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Role of HSP 70 Gene Polymorphism in Diabetic Foot Ulcers

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder that occurs due to defects in insulin secretion, insulin action or both, resulting in high blood glucose levels. Developing countries are found to be at greater risk for diabetes, which is evident from increasing incidences of diabetic cases. By the year 2030, India is estimated to have the largest number of diabetic patients. Diabetic foot ulcers (DFU) will affect 12% to 25% of persons with diabetes mellitus throughout their lives and is the leading cause for hospital admission among diabetic patients in India. Recurrence of foot infection is common and is mainly due to the presence of neuropathy and peripheral vascular disease. The etiology of diabetic foot ulcers is multifactorial and the management of DFU and its consequences put a great strain on both health and social services. This results in huge related costs for treatment, as well as the loss of general economic productivity. Heat-shock proteins (HSPs) are molecular chaperones synthesized under stressful conditions. They are important in physiological and pathological processes and are highly active within the immune system. At normal physiological conditions, HSPs are expressed at low levels. However, in response to cellular stress, there is an increased expression of HSPs. They protect against tissue injury by maintaining synthesis and proper conformation of proteins by repairing damaged proteins and promoting the healing of injured tissue. This process plays an important role in the assembly and transport of newly synthesized proteins within cells.7 Xiao et al showed that the expression of heat shock proteins was triggered when organisms are exposed to a variety of stressful stimuli including hypoxia, ischemia, and oxidative free radicals. Initially, HSPs were thought to be only functional in the cytoplasm and nucleus. However, recently they have been implicated in intercellular signaling and transport after they are released to the extracellular space and bloodstream. The known family members of HSPs are HSP70, HSP90, HSP60, HSP47 and HSP110, which are numbered based on their molecular masses.

KEYWORDS: *diabetes, ulcers, heat shock proteins, genes, chaperones, foot, neuropathy, insulin*

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INTRODUCTION

Wound healing is an innate mechanism of action that works reliably most of the time. A key feature of wound healing is stepwise repair of lost extracellular matrix (ECM) that forms the largest component of the dermal skin layer.[1] But in some cases, certain disorders or physiological insult disturbs the wound healing process. Diabetes mellitus is one such metabolic disorder that impedes the normal steps of the wound healing process. Many studies show a prolonged inflammatory phase in diabetic wounds, which causes a delay in the formation of mature granulation tissue and a parallel reduction in wound tensile strength.[2]

Treatment of diabetic foot ulcers should include: blood sugar control, removal of dead tissue from the wound, wound dressings, and removing pressure from the wound through techniques such as total contact casting. Surgery in some cases may improve

outcomes.[3] Hyperbaric oxygen therapy may also help but is expensive.[3]

It occurs in 15% of people with diabetes,[4] and precedes 84% of all diabetes-related lower-leg amputations.[5]

Diabetic foot ulcerations have been extensively reported as vascular complications of diabetes mellitus associated with a high degree of morbidity and mortality. Diabetic foot syndrome (DFS), as defined by the World Health Organization, is an "ulceration of the foot (distally from the ankle and including the ankle) associated with neuropathy and different grades of ischemia and infection". Pathogenic events able to cause diabetic foot ulcers are multifactorial. Among the commonest causes of this pathogenic pathway it's possible to consider peripheral neuropathy, foot deformity, abnormal foot pressures, abnormal joint mobility, trauma, peripheral artery disease. Several studies reported how diabetic patients show a higher mortality rate compared to patients without diabetes and in particular these studies under filled how cardiovascular mortality and morbidity is 2-4 times higher among patients affected by type 2 diabetes mellitus. This higher degree of cardiovascular morbidity has been explained as due to the observed higher prevalence of major cardiovascular risk factor, of asymptomatic findings of cardiovascular diseases, and of prevalence and incidence of cardiovascular and cerebrovascular events in diabetic patients with foot complications. In diabetes a fundamental pathogenic pathway of most of vascular complications has been reported as linked to a complex interplay of inflammatory, metabolic and procoagulant variables. These pathogenetic aspects have a direct interplay with an insulin resistance, subsequent obesity, diabetes, hypertension, prothrombotic state and blood lipid disorder. Involvement of inflammatory markers such as IL-6 plasma levels and resistin in diabetic subjects as reported by Tuttolomondo et al confirmed the pathogenetic issue of the a "adipo-vascular" axis that may contribute to cardiovascular risk in patients with type 2 diabetes. This "adipo-vascular axis" in patients with type 2 diabetes has been reported as characterized by lower plasma levels of adiponectin and higher plasma levels of interleukin-6 thus linking foot ulcers pathogenesis to microvascular and inflammatory events. The purpose of this review is to highlight the immune inflammatory features of DFS and its possible role as a marker of cardiovascular risk in diabetes patients and to focus the management of major complications related to diabetes such as infections and peripheral arteriopathy.[5,6]

DISCUSSIONS

Foot complications are common in patients with diabetes and include neuropathy, ulceration, infection and gangrene. Foot ulcers and superimposed infection are the commonest cause of hospitalization and leg amputation in diabetics, affecting up to 25 per cent of patients at some time in their life[1]. Foot ulceration in diabetic patients with associated peripheral arterial disease can lead to a limb- or life-threatening infection[2,3]. Diabetic foot ulceration is accompanied by inflammatory processes resulting in increased local levels of tumour necrosis factor (TNF) α and decreased levels of transforming growth factor β, both of which affect wound healing processes[4]. Peripheral blood mononuclear functions (phagocytic activity index and intracellular killing activity) correlate with healing in diabetic ulceration[5]. Treatment with growth factors has been used to assist healing and reduce morbidity in these patients[6,7]. The degree of tissue damage in diabetic foot ulceration is thus determined by the nature and extent of the host inflammatory and reparative responses. Indian patients with type II diabetes mellitus have a low incidence of peripheral arterial disease compared with their Western counterparts[8]; infection is considered to be the major factor responsible for progression of foot complications to amputation. Active treatment at presentation may prevent this progression. The virulence of the pathogen and the severity of the inflammatory response may be determinants of the outcome. Knowledge of the inflammatory phenotype of the individual patient may help in planning treatment.[7,8]

Heat-shock protein (HSP) 70 is involved in the response to stress and in wound repair. In experimental studies, creation of a cutaneous wound induced HSP70 expression in epithelial and inflammatory cells; this response was significantly delayed or attenuated in diabetic animals[9,10]. In addition, HSP70 functions as a critical molecule in the pathways related to inflammation. Excessive production of inflammatory cytokines is implicated in the pathogenesis of severe diabetic foot ulceration[11]. Polymorphisms in the HSPA1B and HSPA1L genes are associated with higher circulating concentrations of the inflammatory cytokines TNF-α and interleukin (IL) 6, and appear to affect the outcome in patients surviving multiple trauma[12]. This prospective study was undertaken to examine the hypothesis that HSP gene polymorphisms may be associated with impaired outcomes in patients with diabetic foot ulceration.[9,10]

METHODS

Consecutive patients with a diabetic foot ulcer managed as an inpatient at the Christian Medical

College, Vellore, between February and October 2008 were enrolled in the study. The department runs three dedicated diabetic foot ulcer clinics each week. Patients with diabetic foot ulcers are managed by a multidisciplinary team consisting of endocrinologists and surgeons who supervise inpatient and outpatient care. All patients were assessed for peripheral arterial disease by clinical examination and documentation of palpable pulses, and measurement of ankle: brachial pressure indices using Doppler ultrasonography. Patients with peripheral arterial disease were excluded from the study, as were those with a history of filariasis, infection with human immunodeficiency or hepatitis B virus, or chronic renal failure, or those who refused or withdrew consent.

Informed consent was obtained from all patients, and samples of venous blood were sent to the laboratory for processing. On admission, demographic details were recorded and the foot ulcer was assessed and graded for severity using the Wagner grading system (Table 1)[13]. Procedures undertaken and the duration of hospitalization were recorded. The decision to discharge the patient from hospital was taken by the same team without any knowledge of the patient's genotype, based on standard criteria: resolution of systemic and wound sepsis, conversion of non-healing to healing ulcers, adequate mobility to manage activities of daily living, and ability to manage the diabetes. Most patients were discharged to step-down or primary care facilities. Patients were then assessed weekly in the outpatient clinic for 3 months. DNA analysis and polymerase chain reaction (PCR), and genotyping were performed in a single batch at the end of the study by an individual unaware of the grading or clinical details.

Table 1 Wagner classification of severity of diabetic foot ulceration[13]

Grade	Lesion
0	No open lesion; may have deformity or cellulitis
1	Superficial diabetic ulcer; partial or full thickness
2	Ulcer extension to ligament, tendon, joint capsule or deep fascia without abscess or osteomyelitis
3	Deep ulcer with abscess, osteomyelitis or joint sepsis
4	Gangrene localized to portion of forefoot or heel
5	Extensive gangrenous involvement of entire foot

RESULTS

It was decided a priori that the primary comparisons between genotypes would be the severity of the foot ulcer (Wagner) grade, the outcome (major amputation) and length of hospital stay. The study protocol was approved by the college's Institutional Review Board.

DNA extraction and analysis of HSP70 polymorphisms

DNA isolated from blood samples was used for the polymorphic analysis using PCR–restriction fragment length polymorphism analysis. Genomic DNA was extracted from mononuclear cells in peripheral blood by phenol–chloro- form extraction and stored at – 20 °C. PCR was performed as described below in a batch at the end of the study, using previously described primers for the HSPA1B A1538G and HSPA1L C2437T polymorphisms[12].

Touch-down PCRs were carried out in a 20-µl reaction mixture with 1·5 mmol/l magnesium chloride, 200 µmol/l dNTPs (Finnzymes Oy, Espoo, Finland), 250 nmol/l forward and reverse primers (Sigma Genosys, Bangalore, India) and 0·2 per cent titanium Taq DNA polymerase (S1792; Clontech, Mountain View, California, USA). The protocol for HSPA1B included initial denaturation at 95 °C for 30 s, 20 cycles of denaturation (94 °C, 30 s), annealing (70 °C, 30 s, reduced by 0·2 °C for each successive cycle) and extension (72 °C, 30 s), followed by 20 cycles of denaturation (94 °C, 20 s), annealing (62 °C, 20 s), extension (68 °C, 20 s), final extension at 68 °C for 10 min and then maintenance at 10 °C for 10 min. The protocol for HSPA1L analysis comprised initial denaturation (95 °C, 30 s), 20 cycles of denaturation (95 °C, 30 s), annealing (65 °C, 30 s, reduced by 0·2 °C for each successive cycle), extension (72 °C, 50 s) and a further 15 cycles with denaturation (94 °C, 20 s), annealing (60 °C, 25 s), extension (72 °C, 50 s), with final extension at 72 °C for 10 min, and maintenance at 10 °C for 10 min. The PCR product sizes were 383 and 705 base pairs respectively for HSPA1B and HSPA1L. The amplified samples were digested (16 h, 37 °C) with restriction enzymes, PstI and NcoI (Fermentas, St Leon-Rot, Germany) for HSPA1B and HSPA1L analysis respectively[12]. The digested products were resolved by 2 per cent agarose gel electrophoresis, and genotype was assigned based on the band patterns.[11,12]

Statistical analysis

Unpublished experience from this hospital suggested that the HSPA1B (AG) polymorphism was present in approximately 60 per cent of the normal population, whereas the HSPA1L (CT/CC) polymorphism was present

in approximately 20 per cent. Assuming that the distribution would be similar in diabetic patients, and examining differences in outcome (reduced length of hospital stay, number of operations, nature of operations and Wagner's grade), it was calculated that examination of 100 patients would provide 90 per cent power to detect an increase in the relative risk (RR) of advanced diabetic foot ulceration by Wagner grading of 1·5 for the HSPA1B polymorphism, and 80 per cent power to detect a similarly increased RR for the HSPA1L polymorphism.

Categorical variables were compared using χ^2 or Fisher's exact test. χ^2 tests for trend were performed for larger contingency tables associating the genotype with magnitude of the surgical intervention. Continuous variables were compared with the Mann–Whitney U test. $P < 0·050$ was considered statistically significant.

Of 106 patients initially enrolled, complete data were available for only 101 patients, who were included in the final analysis. All patients came from the state of Tamil Nadu and shared the same ethnic background. There were 79 men and 22 women, of mean (s.d.) age 55·2(10·2) years. The mean(s.d.) duration of diabetes before admission was 8·7(6·6) years. At presentation, the location of the foot lesions was as follows: only the toes in 46 patients, interdigital in one patient, dorsum in eight, plantar in 12, heel in 21, Achilles tendon in one, ankle in four, and leg in eight. The eight patients with leg ulcers had recently treated foot ulcers.

The lesions had been present for a mean (s.d.) of 10·4(24·8) days before enrolment. Debrided tissue from 69 patients was cultured. Staphylococcus aureus (two methcillin resistant) was isolated in nine cases, Pseudomonas aeruginosa in 11, Escherichia coli in seven, non-fermenting Gram-negative bacilli in seven, Proteus in five and Klebsiella in five. In nine cultures no bacteria were isolated, and 20 cultures grew miscellaneous organisms.

Surgical procedures

The median Wagner grade was 3 (range 1–5). A total of 89 patients had a surgical procedure; 71 had a single procedure, and 18 patients required more than one procedure. Initial procedures performed were debridement (46), amputation of the toes (25), transmetatarsal amputation (five), below-knee amputation (12) and above-knee amputation (one). Nine patients were managed conservatively. Three patients self-discharged against medical advice. The 18 second surgical procedures carried out during follow-up included: further debridement (eight); toe amputation (four); revision amputation (two); transmetatarsal amputation (two); and below-knee amputation (two).

DNA analysis

Of the 101 patients in the final analysis, 70 had a mutant heterozygous pattern (AG genotype) for the HSPA1B mutation and 30 were wild-type homozygous (GG genotype). In one patient, the pattern after amplification was not sufficiently clear to assign a genotype.[13,14]

There was a statistically significant association between the HSPA1B genotype and the severity of diabetic foot ulceration ($P = 0·008$, χ^2 test) (Table 2). When patients with mild disease (Wagner grade 1–3) were compared to those with severe disease (grade 4 or 5), the relative risk of patients with the AG genotype having severe disease was 2·02 (95 per cent confidence interval (c.i.) 1·01 to 4·05) ($P = 0·028$, Fisher's exact test). HSPA1L genotyping showed that the TT genotype was predominant (78 patients) followed by the heterozygous mutant CT genotype (22), whereas CC was uncommon (one patient). The HSPA1L genotype was not associated with the severity of diabetic foot ulceration

TABLE-2

Genotype	Wagner grade					P*
	1	2	3	4	5	
HSPA1B						0·008
AG	12	10	15	26	7	
GG	1	12	10	5	2	
HSPA1L						0·221
CT/CC	3	2	5	11	1	
TT	10	20	20	21	8	

One patient could not be assigned an HSPA1B genotype.

The surgical procedures undertaken in these patients (ranked in ascending magnitude of intervention) in relation to HSP70 genotype are shown in Table 3. The HSPA1B genotype was associated with the size of the intervention; the AG genotype was significantly associated with interventions of greater magnitude ($P = 0·040$,

χ^2 test for trend). The AG HSPA1B genotype was significantly associated with the need for toe or foot amputation (35 of 69 patients) in comparison to the GG genotype (seven of 28 patients) (P = 0·025). The relative risk of a diabetic patient with AG genotype requiring any amputation was 2·02 (95 per cent c.i. 1·02 to 4·01). The HSPA1L genotype had no association with the magnitude of the procedure performed (P = 0·854, χ^2 test for trend). There appeared to be a trend for the CT/CC genotype to be associated with a need for amputation (14 of 22 patients) compared with the TT genotype (29 of 76 patients), but this did not reach statistical significance (P = 0·050).

TABLE-3

	Conservative therapy	Debridement	Limited amputation		Major amputation		P*
			Toe	TMT	BKA	AKA	
HSPA1B							0·040
AG	8	26	19	3	12	1	
GG	1	20	5	2	0	0	
HSPA1L							0·854
CT/CC	2	6	11	2	1	0	
TT	7	40	14	3	11	1	

Of the 101 patients, three self-discharged against medical advice and one could not be genotyped for the HSPA1B mutation; thus, 97 interventions are shown for the HSPA1B genotype and 98 for HSPA1L. TMT, transmetatarsal; BKA, below-knee amputation; AKA, above-knee amputation.

*

χ^2 test for trend.

CONCLUSIONS

This study suggested that a polymorphism in the HSPA1B gene, the AG genotype, was associated with an increased severity of the local lesion, a greater need for toe or leg amputation, and an increased length of hospital stay in these diabetic patients with foot ulceration. Several factors predispose to ulceration, including peripheral neuropathy, peripheral arterial disease and trauma. It is possible that excessive inflammation occurs in a subset of patients, leading to increased morbidity. Although the study was limited in size, it suggests a new potential factor that should be considered in the pathogenesis of this condition.

HSPs are expressed both constitutively and upon induction in response to a number of stimuli. HSP70 is the major HSP expressed in humans and its intracellular expression serves to protect cellular homoeostasis. HSP70 detects proteins that are incorrectly folded or denatured, and promotes further intracellular processing, leading either to correct folding or to proteolytic degradation. Intracellular expression of HSP70 is induced by a wide variety of stimuli including heat, fever, hypoxia, oxygen radicals, endotoxins and cytokines, and has been linked to diverse processes ranging from reproduction to inflammation and cancer. Such intracellular HSP70 expression is a protective response and its pre-emptive induction reduces organ dysfunction and mortality in animal models of sepsis[14].

HSPs have a dual role in human health. HSP70 has both anti-inflammatory and proinflammatory effects depending on the cell type and context, and intracellular or extracellular location. Intracellular effects are often anti-inflammatory, with inhibition of nuclear factor κB signalling. Extracellular effects can lead to inflammatory cytokine production or induction of regulatory immune cells and reduced inflammation[15]. HSP70, although usually intracellular, can be released extracellularly when cells are stressed, particularly when necrosis occurs[16].[15,16]

Polymorphisms in the HSP70 genes are associated both with defects in intracellular HSP70 production and with an imbalance in inflammatory cytokines. HSPA1L gene polymorphisms have been found to be associated with the magnitude of the HSP70 response in monocytes and lymphocytes, with the CC genotype being associated with lower levels of inducible HSP70 than the TT genotype[17]. In that study, HSPA1B gene polymorphisms had no association with intracellular HSP70 response. In contrast, the HSPA1B G allele has been reported to be associated with higher levels of HSP70 in the serum of patients with chronic heart failure[18]. Extracellular levels of HSP70 are also increased in diabetic ketoacidosis[19], but their relationship to the HSPA1B or HSPA1L genotype is not known. In the setting of severe multiple trauma, patients carrying the genotypes HSPA1B AG or HSPA1L CT had significantly higher plasma concentrations of TNF-α and IL-6 than those with GG or TT genotypes[12].[12,13,17,18]

The present study did not examine levels of cytokines or HSP70 specifically. It suggested that the HSPA1B

AG genotype was significantly associated with more severe local disease, amputation and longer hospital stay than the GG genotype. It is likely that this was related to levels of expression of HSP70 (intracellular or extracellular), with possible effects on the outcome of diabetic foot ulceration. This association should be confirmed in similar populations, and its biological basis needs further investigation.[19,20]

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