Use of Mucosal Fluid Collection to Assess Oral Mucositis in Patients Receiving Head and Neck Radiation Therapy: A Feasibility Study

Joel B. Epstein, DMD, MSD, FRCD(C)*
Laura Wan, DMD, DDS†
Peggy Chrisman, BS‡
Beverly A. Dale, DDS, PhD‡
Douglass Jackson, DDS, PhD§

*Department of Oral Medicine and Diagnostic Sciences, College of Dentistry, and College of Medicine, Chicago Cancer Center, University of Illinois, Chicago, Illinois, and British Columbia Cancer Agency, Vancouver, British Columbia
†Private practice in British Columbia
‡Department of Oral Biology, University of Washington, Seattle, Washington
§Department of Oral Medicine, University of Washington, Seattle, Washington

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ABSTRACT

Purpose: Oral mucositis is a common and severe complication of head and neck radiation therapy. Evaluating the progression and severity of radiation-induced oral mucositis is an important aspect of treatment because oral mucositis often debilitates the patient's quality of life and may interrupt or change the cancer treatment. Localized, time-dependent tissue changes that occur during radiation may be conducive to the development of a clinical model of mucosal tissue damage.

Materials and Methods: The secretion of biochemical markers, such as human α-defensins, was examined in the oral mucosa from patients receiving radiation therapy using the ProteinChip array, surface-enhanced laser desorption/ionization technology (Ciphergen Biosystems Inc, Fremont, CA) combined with time-of-flight mass spectrometry. This method has also proven to be effective and feasible in detecting and measuring changes of α-defensins in a small amount of biological fluids.

Results: The concentration of the human α-defensins measured in the exudates from the surface in the field of developing oral mucositis lesions changed during the development and healing processes.

Conclusion: This method may provide the means to assess oral mucositis during development and resolution and to assist in developing an understanding of the pathogenesis of the condition. In addition, this method may provide a

means of assessing developing tissue damage and lead to new approaches to prevent and treat oral mucositis in cancer therapy.

INTRODUCTION

Oral mucositis is a painful condition characterized by redness and ulceration of the lining of the mouth that is a common complication of head and neck cancer therapy. Radiation therapy for head and neck cancer results in the development of oral mucositis in nearly 100% of all patients and ulcerative oral mucositis in approximately 90% of patients.[1] The severity of oral mucositis often debilitates the patient's quality of life. Associated pain and oral dysfunction impacts eating, drinking, the use of oral prostheses, and speaking. The oral complications may lead to the need of opioid analgesics, tube feeding, hospitalization, and interruption in the planned cancer therapy. The development of radiation therapy–induced oral mucositis lesions is directly related to the field, dose per fraction, total dose of radiation, and individual variability.[2-6]

Radiation therapy directly disrupts the mucosal lining, further enhancing the effects of physical, chemical, and microbial insults in the mouth. The development and progression of radiation therapy–induced oral mucositis is well understood. Prior to radiation therapy, most patients have intact intraoral mucosa, with the exception of the presence of the tumor. The first clinical sign of mucosal damage is the whitening of tissue, commonly seen within the first or second week of therapy. Erythema follows, which may progress to ulceration and pseudomembrane formation, reaching maximum hyperalgesia and allodynia. Once the radiation therapy is stopped, the damaged mucosa enters the healing phase, returning to the baseline condition in 4 to 8 weeks.[7]

Due to mucositis, pain may be present, and pain associated with physical, thermal, and chemical stimuli is often enhanced (also known as hyperalgesia). The threshold of painful stimuli is reduced, and the response to suprathreshold is elevated during hyperalgesia.[8] Numerous proinflammatory biochemical mediators are either synthesized at the site of injury or released from various cell types following tissue injury. Bradykinin, histamine, cytokines, leukotrienes, and neuropeptides are among the many local inflammatory mediators found in injured tissue.[9,10] Cytokines such as epidermal growth factor (EGF), tumor necrosis factor alpha (TNFα), and interleukin 11 (IL-11) have a role in the maintenance of normal cellular function and in healing and repair.[11-14] Administration of EGF, which stimulates the growth and differentiation of oral epithelium, has been shown to significantly increase the severity of breakdown oral mucosa and increase the duration of mucositis.[11] TNFα has been shown to regulate a reduction in epithelial cell proliferation.[15] IL-11 stimulates bone marrow cells and subcutaneous tissue and has been shown to decrease oral mucositis in a dose-dependent manner.[14] In addition, human β-defensins, which are small cationic antimicrobial peptides that are newly recognized components of innate responses, have been identified in human oral epithelia. They are secreted from appropriated oral epithelial cells and play a role in the epithelial protective barrier function.[16,17] Human α-defensins, produced by polymorphonuclear leukocytes (PMNs), are also expected to be present at sites of injury and inflammation.[18]

The current management of oral mucositis relies on the reduction of physical, chemical, and microbiological irritants in the mouth. It is important that the patient maintain good oral hygiene and avoid irritating and abra-

Table 1. World Health Organization Mucositis Score

Grade 0	Healthy mucosa
Grade 1	Erythema of the mucosa
Grade 2	Patchy pseudomembranous reaction (patch generally <1.5 cm in diameter and noncontiguous)
Grade 3	Confluent pseudomembranous reaction (contiguous patches generally >1.5 cm in diameter)
Grade 4	Necrosis or deep ulceration—may include bleeding not induced by minor trauma or abrasion

sive foods during radiation therapy so as not to aggravate the symptoms of mucositis. Other palliative measures used to reduce a patient's pain experienced during mucositis include the use of bland rinses, topical anesthetics, and in some occasions, systemic analgesics are prescribed.[2,4,19,20]

Due to the increasing awareness that cytokines are involved in the process of mucosal damage and repair, this study sought to determine if measure of biochemical change is possible in at-risk sites of oral mucosa during the delivery of radiation therapy. The goal was to examine the feasibility of the use of the ProteinChip array, surface-enhanced laser desorption/ionization (SELDI) technology (Ciphergen Biosystems Inc, Fremont, CA) combined with time-of-flight mass spectrometry in order to detect biochemical markers that are associated with tissue damage and repair. This was followed from the time of intact mucosa at the beginning of radiation therapy to the development and resolution of inflammation.[16] A sensitive technology was needed to measure the expected small changes in the quantity of cytokine that may pass through oral mucosa during radiation therapy, potentially reflecting epithelial/connective changes.

METHODS AND MATERIALS

Seven patients at the British Columbia Cancer Agency who planned to receive tumoricidal radiation therapy >4500 cGy for the treatment of head and neck cancer were included in the study. They were examined in the dental clinic prior to radiation therapy. Institutional approved informed consent was given. Tumor staging was completed following UICC (International Union Against Cancer) criteria for oropharyngeal carcinoma. The condition of the mouth tissues was examined. Specimen collection was completed using a filter strip (PerioPaper gingival fluid collecting strips, Oraflow Inc, Plainview, NY), which was placed on the region designated to receive the radiation therapy for collection of tissue exudates. The site was dried and isolated with cotton rolls, and the strip was placed on the mucosa until saturated or for up to 5 minutes. Another collection was performed on the contralateral side not receiving radiation, serving as a within-subject control. These 2 samples served as the pretreatment baseline. Subjective pain reports, lesion assessment (World Health Organization [WHO] mucositis score, Table 1), and exudate samples were collected each week during radiation therapy from the treated area and the contralateral control site until the last day of radiation therapy.

Acid Extraction of α-Defensins

A total of 100 µL 5% acetic acid was added to PerioPaper filter strips. Samples were shaken gently at approxi-

Table 2. Patient Tumor and Treatment Characteristics

Patient No.	Age	Sex	Diagnosis	Radiation Field	Epithelial Stage	Radiation (cGy)	Fractions
1	75	M	Nasopharynx cancer	POP/ant split	T2A N0 MO	6600	33
2	78	F	Right neck metastasis	Right side	T2 N2b Mx	5500	25
3	51	F	Squamous cell carcinoma of tongue	Tongue	T3 N0	6600	33
4	63	F	Right jugulo-tympanic para-ganglioma	Right posterior	N/A	5000	33
5	49	F	Nasopharynx cancer	POP/ant	T1 N0 M0	6600 5000	33 25
6	45	M	Nasopharynx cancer	POP/ ant split	T2 N1	6600	33
7	50	M	Nasopharynx cancer	Ipsilateral	N/A	6000	33

POP/ant split=parallel opposed/anterior split.

mately 100 rpm for 30 minutes. Filter strips were removed and acid solutions were centrifuged at 15,800 *g* for 5 minutes at room temperature. Supernatant was collected and vacuum evaporated. Samples were resuspended in 0.01% acetic acid and stored at -20ºC.

Attachment to Affinity Chip Surfaces
Concentrated acid extracted sample, 5 µL, was adsorbed to WCX2 cation exchange chips (Ciphergen Biosystems Inc.) via interaction with weak anionic carboxylate groups on the chip surface. The Ciphergen system can measure quantities of defensins and other peptides to the femtomolar range.[16] Samples were bound with a 40% ammonium acetate pH 8 buffer, and then washed twice with binding buffer and twice with molecular biology grade water.

Detection of Bound Cationic Peptides
Two applications of matrix (α-cyano-4-hydroxy-cinnamic acid [CHCA, 0.5 µL of saturated solution in 50% acetonitrile, 0.5% trifluoroacetic acid]) were applied to the chip surfaces to assist ionization. Samples were analyzed with the PBS-II SELDI-MS and software (Ciphergen Biosystems Inc) according to the protocol for peptide detection. The instrument was operated in positive ion mode with time lag focusing. Source and detector range were 2.0 and 1.8 kV, respectively. Digitizer rate was 250 MHz, pulse voltage was 3000 V, and pulse lag time was 492 ns. Spot protocol was set to high mass 30K Da, and the optimization range was 1000 to 8000 Da. The starting laser intensity was 154, the detector sensitivity was 10, and the focus was by optimization center.

RESULTS

Patient characteristics, diagnosis, and the radiation therapy provided are shown in Table 2. The mean age was 58.7 years (range 45-78 years). One patient had squamous cell carcinoma of the tongue, 4 had nasopharynx carcinoma, 1 had adenoid cystic carcinoma, 1 had right jugulotympanic paraganglioma, and 1 had a tumor on the right neck from

Table 3. Radiation and Mucositis Scores

Patient No.	Radiation Dosage/# of Fractions (cGy/#)	Exposed Site Where Strips Were Placed	Control Site Where Strips Were Placed	Maximum Mucositis Score During Radiation Period at Exposed Site
1	6600/33	Left buccal mucosa	Upper lip central	3
2	5500/25	Right buccal mucosa	Upper left vestibule	3
3	6600/33	Left buccal mucosa	Upper left vestibule	3
4	5000/25	Right posterior gingiva	Left posterior gingiva	1
5	6600/33 5000/25	Right buccal mucosa	Upper left vestibule	3
6	6600/33	Right buccal mucosa	Upper left vestibule	1
7	6000/30	Left buccal mucosa	Lower left vestibule	3

metastasis. These patients received a mean radiation dose of 5980 cGY (range 5000 to 6600 cGY) over a mean of 30 fractions (range 25-33 fractions). Mucositis scores are shown in Table 3. The most common site exposed to radiation where the filter strip was placed involved the buccal mucosa. The control site most commonly used was in the upper lip vestibule.

Patients 1, 2, 3, 5, and 7 all experienced maximum mucositis and received a score of 3 during radiation period at the exposed site. Patients 4 and 6 suffered from minimal mucositis at the exposed site. None of the control sites developed clinical mucositis.

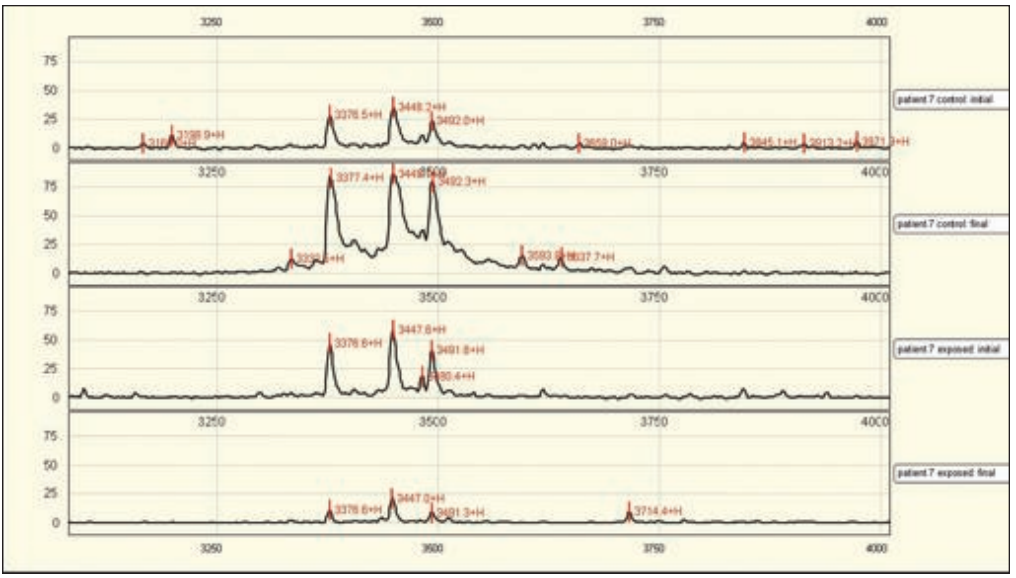
ProteinChip array technology was used to examine patient exudates. The β-defensins, hBD1 and hBD2, were identified as peaks at 5068 Da and 4328 Da, respectively, as previously identified in gingival crevicular fluid.[19] However, these peaks were weak and difficult to assess in a semiquantitative manner. Also detected were peaks consistent with the size of the α-defensins, HNP-1, -2, and -3, at 3442, 3371, and 3486 Da, respectively (Figure 1A).

Changes in the concentrations of human α-defensins measured over time from initial to final collection are shown in Figure 1B. There may be a difference in the amount of the human α-defensins measured in the control and exposed sites, and an increase of 3 α-defensins were seen at the final exposed sites for patients 4 and 6. Patients 4 and 6 developed a minimal tissue reaction to radiation therapy (mucositis score of 1). All of the other patients who developed ulcerative mucositis did not demonstrate consistent changes in the α-defensins throughout the study period.

DISCUSSION

A ProteinChip array and SELDI technology[16] were used to examine the tissue exudate of human α- and β-defensins in patients receiving radiation therapy for head and neck cancer. This technology shows the ease and speed of screening to profile biological samples. Plus, only a small sample size is needed.

Human α-defensins may change during radiation therapy consistent with their proposed role in inflammation and in innate host defense. Two patients (patients 4 and 6) experienced a mild mucositis (WHO mucositis score of 1) and showed the greatest increase of α-defensins over time, and those with ulcerative mucositis (WHO mucositis score of 2 or 3) showed less change, suggesting that α-defensins may play a role in preventing the breakdown of mucosal



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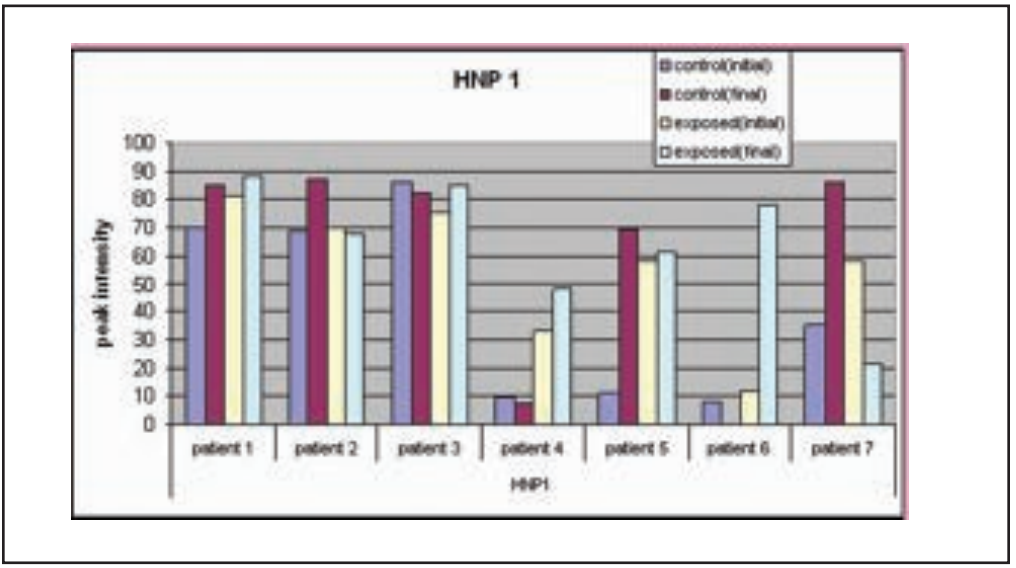

B

Figure 1. Relative changes in the peak intensity of human α-defensins in different patients. A) Detection of α-defensins, HNP-1, -2, and -3 at 3442, 3371, and 3486 Da, respectively, in exudate from patient 7 at exposed and control sites. B) Summary of HNP-1, -2, and -3 peak intensity from each patient. Data were quantitated using the Ciphergen system software from tracings such as shown in A.

tissue. Although the numbers were small and subject to future confirmation, these findings suggest that human α-defensins may be associated with less severe mucositis and may be a part of host defense in these patients. If this is confirmed, studies to assess the topical application of α-defensins may be useful. Other means of stimulating host production and secretion are also strategies that could be pursued.

However, this data must be cau-

tiously assessed due to the limited number of patients and the small changes that can be measured using SELDI. The technique of collection must be conducted carefully as small amounts of contamination (ie, via saliva) may result in large changes (eg, patient 5). Moreover, statistical analysis was not performed due to the sample size in this study. The initial study plan was to assess TNFα because it may play a central role in mucosal damage. However, the standard TNFα applied to the filter strip was not eluted, and it was elected to assess α-defensins instead, as this was an established method.[14]

CONCLUSION

Although this test failed to measure TNFα as planned, the findings in this study suggest that it is feasible to assess small quantities of molecules that may have a role in the pathogenesis of oral mucositis. The findings also suggest that future studies can be conducted to assess molecular changes in tissue damage and repair in an in-vivo human model of tissue.

Future studies can extend to other molecules including other cytokines (ie, EGF, TNFα-blocking agents) and assessment of pain mediators. Ultimately, a more complete understanding of radiation therapy–induced mucosal damage can be developed. This will lead to further exploration of prevention, treatment, or acceleration of healing of damaged mucosa in patients with cancer, and potentially with other causes of mucosal damage.

It is likely that multiple modalities will be employed for the management of mucositis that may require administration at specific times during cancer treatment to reduce injury, reduce the risk of microbial irritation, and to accelerate healing (rather than the current symptomatic/palliative measures). Advances in the prevention and management of mucositis will greatly improve the quality of life of patients with cancer, allow intensification of therapy, and reduce the cost of care. In addition, an improved understanding of pathogenesis of oral mucositis has implications for management of mucosal injury due to other causes and at other body sites.

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