.001) \). The three groups were also significantly different \( (p<0.001) \) in terms of the duration of diabetes (years since diagnosis), and this variable was significantly correlated with the severity of the disease. There was no significant difference between the mean values of microalbuminuria in Groups II and III. Glycosylated hemoglobin values were frankly pathological in all three groups, with no significant inter-group differences.

Fig. 1 and 2 show representative scan results for a control subject and a diabetic patient from Group 1. As shown in Table III, the mean RIs for the three groups of diabetic patients were all significantly different from that of the control group. The RI for Group I was significantly higher than that of controls \( (p<0.001) \) and significantly lower than those of Groups II \( (p<0.001) \) and III \( (p<0.0001) \) (Fig.3). The difference between the RIs of the latter two groups was also significant \( (p<0.0001) \). The PIs of the three groups of diabetic patients were also significantly different and increased with the severity of the disease \( (p<0.001) \). The difference between the PIs of Group I and the control group was significant \( (P<0.05) \) but less so than that of the RIs.

The RI displayed significant correlation with the duration of diabetes \( (p<0.01) \). Serum creatinine levels displayed a highly significant correlation with RI \( (R^2=0.75) \) and a slightly less significant correlation with the PI \( (R^2=0.63) \). Microalbuminuria levels were not significantly related to either of the Doppler indices or to serum creatinine levels. In fact, while the latter three variables (I.R., I.P. and microalbuminuria) all increased progressively from Group 1 to Group 3, this trend was not observed for albumin excretion. The correlation between the RI and PI was highly significant \( (p<0.001) \). The duration of diabetes was significantly associated with the haemodynamic parameters and with renal function (as reflected by serum creatinine).

**DISCUSSION**

Diabetic nephropathy is an important cause of mortality in patients with type-1 and type-2 diabetes mellitus. Although much of the work that has been done in this field has focused on renal disease in type 1 diabetes, the pathophysiology of diabetic nephropathy associated with type 2 disease appears to be essentially the same. In both cases, the renal effects of diabetes are related to the macro- and microvascular complications of altered glucose metabolism. In fact, the initial stage of diabetic nephropathy is manifested by functional alterations...
in renal haemodynamics, which result in glomerular hyperfiltration and increases in the intraglomerular pressure (19).

There is currently no reliable laboratory marker for detection of diabetic nephropathy in this preclinical phase. The onset of microalbuminuria (>30 mg albumin / day) is widely considered to be the first detectable sign of diabetic damage to the kidney (20). Its appearance reflects structural damage to the glomerular membrane resulting in loss of its selective permeability. According to some investigators (21) microalbuminuria represents the first step in an inexorable progression to overt proteinuria and renal failure although this view has been challenged by others (22).

Intraglomerular pressure can not be directly measured in humans, but it can be measured indirectly and in a non-invasive manner by means of color or power Doppler ultrasound studies of the renal vasculature (23). This approach provides us with quantitative indices of renal vascular resistance based on blood-flow velocities in the intrarenal arteries (24).

The scope of the present study is to identify changes in renal vascular resistance associated with the onset and progression of diabetic nephropathy. To this end, we performed power Doppler studies of the intrarenal arteries, with calculation of the RI and PI, on 262 type-2 diabetic patients, who were divided into three groups reflecting their renal status. Compared with 100 healthy non-diabetic controls, the Group I diabetics, who had normal creatinine levels and no signs of microalbuminuria, presented significantly higher renal Ris and Pis. Furthermore, additional significant increases in both indices were observed in diabetic patients of Group II, who had microalbuminuria (mean RI: 0.78+/-0.05), and Group III, with microalbuminuria and elevated serum creatinine levels (mean RI: 0.91+/-0.08). Unlike 24 h albumin excretion rates, which were not significantly different in patients from Groups III and III, the RI and PI displayed significantly higher values in patients whose altered albumin excretion rates were accompanied by signs of altered renal function.

Our study indicates that the renal RI and PI increase progressively with the onset and evolution of diabetic nephropathy.

The significant differences between the RIs of all three diabetic groups and those of the healthy controls indicate that this parameter can also be used to distinguish normal and diabetic populations, regardless of the latter’s stage of disease. The discriminatory power of the IP proved to be slightly lower than that of the RI, in all probability because its calculation is based on the relatively inexact variable, mean blood flow velocity.

These findings suggest that the renal RI might be used as a marker of early (pre-microalbuminuric) renal involvement in type 2 diabetes. It is important to note that the patient and control populations analyzed in this study were carefully selected to minimize the risk of confounding effects on renal vascular resistance due to non-diabetic factors.

Discordant reports have been published on the time of appearance of laboratory changes in diabetics (25). Nonetheless, microalbuminuria seems to be extremely rare during the first five years of diabetes. Our data confirm this observation and indicate that the duration of the disease is directly correlated with the stage of nephropathy. In fact, the mean number of years from diagnosis increased significantly from Group I through Group III. On the average, microalbuminuria developed approximately seven years after the diabetes was diagnosed, whereas outright renal failure developed only when the diabetes had been present for around 20 years. Group III patients were significantly older than those of Group I but not those of Group II. These differences do not diminish the value of the RI as an index of the severity of diabetic nephropathy although it is clear that intrarenal haemodynamics are physiologically influenced by age.

Glycosylated hemoglobin levels showed no correlation with albumin excretion rates or serum creatinine levels. In fact, there were no significant differences between the mean HbA1c values observed in the three groups.

In conclusion, although the mean serum creatinine levels and albumin excretion rates of our Group 1 diabetic patients were clearly within normal limits, the RI for this group was significantly higher than those of the non-diabetic control group. This finding suggests that the intrarenal arterial RI might be used as a reliable predictor of latent diabetic renal damage. Compared with albuminuria
levels, the RI also appears to be a more sensitive index of the progression of diabetic renal disease. Nonetheless, our findings support the hypothesis that these Doppler indices can be used as markers of early-stage diabetic nephropathy. This does not mean that all of our Group I patients will go on to develop albuminuria and outright renal failure, since the onset and progression of diabetic nephropathy also depends to some extent on genetic and environmental factors alone. The follow-up of these patients will be important to determine the incidence of complications and verify the value of renal RI and PI in identifying early stages of diabetic nephropathy.

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LETTER TO THE EDITOR

DELAYED INTERVAL DELIVERY OF A SECOND TWIN: A CASE REPORT AND REVIEW OF THE LITERATURE

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We report a case of diamniotic, dichorionic pregnancy that presented at 26 weeks with premature rupture of the first amniotic sac. Nine days later, premature labour and delivery of the first male twin took place, with death of the first twin. The second twin was left in utero. The management included combination of tocolytics, antibiotics and cervical cerclage. Caesarean section was performed 48 days later, at 34 weeks due to breech presentation and contractions. We delivered a live male infant with apgar scores 4/1 and 7/5 and 1680 gr weight. The infant was discharged home 29 days later.

The implementation of assisted reproduction during the last ten years has increased the incidence of multiple pregnancies. In some cases one or more infants must be born due to intrauterine risks or stillbirth. According to the relevant literature there is an absence of unanimity on the best management for these pregnancies. The aim of this report is to add our experience to the currently limited literature.

A 31 year-old nullipara woman was admitted to the hospital at the 26th week of a twin dichorionic, diamniotic pregnancy after in vitro fertilisation (IVF), because of premature rupture of the membranes of the first amniotic sac. The ultrasound examination revealed cephalic presentation of the first female fetus, whereas the second male fetus was breech. Both had normal amniotic fluid index and growth. Two independent placentas were also visualized.

The patient was treated with bed rest, erythromycin 250 mg three times per day (TID) for 7 days, atociban iv for 48 hours and 24 mg betamethasone in two separate doses. Seven days later, the first infant was born, weighting 780 g, but died seven days later at the Neonatal Intensive Care Unit (NICU) because of severe lung prematurity. The contractions ceased after the delivery of the first fetus. A ligation of the umbilical cord was performed, as high in the cervix as possible, in aseptic conditions, and the placenta was left inside the uterus. A McDonald cervical cerclage was also performed. During the procedure a course of iv coamoxyclav 1.2 gr was administered. In addition, prophylactic ritrodine iv was administered for 48 hours.

The patient was kept in the hospital for close monitoring which involved daily auscultation of the fetal heart and measurement of body temperature, twice weekly full blood count, CRP and clotting screen and ultrasound examination for growth and doppler once weekly. Pregnancy was terminated by caesarean section 48 days later (34th week) due to uterine contractions and breech presentation. A male

Key words: twin pregnancies, delayed interval delivery, cervical cerclage

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infant was born weighing 1,680 g, with Apgar scores of 4 and 7 at 1 min and 5 min, respectively. Two placentas were delivered, one of which was small, fibrous and calcified. The post operative recovery of the mother was uneventful. The neonate stayed in the hospital for 29 more days and was dismissed in excellent condition.

Due to a surge in the availability of assisted reproductive techniques (ART), the incidence of multiple pregnancies is increasing. Patients with multiple pregnancies have an increased risk of preterm labor and delivery with the associated high prenatal morbidity and mortality. Obviously, as the number of multifetal pregnancies increases (due primarily to the use of assisted reproductive treatments), a rise in the unexpected birth of one or more siblings at premature or previable stages is observed. Delaying delivery of the remaining fetus(es) in a multifetal pregnancy is feasible in some cases (1-3).

After the birth of the first fetus delayed delivery in multiple pregnancies can be successful in selected cases. There is absence of agreement regarding the best management of these pregnancies. Each case is a unique medical situation that must be met with the best possible solution. The main problem appears to be preterm labor and preterm prelabor rupture of the membranes, with one condition often leading to the other. A possible reason for the premature rupture of the membranes could be an ascending infection from the vagina or the cervix into the uterine cavity. Both infection and rupture of the membranes can lead to uterine contractions and subsequent delivery. Modern management procedures, including tocolytics, corticosteroids, antibiotics and cervical cerclage would appear to be important in the overall success of treatment in a retained twin. Although both tocolytics and cerclage appear to prolong the mean delivery interval, no statistical difference in survival was found concerning cerclage, antibiotic therapy, tocolysis and hospitalization (1, 3-4). The use of prolonged bed rest, cervical cerclage, tocolysis, antibiotics and corticosteroids compose complex, frequently debatable issues. It is not clear whether the management described in this case report is the most adequate treatment and if all the possibilities mentioned above are really necessary to increase the rate of success (5-6). Early tocolytic and antibiotic therapy may delay delivery and, in combination with antenatal glucocorticoids to stimulate lung maturation, may thereby improve the condition of the second twin (7-8). Tocolysis may be used precautionarily after the first twin’s birth, or only later during uterus contractions, but never in the presence of a well established chorioamnionitis. After premature rupture of the membranes, the suspicion of an infection could be raised on the basis of a rise of the temperature, of the white blood cell count and of the C-Reactive Protein (CRP). CRP is considered as a good prognostic index for incipient chorioamnionitis (3). In cases of high CRP, tocolysis cannot achieve the prolongation of pregnancy.

The role of cervical cerclage remains controversial (9-10). There is an important publication bias in the literature due to under-reporting of the failed attempts of delayed deliveries. Cervical cerclage after the first delivery is associated with a longer inter-delivery interval without increasing the risk of intrauterine infection (9-10). However, the impact of this procedure on infant survival is unclear. If cervical cerclage is decided, it is advisable to be carried out in aseptic conditions, during the first two hours after the birth of the first fetus, and of course when there is no evidence of infection. To avoid ascending infection, the cord of the first born twin should be ligated with an absorbable suture as close to the cervix as possible, under aseptic conditions. Patients with previous cervical cerclage(s) during the index pregnancy are less likely to achieve significant latency intervals (9, 11). Delayed delivery of the remaining fetus(es) for 2 or more days before 30 weeks of gestation was associated with improved infant survival (10-11). When a first twin was delivered at 22 to 23 weeks, delayed delivery of the second twin was associated with reduced perinatal and infant mortality of the second twin if the interval was less than 3 weeks (11-12). Delayed delivery of the second twin when the first was delivered at \( \geq 24 \) weeks had no benefit on mortality (9, 11). Interval delivery of the fetuses in multiple gestations has been shown to increase perinatal survival (9-11). Earlier delivery of the first twin and premature rupture of membranes for the second twin were significantly related to a longer interval between deliveries.

In reviewing the literature, in most cases the outcome of the second twin was favorable, in contrast to the bad outcome of the first twin. Survival of the first born was clearly linked to its gestational age and birth-weight (13). The survival of the second born was
dependent upon a number of factors, including the delivery interval between the first and second twin and the presence of obstetric problems appearing during the latency period; the longer the interval, the greater was the chance for surviving (10-11, 14). Likewise, the absence of significant obstetric problems in the latency period also improved survival. In cases where the membranes of the second twin remained intact with no evidence of ongoing labor or other obstetric risk factors, a conservative approach could be adopted (4-5, 8). Vaginal examinations should be avoided; however the length and dilatation of the cervix should be followed ultrasonographically (13). There is no evidence that retention of placenta causes disseminated intravascular coagulation (11, 15). Even a large placental mass can be retained in the uterus and produce no demonstrable clinical symptoms (12, 15). Monitoring should be carried out and limited to a weekly full blood count, CRP, prothrombin time and fibrin degradation product (FDP) (13).

In conclusion, delayed delivery in multifetal pregnancies can be successful if there are no contraindications and these pregnancies are managed in a tertiary perinatal center. Possible risks should be thoroughly discussed with the patient as well as possible future problems of the child (16). In high-order multiple pregnancy, delayed interval delivery is strongly recommended, provided that there are no signs of chorioamnionitis, fetal distress or maternal compromise. In multiple pregnancies with imminent preterm birth, delayed delivery may offer survival chances and a favorable outcome for the remaining fetus(es) (11-13). The results gained from pregnancy prolongation are a clinically significant benefit to the second-born twin, without significant morbidity in the mother. Even modest intervals between births of siblings at critical gestational stages can improve neonatal survival and decrease neonatal morbidity. With careful patient selection and thorough patient counseling, delayed delivery offers a safe and beneficial management option for selected multiple gestations.

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