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COMPARATIVE STUDY OF CONNEXINS EXPRESSION IN DIABETIC AND NONDIABETIC WOUNDS

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Wound healing is the synchronized interplay of interaction of several cellular and biochemical components. In case of diabetes this normal course of wound healing is delayed. The mechanism behind the delayed wound healing in diabetes remains unexplored. The passage of various apoptotic and inflammatory signals via gap junctions play an important role in tissue remodeling during diabetic wound healing. In this study, we compared the expression of the following connexins (Cx) namely Cx26, Cx30.3, Cx31, Cx31.1, Cx37, Cx40, Cx43 in diabetic and non-diabetic wounds. A significant increase in the levels of Cx26, Cx30.3, Cx31, Cx31.1, Cx43 with the commencement of wound repair was observed in diabetic wounds as compared to non-diabetic wounds. In contrast, Cx37 and Cx40 were not expressed in either in diabetic or non diabetic wounds. The results of this study suggest that cellular cross talking via gap junctions is as much vital during proper wound healing, and an up regulated connexin expression might leads to improper gap junctions formation attributing to the passage of various, apoptotic and inflammatory signals thereby resulting in delayed healing of chronic diabetic ulcers.

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1. Introduction

According to studies carried out by World Health Organization (WHO), approximately 220 million people are suffering from diabetes worldwide, and India has the largest number of diabetic patients and the number may even go up to 69.9 million by the year 2025 [1, 2]. Chronic and persistent wounds are the common secondary complications in uncontrolled diabetes associated with significant morbidity and ailment. Diabetic foot ulcers alone are estimated to occur in 15% of total diabetic patients [3, 4]. Despite several scientific studies conducted worldwide, the etiology and underlying mechanisms of this disease have yet not been completely explored.

Wound healing is the key survival process in all organisms, which involves orchestrated interplay of several cell types, proteins, proteinases, cytokines, angiogenic factors. It involves several biochemical processes of tissue repair like granular tissue formation, angiogenesis and reepithelialization [5]. All these events involve active participation of various cell types like endothelial and fibroblast cells, keratinocytes and their cross talk through gap junctions play an important role during wound healing process [6]. Connexins (Cx), the gap junction proteins, form channels between two adjacent cells and their expression is highly regulated after wound

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formation at the transcriptional, translational and post translational levels [7]. Till date nearly 20 connexin genes have been identified in mouse genome and 21 in the human genome [8]. Gap junctions are transmembrane hydrophilic channels that are formed by two connexons joined end to end in the extra cellular space and each connexon has a hemichannel made up of six protein subunits known as connexins [9]. These intercellular channels mediate the direct transfer of low molecular weight metabolites such as ATP, nutrients like glucose along with second messengers such as IP_3, Ca^{2+} etc [10] and signaling of different apoptotic and inflammatory factors [11, 12].

In diabetic patients the normal course of wound healing is impaired and the mechanisms underlying this impairment are not yet fully understood [13]. However, it has been suggested that gap junction mediated intercellular communication (GJIC) coordinates migration and proliferation of apposite cell populations during the process of wound repair [14]. The diabetic wounds are also marked by decreased levels of growth factors like VEGF and angiogenin that may lead to bleak angiogenesis and delayed tissue repair [15 -17]. Presently available evidences indicate that decreased levels of VEGF may up-regulate connexin expression leading to increased GJIC activity [18]. This may result in increased passage of apoptotic signals to the wound site thereby causing enhanced endothelial cell apoptosis, delayed fibroblast migration and decreased rate of angiogenesis, ultimately resulting in excessive blood vessel regression at the wound site. Thus, this study was mainly focused at exploring the differential expression of various connexins viz.Cx43, Cx26, Cx30.3, Cx31, Cx31.1, Cx37, Cx40 in diabetic and non-diabetic wounds.

2. Materials and methods

All experiments were performed on 7 to 8 week old male, inbred swiss albino mice with average weight of 26±1 grams. The animals were maintained individually under controlled laboratory conditions at the Center for Experimental Medicine and Surgery, Institute of Medical Science (Banaras Hindu University). They fed with standard laboratory food and water. The study was conducted with the prior approval of the institutional animal ethical committee.

The mice were divided into two groups with four animals in each group. One of the groups of mice was rendered diabetic by injecting them with intraperitoneal streptozotocin injection (40mg/kg body weight) for five consecutive days [19]. After five days, diabetes was confirmed by estimating serum glucose levels using glucose test kit (Span Diagnostics, India). Another group of mice was taken as non-diabetic control. A single full thickness excision wound of 1 cm diameter was made at the superficial level on the mid dorsum of each diabetic and non diabetic mouse. The wound tissue was collected after 72 hrs post injury for the detection and quantification of relative levels of different connexins by RT-PCR.

RT-PCR: Total RNA was isolated from diabetic/non-diabetic wound tissues (50mg) using TRI® reagent (Sigma Aldrich, USA), chloroform and isopropanaol. The RNA was subjected to RNase free DNase (Fermantas, Germany) treatment before use. The cDNA was prepared using 500ng of total RNA subjected to reverse transcription using MMLV reverse transcriptase (Fermantas, Germany), dNTPs (New England Biolabs, USA), RNasin (Fermentas, Germany) and random hexamers (Fermentas, Germany). For PCR amplification, the cDNA (2µl) was added to 25µl of a reaction mixture containing 10XPCR buffer, 0.5mM MgCl2, 200µM dNTPs, 1U Taq DNA polymerase (New England Biolabs, USA) and mice specific forward and reverse primers (3.2µM) for each connexins [20]. The β-actin primers were used as internal control. The PCR amplification was performed in a thermal cycler (Labnet, USA) programmed for 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 30s, which were preceded by initial denaturation at 94°C for 2 min. Final extension was done for 5 min at 72°C. The amplicons were analyzed by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 g/ml) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and photographed under illumination on a gel documentation system (Alpha Imager EP, Alpha Innotech Corporation, USA). The mRNA expression levels were analyzed by Image Analysis Software (Alpha ViewTm, Alpha Imager EP, Alpha Innotech Corporation, USA).

Histopathological analysis

The wound tissues from diabetic and non diabetic mice were excised and fixed in 4% buffered formalin. The tissues were washed with PBS, embedded in paraffin, cut into 6μm thick sections and then stained with haematoxylin and eosin for observing morphometric tissue changes.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using Sigma Stat 3.5. The $p < 0.05$ were considered to be significant. Data were presented as mean ± standard deviation.

3. Results

The differential expression of connexins was analyzed in wound tissues extracted from diabetic and non diabetic groups of mice (Fig.1 and Fig.2). The expressions of Cx26, Cx31, Cx31.1 and Cx43 were increased in diabetic wounds. However, the expression level for Cx43 and Cx31.1 was more significant ($p<0.001$) as compared to Cx31 ($p<0.05$) and Cx26 (NS). The Cx30.3 was significantly ($p<0.001$) expressed only in diabetic wounds whereas Cx37 and Cx40 were not expressed in diabetic as well as non diabetic wounds (Fig.1 and Fig.2).

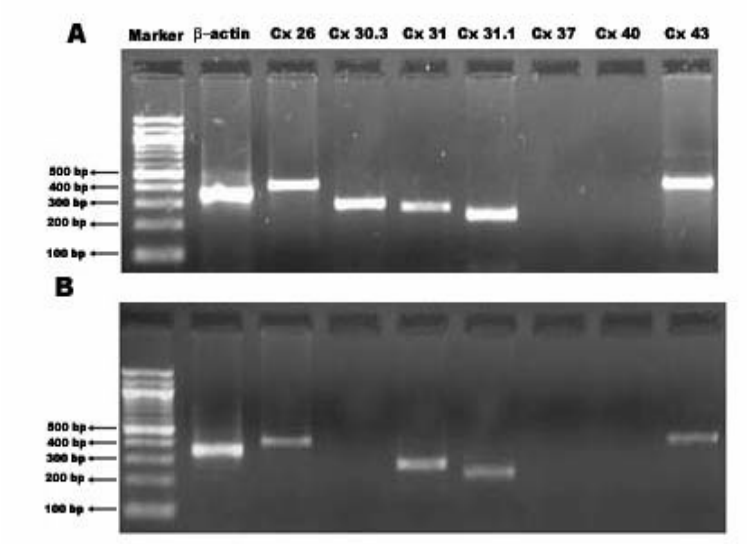

Fig. 1. Expression of various connexins in wound tissue extracted from diabetic (panel A) and non-diabetic mice (panel B). β Actin was used as internal control gene. Marker-100bp ladder.

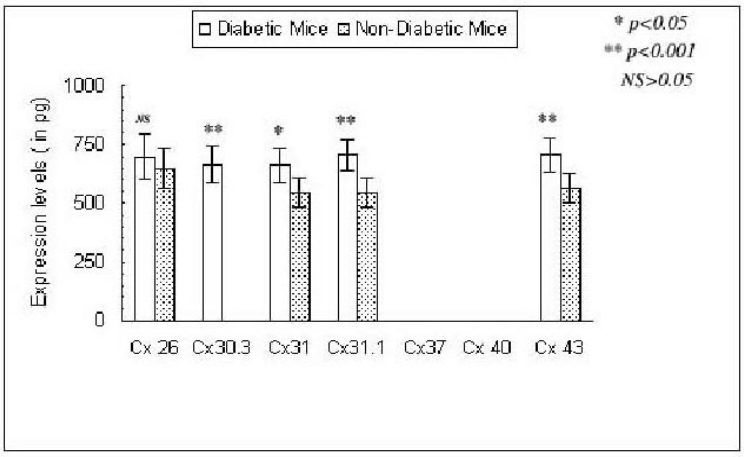

Fig. 2. Levels of expression of different connexins in diabetic and non-diabetic mice.

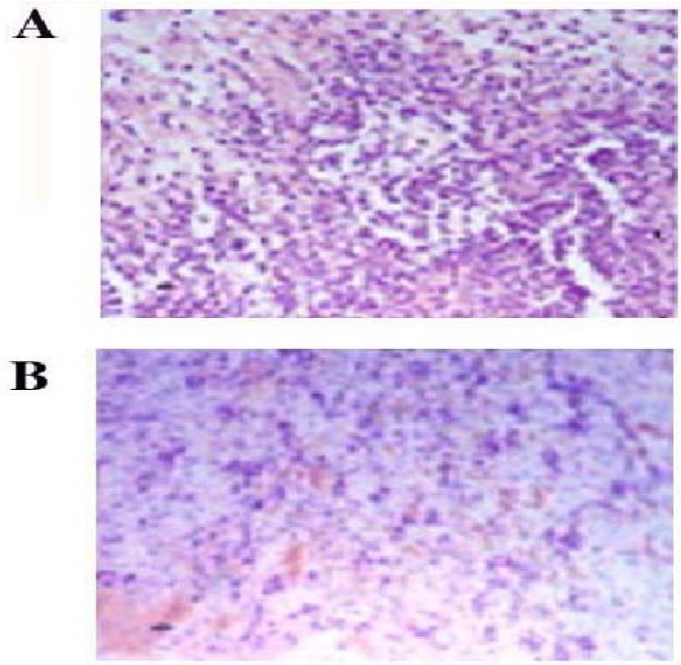

Fig.3. Eosin and hematoxylin stained histopathological slides of wound tissue extracted from (A) diabetic (B) non-diabetic mice showing angiogenesis (Original magnification 60X). The blood vessels are eosin stained (red). The (B) non-diabetic mice presents more angiogenesis with more blood vessel formation than (A) diabetic mice.

The histopathogical analysis revealed an increased angiogenesis in terms of number of blood vessels in non diabetic mice as compared to the diabetic mice (Fig.3).

4. Discussion

This study shows that the connexins expression in diabetic wounds is significantly increased with decreased angiogenesis as compared to the non diabetic wounds.

In diabetes, improper tissue remodeling is often associated with increased endothelial cell apoptosis [21], delayed fibroblast migration, poor extracellular matrix deposition [22], decreased levels of growth factors [23] and collagen synthesis, which lead to chronic wound formation [24]. It is now established that *in vitro* knock down of Cx43 in fibroblast cells increases the expression of TGF-β, collagen α-1 and keratinocyte proliferation; whereas it reduces the levels of chemokine ligand 2 and TNF-α along with neutrophil and macrophage infiltration leading to early wound closure in normal wounds [25]. Further, it has also been found that Cx43 down regulation at the wound site enables healing of the diabetic wound with a faster rate of re-epithelization [7].

During the wound healing, angiogenesis plays an important role in tissue re-epithelization [26]. This study indeed demonstrates improper angiogenesis in diabetic wound and that may be correlated with increased endothelial cell apoptosis in diabetes. The etiology of increased endothelial cell apoptosis is unknown in diabetes; however, the role of connexins and GJIC cannot be ruled out. The GJIC mediated cell death has been observed in many cases like Cx 43 mediated myocyte apoptosis in post ischemic cardiac dysfunction [27, 28] and in rat bladder carcinoma cell lines (BC-31 cells) [29]. The Cx43 linked apoptosis has also been shown to be mediated through, bcl-2 down regulation, an anti-apoptotic factor, in human glioblastoma cells [30].

In addition, this decreased angiogenesis may occur partly due to decreased level of VEGF [31]. In this study we have also estimated the levels of VEGF and angiogenin, and both of them were found to be decreased in diabetic wounds as compared to the non diabetic (data not shown). The reduced level of VEGF might result in the increased gap junction activity leading to increased passage of apoptotic, proinflammatory and toxic signals from local injury to adjacent healthy parts via the gap junctions causing an excessive damage to various cell types at the wound site that eventually results in delayed wound healing [18]. However, the factors that regulate connexin expression by various cell types like keratinocytes, endothelial and fibroblast cells in diabetes still need to be investigated. In addition, the expression of connexins by immunocompetent cells like macrophages, neutrophils, mast cells and lymphocytes in correlation with increased levels of inflammatory and immunomodulatory molecules like interleukin-1 (IL-1) and interleukin-6 (IL-6), in normal wound tissues strongly suggests their essential functional role in diabetic wound healing process that necessitates further investigation [32].

The results of this study demonstrate the dynamic expression of different connexins together, which might participate in tissue homeostasis and signaling in diabetes. However, there still remains a scope for more extensive research to ascertain the role of connexins and their regulation at wound site in the context of tissue remodeling during diabetic wound healing.

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