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RESEARCH ARTICLE

# STROMAL CHANGES IN LEUKOPLAKIA: EXPLORING NEW VISTAS

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**ABSTRACT**

**Introduction:** Leukoplakia is a potentially malignant disorder which may histologically present with hyperplastic or dysplastic changes in the epithelium. There is an increased potential for malignant transformation with higher grades of epithelial dysplasia. The stimulus produced by the dysplastic cell may evoke a connective tissue response in the form of changes in vascularity, collagen fiber activity and inflammatory cell infiltration.

**Aim and Objectives:** This study is designed to analyze and compare stromal changes involving inflammatory cell infiltration, vascularity and collagen fibers in leukoplakia with and without dysplasia with an aim to determine whether the changes in the connective tissue could be related to the dysplastic changes in the epithelium.

**Materials and method:** The study group comprised of 40 specimens of clinically diagnosed leukoplakia. These were divided into two groups (20 cases of leukoplakia without dysplasia and 20 cases of leukoplakia with dysplasia). Hematoxylin and eosin stained sections were evaluated for inflammatory cell infiltrate and vascularity while picrosirius red stained sections were used to study collagen under polarizing microscope. Statistical analysis was performed by employing ANOVA test using SPSS version21 ($p<0.05$)

**Result:** There was a significant increase in the number of thick green fibers and decrease in number of red fibers in leukoplakia with dysplasia as compared to non-dysplastic leukoplakia.. Also a significant increase in the number of blood vessels and inflammatory cells was noted in dysplastic leukoplakia.

**Conclusion:** Stromal modifications in the form of collagen fiber remodeling indicated by an increase in the number of green fibers and a decrease in the number of red fibers along with an increase in inflammatory cells and vascularity, suggests an important role of connective tissue components in leukoplakia with epithelial dysplasia.



## INTRODUCTION

Oral leukoplakia with dysplasia is a common white lesion of oral mucosa with varying histopathological changes ranging from simple hyperplasia to severe dysplasia. The relevance of this lesion is in its malignant transformation which is of prime importance in determining its management protocol. In a recently published paper, leukoplakia has been defined as ‘‘a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”. [(1)] Nevertheless, the most widely used definition of leukoplakia is, the one proposed by the World Health Organization (WHO) in 1978, which states that “leukoplakia is a predominantly white patch that cannot be characterized clinically or histopathologically as any other definable lesion”. [(2)]

Although histological changes of clinical leukoplakia are primarily evident in the epithelial tissue, the connective tissue may undergo modification owing to stromal response to the overlying epithelial changes. Collagen forms the major portion of the connective tissue and provides it with a unique combination of flexibility and tensile strength and helps in maintaining the structural integrity and the tissue function. [(3,4)] The process of collagen deposition and degradation is well regulated , not only to control the amount of collagen produced but also to control the fiber architecture. Collagen synthesis is determined by a variety of mediators, which include hormone, growth factors, cytokines and lymphokines. However TGF-β plays a pivotal role in the production of collagens and other matrix components.

The connective tissue integrity is maintained by both thick and thin fibres thus emphasizing the importance of demonstrating

both these fibers in the evaluation of connective tissue changes.

Picrosirius red, a special stain was found to be promising in demonstrating both thin and thick fibres. It stains the thin fibres intensely and increases their birefringence.[5] Junqueira *et al. (1979)*[6] in their study using Picrosirius red staining followed by polarizing microscopy, selectively demonstrated collagen with an observable difference in the polarizing colors caused by fibers thickness as well as by the packing of the collagen. The examination of collagen fibres by this method served as a procedure to differentiate between intermediate, procollgen and pathological collagen fibres. [7, 8]

Dynamic interplay between epithelium and stroma composed of fibroblasts, vasculature and resident immune cells determines potential for malignant transformation. Mononuclear cell infiltration has been observed in leukoplakia by most workers, but little significance has been ascribed to it. Authors have found that carcinomatous transformation may be associated with some immunological changes. Angiogenesis is a fundamental process in tumor growth and metastasis. The induction of angiogenesis is mediated by positive and negative regulatory molecules released by both the tumor and host cells and depends on a net balance between positive and negative angiogenic factors.[9] It is found that an increase in relative vascular volume in the stroma of premalignant lesion and malignant lesions of the oral cheek lesions is accompanied by both angiogenesis and vasodilatation of the blood vessels which may reflect the increasing nutrient requirements of actively growing and dividing cells.[10,11]

It is now well accepted that the coordinated activity of epithelial cells with their stroma is fundamental in controlling growth and differentiation in normal and pathological situations. This study was aimed to analyze and compare stromal changes involving inflammatory cell infiltration, vascularity and collagen fibers in leukoplakia with and without dysplasia and to correlate these stromal changes with that of overlying epithelial dysplasia.

## METHODOLOGY

The present study included a total of 40 clinically diagnosed cases of leukoplakia from the archives of our department. The tissue specimens of these cases were histologically assessed and were grouped into leukoplakia without dysplasia (Group I, n=20) and leukoplakia with dysplasia (Group II n=20). The slides of leukoplakia with dysplasia were further subdivided into mild dysplasia, moderate dysplasia and severe dysplasia according to WHO classification.[9,12] Two consecutive sections of 3.5µ thickness each were cut from the paraffin embedded tissue blocks. One of the sections was stained with Hematoxylin and Eosin (H&E) [13]) and the other with picrosirius red stain using standard procedure.[7]

Three groups of polarizing colors for collagen fibers were observed which included green to greenish yellow (GGY), yellowish orange (YO) and orange red to red (ORR).The collagen fibers with thickness less than or equal to 0.8µ were considered as thin fibers and those between 1.6-2.4µ, were considered as thick fibers. [14]Total of 100 collagen fibers were randomly selected under high power field in lamina propria of connective tissue. The thickness and color of the collagen fibers were measured and analyzed using the image analysis software Motic image, Plus Version 2.0 software. The number of inflammatory cells and blood vessels were counted in the lamina propria of connective tissue in 5 random high power fields by moving the microscopic stage in "Z" shape to avoid recounting of the same cell. Blood vessels with well formed lumen only were counted. The images were uploaded for computer analysis using Motic image plus software and the mean of five fields were counted and it was considered for each slide.

## RESULTS

In the present study, picrosirius red stained tissues under polarizing microscopy showed different birefringence depending on thickness of collagen fibers. Enhanced birefringence of collagen fibers enabled differentiation between thin and thick fibers and change in polarizing colors helped studying the compactness of collagen. All the data was recorded and statistically analyzed using one way ANOVA test. (SPSS version 21)

The numbers of ORR fibers were more in non-dysplastic leukoplakia than leukoplakia with dysplasia (p=.00) (Table 1) while mean number of GGY fibers were more in dysplastic leukoplakia. (p=.00) (Table 1) The mean numbers of thick GGY fibers were significantly increased in leukoplakia with dysplasia. (p=.00)(Table 2)

**Table1** Showing Mean and standard deviation for number of collagen fibers, blood vessels and inflammatory cells in two groups.

| | | Group I | Group II | p-value | significant |
|---|---|---|---|---|---|
| Collagen fibers | ORR | 64±8.96 | 26.7±22.38 | .00 | significant |
| | YO | 21±9.10 | 24±16.37 | .471 | Not significant |
| | GGY | 15±7.19 | 46±25.10 | .00 | significant |
| Blood vessels | | 18.5±15.06 | 27.15±11.43 | .048 | significant |
| Inflammatory cells | | 172.1±150 | 314±194.6 | .014 | significant |

**Table 2** Mean and standard deviation for thick and thin collagen fibers in two groups

| Type of parameter | | Mean±SD | | p-value | Significant |
|---|---|---|---|---|---|
| | | GROUP I | GROUP II | | |
| G-GY | Thick | 2.85±3.31 | 33.40±27.74 | .000 | Highly significant |
| | Thin | 11.95±5.12 | 12.60±15.32 | .858 | Not significant |
| YO | Thick | 16.50±8.54 | 14.30±13.78 | .585 | Not significant |
| | Thin | 4.70±2.40 | 9.60±6.15 | .001 | Significant |
| OR-R | Thick | 57.25±9.64 | 11.11±10.92 | .000 | Highly significant |
| | Thin | 7.16±4.33 | 16.20±14.94 | .016 | significant |

When we studied birefringence in the three subgroups of group leukoplakia with dysplasia, there was a change in the birefringence from ORR to YO to GGY with increasing grades of dysplasia. However, the data was statistically insignificant between these subgroups. (p>.05) (Table 3)

**Table 3** ANOVA test result showing comparison in subgroups of leukoplakia

| | Mean± SD | | | P value | significant |
|---|---|---|---|---|---|
| | Mild dysplasia | Moderate dysplasia | severe dysplasia | | |
| ORR | 28.29 | 29.71 | 21.33 | >0.05 | Not significant |
| YO | 33.14 | 14.57 | 23.33 | | |
| GGY | 37.86 | 48 | 53 | | |

Mean number of blood vessels (p=.048) and inflammatory cells (p=.014) were significantly higher in leukoplakia with dysplasia than those without dysplasia. (Table 1)

## DISCUSSION

The extracellular matrix (ECM) is a key regulator of cell and tissue function and has been thought of primarily as a physical scaffold that binds cells and tissues together.[15] It is a dynamic structure that interacts with cells and generates signals through feedback loops to control their behavior.

Thus, ECM macromolecules are bioactive and modulate cellular events such as adhesion, differentiation, migration, proliferation, and survival.[16] Even minor alterations in a single ECM component can lead not only to altered physicochemical properties of tissues but also to changes in cellular phenotype and cell-matrix interactions. It has been proposed that these changes in ECM structure and bioactivity in tissue function ultimately lead to development of disease.

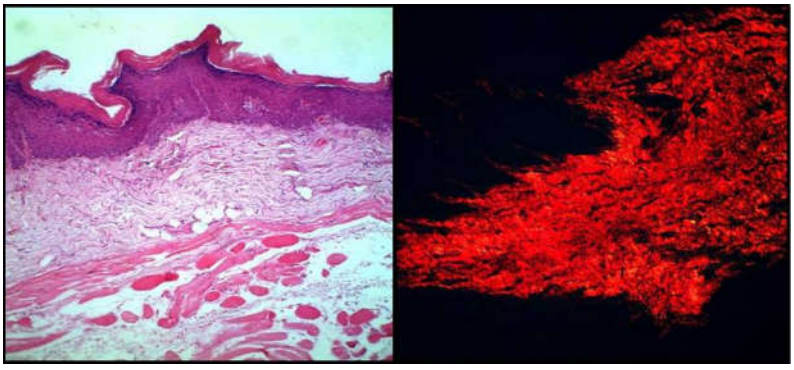

**Figure no.1** photomicrograph [A) H & E 10x and (B) Picrosirius red 10x] showing leukoplakia without dysplasia.

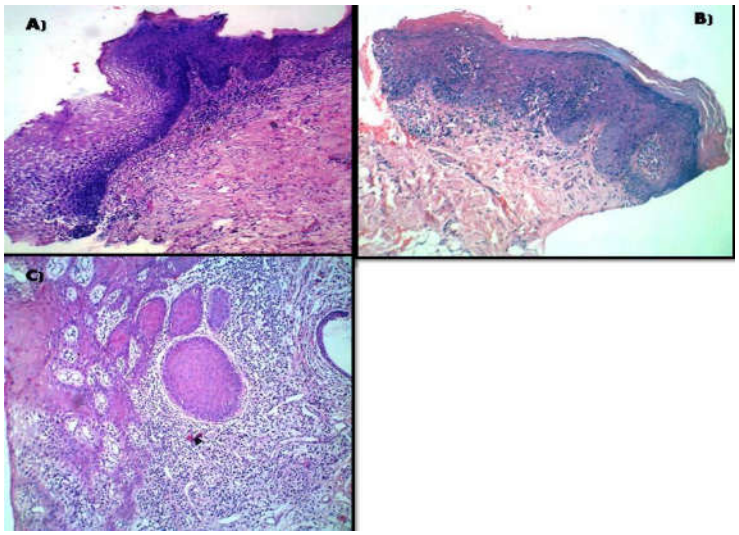


**Figure no.2** photomicrograph H & E 10x, A) leukoplakia with mild dysplasia, B) leukoplakia with moderate dysplasia, C) leukoplakia with severe dysplasia

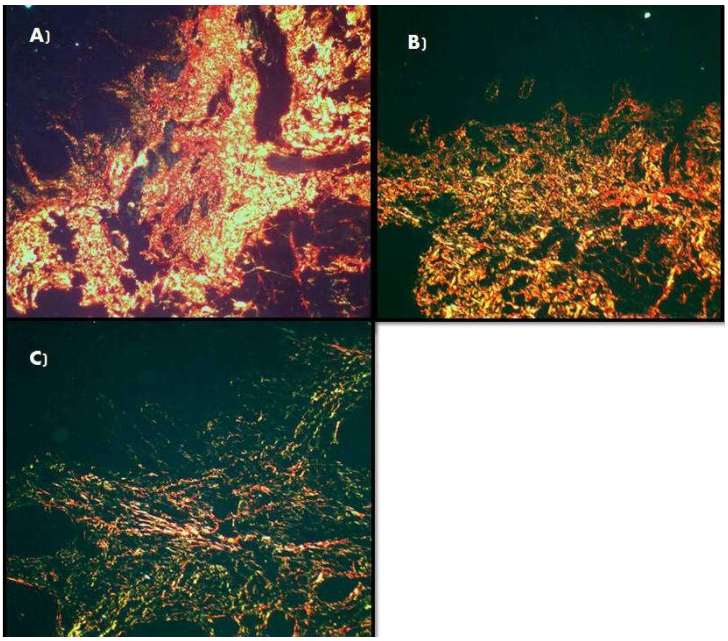


**Figure no.3** photomicrograph Picrosirius red 10x, A) leukoplakia with mild dysplasia, B) leukoplakia with moderate dysplasia, C) leukoplakia with severe dysplasia

Similarly, during transition from normal tissue to in situ to invasive carcinoma in oral cavity, the stromal environment may also play an important role. Collagen forms a major component of ECM and changes in collagen fiber composition represent a major disorganization of "basic skeleton" of ECM. Many researchers have observed that Sirius red stain enhances the normal birefringence of collagen fibers in tissue sections. Thin collagen fibers exhibit green to greenish yellow polarizing colors, whereas thick fibers reveal yellowish-orange through orange to red polarizing colors. *(17)* Green to greenish-yellow colors indicates poorly packed collagen whereas orange red color denotes tightly packed fibers.[18,19]The color and intensity of birefringence are due to the difference in their pattern of physical aggregation and thickness of collagen fibers. Junqueira *et al*. (1978) and Montes *et al*. (1980) stated that the thick fibres were Type I collagen fibres and exhibited an intense birefringence of red, orange and yellow color by polarizing microscopy and a weak birefringence of green when the fibres were thin fibrillar, thus constituting Type III collagen. [20,21] It is stated that transformation of tissue from preneoplastic state into carcinomas is associated with an increase in collagenolytic enzyme activity. As type I and III collagen are the most abundant component in extracellular matrix of dermal and oral submucosal connective tissue and cancer cells producing collagenases which degrade this collagen. Hence, it is likely that ability to degrade collagen is essential for invasion and metastasis of neoplastic cells. By increased formation of collagenases, the invading tumor cells are capable of dissolving collagen in connective tissue which obstructs its course. [22]

In our study we observed a significantly increased number of GGY fibers and an increase in thick GGY fibers in leukoplakia with dysplasia as compared to non-dysplastic leukoplakia. This change in polarizing colors of the thick fibers from yellow-orange to greenish-yellow is considered to be due to loosely packed fibers which might be composed of procollagens, intermediates or pathologic collagen rather than normal that of tight packed fibers. [23]

Further, nuclear resonance studies on the physical aggregation of the collagen fibres by Sharf *et al*. (1977).[24] have also revealed a color profile of orange to red, which corresponded to the well packed fibres and the green to greenish yellow to poorly packed fibres.

In our study, we observed that mean number of GGY fibers were more in leukoplakia with dysplasia than without dysplasia which was supported by Yokoyoma M. *et al*. (2011) [25] who found that there was a decrease in mature collagen fibres with progression towards advanced dysplastic grading.
Angiogenesis in the adult organism is limited normally to conditions of tissue repair or remodeling such as exist during menstruation, mammary gland involution, wound healing, inflammation, and neoplasia. Angiogenesis involves the activation, proliferation, and migration of endothelial cells in concert with localized proteolytic modification of the ECM. [26] Angiogenesis is fundamental process in tumor growth and metastasis. It is found that an increase in vascular volume in the stroma of premalignant lesion is accompanied by both angiogenesis and vasodilatation of blood vessels. This may reflect increasing nutrient requirements of actively growing and dividing cells.[27] We found statistically significant difference between mean number of blood vessels from leukoplakia without dysplasia to leukoplakia with dysplasia. This explains that angiogenic switch seems to be turned on in the early stage of dysplasia. Dysplastic lesions contained dilated and engorged capillaries that are increased in number, and blood capillaries are localized proximal to the epithelial basement membrane and concluded that this pattern is indicative of an angiogenic switch from vascular quiescence to modest neovascularization in early low-grade lesion (hyperplasia), followed by striking upregulation of angiogenesis in high grade lesions(dysplasia).[26]

A number of authors have stated that the presence of inflammatory cell infiltrate is common in tumors of the oral cavity. Chronic inflammation as a risk factor for cancer was first conceived by Virchow in the early 19th century and reported by Weitzman and Gordon in the association of various chronic inflammatory diseases, including irritable bowel syndrome, chronic colecistitis, atrophic gastritis, and reflux esophagitis, with the development of cancer. [28] Till now most studies have addressed the function of immune cell infiltration in established tumor. Inflammatory cell infiltration has been observed in leukoplakia by most workers but little significance has been ascribed to it. We found mean no. of inflammatory cells were more in leukoplakia with dysplasia than without dysplasia significantly. Also, mean number of inflammatory cell were significantly increased with increased grades of dysplasia. Similar observations were made by T Lehner *et al*. (1970) [29] who concluded that, carcinomatous transformation may be associated with some immunological changes. G GANNOT *et al*.(2000)[30] found that inflammatory mononuclear cell infiltrates were associated with oral premalignant lesion and squamous cell carcinoma. Others found that no. of CD8T positive cells correlated with grades of dysplasia. Comparative studies of dysplasia and carcinoma have showed significant molecular alterations, which do not occur between the histological grades of carcinoma. Study carried by MR Piva *et al*. (2011) [31] found, a greater expression of CD8 in the case of dysplasia than in those of carcinoma suggesting an initially protective function of the inflammatory infiltrate,

## CONCLUSION

Leukoplakia with dysplasia showed modifications in the stroma in the form of increased number and thickness of immature collagen fibers, increase in vascularity as also inflammatory cell density. The connective tissue changes concurrent with incipient dysplastic epithelial changes may explain ontogenic aspects of stromal epithelial relationship. Based on our study results we can assume that changes in connective tissue stroma in the form of collagen fibers, vascularity and inflammatory cell infiltration are concurrent with changes in the overlying epithelial dysplasia. Alterations in stromal biology may precede and stimulate neoplastic progression in preinvasive disease. This study demonstrates the etiological importance of connective tissue in the development of cancer, and emphasis should be given to stromal changes in diagnosis of dysplasia and its relevance to malignant transformation.

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