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Genetic and epigenetic alterations in Toll like receptor 2 and wound healing impairment in type 2 diabetes patients

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ABSTRACT

Aim: Persistent hyperglycemic microenvironment in type 2 diabetes mellitus (T2DM) leads to the development of secondary complications like wound healing impairment. Proper co-ordination of innate immune system plays an integral role in wound healing. Toll like receptors (TLRs) are prominent contributors for the induction of the innate immune and inflammation response. TLR2 is an important extracellular member in mammalian TLR family and has been shown to be a potent player in the wound healing mechanism.
Methods: Expressional status of TLR2 was seen in wounds of T2DM cases with respect to the severity of wounds in 110 human lower extremity wounds. The methylation status of TLR2 promoter was also examined.
Results: Although TLR2 transcripts were downregulated in T2DM wounds compared to control, their levels tend to increase with the severity of T2DM wounds. The methylation status of TLR2 gene promoter was not significantly different among different grades of wounds in T2DM subjects. The CpG sites investigated were totally or partially methylated in majority of DFU cases.
Conclusion: TLR2 down regulation in wounds of T2DM patients compared to non diabetic patients may lead to development of non healing chronic ulcers in them.

1. Introduction

The cascade of wound healing in higher organisms generally exhibits an integration of several mutually coherent steps ranging from (i) wound homeostasis through (ii) acute inflammation, (iii) proliferation and finally leading to (iv) remodeling of the affected part (Singh, Agrawal, Gupta, & Singh, 2013a). These steps should be tightly regulated spatially and temporally as any type of imbalance may lead to non healing chronic ulcers (Whitney, 2005). The immune system, both innate and adaptive, plays an integral function in the process of wound healing, as evident by the secretion of signaling molecules like cytokines, lymphokines and growth factors (Singer & Clark, 1999; Werner & Grose, 2003). Innate immune system provides the first line of defense against foreign invaders during the process of wound healing (Park & Barbul, 2004). Signaling receptors like Toll like receptors (TLRs) are one of the most prominent contributors for the induction of the innate immune and inflammation response (Takeda, Kaisho, & Akira, 2003). TLRs are the family of transmembrane proteins, expressed on almost every immune cell like macrophages, neutrophils and dendritic cells, where their main function is to serve as a pathogen recognizing receptor (PRR) and to sense the pathogen associated molecular patterns (PAMPs) over a plethora of microbes invading during open wounds (Akira, Uematsu, & Takeuchi, 2006). After binding with these PAMPs, TLRs initiate signaling pathways that ultimately lead to activation of two main transcription factors: Nuclear factor kB (NF-kB) or type I Interferon (IFN) (Dasu & Isseroff, 2012). The TLR induced inflammation may be either agonist or antagonist of wound healing, depending upon the timing and the extent of these transcription factors which critically determine the fate of the healing wound (Dasu & Isseroff, 2012).

TLR2 is an important extracellular member in mammalian Toll family of leucin rich receptors. TLR2 is known to be a signaling receptor for many microbial products including whole gram positive bacteria, microplasma, peptidoglycan and lipoteichoic acid derived from Gram-positive bacteria (Flo, Halaas, & Torp, 2001). Anti infectious property of TLR2 is evident from the fact that the TLR2-deficient mouse strain is more prone to infection with Gram-positive bacteria *S. aureus* and shows defective clearance of spirochetes after infection by *Borrelia burgdorferi* as compared to their wild type counterparts (Kuo et al., 2013). TLR2 has an ability to form heterophilic dimers with other structurally related TLRs like TLR1 and TLR6, due to which it can recognize a wide spectrum of microbial

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Table 1
Biochemical and demographic parameters of DFU patients (N = 102) and controls (N = 8). Data are presented as mean ± SD or as number (percentage).

Parameters	DFU (N = 102)	Control (N = 8)	p-value
Age in years; mean ± SD	54.12 ± 8.72 years	56.27 ± 3.42	0.60
BMI in kg/m^2; mean ± SD	21.45 ± 2.23 kg/m^2	23.45 ± 1.95	0.17
Duration of T2DM in years; mean ± SD	9.78 ± 4.25 years	N/A	–
Male	70 (68.62%)	5 (62.5%)	0.84
Female	32 (31.38%)	3 (37.5%)	0.87
HbA1c levels (%) (Mean, range)	10.1% (8.9%–12.5%)	N/A	–
Family history present; n (%)	13 (12.74%)	N/A	–
Nephropathy present (Serum creatinine > 1.4 mg/dl); n (%)	31 (30.39%)	N/A	–
Neuropathy present (by monofilament test); n (%)	62 (60.78%)	N/A	–
Hypertension present (systolic BP > 140 mm of Hg); n (%)	35(34.31%)	N/A	–
Retinopathy present; n (%)	11 (10.78%)	N/A	–
Dyslipidemia present (Serum cholesterol and Tgy levels > 200 mg/dl); n (%)	15(14.70%)	N/A	–
Infection present (Wound culture positive for microbes); n (%)	56(55.88%)	N/A	–
Bone involvement (Osteomyelitis); n (%)	36 (35.29%)	N/A	–

components. Alteration in the methylation pattern of TLR2 has been also described in certain epithelial diseases including carcinoma and cyst formation (Furuta et al., 2008). TLR2 has been shown to be a potent player in the wound healing mechanism. TLR2 activation after acute ischemic injury promotes the process of angiogenesis by inducing endothelial cell migration and adhesion to the wound site (Xu et al., 2013). TLR2 has been also shown to modulate the synthesis of Connexin-43 (Cx43), a gap junction protein, another potent wound healing agent (Ey, Eyking, & Gerken, 2009).

Persistent hyperglycemic microenvironment in type 2 diabetes mellitus (T2DM) leads to the development of secondary complications like cardiovascular disease, neuropathy, nephropathy, retinopathy and impairment of wound healing in patients. Wound healing impairment is a serious secondary complication of T2DM which contributes to a huge percentage of total amputations performed worldwide. As per recent data, around 25% of T2DM patients develop non healing wounds once in their life time (Singh, Agrawal, Gupta, & Singh, 2013b). The reason for this observation is that the hyperglycemic conditions in T2DM cases lead to decrease in cytokines and growth factors essential for healing of wounds (Werner & Grose, 2003). The immune response is also compromised in T2DM cases which generally leads to prolonged inflammation and unresolved infection in the wound microenvironment, thereby resulting in chronic wound which either takes a long time to heal or does not heal at all. TLRs are one of important members of immune system which is affected significantly in the T2DM individuals (Kanhaiya, Agrawal, Gupta, & Singh, 2013). Recently our group has shown that genetic and epigenetic alterations in TLRs, especially, TLR4 lead to impairment of wound healing in T2DM cases (Kanhaiya et al., 2013; Singh, Singh, Agrawal, Gupta, & Singh, 2013, 2014). In the present study we tried to look upon the expressional status of TLR2 in wounds of T2DM cases with respect to the severity of wounds. The methylation status of many CpG dinucleotides situated near the regulatory sequence in the promoter region of TLR2 gene was also examined to see the relationship of epigenetic regulation of TLR2 in the patho-physiology of diabetic wound healing impairment.

2. Materials and methods

2.1. Subjects

In this hospital based case control study, a total of 110 lower extremity wounds of different grades were analyzed, out of which 102 were diabetic foot ulcer (DFU) cases and 8 were controls. Patients were recruited from the outpatient department (OPD) clinics and operation theaters of Department of Endocrinology and Metabolism and Department of Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, during the period of July 2010 to December 2013. Patients were advised to undergo a standardized clinical and laboratory evaluation (Table 1). Family history, habits (smoking, alcoholism etc.) and disease status of each patient were recorded through a questionnaire. Previously diagnosed T2DM cases having non-healing wounds of > 4 weeks were included in the study as DFU cases. The exclusion criteria of the study included presence of co morbid disorders such as thyroid dysfunction and patients not belonging to north India. All the DFU cases had lower extremity wounds, 90% of which were located on the foot alone and the remaining 10% involved both foot and lower leg. Both the plantar and dorsal aspects of the foot were involved in majority of the cases. Age and sex matched controls were recruited by full thickness wound biopsies of post cellulitic chronic ulcers of the foot and the distal leg from the general north Indian population residing in Varanasi belonging to same ethnicity, having controlled fasting or postprandial sugar levels, no family history of T2DM. These were non-healing ulcers present for 4 weeks or more following cellulitis of the lower limb. The samples were collected at the first visit of the patients to the Diabetic foot clinic. Biopsies were taken from the wound margins during the debridement process and the histological analysis was performed to determine the cell types. Tissue samples were collected in RNAlater solution (P/N AM7020, Ambion, Inc., Austin, TX, USA) and phosphate buffer saline (PBS) for RNA and DNA isolation respectively and kept frozen at −80 °C until use. For immunohistochemical staining, samples were collected in Boiun's fixative solution and kept at room temperature. Biochemical markers, such as serum creatinine and cholesterol levels were measured using biochemical autoanalyzer (Beckman Coulter) at the clinical laboratory of the Department of Endocrinology and Metabolism. Diagnosis criteria of T2DM cases were on the basis of World Health Organization (WHO) criteria i.e. fasting plasma glucose ≥ 126 mg/dl and 2 h plasma glucose ≥ 200 mg/dl. Poor glycemic control was assessed by measuring HbA1C levels. The mean HbA1c levels of DFU subjects were 10.1% (Range 8.9% to 12.5%). Screening for neuropathy was done by taking a history of sensory loss and other symptoms such as burning sensation or paresthesias. Clinical neurological examination included assessment of the vibratory threshold perception using a 128 Hz tuning fork and assessment of pain and fine touch with a pin and 10 g monofilament respectively. The tendon reflexes and muscle power were measured in patients with sensory neuropathy. Screening for vascular involvement included detailed history of vascular insufficiency, clinical examination for signs of chronic ischemia and assessment of all lower limb pulses. A bed side hand held Doppler study was carried out in all clinically suspicious cases and ABPI (ankle brachial pressure index) of < 0.9 was considered indicative of peripheral vascular disease. Classification of wounds was done on the basis of the Wagner Grading System (Table 2). The study was approved by the Institutional Human Ethics Committee of Institute of Medical Sciences, Banaras Hindu University,