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Profiling of Pro-Inflammatory Cytokines in Radiation Induced Oral Mucositis (RIOM) among Indian patients

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Abstract

Radiation-induced oral mucositis (RIOM) is aimed at evaluating the expression of NF-κβ, IL-1α, IL-6, IL-8 and TNF-α in patients with RIOM so as to validate their role in the pathobiology of the disease. Blood samples were collected and serum of 45 patients isolated with clinical signs and symptoms of mucositis and 10 healthy controls were also included in the study. The expression level of NF-κβ, IL-1α, IL-6, IL-8, TNF-α was investigated using ELISA. Mann Whitney U test was applied to find the significance of the expression of these markers in RIOM patients as compared to normal healthy controls and significant expression ($P < 0.05$) for NF-κβ, IL-6, TNF-α and non-significant expression ($P > 0.05$) IL-1α and IL-8 was found. No significant change in the expression level of the cytokines was observed for patients undergoing chemotherapy and radiation therapy as well as those receiving only the radiation therapy as a part of their treatment. We have also found less expression in grade 1 of mucositis as compared to grade 4. Pro- inflammatory cytokines indeed play a vital role in the pathogenesis as well as progression of RIOM.

Keywords: Mucositis, NF-κβ, Proinflammatory cytokines, Radiation therapy

Introduction

Incidence of various cancer types have been reported to increase multifold in recent times[1,2] with oral mucositis being one of the most recurrent, symptomatic, and troubling complication of the conventional cancer treatments, viz., radiation and/or chemotherapy[3,4]. It can be defined as inflammatory and/or ulcerative lesions of the oral and/or gastrointestinal tract usually caused by cancer therapies[5]. It is mostly associated with pain and increased risk of infection thus leading to impaired nutritional status and inadequate hydration[3,6]. It may also be associated with increased risk for poorer outcome of cancer treatment due to the need for treatment interruption in some patient cohorts[6,7]. The incidence of oral mucositis can be close to 80% in patients receiving radiation therapy, with or without chemotherapy having head and neck cancer[8,9] with 25-45% of the patients reporting with grade 3 or 4 mucositis[10].

The process of mucositis occurs in five stages or phases: initiation, message generation, signal amplification, ulceration, and healing[11,12]. There are numerous transcription factors involved in the establishment of mucositis, one of the most important is nuclear factor-kappa β (NF-κβ). NF-κβ activation can upregulate the expression of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α),

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interleukin-1α (IL-1α), IL-6 and IL-8[13,14]. The increased levels of these cytokines induce inflammatory reactions in oral mucosa and promote the damage of the underlying connective tissues reduce epithelial oxygenation and ultimately result in epithelial basal cell death and injury[4,15]. Since TNF-α, IL-1α and IL-6 are efficient activators of NF-κβ and the repeated NF-κβ activation by them may amplify the mucosal damage in a vicious circle[4,13,14] which hence would ultimately lead to the development of mucositis as an after effect of radiation therapy.

The aim of this study is to analyze the cytokine expression of NF-κβ and its associated cytokines (IL-1α, IL-6, IL-8, TNF-α) in the serum of patients with radiation induced oral mucositis after undergoing radiation therapy for head and neck cancers so as to validate their role in the pathobiology of the disease.

Materials and Methods

Sample selection

45 patients (Group I) reported to Department of Radiation therapy, LNJP Hospital, Delhi with head & neck cancer undergoing radiation therapy with/without chemotherapy and showing clinical symptoms of oral mucositis were selected for the study. Around 2 mL blood was collected and centrifuged at 5000rpm. Their serum was separated and stored at -80°C until further use. Serum samples from 10 healthy volunteers with no history of cancer or previous radiation therapy served as the control group (Group II). Approval from institutional ethical committee & prior informed consent was taken from all the patients.

Patient Characteristics

In group I 33 male and 12 female with a mean age of 49.82 years were enrolled. Group II included 10 healthy controls (5 male and 5 female) with a mean age of 44.9 years with no prior history of undergoing any radiation treatment.

39 out of 45 patients developed symptoms of mucositis during /after the treatment therapy while about 6 patients showed no visible signs of any such complications (grade 0). 27 out of 39 patients had developed early signs of mucositis (grades 1 &2) with symptomatic redness and erythema, loss of taste, mouth dryness. 12 out of 39 patients had developed severe signs of mucositis (grades 3 & 4) during the course of their treatment, with painful contiguous pseudo membraneous lesions developed along with associated dysphagia and decreased oral intake. 26 out of 45 patients had undergone chemotherapy along with the radiation treatment, while 19 out of 45 patients undertook just the radiation therapy as a part of their cancer treatment.

Detection of serum Levels of NF-κβ, IL-1α, IL-6, IL-8 and TNF-α

The concentration of the studied biomarkers were assessed in the serum collected from patients and healthy subjects by ELISA by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Ray Biotech, Inc.). This assay employs the quantitative sandwich enzyme immunoassay technique performed using human recombinant standards according to the manufacturer's instructions. A monoclonal antibody specific for the respective cytokines was precoated onto the microplate. Standards and samples (100 µL each) were pipetted into the wells in duplicate and incubated for 2.5 hrs. so that any of the proteins, *i.e.*, NF-κβ, IL-1α, IL-6, IL-8 and TNF-α, if present in the sample can bind to the immobilized antibody. After washing away any unbound substances biotinylated antibody (100 µL) was added to the wells and incubated again for an hour. After washing 100 µL of HRP-streptavidin solution was added to each well followed by 45 min. incubation. Following a wash to remove any unbound antibody-enzyme reagent a substrate solution (100 µL) was added to the wells and incubated for 30 min. The color developed in proportion to the amount of protein bound to the immobilized antibody in the initial step. The yellow color development was stopped and the intensity of the blue color was measured by determining its absorbance at 450 nm using an ELISA plate reader (Bio-Rad). The concentration of proteins in the samples was calculated from the standard curve and the results were presented in picogram per milliliter (pg/mL) for IL-1α, IL-6, IL-8 and TNF-α and in nanogram per milliliter (ng/mL) for NF-κβ. The standard curves for NF-κβ, IL-1α, IL-6, IL-8 and TNF-α ranged from (0–500 ng/mL), (0–300 pg/mL), (0–1000 pg/mL), (0–1000 pg/mL) and (0–600 pg/mL) respectively.

Statistical Analysis

Data were analyzed with descriptive statistical methods (frequency percentage and mean ± standard deviation) and Mann–Whitney *U* test (a non-parametric method) using SPSS (statistical package for social sciences) software version 19.0 for windows. Statistical significance was defined at $P < 0.05$.

Results

Serum levels of NF-κβ and pro-inflammatory cytokines

Detectable levels of NF-κβ, IL-1α, IL-6, IL-8 and TNF-α proteins were present in serum samples obtained from the control group (group II). Serum levels of NF-κβ, IL-1α, IL-6, IL-8 and TNF-α were elevated in 100% (45/45), 60% (27/45), 100% (45/45), 53.34 % (24/45), 100% (45/45) of the patients respectively as compared to their concentration in the serum of healthy subjects. The mean values of NF-κβ (262.65 ± 169.91 ng/ml), IL-6 (112.56 ± 309.07 pg/ml) and TNF-α (372.28 ± 472.39 pg/ml) in serum of the patients were significantly ($p < 0.05$) higher in comparison with the values obtained from control subjects that were 25.66 ± 9.502 ng/ml, 4.880 ± 2.95 pg/ml and 12.00 ± 7.0590 pg/ml for NF-κβ, IL-6 and TNF-α, respectively, as revealed by Mann–Whitney *U* test[2] (Figure 1)

Mean values for IL-1α (38.919 ± 69.99 pg/ml) and IL-8 (53.38 ± 137.88 pg/ml) were also increased as compared to their values in the control group (IL-1α = 26.790 ± 10.055 pg/ml and IL-8 = 8.690 ± 2.534pg/ml) but statistically a non-significant difference was obtained between the two groups (Figure 1).

Table 1 - Clinical detail of patients.

S.No.	Sex	Age	Tumour Type	Radiation Therapy	Chemo-therapy	Mucositis	Grade
1	F	40	Tongue	Yes	No	No	0
2	M	30	Tongue & fossa	Yes	Yes	Yes	4
3	F	64	Larynx	Yes	Yes	Yes	1
4	M	60	Tonsil-R	Yes	Yes	Yes	1
5	F	55	Tonsil-L	Yes	Yes	Yes	1
6	M	26	Buccal mucosa	Yes	No	Yes	2
7	M	40	Gingivae	Yes	No	Yes	2
8	M	65	Base of tongue	Yes	Yes	Yes	4
9	M	50	Soft palate	Yes	No	Yes	3
10	M	48	Larynx	Yes	Yes	No	0
11	F	60	Base of tongue	Yes	Yes	Yes	3
12	M	35	Base of tongue	Yes	No	Yes	3
13	M	76	Base of tongue	Yes	No	No	0
14	F	45	Tongue	Yes	Yes	Yes	3
15	M	65	Body of tongue	Yes	Yes	Yes	1
16	M	40	Pharynx	Yes	Yes	Yes	1
17	M	68	Tonsil	Yes	Yes	Yes	1
18	M	32	Buccal mucosa & border of tongue	Yes	Yes	Yes	1
19	M	63	Soft palate	Yes	No	No	0
20	F	45	Larynx	Yes	Yes	Yes	1
21	M	28	Tongue	Yes	Yes	Yes	3
22	M	45	Nasopharynx	Yes	Yes	Yes	1
23	M	36	Buccoalveolar region-R	Yes	No	Yes	4
24	M	22	Buccal mucosa & border of tongue	Yes	Yes	Yes	1
25	M	40	Larynx	Yes	Yes	Yes	1
26	M	62	Larynx	Yes	Yes	Yes	1
27	F	50	Right tonsil	Yes	Yes	Yes	1
28	M	68	Left tonsil	Yes	No	No	0
29	M	70	Buccal mucosa-left	Yes	No	Yes	1
30	M	63	Buccal muco-sa-right	Yes	No	Yes	3
31	M	55	Base of tongue	Yes	Yes	Yes	1
32	M	54	Buccal mucosa-left	Yes	No	Yes	3
33	F	55	Recurrent adenoid cystic carcinoma with secondary metastasis	Yes	Yes	Yes	1
34	M	62	Base of tongue	Yes	No	No	0
35	F	48	Tonsil	Yes	No	Yes	1
36	M	27	Buccal mucosa	Yes	Yes	Yes	3
37	F	55	Tongue	Yes	Yes	Yes	1
38	M	71	Tongue	Yes	Yes	Yes	1
39	F	43	Larynx	Yes	No	Yes	1
40	F	45	Tonsil- left	Yes	No	Yes	2
41	M	42	Floor of mouth	Yes	Yes	Yes	2
42	M	65	Soft palate	Yes	No	Yes	2
43	M	40	Base of tongue	Yes	Yes	Yes	2
44	M	53	Larynx	Yes	No	Yes	2
45	M	36	Base of tongue	Yes	No	Yes	4

Mean values of Cytokine concentration in serum of mucositis patients and healthy controls.

Cytokines	Mean±S.D.		Median		Percentage of patients showing upregulation in mucositis group as compared to control group	P-value
	RIOM patients (Group I)	Control group (Group II)	RIOM patients (Group I)	Control group (Group II)		
NF-κβ (ng/ml)	262.65±169.91	25.66±9.502	240	28.00	100% (45/45)	.001 (S)
IL-1α(pg/ml)	38.919±69.99	26.7907±10.5	29	25.7	60% (27/45)	.115(NS)
IL-6 (pg/ml)	112.56±309.7	4.880±2.95	45.7	5.450	100%(45/45)	.001(S)
IL-8 (pg/ml)	53.38±137.88	8.690±2.354	9.8	9.050	53.34%(24/45)	.952(NS)
TNF-α (pg/ml)	372.28±472.39	12.00±7.0590	256	9.100	100% (45/45)	.001(S)

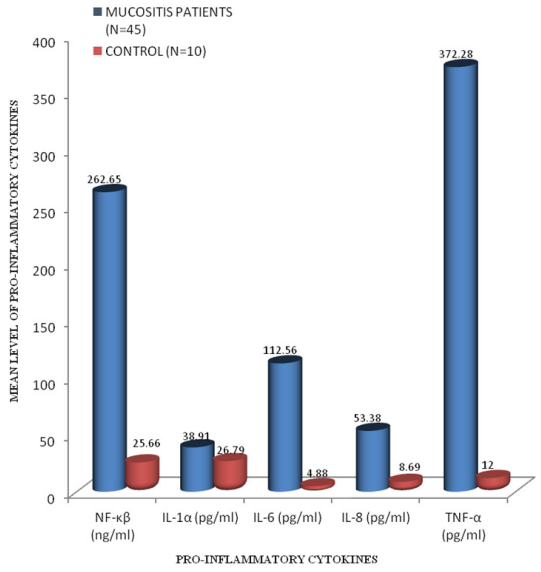

Fig. 1 - Mean serum concentrations of pro-inflammatory cytokines in control subjects and mucositis patients.

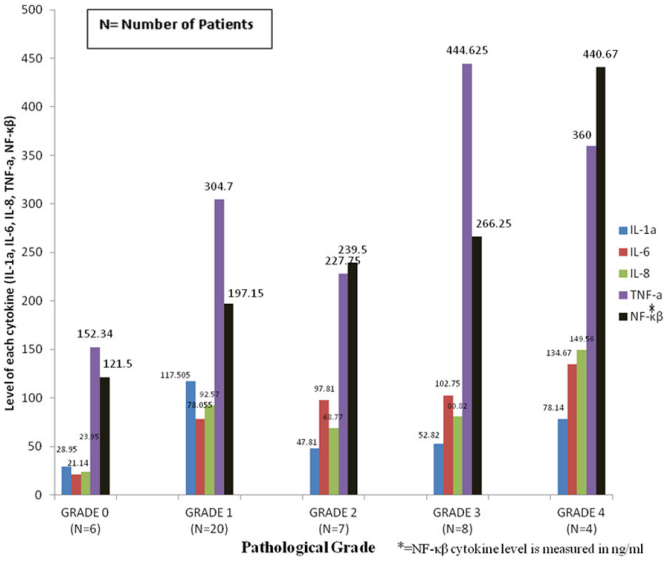

Fig. 3 - Comparison of expression of different cytokines-NF-κβ, IL-1α, IL-6, IL-8 and TNF-α in different grades of mucositis.

On comparing the expression level of different pro-inflammatory cytokines of the patients receiving chemotherapy along with the radiation therapy and those undergoing just the radiation therapy no significant difference ($p > 0.05$) was observed in the expression of these cytokines as depicted in figure 2 thus implying that radiation and chemotherapy treatments whether given alone or in combination as a part of treatment regimen for head and neck cancer have almost the same effect on the expression level of the studied pro-inflammatory cytokines.

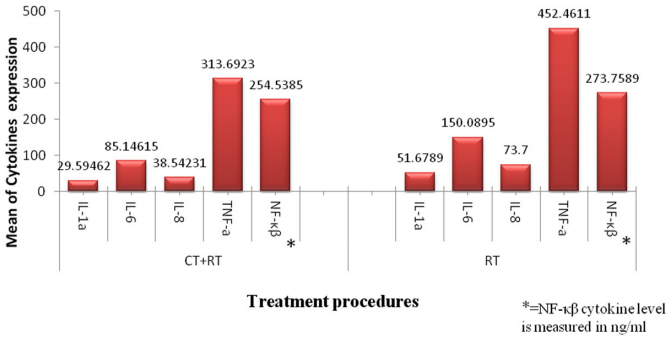

Fig. 2 - Graph depicting the mean of expression of different pro-inflammatory cytokines for patients undergoing radiation therapy along with chemotherapy (CT + RT) and those undergoing only the radiation therapy (RT).

Furthermore, analyzing the expression of different cytokines (TNF-α, NF-κβ, IL-8, IL-6, IL-α) with respect to the different grades of mucositis we have found that the expression level of studied cytokines increases with the progression of the mucositis (grade 1 to grade 4) as depicted in figure 3.

Discussion

Oral mucositis is an undesirable painful outcome endured by patients receiving radiation and/or chemotherapy for head and neck cancer treatment. Radiation and chemotherapy are effective activators of several pathways in endothelia, fibroblasts and epithelia thus leading to the production and upregulation of certain pro inflammatory cytokines[16,17] which have been reported to play an important role during inflammation and tissue damage[18].

The present study examined the expression level of NF-κβ and its associated pro-inflammatory cytokines (IL-6, TNF-α, IL-1α and IL-8) in head and neck cancer patients undergoing radiation and/or chemotherapy as a part of their treatment. Our results showed a significant increase in the expression of NF-κβ, IL-6 and TNF-α (P value < 0.05) in the serum of all the patients enrolled for the study as compared to the control group. However, nearly 60 % and 52.5 % of the patients showed an increase in the expression of IL-1α and IL-8 (P value > 0.05) as compared to the control group (healthy volunteers).

A marginal increase has been seen in the expression of NF-κβ due to the exposure of the tissue to chemotherapy or radiation therapy causing the production of reactive oxygen species (ROS) that ultimately results in tissue injury and cell death[4,11,14,16]. Previous studies have reported the transcription factor NF-κβ as being a regulator of inflammation signal and immunity as well as also being involved in the progression of mucositis[4,11]. NF-κβ regulates the expression of approximately 200 genes many of which may play the role in the pathogenesis of mucositis[4,13,19] including those encoding COX-2[15,19-21] and proinflammatory cytokines such as TNF-α, IL-6, and IL-1β[4,11,14,22]. COX-2 intensifies and prolongs mucositis by interacting with a variety of apoptotic pathways and by providing positive feedback to NF-κβ. The increase in the expression of COX-2 can be an

initial sign of the inflammatory cascade which leads to the production of prostaglandins and further tissue damage[4,11,15,19].

Further, upregulation in the expression of proinflammatory cytokines by the action of NF-κβ triggers the initiation of various pathways that damage epithelial cells and surrounding fibroblasts. The feedback loop formed between various proinflammatory cytokines such as TNF-α and NF-κβ promotes the cycle of inflammation, pain, and functional impairment[11,13,14,17,23,24]. The presence of TNF-α has been reported by previous studies related to the stimulation of early damage to connective tissue and endothelium thus initiating mesenchymal-epithelial signaling and reducing epithelial oxygenation ultimately resulting in epithelial basal cell death and injury[11,15].

Several studies have also reported the increase in the expression of IL-6 and IL-8 as a downstream product of NF-κβ activation. The upregulation of these proinflammatory cytokines has been known to mediate neutrophil extravasation and tissue infiltration during inflammation thereby playing an important role in the migration of leukocytes into the site of inflammation[15,25-27]. The amplification of these biological events via positive feedback loops as well as stimulation by bacterial cell wall products results in the widespread tissue damage as seen in the clinical setting as ulceration. This ulcerative phase is primarily responsible for the main clinical symptoms of mucositis (pain, inflammation, and loss of function)[11,17,28].

A study by Ong et al., in 2010 partially related to our study was done to characterize the expression of pro-inflammatory cytokines in the gastrointestinal tract using a rat model of fractionated radiotherapy-induced toxicity. Ong found that a significant upregulation of IL-1β, IL-6 and TNF mRNA levels in the jejunum and colon thus concluding the role of pro-inflammatory cytokines in radiotherapy-induced gastrointestinal mucositis[22].

Another study[29] implicated the role of NF-κβ, pro-inflammatory cytokines, COX and MMPs in the pathogenesis of mucositis as these factors were being expressed at elevated levels in both serum and tissue following radiotherapy and/or chemotherapy[29]. Our findings have also been supported by studies done on an animal model by Logan et al that reported the changes in the expression of NF-κβ, TNF, IL-1β and IL-6 in the mucosa and serum following chemotherapy orated a statistically increased oral mucosal stain[17,30]. Another study done by Logan et al., on a human biopsy demonstrated the increased level of NF-κβ and COX-2 in patients with chemotherapy as well as radiotherapy[17]. However, Ikebe et al., observed the expression of NF-κβ to be reduced after chemo radiotherapy and concluded that the vulnerability of oral mucosa undergoing chemo radiation may be associated with reduced NF-κβ expression and impaired growth activity[31]. The role of systemic inflammation in the development of mucositis was further supported by studies showing that the therapy targeted to alter cytokine expression is able to modify the course of mucositis[32]. Animal[33] and human studies[34] have demonstrated a decrease in the occurrence or severity of mucositis following the administration of TNF inhibitors.

The cytokines are reported to be unregulated when individually subjected to radiation treatment[11,15,19,20,22,35] and chemotherapy[24,30,36-41]. However, no such studies have been made on patients undergoing chemotherapy and radiation therapy simultaneously. We evaluated the level of cytokines (TNF-α, NF-κβ, IL-8, IL-6, IL-α) in patients receiving radiation treatment and those receiving chemotherapy along with the radiation treatment. This study for the first time reveals that there is no significant variation in the levels of cytokines in the patients undergoing radiation as well as chemotherapy and those undergoing just the radiation treatment. This finding demonstrated that the severity of mucositis does not depend on the type of treatment (radiation and/or chemotherapy) being administered to the patient and that both the treatments have more or less the same effect on the level of these cytokines.

We further correlate our study to the clinic-pathological parameters (grading of mucositis). Interestingly we found that advanced stage (grade 4) mucositis demonstrated the increase in the expression of cytokines (TNF-α, NF-κβ, IL-8, IL-6, IL-α) as compared to early stage (grade o & 1) of mucositis as well as healthy control. This finding was supported by Ong et al., who also reported the importance of these proinflammatory cytokines in the development and severity of radiotherapy induced gastro-intestinal mucositis in rats[22].

It is therefore very clear to understand that inflammatory cytokines (TNF-α, NF-κβ, IL-8, IL-6, IL-α) play a vital role in the progression of radiation induced oral mucositis.

Conclusion

On the basis of above findings, we are able to conclude that NF-κβ and its associated cytokines (TNF-α, IL-8, IL-6, IL-α) are involved in the pathogenesis of radiation induced oral mucositis. We further provide evidence for the association of these pro-inflammatory cytokines with the progression of this disease into more advanced stages. Continued research will undoubtedly provide a better understanding of the dynamics leading to mucosal injury and to more effective measures for prevention and treatment. Refinement of the five-stage model of mucositis pathogenesis may aid in the rational use of therapies to maximize their efficacy. Further studies are necessary to elucidate the exact role of these cytokines as biomarkers for this disease.

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Conflict of Interest

None Declared.

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