RESEARCH ARTICLE

ESTIMATION OF LYMPHOCYTE APOPTOSIS IN PATIENTS WITH CHRONIC NON-HEALING DIABETIC FOOT ULCER

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ABSTRACT

Background: Lymphocytes play an important role in wound healing and the removal of circulating T lymphocytes inhibits the healing cascade. Decreased stimulation of survival factors and increased levels of dead signals may lead to the malfunction of many cells, including lymphocytes.

Aims & Objective: To explore lymphocytes involvement in wound healing.

Material and Methods: Study participants were divided into three groups: group A, group B and group C (30 participants each). Annexin-V-FITC+CD-3-PE kit were used for the lymphocyte apoptosis estimation in diabetic foot patients by florescent activated cell sorter (FACS).

Results: We find out significantly higher total T cell apoptosis in type 2 diabetes mellitus patients having chronic, non healing diabetic foot ulcer as compared with healthy individuals. CD-3 + Annexin-V-FITC positive lymphocytes were statistically significant in group C ($P<0.01$) and group B ($p<0.001$) when compared from group A.

Conclusion: This study suggests the importance of T-lymphocytes in wound healing. From the present study we can suggest diabetic patients to maintain their immune system for the normal wound healing.

Key-Words: Diabetic Foot; Apoptosis; Lymphocyte

Introduction

Diabetes mellitus (DM) is one of the most common non-communicable diseases affecting majority of population globally. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genito-urinary, and cardiovascular symptoms and sexual dysfunction.[1]

The process of wound healing is very complex; it consists of haemostasis, inflammation, proliferation, and remodelling. Large number of cell types including neutrophils, macrophages, lymphocytes, keratinocytes, fibroblasts, and endothelial cells are involved in this process.[2] Lymphocytes are part of the adaptive immune response which plays a crucial role to maintain normal immune functions. These cells are carefully regulated through the equilibrium between cell birth and cell death. The participation of lymphocytes to the healing process is largely associated with their production of cytokines and growth factors (lymphokines). The role of lymphokines in wound healing has been studied in a variety of in vitro and in vivo studies.[3] Their regulatory effect on fibroblast activity and wound fibroplasia is well known.[4]

Materials and Methods

All participants involved in this study were divided into three groups. Group A included thirty healthy individuals of age group (51.33 ± 6.55 years) [mean ± SD]; group B included thirty patients with T2DM without neuropathic diabetic foot ulcer (52.50 ± 7.04 years) and group C have thirty T2DM patients with non-healing neuropathic diabetic foot ulcer (55.40 ± 7.25 years) attending SS hospital, BHU, Varanasi, India was recruited. Patients having any other complications with neuropathic diabetic foot ulcer were excluded from the study. T2DM was diagnosed according to American Diabetes Association (ADA) criteria i.e. fasting plasma glucose level (≥ 126 mg/dL, i.e., 7.0 mmol/L) on two occasions, or an oral glucose tolerance test (OGTT) yielding ≥ 200 mg/dL (11.1 mmol/L) after 2 h, or symptoms of uncontrolled diabetes with a random plasma glucose level ≥ 200 mg/dL (11.1 mmol/L). Study entrance criteria included

presence of poorly controlled type-2 diabetes {glycosylated haemoglobin (HbA1C %) ≥8.0}. Participants involved in this study were gone through detail clinical history and physical examination. This included age, sex, duration of diabetes, symptoms suggestive of diabetes mellitus and family history of diabetes. Physical examination included anthropometric measurements such as, height, weight, body mass index and waist circumference.

The Institutional ethical committee approved the study and written informed consent was obtained from each participant. We have followed the Helsinki guidelines, developed by the World Medical Association (WMA) on good clinical practices. Human blood samples were obtained by vein puncture in the morning while the patients were in the fasting state and kept in a heparinised tube. Peripheral blood monocytes (PBMC) were prepared by centrifugation using Ficoll-Paque™ plus. The number of viable cells (>95%) was determined in by Trypan blue dye exclusion test. Equal numbers of cells (1 x 106 cells/ml) were resuspended into 1X binding buffer and 100 µl was taken in 5ml falcon tube for staining. Staining was performed using annexin-V-FITC kit and CD-3-PE antibody. 5 µl propidium iodide (PI), 5µl annexin-V-FITC and 10 µl CD-3-PE were added and the cells incubated for 15 min in the dark, after which 400 µl binding buffer was then added to each tube and the cells analyzed by flow cytometry within 30 min. Flow analysis was performed on a FACScan (Becton Dickinson, CA) with a 488-nm argon laser. A quantity of 10,000 cells was collected and analyzed.

Total lymphocytes undergoing early and late apoptosis in T2DM patients with and without ulcer were compared with those of the control subjects. Statistical analysis was performed within these three groups by One-way analysis of variance (ANOVA). Newman-Keuls post-test was used for multiple comparisons. The differences were considered statistically significant when p <0.05.

Results

Significance difference was observed in FBG, PPBG and HbA1c whereas there was no significance difference in the age and body mass index (BMI) in between the all three groups. The percentage of CD-3 + annexin-V-FITC-positive cells was 14.76 ± 4.61% in group A, 22.52 ± 8.47% in group B, and 28.36 ± 15.41% in group C. The percentage of CD-3 + PI-positive cells was 0.44 ± 0.38 in group A, 0.90 ± 0.53 in group B, and 1.17 ± 0.26 in group C. CD-3 + Annexin-V-FITC positive lymphocytes were statistically significant in group C (P<0.01) and group B (p<0.001) when compared from group A. The value of significance was p<0.05 when compared between group B and group C.

Table-1: Baseline Characteristics of the Study Participants

Parameter	Groups (Mean ± SD)			p value	
	A	B	C	A vs B & C	B vs C
Number	30	30	30		
Age (Years)	51.33 ± 6.55	52.50 ± 7.04	55.40 ± 7.25	NS	NS
Duration of DM (Months)	-	30.42 ± 18.32	59.57 ± 9.51		<0.001
BMI (kg/m²)	23.57 ± 3.60	23.91 ± 1.66	24.88 ± 2.09	NS	NS
FBS (mg/dl)	93.67 ± 5.62	152.03 ± 13.59	170.63 ± 16.41	<0.001	<0.001
PPBS (mg/dl)	128.66 ± 5.56	232.16 ± 20.66	252.03 ± 19.55	<0.001	<0.001
HbA1c (%)	5.93 ± 0.34	8.81 ± 0.81	10.06 ± 1.99	<0.001	<0.001

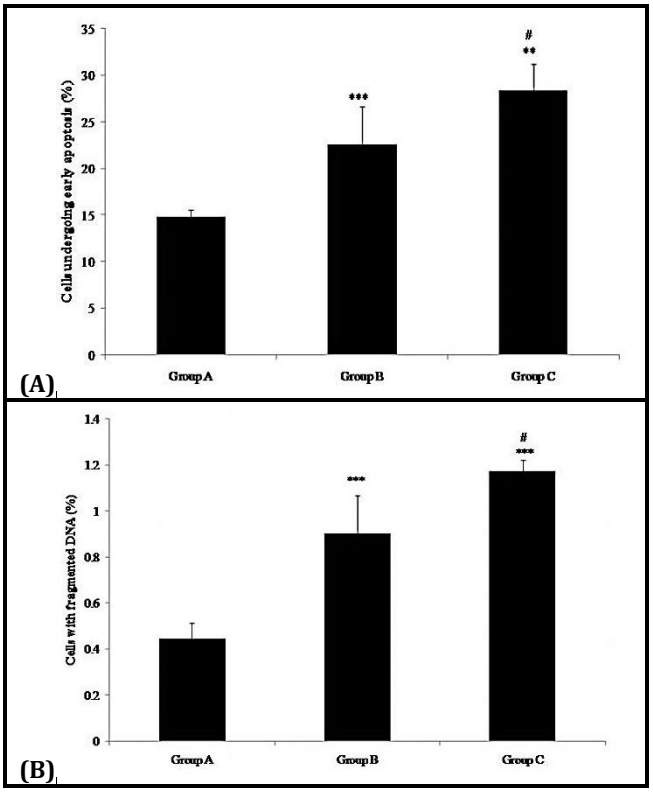

Figure-1: (A) Percentage of Annexin-V-FITC-Positive and PI-Negative (B) Percentage of Annexin-V-FITC-Negative and PI-Positive CD-3 cells in group A, group B and group C (* comparision of group A with group B and group C, # comparision between group B and group C)

CD-3 + PI positive lymphocytes were highly significant in group C and group B when compared from group A ($p<0.001$). The value of significance was $p<0.05$ when compared between group B and group C (Figure 1 A & B).

Discussion

Lymphocytes provide cell mediated immunity and are capable of producing growth factors to take part in the setting of wound repair.[5] T cell-dependent immune system plays an active role in the process of wound healing; its depletion leads to impaired wound healing.[6] Several studies suggest that wound healing is delayed due to decreased T-cell infiltration and concentration in the wound site where as others have reported that CD 4+ cells have a positive role and CD8+ cells play an inhibitory role in wound healing.[7] Wound healing impaired by radiation reflects reduced expression of lymphocyte subtypes.[8] The role of T lymphocyte in diabetic foot ulcer is not completely understood and is a current area of intensive investigation. In this report, we demonstrated that the total T-cell apoptosis is significantly higher in T2DM with diabetic foot ulcer as compared with T2DM without ulcer and healthy control. Our previous studies show that increased oxidative stress in diabetics with chronic non healing wound induces lymphocyte apoptosis.[9] Lymphocytes regulated the formation of fibrous tissue at wound site and alteration in host immune response alters the course of fibroplasia after wounding.[10] T lymphocytes play a crucial role in the wound healing process and its removal inhibits the healing cascade.

Conclusion

Our study provides basic information regarding increased T lymphocyte apoptosis in diabetic foot patients and shows that increased T lymphocyte apoptosis in diabetic foot patients is responsible for delayed wound healing with other factors. A detailed and thorough research is required to understand the mechanism by which T cells up regulate wound healing in diabetic foot patients.

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