Original Article

Erosive oral lichen planus inflicts higher cellular stress than reticular type

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Abstract

Background: Lichen planus is a chronic inflammatory mucocutaneous disease which frequently involves the oral mucosa. The most common types of oral lichen planus (OLP) are reticular (asymptomatic) and erosive (ulcerative) with malignant potentiality. The aims of the present study are to assess the cellular stress level in both types of OLP lesions with respect to oxidative stress, DNA damage and inflammation.

Materials and Methods: Freshly diagnosed untreated 25 OLP reticular type and 25 OLP erosive (OLP-E) type patients aged 35–55 years were enrolled in the study along with age and sex-matched 25 healthy subjects as control. Tissue antioxidant enzymes were measured biochemically, single-cell DNA damage was measured by comet assay and the molecular markers for inflammation were assessed by using semi-quantitative reverse transcriptase–polymerase chain reaction. Statistical analyses were performed using one-way ANOVA and Tukey's *post hoc* test.

Results: Oxidative stress was significantly greater in OLP-E type compared to the reticular. mRNA expression of cyclooxygenase-2 was significantly elevated ($P < 0.0001$) in erosive form, but such expression of nuclear factor kappa beta, tumor necrosis factor-alpha, Interleukin-6 and inducible nitric oxide synthase did not significantly differ between the two disease groups. Comet assay revealed a higher degree of DNA strand breakage in erosive lesions.

Conclusions: The unhandled free radicals may imbalance the homeostatic network toward pro-inflammatory, DNA damaging responses, creating a vicious cell-damaging spree resulting in stress. Molecular analyses showed that erosive lichen planus is more under stress than the reticular form.

Keywords: DNA damage, inflammation, oral lichen planus, oxidative stress

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INTRODUCTION

Oral lichen planus (OLP) is a mucocutaneous, chronic inflammatory disease of unknown etiology. The disease is characterized by T-cell-mediated autoimmune response and altered epithelial keratinization cycle.[1] OLP lesions are mostly painful at the central red area with erosions and ulcerations, whereas some are less painful/painless with radiating white striae (Wickham striae) form having papular or reticular patches.[2] The lesions are commonly located on the posterior buccal mucosa, although it may found on lips, tongue and gingiva.[3] Clinically, OLP may also be classified into reticular, papular, atrophic and erosive lesions.[4] The reticular is the

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most common painless lesion and erosive form is the second most common painful lesion with malignant potentiality in the general population.[5] The pathogenesis of OLP has not yet been delineated comprehensively.

Different antigen-specific and nonspecific inflammatory mechanisms have been put forward to clarify the pathogenesis process.[6] Chronic stimulation from the inflammatory and stromal cells of OLP lesions can alter the growth of epithelial cells through oxidative and nitrative products, provoking DNA damage resulting in neoplastic changes.[7,8] Many such hypotheses regarding the pathogenesis of OLP have been proposed, but the mechanism is yet to be understood. Nevertheless, sparse studies have delved into the comprehensive molecular differences in clinically differentiated OLP of reticular (OLP-R) type and OLP erosive (OLP-E) type, with respect to oxidative stress, DNA damage, inflammation, and thus the propensity of cellular stress incurred.

Thus, the aim of the present study is to uniquely differentiate the level of oxidative stress, DNA damage and inflammatory modification between the OLP-R and OLP-E at the oral tissue level to understand the pathogenesis mechanism of different types of OLP lesions.

MATERIALS AND METHODS

Subjects

To ensure proper diagnosis of OLP, key histopathological features from biopsy specimen and clinical characteristics were correlated.[9] A total of 25 untreated OLP-R and 25 OLP-E patients aged 35–50 years were enrolled for oxidative stress and inflammatory study, along with 25 age and sex-matched healthy individuals. Punch biopsy was performed to collect the patient tissue sample from buccal mucosa, gingiva and tongue areas, from the Department of Oral Medicine and Radiology, PMS College of Dental Science and Research, Kerala. Normal healthy samples were the discarded tissues from surgical treatments of impacted tooth where the normal mucosal tissue margin was trimmed (excised) and discarded to facilitate primary closure. This was collected from the aforesaid institute. This study was conducted and approved by its Institutional Ethical Committee. Written consent was obtained from the OLP patients and healthy control subjects who were included in this study. Institutional Ethical Committee No. PMS/IEC/12/24.

Inclusion criteria

Biopsy samples were obtained from the patients initially diagnosed with OLP lesions and not undergone through any treatment before for the same.

Exclusion criteria

OLP patients and normal individuals with, oral epithelial dysplasia, oral lichenoid lesions, asthma, hypertension, diabetes, cardiac disorder, bleeding or clotting disorders, psychiatric illness, hepatitis, acquired immunodeficiency syndrome and malignancy, were not included in this study. Those with habits of tobacco chewing, smoking and alcohol consumption were eliminated from this study.

Measurement of oxidative stress

Tissue biopsy samples rinsed with phosphate buffer saline (PBS) for the removal of red blood cells and clots were later homogenized separately using a Potter-Elvehjem tissue homogenizer. One part of the tissue sample was homogenized in 50 mM PBS, pH 7.4, for estimation of glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD) enzyme activities, reduced glutathione (GSH) and nitrite level. The other part of the tissue was homogenized in 10 mM potassium phosphate buffer, pH 7.4, for estimation of lipid peroxidation product malondialdehyde (MDA), glutathione reductase (GR) and glutathione peroxidase (GPx) activity. Total protein content in tissue homogenate was measured.[10] Biochemical estimation of GSH level,[11] GST,[12] GR,[13] GPx,[14] SOD,[15] CAT activity,[16] MDA[17] and tissue nitrite level[18] was measured spectrophotometrically.

Single-cell gel electrophoresis or comet assay

Brush biopsy technique was used to collect epithelial cells from the buccal mucosa and gingiva.[19] Cells adhered in brush were collected by agitating in 5.0 ml of Dulbecco's PBS in a 15-ml centrifuge tube and centrifuged at 270 g for 5 min at 4°C.

Single-cell suspension was prepared by tissue mincing and digesting with proteolytic enzymes for 10 h.[20] Isolated epithelial cells' suspensions were mixed properly into 10 µl of low melting point agarose (LMA) and placed on a layer of normal melting point agarose (NMA) on a clean glass slide. NMA- and LMA-coated slides were placed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris at pH 10 and 1% SDS + 10% DMSO + 1% Triton × 100) for 1 h. The slides were placed in electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH 13) and set aside for 20 min to allow unwinding of DNA. The comet assay results were observed using a fluorescent microscope (Olympus CKX 41) and the DNA damage was measured quantitatively using Tritek Comet Scoring software.[21]

Semi-quantitative reverse transcriptase–polymerase chain reaction

mRNA expression of inflammation-associated genes, nuclear factor kappa beta (NF-κβ), tumor necrosis factor-alpha (TNF-α), interlukin-6 (IL6), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were evaluated by two-step semi-quantitative reverse transcriptase–polymerase chain reaction (RTPCR). The total RNA was isolated from the oral tissue sample using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. RNA strand was first reverse transcribed into its DNA complement (cDNA) using the enzyme reverse transcriptase and the resulting cDNA was amplified using RT-PCR. cDNA was synthesized according to the kit supplied by ThermoScript™, Invitrogen, USA. The synthesized cDNA was amplified using a Platinum Taq DNA polymerase kit (Invitrogen, USA). The stained gel was observed using E-gel imager system (Invitrogen, Life Technology, USA) Table 1.

Statistical analysis

The results were expressed as mean ± standard deviation and the statistical analyses were performed using one-way ANOVA and Tukey's *post hoc* test by SPSS software version 22.0 (SPSS Inc, Chicago, IL, USA). Differences were considered to be significant at $P < 0.05$.

Table 1: Gene-specific forward and reverse primer sequences of associated genes

Gene	Primer sequences
NF-κβ	Forward 5'-CCCACACTATGGATTTCCTACTTATGG-3'
	Reverse 5'-CCAGCAGCATCTTCACGTCTC-3'
TNF-α	Forward 5'-CCCAGGCAGTCAGATCATCTTC-3'
	Reverse 5'-AGCTGCCCCTCAGCTTGA-3'
IL6	Forward 5'-GGTACATCCTCGACGGCATCT-3'
	Reverse 5'-GAGGATACCACTCCCAACAGACC-3'
COX-2	Forward 5'-GGAGAGACTATCAAGATAGT-3'
	Reverse 5'-ATGGTCAGTAGACTTTTACA-3'
iNOS	Forward 5'-AATGGCAACATCAGGTCGGCCATCACT-3'
	Reverse 5'- CTGTGTGTGTCACAGAAGTCTCGAACTC-3'
GAPDH	Forward 5'-GAAGGTGAAGGTCGGAGTC-3'
	Reverse 5'-GAAGATGGTGATGGGATTTC-3'

NF-κβ: Nuclear factor kappa beta, TNF-□: Tumor necrosis factor-alpha, IL-6: Interlukin-6, COX: Cyclooxygenase-2, iNOS: Inducible nitric oxide synthase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

RESULTS

The collected tissue was analyzed for the levels of oxidative stress, inflammation, DNA damage and the difference among OLP-E, OLP-R and healthy subjects was compared for pathogenicity [Table 2].

The oxidative stress-resisting enzymes were assayed along with such stress markers. Tissue GSH, GR, GPx, GST, MDA, SOD and CAT levels were assayed, namely healthy control, OLP-R and OLP-E subjects. Reduction of oxidative stress by antioxidant enzymes is a chronic method of eradicating the cumulative accumulation of toxic radicals in the physiological system. One-way ANOVA shows that tissue GSH levels ($P < 0.0001$), GR ($P < 0.0001$), GPx ($P < 0.0001$), GST ($P < 0.0001$), SOD ($P < 0.0001$), CAT ($P < 0.0001$) activity, MDA ($P < 0.0001$) and nitrite level ($P < 0.0001$) were significantly differ among the study groups.

GSH,[22] MDA[23] and nitrite[24] levels were found to be in cohesion with our studies, in OLP patients. Salivary,[8] serum[25] and plasma[22] MDA levels were high in OLP patients.

Comparison by Tukey's *post hoc* test revealed that tissue GSH level ($P < 0.03$), GR ($P < 0.02$), GPx ($P < 0.01$) and GST ($P < 0.02$) activity significantly reduced in erosive lesions compared to reticular and control, whereas SOD ($P < 0.0001$) and CAT ($P < 0.02$) activities were significantly higher in the erosive/ulcerative lesion compared to the reticular and control. MDA ($P < 0.001$) and tissue nitrite ($P < 0.0009$) levels in erosive group were significantly elevated when compared with the reticular. Interestingly, the SOD and CAT activities were not significantly differed between the control and reticular

Table 2: Oxidative stress and antioxidant enzyme activity in asymptomatic (reticular) and symptomatic (erosive) oral lichen planus lesions compared to samples from control subjects

Oxidative stress marker	Mean±SD		
	Control	OLP-R	OLP-E
Reduced GSH (μmol/mg of tissue)	5.78±1.4	2.69±0.8	0.81±0.68
GR (nmol NADPH oxidised/min/mg protein)	8.81±1.02	3.66±1.03	1.94±0.61
GPX (nmol NADPH oxidized/min/mg protein)	4.27±1.11	2.25±0.80	0.50±0.15
GST (nmol/min/mg of protein)	9.58±2.11	5.32±1.05	2.38±1.04
SOD (Unit/mg protein)	0.44±0.24	1.07±0.36	2.25±0.68
CAT (μmol/min/mg of protein)	35.74±5.42	60.26±7.55	76.06±10
Lipid peroxidation (MDA) (nmol/mg protein)	1.12±0.36	3.07±0.68	6.35±1.72
Nitrite level (nmol/mg of tissue)	410±52.34	524.2.4±49.1	666.4±34.76

OLP-R: Oral lichen planus-reticular, OLP-E: Oral lichen planus-erosive, GR: Glutathione reductase, GPX: Glutathione peroxidase, GST: Glutathione-s-transferase, SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, MDA: Malondialdehyde, SD: Standard deviation, NADPH: Nicotinamide adenine dinucleotide phosphate

groups. The present result implies that the activity of antioxidant defense system has reduced in response to chronic oxidative stress Figure 1.

Cellular stress is related to DNA damage which if detectable may be quantified by single-cell gel electrophoresis or comet assay. The intensity of the comet tail relative to the head reflects the number of DNA strand breaks and thus the intensity of DNA damage.[26] Comet length (μm) in control group, OLP-R and OLP-E groups was 56.4 ± 10.7, 88 ± 7.2, 113 ± 6.1 and tail length (μm) was 0.09 ± 0.05, 1.95 ± 0.59 and 5.11 ± 1.51, respectively. Statistically, comet length and tail length significantly ($P < 0.0001$) differ between control, reticular and erosive OLP groups. The comet length ($P < 0.001$) and tail length ($P < 0.0004$) in OLP-E were significantly higher compared to that of OLP-R patients Figure 2.

Stress is contributory to inflammation and *vice versa*. The general stress markers were analyzed to observe the potency of the OLP forms. mRNA expression of NF-κβ, TNF-α, IL6, COX-2 and iNOS significantly ($P < 0.0001$) differed among the study groups. Significantly elevated mRNA expression of NF-κβ, TNF-α, IL6, COX-2 and iNOS was observed in both types of OLP patients when compared with control. Immunohistochemistry study[27] has earlier shown high COX-2 mRNA expression in OLP lesions and lichenoid reactions. Although corroborated similar findings with iNOS,[28] contradictory findings have also been recorded.[29] We have not found any significant difference in expression of NF-κβ, TNF-α, IL6 and iNOS between OLP groups, whereas significant elevated expression of COX-2 ($P < 0.0001$) was observed in OLP-E compared to OLP-R.

DISCUSSION

The detail pathogenesis mechanism of chronic inflammatory disease like OLP is not well understood. OLP is a mouth lesion affecting systemic homeostasis. Any comprehensive mechanism of it is rarely available.[24] Different forms of stress may be caused by upsurge of molecules and hematological parameters[30] from deviated pathways. This may be assessed from its level inflammation, oxidative stress and finally DNA damage. Firing of pro-inflammatory signaling pathways initiates changes in the human system inducing a gradual shift in homoeostatic set point.[31] The stress inducers and pro-oxidants compel the reticular and erosive forms of the disease.

Oxidative stress induced by overproduction of reactive oxygen species (ROS) is encountered by a variety of enzymes, of which GSH constitutes more than 90% of the total glutathione pool. It is capable of preventing

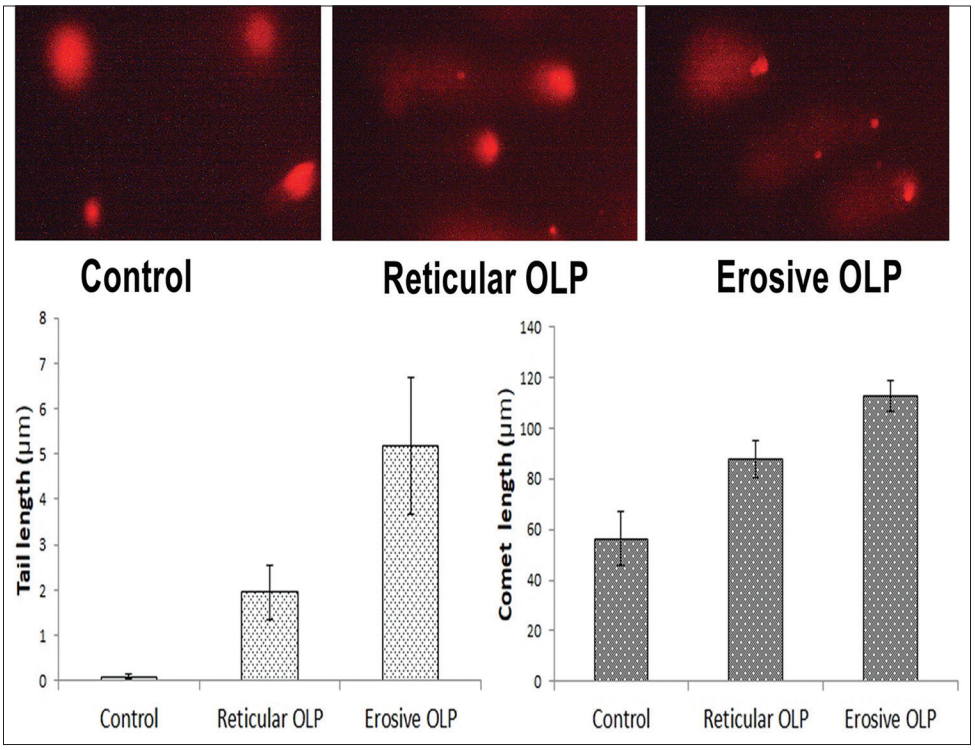

Figure 1: Comet assay image shows DNA damage pattern of oral lichen planus lesions and healthy individuals. Comet length (μm) and tail length in OLP reticular and OLP erosive patients have been compared with healthy individuals; the standard deviation is shown for each bar graph. (C: Control)

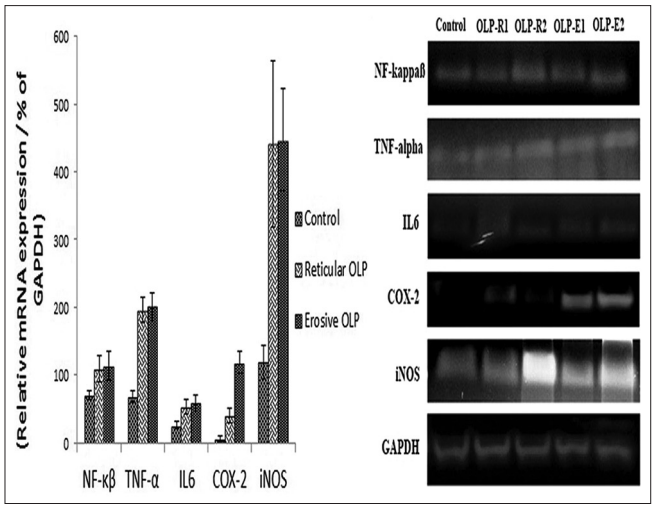

Figure 2: Reverse transcriptase–polymerase chain reaction results shows mRNA expression of nuclear factor kappa beta, tumor necrosis factor-alpha, interlukin-6, cyclooxygenase-2 and inducible nitric oxide synthase in control, OLP reticular and OLP erosive, the standard deviation is shown for each bar diagram. (C: Control)

damage to cellular components caused by ROS and its level is reduced in commonly observed severe diseases. Various studies showed that oxidative stress is involved in the pathogenesis of OLP.[23,24]

The endogenous enzymatic antioxidants are represented by glutathione system, SOD and CATs. Although it is critical for the cells to maintain high levels of GSH, GR, GPx and GST in their response to fight stress, they show depletion in their activity as shown in the present study (in OLP-E compared to OLP-R and control). Reduced GST might indicate reduced detoxification ability in OLP-E compared to OLP-R possibly due to over usage and exhaustion. The glutathione enzyme system also consists of GR and GPx which are critical in resisting oxidative stress through oxidative burst, thus detoxifying compounds and peroxides.[32] SOD is an antioxidant enzyme that accelerates the dismutation of toxic superoxide radicals produced during the oxidative processes, into hydrogen peroxide and molecular oxygen.[33] CAT is an antioxidant enzyme which breaks down H_2O_2 to yield oxygen and water.[34] In the present study, SOD and CAT activity increased significantly in erosive lesions when compared with reticular. Overall, these indicate that higher oxidative stress is encountered in OLP-E than in OLP-R.

iNOS enzyme is expressed in response to cytokines, enabling enhanced NO production, which may be beneficial in the modulation of immune response[35] but is a highly reactive free radical.[36] In inflamed tissues, it might potentiate malignant transformation through the ability of NO to promote mutagenicity through DNA oxidization and protein nitrosylation.[37] iNOS can thus activate this inflammatory cytokine which has damages cellular proteins, DNA and lipids eventually leading to cell death. Higher serum levels of NO in OLP patients might activate cellular immunity, thus implicating NO in pathogenesis of the disease.[38,39] The tissue nitrite level in OLP-E surpassed that in OLP-R; its metabolism is oxygen dependent and under conditions of oxygen deficiency is converted to stressor nitric oxide.

Oxidative damage of membrane phospholipids causes lipid peroxidation with MDA as an end-product[40] which is also more elevated in OLP-E.

Apoptosis triggers cell death on encountering rare survival hope despite corrective action of revival machinery. Considerable DNA damage is observed in leukoplakia and squamous cell carcinoma blood samples.[26] The present study indicates greater comet length and tail in OLP-E, indicating a higher degree of apoptosis in OLP-E type of the disease. Despite the severity of DNA damage, it gives the redundant cells an opportunity to die in anticipation of a flawless newer cell imparting disease-free life to the body. These indicate that the consequences in OLP-E are more severe than in the OLP-R type.

The human system is exposed to multiple xenobiotics which is evident from high level of expression of P-glycoprotein, an efflux transporter[41] in the inflicted tissues. The broad substrate specificity of GSTs allows them to protect cells against a wide range of toxic chemicals.[42] Inflammation is a protective mechanism employed by tissues against all onslaughts. Inflammatory cells produce soluble mediators, cytokines and chemokines, which further recruit such cells to the site of damage and produce more ROS. These mediators also activate signal transduction cascades and induce changes in transcription factors, such as NF-κβ which mediate immediate cellular stress responses[43] through overexpression of IL6, TNF-alpha, iNOS and COX-2.[44,45] Increased expression of TNF-α and IL6 was observed in OLP-E.[46,47] Though the present study could not reveal significantly different levels of NF-κβ, TNF-α, IL6 or iNOS between OLP-R and OLP-E, the expression of COX-2 increased significantly in erosive lesion compared to the OLP-R type.

Higher expression of COX2, at both mRNA and protein levels, enhanced the synthesis of prostaglandins, stimulate proliferation of neoplastic cells, angiogenesis and suppress immune surveillance. Therefore, elevated expression of COX2 may be an important predictor for the clinical outcome in oral cancer.[48] Thus, a wide variety of chronic inflammatory conditions predispose susceptible cells to

stress. Chronic inflammation often leads to cancer.[49] The pathways from homeostasis to stress may be due to predisposition to chronic high levels of psychological, environmental or xenobiotic-induced stressors. These sequences of events gradually shift from health to stress to disease, culminating in OLP. In this ruptured homeostatic condition, the cells appear to have lost their ability to regain normal set points and thus settle for perpetually new created set point for survival in the stressful condition.

Nevertheless, the increased expression of some of the stress molecules in OLP-E compared to that in OLP-R indicates its acute propensity toward a more severe condition. The homeostatic elastic balance in OLP-E exists at the verge of rupture while that of OLP-R lingers in the edge of the elastic zone.

CONCLUSIONS

So far, sparsely any molecular data has revealed the differential aspects between OLP-E and OLP-R. The OLP-R type has been shown to possess less intensity with respect to pro-inflammatory, apoptotic, oxidative stress-inducing level of expression or activity of the chemokines, compared to the erosive form. We have uniquely assessed the graded degree of severity of OLP from the reticular to the OLP-E type and have aggregately quantified, the level of molecular factors derived from the affected tissue samples. Despite that, it may be inferred that the apparent severity of the disease is determined by changes in interplay of cellular factors which is further determined by the critical level of some components of the biologically active molecular network responsible for the deviation in homeostasis. Mere genetic expression of some proteins does not determine the fate of the cell but their interaction and the specific pathway in action play a vital role in OLP pathogenesis. Thus, the present study shows that the OLP-R type of OLP is less intense with respect to the level of oxidative stress, inflammation and DNA damage, compared to the erosive form. The onslaught of multiple ROS instigates oxidative stress and pro-inflammatory condition, along with DNA damage, which further stimulate apoptosis and cell death signaling pathways compelling a vicious damaging process.

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Conflicts of interest
There are no conflicts of interest.

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