ORIGINAL RESEARCH

Melanin: A scavenger in gingival inflammation

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ABSTRACT

Background: One of the major direct or indirect targets of ultraviolet exposure of skin is the melanocyte or the melanin -forming cell. Epidermal melanocytes act as a trap for free radicals. Based on the protective role of melanocytes in medical literature, the role of melanin pigmentation in gingiva needs to be elucidated. Periodontal pathogens and their products demonstrate the ability to induce the generation of reactive oxygen species. Hence purpose of this study was to unravel the protective role of melanin (if any) against the gingival inflammation.

Materials and Methods: A total of 80 subjects; 20 in each group were selected. The selection of subjects regarding gingival pigmentation was based on Dummett's scoring criteria 0, 3. A complete medical, dental history and an informed consent were obtained from the patients. After evaluation of clinical parameters the GCF was collected using microcapillary pipettes at the selected sites. IL-1β levels were quantitated using ELISA.

Results: In non-pigmented healthy and gingivitis groups, there was a positive correlation between plaque index, gingival index and bleeding index versus IL-1β level: indicating an increase in the biochemical mediator of inflammation corresponding to an increase in the clinical parameters of inflammation. Also a positive correlation was found between the gingival index and bleeding index versus the IL-1β levels in the pigmented healthy group. The pigmented gingivitis groups showed a negative correlation between the plaque index, gingival index and bleeding index .

Conclusions: The clinical markers of inflammation such as gingival index, bleeding index was of low numerical value in pigmented group than in the non-pigmented group, supposedly due to the protective action of melanin. The negative correlation of clinical markers of inflammation to the IL-1β levels in the pigmented gingivitis group could possibly be attributed to the protective role of melanins.

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Epidermal melanocytes have been shown to be responsive to many immunological mediators and are also protective against ultraviolet irradiation, by not only diffusing and absorbing light but also as a trap for electrons and possibly free radicals.[1]

In vitro measure of scavenging action of melanins is reported in medical literature. Bustamante *et al.* studied the protective role of melanin, either synthetic or derived from a metastatic lung melanoma nodule, in terms of its ability to interact with active oxygen species (O^{2-}, H_2O_2, RO, ROO, etc.). Both melanins showed the ability to react with O^2.[2]

The production of reactive oxygen species (ROS) in periodontal environment can be attributed to microbial induced inflammation. Free radicals associated with superoxide anion, which is generated as a result of 'respiratory burst' of phagocytes during bactericidal killing are scavenged by melanins.[1] It has been hypothesized that oral mucosa melanin provides a defense barrier by acting as a binder of toxic products such as free radicals and polycyclic compounds.[3] Therefore, the oral melanocytes may act as scavenging antioxidants and prevent oxidative stress.

Studies on melanin pigmentation of the gingiva have concentrated mainly on the incidence of gingival pigmentation in different population groups and races[4,5] and on making logical quantitative observations by microscopy and ultramicroscopy using histochemical techniques.[6--8]

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The information on oral mucosal melanocytes is mainly extrapolated from the results of investigations on epidermal melanocytes. The possible defensive role of oral melanin to the plaque-induced inflammatory response by the clinically melanin-pigmented gingiva has not been studied so far. With the continued microbial -induced inflammation and ROS of the gingival/periodontal environment, whether the pigmented gingiva express better defensive role in terms of decreased inflammatory signs and symptoms as compared to non-pigmented gingiva requires to be investigated.

A MEDLINE search was done with the key words "melanin", "oral mucosal melanocytes", "pigmentation", "gingiva", "gingivitis", and/ or "ROS" it revealed a limited number of articles with regard to inflammation and melanin pigmentation many of them pre-1960 and only one article on scavenging role of oral mucosal melanocytes.[3]

Based on the lack of information regarding scavenging role of oral mucosal melanins, this study was initiated to clinically and biochemically elucidate the defensive role of melanins in terms of gingival pigmentation to gingival inflammation.

Hence, the purpose of this study was to evaluate and compare the clinical response of non-pigmented and pigmented gingiva using gingival index and bleeding index ; and estimation of inflammatory marker such as Interleukin-1β levels in non-pigmented versus pigmented healthy and gingivitis cases.

MATERIALS AND METHODS

Ethical considerations

The review committee constituted by Rajiv Gandhi University of Health Sciences approved the protocol for human subjects. All volunteers were informed about the aims and methods of the present study and gave written consent to participate.

Subjects

Eighty systemically healthy Indians participated in this study. The patients were age matched between various groups in the present study, to avoid its influence. The study design was an observational case-control single blind study. Volunteers were grouped into four depending upon the melanin pigmentation and health of the gingiva.

Group I: Non-pigmented (DOPI 0) healthy gingiva
Group II: Non-pigmented (DOPI 0) with gingivitis
Group III: Pigmented (DOPI 3) healthy gingiva
Group IV: Pigmented (DOPI 3) with gingivitis

Subjects in the non-pigmented groups served as controls and those in the pigmented group served as cases.

No volunteer had taken any over the counter or prescribed pharmaceutical agent for at least the preceding 2 months of the study. The subjects gave their informed consent. Patients suffering from any systemic condition that are known to cause gingival melanin pigmentation, smokers and pregnant or lactating female patients were excluded from the study.

Clinical assessment

Clinical assessment was carried out single handedly by a qualified clinician. Dummetts oral pigmentation[9] was recorded for each patient to assess the degree of gingival melanin pigmentation. Assessment of plaque was done with plaque index (Silness and Loe[10] on the basis of plaque at the gingival area of the tooth. The presence or absence of gingival bleeding was determined by gentle probing of the gingival crevice with a UNC 15 periodontal probe (UNC 15 periodontal probe). Examinations were performed on four sites in each tooth (distofacial, facial margin, mesiofacial and the entire lingual margin). The presence of bleeding within 10 seconds indicated a positive score, which was expressed as a percentage of the total number of gingival margins examined. (Gingival Bleeding Index,[11] Ainamo and Bay, 1975). Gingival Index[12] (Loe and Silness, 1963) was recorded (4 gingival scoring units per tooth) and subjects with mild to moderate degrees of gingivitis were included. The same investigator carried out all clinical measurements.

GCF collection and analysis

The GCF samples were collected using microcapillary pipettes (Sigma Aldrich Germany). The experimental area was isolated with cotton rolls and gently air-dried. The supragingival plaque was removed carefully without touching the gingiva, which might stimulate fluid flow, to minimize plaque contamination of the microcapillary pipettes.[13] The micropipettes were placed just at the entrance of the gingival sulcus till 2.5 µl of the GCF was collected. The GCF was stored in 100 µl.of phosphate-buffered saline (PBS) at -20°C. Total amounts of the pro-inflammatory cytokine IL-1β was analyzed by the enzyme -linked immunosorbent assay (ELISA) techniques following the manufacturer's instructions (Immunotech France) by a qualified microbiologist who was blinded to the groups. IL-1β was expressed in nanograms.

Statistical analysis

Results are presented as mean standard deviation, median and range values. Since the observations showed moderately skewed, comparisons were done with nonparametric tests. Kruskall-Wallis ANOVA was used for simultaneous multiple group comparisons followed by Mann-Whitney test for two group comparisons. To assess the relationship between clinical parameters and IL-1β levels, Pearson's Correlation Coefficient was determined.

For all the tests, a *P* value of 0.05 or less was set for statistical significance.

RESULTS

A total of 80 healthy volunteers age matched 15 years and above of both sexes were recruited for the study. General information of the patients included in the control and the experimental group is presented in Table 1.

Although there was significant difference between various clinical parameters of gingival inflammation, the IL-1 β levels did not show any significant difference in the four groups.

The overlapping values of IL-1 β in different groups did not signify healthy or diseased state.

There was no significant correlation between the clinical parameters and IL-1β levels in non-pigmented healthy and gingivitis groups (Groups I and II).

Pigmented healthy (Group III) showed no significant correlation. Pigmented gingivitis (Group IV) showed an inverse relationship, although not significant [Table 2].

DISCUSSION

Melanin is light-absorbant polymeric pigments found widely dispersed in nature. They possess many interesting physicochemical properties. One of these is the expression of stable free radicals that appear to have a protective action in cells, probably by acting as a sink for diffusible free-radical species.[14] Free radicals associated with superoxide anion, which is generated as a result of 'respiratory burst' of phagocytes during bactericidal killing are scavenged by melanins.[3]

The damaging role of ROS and the scavenging action of melanin has been discussed in the medical literature. A major function of melanocytes, melanosomes and melanin in skin is to inhibit the proliferation of bacterial, fungal and other parasitic infections of the dermis and epidermis.

Although they have not been investigated in great detail in the inflamed periodontium, free radicals may have the potential to play a role in matrix destruction in the inflamed periodontium. For example, oxygen-derived free radicals may depolymerize gingival proteoglycans and hyaluronan,[15] active neutrophil collagenase, and thus initiate matrix degradation.[16]

It has been hypothesized that oral mucosal melanin provides a defense barrier by acting as a binder of toxic products such as free radicals and polycyclic compounds.[4] Therefore, the oral melanocytes may act as scavenging antioxidants and prevent oxidative stress by literally scavenging radicals as they form. This raises a question of whether oral mucosal melanocytes have a role in protection of the oral mucosa from noxious substances potentially involved in oral mucosal tumors/ lesions. It remains to be seen whether the role of melanin in scavenging free radicals in oral mucosa is limited by the apparent metabolic inactivity of the oral mucosal melanocytes, or whether oral mucosal melanocytes adapt to the environmental changes as they occur.[3]

Variations in the pigmentation of oral tissues recently received attention when the first symposium on oral pigmentation was held in Tukegee, Alabama.[17] It is now well-established that pigmentation normally occurs in the oral mucous membrane of many ethnic groups, although the relevant literature is not extensive. The studies indicate that pigmentation of the oral tissues more frequently and in more intense patterns within the darker ethnic groups but are by no means confined to these people.

Although little is known of the factors involved in the melanin formation and deposition in oral mucosa, it is reasonable to assume that these processes are essentially similar to those operating in other tissues. Therefore, until additional information is available, the results of the present study should be interpreted with reference to current views on melanogenesis in epithelial tissues generally.

The distribution of oral melanin pigmentation is 61%, in the hard palate; gingiva, 60%; mucous membrane, 22%; and tongue, 15%; the gingival pigmentation occurs as a diffuse, deep purplish discoloration or as irregularly shaped brown and light brown patches in the gingiva as early as 3 hours after birth, and often is the only evidence of pigmentation.[18] Considering the distribution of oral melanin pigmentation, the gingival pigmentation provides a suitable platform to study the possible defensive role of melanins in the oral

Table 1: General information of patients in the four groups

	Group I	Group II	Group III	Group IV
No. of subjects	20	20	20	20
Mean age ±S.D.	22.0 ± 4.9	23.8 ± 7.2	19.6 ± 3.4	20.6 ± 4.6
Range	18--38	15-38	15-26	16-32
Male	7	14	13	11
Female	13	6	7	9

Table 2: Relationship between clinical parameters and IL-1 β

Correlation between	Group I *r*-value	Group I *P* value	Group II r-value	Group II *P* value	Group III r-value	Group III *P* value	Group IV r-value	Group IV *P* value
PI and 1L-1 β	0.40	0.08	0.07	0.78	-0.25	0.29	-0.25	0.29
GI and 1L-1 β	0.31	0.18	0.06	0.77	0.16	0.48	-0.03	0.91
BI and 1L-1 β	0.30	0.19	0.07	0.78	0.17	0.47	-0.04	0.88

r = Pearson's correlation coefficient; *P*>0.05 not significant; BI - Bleeding index; GI - Gingival index; PI - Plaque index.

environment. The criteria of Dummett score 0 (Pink tissue-no clinical pigmentation) suited the clinical assessment of non-pigmented and the score 3 (Deep brown or blue/black tissue -heavy clinical pigmentation) suited the selection of pigmented gingiva for the present study.

General agreement on many questions was reached, but the conflicting statements concerning several basic points, and insufficient data concerning others were found in recent literature when we became interested in the mechanism of protective role of melanin during inflammation. When the question was being investigated, it also became necessary to concern us with the not so often debated problem of the relation of the melanins with the exact role in inflammation. Based on the lack of information regarding scavenging role of oral mucosal melanins, this study was initiated to clinically and biochemically elucidate the defensive role of melanin (in terms of gingival pigmentation) to gingival inflammation.

The objective of the study was to evaluate and compare the clinical markers of gingival inflammation in the four groups using plaque index, gingival index and bleeding index. The present study is based on clinical observations of gingival inflammation and estimation of biochemical marker IL-1β using ELISA.

The comparison of plaque index, gingival index and bleeding index between non-pigmented healthy and gingivitis groups was significant. Gingival inflammation has generally been described clinically and histologically as a relatively homogenous response characteristic of acute inflammation with a primary neutrophil infiltrate. Experimental gingivitis studies have ascribed this host response directly to the accumulation of plaque mass supragingivally and at the gingival margin.[19] The plaque index is one of the parameters of the existing oral hygiene and a low plaque index suggests good oral hygiene, but is inconclusive of the extent of inflammation.[20]

The IL-1β levels in our study were in the range of 0.48-137.04 ng/ml (34.16±46.65) and 1.23-246.60 ng/ml (65.78± 64.6) between non-pigmented healthy and gingivitis groups and did not differ significantly.

A study in the literature has shown the IL-1 activity in GCF obtained from inflamed gingiva to be higher than that from healthy gingiva.[21] Stashenko *et al*.[22] reported that the mean levels of IL-1β was higher at sites not exhibiting plaque and redness, suggesting dissociation between the production of this cytokine and marginal gingivitis and that the IL-1β levels were inversely related to supragingival plaque or redness. These findings indicate that IL-1β production does not simply reflect the presence of gingival inflammation and are consistent with, the previously reported divergence between clinical signs of inflammation and disease activity.[23] In the latter study, plaque, redness, bleeding and suppuration were present in only a minority of active sites. This could possibly be due to, supragingival micro-organisms, which are predominantly Gram positive[24,25] and may not effectively stimulate IL-1β production, in contrast to Gram negative periodontopathogens located subgingivally which possess LPS.[26] In addition, certain mechanisms associated with inflammation, e.g., the synthesis of prostaglandins that can down-regulate IL-1β production,[27] could also account for this observation.[12,22]

IL-1 was originally considered to be a product of mononuclear phagocytes, recent evidence suggests that keratinocytes and gingival fibroblasts can also produce IL-1 in response to stimulation by bacterial products.[28,29] The accumulation of dental plaque might provide the appropriate trigger for gingival cells or monocyte/macrophages to produce IL-1, which would then propagate an inflammatory response.[30] The amount of IL-1β in GCF depends upon the site sampled. Most of the sites tested had varying but measurable levels of IL-1, while at other sites of the same patient were not detectable. This lack of uniform expression of IL-1 argues against a systemic origin of this cytokine (i.e., serum derived). More likely, production and release of IL-1 occurs locally in response to the prevailing disease activity at each site.[31,32] Studies have reported the IL-lβ level to be 7.4±1.5 ng/ml in the healthy sites,[22] in another study the IL-lβ concentration in the healthy control group ranged from 22.8 to 150 ng/ml.[33] IL-1β levels in experimental gingivitis study were in the range of 229.25--526.13 ng/µl.[34] The level has also been reported to be 139± 26.90 ng/µl. in gingivitis sites.[35] A wide spectrum of concentrations and overlapping values between healthy and gingivitis of IL--1 was observed in this study.

The various reasons for the wide range and overlapping values of IL-1β could be due to variation in individual immunological response.[33] Stress of any kind has also been shown influence the Il-1β levels.[36] Influences of factors on IL-1β levels such as genetic polymorphism, various source of synthesizing cells include PMN within tissues and in the crevicular fluid, the keratinocytes of the junctional and sulcular epithelium and macrophages and fibroblasts in the connective tissue,[37] intrinsic factors (like stress, antigen antibody complex), synergistic factors (other cytokines), antagonistic agents (PGE_2, IL-Ira), extrinsic factor (like LPS from Gram- negative bacteria).[33] Further studies should be directed to include combination of various factors influencing IL-1β production to better understand the cytokine levels. Based on these reports, the measurement of IL-1β level, as an inflammatory marker is questionable unlike enzyme levels that are dependable to define healthy and diseased states.

On comparison between the pigmented healthy and gingivitis groups, the plaque index was significant (P<0.006). The bleeding index and gingival index were highly significant (P<0.001). The IL-1β levels were not significant. The intergroup comparison of the plaque index, gingival

index and bleeding index between non-pigmented healthy and gingivitis versus pigmented healthy and gingivitis groups revealed no significant difference.

Similarly the IL-lβ levels were not significant [Table 2]. The wide range and overlapping values of IL-lβ in different groups did not signify healthy or gingivitis states of the subjects in the present study. Unfortunately, a lacuna exists in the literature to cite comparison between non-pigmented and pigmented gingiva.

This study attempted to correlate the IL-lβ levels with various clinical parameters. The Pearson's correlation coefficient comparing the clinical parameters and IL-1β showed a positive correlation, but did not reach statistical significance for the plaque index and IL-1β in non-pigmented healthy and gingivitis groups . Studies in the literature, have also reported "no significant correlation" between the clinical parameters and IL-1β.[34,38] It is suggested that the plaque accumulation per se is not the major determining factor in gingival IL-1 production. In an experimental gingivitis study, the increase in IL-1 concentration in the crevicular fluid was found prior to an observable inflammation of the gingiva, but closely followed the plaque index. Whereas the plaque and inflammation indices continued to increase while oral hygiene remained withdrawn, the concentration of IL-1 declined after the seventh day. This early decline was attributed to an abnormal regulation of IL-1 production, or due to inhibition or degradation by host or bacterial factors.[39] Nonspecific inhibition of IL-1 bioactivity might result from proteolytic digestion by phagocyte and microbial factors present in crevice fluid.[40] In the gingival sulcus IL-1 might also be affected by an inhibitor produced by human gingival epithelial cells.[41]

A positive correlation, (though not significant) was demonstrated between gingival and bleeding indices to IL-1β. One study reported that in the non-inflamed gingival crevices of the healthy subjects (0.5 ±2 μl of the gingival crevicular fluids), IL-lα, IL-lβ or IL-1ra was not detected. There was a good relationship between the relative amount of IL-1α and IL-1β in GCF and the severity of disease, confirming the concept that the levels of IL-1 in GCF are symptomatically valuable in detecting the inflammation of periodontal tissues and its extents.[42] A study, which compared clinically non-inflamed with diseased sites of 11 untreated patients observed that the amount of IL-1β increased almost three times when sites with GI=2 were compared with sites showing a GI=2.[43]

With the exception of the plaque index; the gingival index and bleeding index in pigmented healthy group was positively correlated to the IL-1β levels. Pigmented gingivitis group showed a negative correlation of plaque index, gingival index and bleeding index to IL-1β levels (though not statistically significant).

The negative correlation of inflammatory markers of clinical inflammation with the IL-1β in pigmented gingiva is thought provoking.

In lieu of dearth of studies seeking a possible explanation for decreased inflammation in subjects with clinically pigmented gingiva, we came up with a question as to what if melanins could serve as a host tissue modifier, thus providing defensive role against ROS-induced inflammation. Hence we postulated that subjects with clinically pigmented gingiva could have lesser degrees of gingival inflammation and bleeding index and decreased levels of GCF IL-1β. With this logic in mind, we designed our study to measure gingival index and bleeding index to GCF IL-1β levels in clinically pigmented and non-pigmented gingiva with and without gingivitis. The non-pigmented gingivitis group showed a positive correlation to the similar plaque levels. The inverse relationship between the biochemical marker and the inflammatory parameters in the pigmented gingivitis group when compared to the non-pigmented gingivitis to same plaque levels forces us to speculate that the presence of pigmentation might have a role in reducing the clinical expression of inflammation.

The confounding problem of this study is that the inflammation of the gingival corium is bordered by the sulcular epithelium on the inner aspect, which is poorly melanized[8] as compared to highly melanized outer epithelium. The clinical gingival inflammation is assessed on the outer epithelium for the visual color change and the bleeding index is elicited from the sulcular epithelium. Hence the net role of melanin in the gingival environment is "questionable". The scoring of gingival index was mainly dependent on bleeding tendency after probing the sulcular area. The anatomical distribution of melanin pigmentation and problems in assessment of clinical markers of gingival inflammation implies that evaluation of defensive role melanins by using gingival index and bleeding index is questionable. An answer to this problem can possibly be derived by ultramicroscopy using histochemical techniques.

The smaller sample size is a drawback to confirm the hypothesis of this study using only clinical markers and IL-1β levels. Further studies directed with larger sample size, including clinical, histological evaluation for melanin scavenging/antioxidant action and biochemical evaluation is mandatory. For all we know, the unraveling of the antioxidant effect of the gingiva would be the genesis of a new outlook to the unanesthetic-pigmented gingiva, which may be welcomed with a smile.

CONCLUSIONS

Pigmented gingivitis group (Group IV) showed a negative correlation of clinical markers of inflammation to the IL-1β levels. The pigmented gingiva demonstrated lower gingival and bleeding scores to the similar plaque levels unlike

the non-pigmented gingiva. However, the IL-1β levels were of wide range that was overlapping in healthy and gingivitis cases, questioning its quantification as a definite inflammatory marker.

Finally, it should be considered such that this data of clinical findings may represent a previously unrecognized or unreported phenomenon. Only time and continuing observation will help answer the possibility of the present study hypothesis.

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