Original Article

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Dentistry Section

Immunolocalization of CD34 Positive Progenitor Cells in Diabetic and Non Diabetic Periodontitis Patients – A Comparative Study

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ABSTRACT

Background: Little research has been documented to determine the CD34 positive cells in healthy periodontium, chronic periodontitis and in chronic periodontitis with diabetes mellitus individuals.

Aim: The aim of the present study was to evaluate and compare the CD34 positive progenitor cells of the gingiva in patients with healthy periodontium, chronic periodontitis and chronic periodontitis with Diabetes Mellitus.

Materials and Methods: A total number of 75 patients were divided into 3 groups which included Group I (healthy periodontium), Group II (chronic periodontitis) and Group III (chronic periodontitis with diabetes mellitus). Periodontal examination included Plaque index, Gingival index, Gingival bleeding index, Probing pocket depth and Clinical attachment levels. Gingival biopsies were collected from each participant and they were fixed in formalin embedded in paraffin which was later subjected to immunohistochemical procedure with anti-CD34 (a stemness marker). T-Test and Regression analysis (R-square) was used to analyse the data.

Results: The mean number of CD34 positive cells were higher in group III (5.71±1.97) compared to Group chronic periodontitis group I (4.98± 2.08) and II (4.48± 1.24).

Conclusion: Although CD34 is a non specific stemness marker, results suggest that there is a significant difference in the number of CD34 positive progenitor cells between Group II and Group III but no significant difference was observed between Group I, II and Group I, III.

Keywords: CD34, Chronic Periodontitis, Diabetes Mellitus, Mesenchymal Progenitor cells, Paravascular and Stromal Progenitor cells

INTRODUCTION

Periodontitis being a chronic inflammatory disease is characterized by periodontal pocket formation, connective tissue attachment loss and resorption of the alveolar bone resulting in eventual tooth loss [1]. Amongst the various systemic diseases such as cardiovascular diseases, respiratory conditions and diabetes mellitus [2,3] which were shown to have positive relationship to periodontitis, periodontal disease has a two-way relationship with diabetes mellitus which has been extensively studied and its importance of inflammation in periodontal disease, has been well documented [4,5].

Gingiva being a tissue with high vascularity and intact vascular system is essential for maintaining proper tissue functioning. The perivascular cells and endothelial cells together, are necessary for the formation of functional and stable vascular networks. The Endothelial Progenitor Cells (EPC) and mesenchymal progenitor cells(MPC) originate from the vascular stem cells (VSC), where in EPC are identified by stemness markers (CD34, CD133, SCA-1, C- Kit) and endothelial markers (VEGR-1, VEGR-R (KDR), VEGFR3, CD31, VWF, VE-Cadherin, Tie-2) [6].

Cluster of differentiation molecules as recognized by specific set of antibodies are the markers on cell surface used in identifying the cell type, stage of differentiation and cell activity. CD34, is a marker of human hematopoietic cells is a 11 KDa transmembrane cell surface glycoprotein with its gene being located on chromosome 1 in the region of 1q32 [7].

This membrane phosphoglycoprotein CD34 has been proven to be a useful marker of human hematopoietic stem/ progenitor cells and it is widely recognized as the principal marker used to isolate HSC. CD34, a sialomucin is expressed on a variety of mesoderm progeny including blood, endothelial and fibroblast cell and by numerous epithelial lineages [8]. The CD34 family of cell surface transmembrane proteins comprising the hematopoietic progenitor cell antigen CD34, podo-calyx and endoglycan has been proposed to promote proliferation, block the differentiation of progenitor cells which is also widely used as a marker of vascular endothelial cells and haematopoeitic stem and progenitor cells. This CD34 positive cells are located in gingival stromal, paravascular location and basal layers of gingival epithelium with progenitors in the gingiva may be arising from the peripheral blood hemopoeitic stem cells [9].

Little research has been documented to determine the CD34 molecule in healthy periodontium, chronic periodontitis and in chronic periodontitis with diabetes mellitus individuals. Hence this study was intended to compare the number of CD34 positive progenitor cells in the healthy periodontium, chronic periodontitis and chronic periodontitis with diabetes mellitus and also correlating the same with various clinical periodontal parameters.

MATERIALS AND METHODS:

Study Design

The study sample included 75 patients visiting the Department of Periodontics, Vishnu dental college and hospital, Bhimavaram and was scheduled from 2011 July to 2012 August. The subjects included were divided into 3 groups of 25 patients each. The patients were informed about the nature of the study. Ethical clearance approval was obtained from the institutional review board, Vishnu dental college, Bhimavaram

Selection Criteria

Patients who had not been treated for periodontitis over the previous 2 years and had not taken antibiotics in the past 6 months and with

Age	Mean±SD
Healthy periodontium	29.44±9.11
Chronic periodontitis	47.48±9.19
Chronic periodontitis with diabetes mellitus	53.88±8.96

[Table/Fig-1]: Mean age of the study population

Groups	Male n (%)	Female n (%)	Total
Healthy periodontium	11 (44)	14(56)	25(100)
Chronic periodontitis	13 (52)	12 (48)	25(100)
Chronic periodontitis with diabetes mellitus	15(60)	10(40)	25(100)
Total	39(52)	36(48)	75(100)

[Table/Fig-2]: Gender wise distribution of the study population

Average number of cells	Mean ± SD	Mean Difference	t value	p value
Healthy periodontium	4.98 ± 2.08	0.50±0.06	0.831	0.410
Chronic periodontitis	4.48±2.14			
Healthy periodontium	4.98± 2.08	0.73± 0.11	-1.284	0.205
Chronic periodontitis with diabetes mellitus	5.71± 1.97			
Chronic periodontitis	4.48±2.14	1.23±0.17	-2.118	0.039
Chronic periodontitis with diabetes mellitus	5.71± 1.97			

[Table/Fig-3]: Comparison of average number of cells between the groups, *Statistical Analysis: unpaired t test. Statistically significant at p<0.05*

a minimum of 20 natural teeth were included. In the study patients with systemic complications other than diabetes mellitus and with hemoglobinopathies were excluded for the study.

Clinical evaluation of subjects

After obtaining a thorough medical and dental history which included chief complaint and clinical examination evaluation of subjects were performed by using a sterile mouth mirror and Williams graduated periodontal probe by using Plaque Index [10], Gingival Index [11], Gingival Bleeding Index [12], Mean Probing Pocket Depth, and Clinical Attachment Level. The total of 75 individuals were divided as following which compromised of:

Group I – 25 patients of healthy periodontium with no features of gingival inflammation, undetectable bleeding on probing and probing depth of less than 3mm with demonstrable loss of attachment.

Group II – 25 patients of chronic periodontitis with a probing depth greater than or equal to 3mm and CAL greater than or equal to 3mm which was confirmed by demonstrable IOPA's in at least 6 teeth.

Group III –25 patients of chronic periodontitis with a probing depth greater than or equal to 3mm and CAL greater than or equal to 3mm which was confirmed by demonstrable IOPA's in at least 6 teeth having diabetes mellitus.

Procedure for obtaining gingival tissues

The patients were informed about the nature of the study and after taking their consent all the recordings were completed in one sitting. In patients with healthy periodontium gingival biopsies were collected from the subjects undergoing crown lengthening procedure, whereas in patients with chronic periodontitis and in chronic periodontitis with diabetes mellitus biopsies were collected from teeth undergoing extraction with Grade III mobility and in cases where in the prognosis was considered hopeless. The gingival biopsies were stained immunohistochemically for comparison of CD34 positive progenitor cells. Anti CD34 (endothelial cell) BiogeninTM Kit used.

Regression parameters	Healthy periodontium		Chronic periodontitis		Chronic periodontitis with diabetes mellitus	
	Regression coefficients	p-value	Regression coefficients	p-value	Regression coefficients	p-value
Average Number Of Cells	Dependent variable	******	Dependent variable	******	Dependent variable	*****
Constant	4.436	0.150	-1.492	0.774	5.155	0.215
Age	-0.035	0.515	-0.009	0.867	-0.008	0.882
Sex	0.461	0.626	0.133	0.893	-0.053	0.963
Plaque Index	-0.549	0.708	-1.262	0.324	0.168	0.814
Gingival index	0.000	0.000	3.986	0.046	0.399	0.662
% Gingival Bleeding Index	0.000	0.000	0.000	0.000	0.032	0.156
Mean Probing Depth	-0.926	0.547	0.309	0.492	-0.042	0.869
CAL	2.461	0.099	0.029	0.959	-0.287	0.552

[Table/Fig-4]: Regression analysis between average number of cells and different variables in three groups
Statistical Analysis: Regression analysis. Statistically significant at p<0.05

Tissue preparation

Formalin fixed paraffin embedded tissues were sectioned at 4µm and mounted on positively charged slides. Deparaffinization was done by keeping the slides in Xylene I for 15 min, same time in Xylene II, 100% Ethanol for 15 min, same time respectively in 80% Ethanol and 70% Ethanol. Later slides were washed in deionized H_2O for 1 min. Antigen retrieval was done by using citric acid buffer in microwave oven for 5 min at 95^0. The solution was allowed to cool in buffer at room temperature for 20 min. Then slides were washed with deionized water three times for two minutes each on stir plate. Excess liquid was removed from the slides. Tissues should not be allowed to dry out at any time during the procedure. Endogenous peroxidase activity is carried by incubating specimens for 5 min in peroxide block. Slides were then washed in PBS for 2 min on stir plate and again excess liquid was removed from the slides.

Slides were treated with protein power block for 20 min and were blot drained. Later they were incubated with primary antibody (CD34) for 1 hour and , were washed with wash buffer (PBS) for 3 times. Super enhancer was added to the slides and incubated for 20 minutes. Slides were again washed with wash buffer for three times. Then, slides were incubated with secondary antibody SS and labelled for 30 minutes. Later they were washed with wash buffer for three times. During the above incubation step, DAB reagent was prepared in the substrate mixing bottle as follows: the tip was removed from mixing bottle and combined with 1.6 ml deionized H_2O, 5 drops 10x substrate buffer, 1 drop 50x DAB buffer and 1 drop 50x peroxidase substrate. Then, slides were incubated with DAB chromogen for 10 min, rinsed with deionized water for 2 min and counterstained with Gills haematoxylin for 5-10 sec followed by keeping in running tap water and were air dried. After that slides were dipped in 70% alcohol for five times, 80% alcohol for five times and 100% alcohol for five times. They were also dipped five times in xylene 1, five times in xylene 2. Then slides were dried and mounted using DPX.

Immuno-histochemical evaluation of CD34 positive cells

Following the immunohistochemical staining all the stained sections of the gingiva which included healthy, chronic periodontitis and chronic periodontitis with diabetes mellitus were studied under research microscope. Brown staining was considered as positive staining. Pyogenic granuloma was taken as positive control. Whereas, CD34 positive cells within the blood vessels served as the positive internal control for the specificity of the stain. The positive staining associated with endothelial cells of the vascular channels were not evaluated. Only stromal and paravascular cells that were stained positive were considered and were counted for quantitative

analysis. Each specimen which is immunohistochemically stained was observed under 10X, and latter 40X of research microscope. Photographs of 5 areas of clearly identifiable brown colored paravascular and stromal cells (CD34 progenitor cells) were taken and saved in the computer which is attached to the research microscope. Counts were performed on Olympus microscope using image pro plus image analysis software. Representative fields were chosen for each section at 40x magnification and the total number of positive cells for all five examined fields per case was calculated. This allowed calculation of the mean number of CD34 positive cells per field. Results were presented as the mean number of CD34 positive cells per field.

STATISTICAL ANALYSIS

Data collected were subjected to statistical analysis using SPSS 16.0 and the p value <0.05 was considered statistically significant for the analysis. T-Test and Regression analysis (R-square) was used to analyse the data.

RESULTS

The age of the study population ranged between 15-76y with mean age of 29.44 y in Group I, 47.48 y in Group II and 53.88 in Group III [Table/Fig-1]. [Table/Fig-2] shows the distribution of study population according to gender. All the 25 patients had controlled blood sugar levels with an average HbA1c of 6.35. The mean number of cells between the three groups were not statistically significant (p>0.05). The mean number of CD34 positive cells were higher in group III (5.71±1.97) compared to group I (4.98±2.08) and group II (4.48±1.24). Although CD34 is a non specific stemness marker, results suggest that there is a significant difference in the number of CD34 positive progenitor cells between Group II and Group III but no significant difference was observed between Group I, II and Group I, III [Table/Fig-3]. The correlation with average number of CD34 positive progenitor cells with gingival index and % gingival bleeding index was statistically significant in Group I and Group II [Table/Fig-4].

DISCUSSION

The conversion of groups of multipotent cells to cells that form differentiated, highly specialized and very narrowly functioning tissue is the hallmark of the development process. This sequence of turnover demands the existence of progenitor cells that give rise to various mature phenotypes observed in complex tissues and organs. This progenitor cells in the gingiva are located in the basal stromal and the paravascular layers.

To the best of our knowledge there are no studies done immunohistochemically to compare the CD34 positive stromal and paravascular progenitor cells in the gingival biopsies of healthy periodontium, and chronic periodontitis with diabetes mellitus whereas previous study in the literature included immunolocalization of CD34 positive cells in healthy human gingiva only.

The present study revealed the presence of CD34 positive cells in the paravascular location of the healthy gingival which could be the fibroblast progenitors, with the possible precursors being the peripheral blood stem cells. However, a study conducted by Tomar GB et al., concluded that human gingival connective tissue derived MSC's (GT-MSC) have the ability to proliferate faster and differentiate into fat, bone and cartilage when compared to the bone marrow derived mesenchymal cells (BM-MSCs). These gingival MSC's which are uniformly shaped fibroblast like cells showed all the characteristics of human MSC's and are easy to isolate, homogenous and proliferate faster than bone marrow MSC's [13].

Various studies from clinical point of view, revealed that human gingiva is also a better source of MSC's than bone marrow and large number of functionally clinical grade MSC's can be generated in short duration for cell therapy in regenerative medicine and tissue engineering [13].

In our study we observed that there is a decrease in the average number of cells with age in healthy, chronic periodontitis and chronic periodontitis with diabetes mellitus groups but the relation is not significant.

Adipose tissue derived mesenchymal stem cells (ASCs) from healthy young, middle aged, and aged volunteers were evaluated, and it was clearly demonstrated that a correlation between age related decrease in quantity of tissue resident stem cells and their impairment in self renewal and differentiation capabilities existed [14]. There was substantial reduction in the quantity of available resident stem cells in aged volunteers. When the differential gene expression was investigated in primary human MSC and HPC derived from different age groups it demonstrated age related gene expression changes in both the cells but there is a moderate and significant concordance in the expression profiles upon aging in vivo and replicating senescence in vitro concluding that the stem and progenitor cells are not protected from aging [14].

However in our study we observed that the average number of CD34 positive progenitor cells was significant in between chronic periodontitis (Gr II) and chronic periodontitis with Diabetes mellitus (Gr III). However the chronic periodontitis with Diabetes Mellitus group included patients with properly controlled blood sugar levels. This may be attributed to homing of the progenitor cells to repair the destructed connective tissue components in Diabetes Mellitus with Chronic Periodontitis (GroupIII) When compared to chronic periodontitis (GroupII) alone [15].

These findings are in agreement with the findings of a previous study, wherein the available data suggests that stabilized glucose levels and life style change by metabolic intervention is able to improve endothelial progenitor cell biology [16]. The findings of a study by Gian paolo Fadini et al., elicit that it is not clear to what extent the defective progenitor cells are related to type 2 diabetic patients [17].

In our study there is no significant correlation between average number of CD34 positive progenitor cells and plaque levels, mean probing depth and clinical attachment level. These findings are in agreement with the findings of Singer NG and Caplan AL which may be due to anti – inflammatory and immunomodulatory functions of these gingival mesenchymal cells.

It is important to consider the limitations of the present study. Primarily, as the study undertaken here included patients with known diabetes and those who were under control, the effect of medication on progenitor cells could not be differentiated. Secondarily, the results cannot be generalized because of the lesser sample size. Hence, further studies should reflect into the deeper aspects of the effect of medication on progenitor cells with larger sample size.

CONCLUSION

Results suggest that there is a significant difference in the number of CD34 positive progenitor cells between Group II and Group III but no significant difference was observed between Group I and II, Group I and III. However, there is no pattern to establish a relationship between CD34 positive progenitor cells and various periodontal parameters. Hence, further studies need to be conducted to suggest a possible role of positive progenitor cells in patients with chronic periodontitis and also on advanced glycation end products on progenitor cells.

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