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FUNCTIONAL ACTIVITIES AND IMMUNOHISTOCHEMICAL DISTRIBUTION OF SUPEROXIDE DISMUTASE IN NORMAL, DYSPLASTIC AND SQUAMOUS CELL CARCINOMA ORAL TISSUES

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Masters of Science in the

Graduate School of The Ohio State University

By

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ABSTRACT

Although reactive oxygen intermediates (ROI) fulfill physiological roles e.g., intracellular signaling and protection against microbes, sustained ROI levels result in oxidative stress and its deleterious sequelae e.g., DNA mutations to include transmissions and translations (Wink, et al., 1998). Consequently, aberrant antioxidant enzyme function has been speculated to be a key contributor in carcinogenesis. Levels of superoxide dismutase (SOD), the antioxidant enzyme responsible for the dismutation of superoxide to hydrogen peroxide and oxygen, can vary greatly depending upon tissue site and donor status. The purpose of this study was to determine the functional SOD activities [total, mitochondrial (Mn), and cytosolic (CuZn)] in histologically confirmed non-inflamed normal oral mucosal, inflamed normal oral mucosal, and oral squamous cell carcinoma tissues (SCC) and to characterize the *in vivo* cellular distribution of MnSOD and CuZnSOD in representative sections of the above tissues, as well as, oral epithelial dysplasia. Materials and methods: Total (tot), cytosolic (CuZn), and mitochondrial (Mn) SOD activities were spectrophotometrically determined (25°C, 550 nm) by calculating the rate of inhibition of reduction of acetylated cytochrome c. Immunohistochemical studies using standard techniques (avidin-biotin-peroxidase) and commercially available CuZnSOD and MnSOD antibodies were completed. Results of functional SOD assays: 1) all of the samples showed the highest proportion of SOD

activity located within the mitochondria, 2) both the inflamed normal and SCC tissues contained a heavy influx of host inflammatory cells, 3) the overall data trends show a SCC > inflamed normal > non-inflamed normal distribution in SOD activities. Immunohistochemical results: 1) epithelial tissues (either of surface or salivary gland origin) demonstrated markedly greater SOD staining relative to fibrovascular connective tissues, 2) only scattered cells within the connective tissues (inclusive of endothelial cells and phagocytes) showed SOD staining positivity, 3) CuZn staining characteristics: a) non-inflamed normal tissues: full epithelial thickness staining distribution, with most intense staining in the granular layer epithelial cells, b) inflamed normal tissues: same full thickness staining distribution, increased intensity at granular layer, c) dysplastic tissues: while staining intensity remained greatest at the granular layer, there was an overall reduction in SOD protein expression, d) SCC tissues: highly variable SOD protein demonstrated within lesional SCC cells, foci of intense staining noted within occasional well-differentiated SCC tumor cells, and when present , the overlying surface epithelium showed higher staining intensity relative to SCC tumor cells. 4) Mn staining characteristics: a) non-inflamed normal tissues: intense staining noted at the basal layer keratinocytes, and in a granular, perinuclear distribution in the granular layer epithelial cells, b) inflamed normal tissues: intense staining not restricted to basal layer keratinocytes, a more uniform, epithelial full-thickness, perinuclear granular distribution was observed, c) dysplastic tissues: an overall dramatic reduction in Mn staining, with foci of positivity noted in scattered granular layer epithelial cells, and d) relative to the overlying dysplastic tissues, dramatically reduced Mn staining intensity in SCC tumor

cells was noted. Scattered foci of positive tumor cells were observed within more well differentiated tumor cells e.g., those showing keratin pearl formation. Conclusions: 1) Proportionately higher levels of SOD in oral mucosa indicate that the oral cavity is possibly a site of high extrahepatic xenobiotic metabolism. 2) SOD functional activities reflect cellular adaptation to ongoing oxidant stress. 3) Reduced SOD expression in dysplastic samples is permissive for the progression to a pre-malignant/malignant phenotype. 4) Decreased MnSOD expression in dysplasia and SCC reflects reactive species enzyme inactivation, possibly by tyrosine nitration as reported by MacMillan-Crow et al., 1996.

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CHAPTER 1

INTRODUCTION

Oral cancer is the sixth most common cancer in the world in men and the ninth most common in women.[1] An estimated 30,100 new cases of oral cavity and pharyngeal cancer will be diagnosed and an estimated 7,800 people will die of oral cavity and oropharyngeal cancer in the United States during 2001.[2] Squamous cell carcinoma (SCC) accounts for, by far, the majority of all cancers arising from oral mucosal tissue (90% according to the American Cancer Society).[3] Numerous studies have shown cancer tissues demonstrate variable levels of MnSOD activity and corresponding low CuZnSOD activity.[4,5] Abnormal levels, ranging from very low to very high, and abnormal regulation of antioxidant enzymes in all tumor cell types has been reported[5] although, this is very tumor dependent. Virtually all eucaryotic cells possess SOD, which is responsible for the conversion of superoxide to hydrogen peroxide and oxygen, and SOD plays the role of cytoprotective enzyme as an antioxidant.[6,7] By determining the functional activity and the cellular distribution of SOD in oral mucosal tissues obtained from histologically confirmed normal, dysplastic, and SCC sites, it is hoped to gain a better understanding of preneoplastic/neoplastic biochemistry and to provide a scientific basis for lesional specific chemoprevention (e.g. antioxidant therapy).

There are two forms of intracellular SOD that exists: a CuZn dependent form, which functions mainly in the cytosol, and an Mn dependent form, which functions in the mitochondria. There is also an extracellular form of SOD (EC-SOD), which is also CuZn dependent. It is found in the tissue interstitium,[8] the connective tissue matrix and on the cell surfaces.[9,10] EC-SOD provides cytoprotection within the extracellular space. The superoxide anion radical substrate poorly penetrates membranes. Therefore, the SOD isoenzymes primarily exert their protective functions within their respective compartments.[11] Due to the ongoing inflammation (and associated potentially carcinogenic oxygen metabolites) in the oral cavity, SOD may play a key role in the suppression (when increased) or promotion (when reduced) of oral SCC. Several investigators have found that SOD in human carcinoma tissues most likely serves as a protective mechanism against superoxide radicals, as well as tumor necrosis factor toxicity.[12,13,14] Also, Lam et al.[15] found MnSOD to display tumor suppressor gene-like activity in the hamster cheek pouch model system.

Reactive oxygen intermediates (ROI), such as superoxide, hydroxyl, peroxyl radicals, hydrogen peroxide and singlet oxygen, are oxygen metabolites. These oxygen metabolites are generated during normal cellular oxidative metabolism (e.g. mitochondrial respiratory chain), and also during xenobiotic (alcohol, tobacco) metabolism. ROI are also generated as a component of the phagocytic cell oxidative burst. The inflammatory reaction is an important source of oxygen-free radicals. Superoxide radicals are formed as a byproduct during prostaglandin and leukocyte biosynthesis.[16] Large amounts of superoxide radicals are secreted by activated

phagocytic leukocytes.[17,18,19] ROI can be cytotoxic at high concentrations, beneficial against microbes, deleterious against host cells. Under normal conditions, cells are protected from ROI by a variety of defenses including antioxidant enzymes: SOD, catalase (CAT), and glutathione peroxidase (GPO).[20] SOD converts superoxide radical into hydrogen peroxide, while CAT and GPO convert hydrogen peroxide to water. Therefore, superoxide radical and hydrogen peroxide (both toxic) are converted into harmless products. These enzymatic functions are necessary for life in all oxygen-metabolizing cells.[7]

Numerous articles have been published on the role of antioxidant enzyme levels in cancer. Lowered MnSOD activity has been observed in human and various rodent cancer cells, either spontaneously transformed[21] or transformed by viruses, chemicals, ionizing radiation, or hormones.[22] High levels of MnSOD compared to their non-malignant counterparts have been reported in thyroid,[23] kidney[5] central nervous system,[24] gastrointestinal tract[25] and malignant mesothelioma[26] and increased MnSOD and CuZnSOD in human lung cancer tumors.[27] The levels of SOD activity may indicate the extent of a specific cytoprotective enzyme expression in cancer tissue.[28,29,30] Although the levels of antioxidant enzymes in cancer tissues are extremely variable, the consensus of these studies is that antioxidant levels are low in most animal and human cancers. Oxidant stress results from these lowered levels of antioxidant enzymes. Oxidant stress is defined as the condition where levels of ROI exceed intrinsic cellular or tissue capacities for inactivation and degradation. H_2O_2 is one ROI in particular that is not detoxified by some cancer cells.[5] High levels of ROI have shown to be cytotoxic and

have been implicated in the formation of chromosome aberrations[31] and DNA mutations (translations and transmissions)[32] that initiate the development of oral SCC. Irreversible genetic damage is one potential outcome of oxidant stress.[31] However, on a subtler, but equally important level, ROI are also implicated as secondary messengers in cellular signal transduction. Therefore, inappropriately sustained signal transduction and increased gene expression are potential outcomes of reduced ROI scavenging enzymatic activity.[33]

Due to its location and function, the oral cavity is exposed to a variety of potential pathogens. This exposure results in the initiation of an inflammatory response, which is necessary to prevent infections as well as for wound healing and repair. A possible correlation between the intensity of the histological inflammatory reactions and the reactivity for MnSOD in pituitary lesions has been reported.[34] In order to avoid injury to the host cells and yet destroy the microorganisms at the inflammation sites, it is necessary to balance this inflammatory response. Just as important as the process of inflammation is the cellular/tissue capacity to avoid cellular damage by upregulation of cytoprotective enzymes. The purpose of this study was to concurrently investigate the SOD functional activities (total, mitochondrial-Mn, cytosolic-CuZn) in histologically confirmed non-inflamed normal, inflamed normal and oral SCC tissues and to characterize the *in vivo* cellular distribution of MnSOD and CuZnSOD in formalin-fixed, paraffin-embedded matched sections of the tissues used for SOD functional assays as well as samples of oral epithelial dysplasia. The percent of epithelium in each sample was determined in order to adjust the functional activity levels based on the immunohistochemical finding that SOD

expression was cellular-specific, with the most intense staining in epithelial and endothelial cells.

CHAPTER 2

NULL HYPOTHESES

H_o1: There is no difference between the SOD (Total, CuZn, Mn) functional activities of the three human oral mucosal tissue types (non-inflamed normal, inflamed normal and SCC).

H_o2: There is no difference in the cellular tissue specific localization of SOD (epithelial verses mesenchymal) in the four human oral mucosal tissue types (non-inflamed normal, inflamed normal, dysplasia, and SCC).

H_o3: There is no difference in the immunohistochemical staining intensity for SOD (Total, CuZn, Mn) in the four human oral mucosal tissue types (normal, inflamed, dysplastic, SCC).

CHAPTER 3

MATERIALS AND METHODS

Tissue Samples

Human oral mucosal tissue samples (n=23) that were divided into non-inflamed normal and inflamed normal groups after histological examination were collected from patients treated in the Oral Maxillofacial Surgery (OMFS) Clinic and the Graduate Periodontal Clinic at The Ohio State University (OSU). Squamous Cell Carcinoma (SCC) tissue samples (n=14) were procured from the NIH funded Tissue Procurement Bank of The Ohio State University. Tissue samples were divided into two portions: one portion was placed in 4% formalin in phosphate buffered saline (PBS) for not greater than 24 hours and then paraffin embedded for use in immunohistochemistry studies and for H&E staining, the second portion was placed in RNA/later and immediately frozen at -80° C to be tested for SOD functional activity. Paraffin embedded sections (n=13) of dysplastic oral mucosal tissues were obtained from the Oral Pathology Department of The Ohio State University. These dysplastic samples were obtained from OSUOMFS patients whose tissues were processed the day of the biopsy. Therefore, the dysplastic samples were exposed to formalin at times comparable to both the normal and SCC

samples (<24 hours). A patient history including the cancer risk factors, smoking and alcohol, was gathered at the time of surgery.

Tissue Preparation

Frozen tissues were thawed and a wet weight obtained. The tissues were minced with scissors and placed in 2 ml of 50 mM potassium phosphate buffer, pH 7.8. The minced tissues were then homogenized using a polytron until no visible particles remained. Homogenization was performed on ice. The homogenized tissues were frozen using liquid nitrogen and vortexed three times in order free the proteins by lysis of the cells. Cell lysis was confirmed by microscopic examination. The homogenized tissues were then centrifuged to remove cellular fractions and the supernates were carefully measured, volumes recorded and frozen at -80° C. To prevent the loss of SOD activity as a result of proteolytic enzymatic degradation, aprotinin (Sigma), 100 kallikrein inhibitory U/ml, was incorporated into the washing and buffer solutions used during tissue preparation.

Assessment of SOD Activity

Tissue SOD activity was determined using a modification of the method of Flohe and Otting (1984),[35] by calculating the rate of inhibition of the reduction of cytochrome c. The assay was conducted on a DU 7400 Beckman spectrophotometer, equipped with a Peltier temperature-regulating device, at 550 nm, 25° C, using a xanthine/xanthine oxidase system to generate superoxide. Acetylated cytochrome c was used to increase the specificity of the assay, as this form of cytochrome c can still be reduced by superoxide, but can no longer undergo redox enzymatic reactions. A five-point SOD

standard curve (0.05, 0.10, 0.50, 0.75, 1.00 U/ml) was conducted with each assay, with one unit of SOD activity defined as the amount of enzyme that results in a 50% inhibition of the reduction of acetylated cytochrome c. To determine mitochondrial MnSOD activity, 2 mM (final concentration) of KCN was added to the MnSOD samples to inhibit cytosolic CuZnSOD activity. CuZnSOD activity was then calculated by subtraction of the MnSOD levels from the total (samples without KCN) SOD activity. Tissue SOD activity was reported in units of activity/mg of tissue protein.

Protein Determination

Protein content was measured by the method of Lowry et al., (1951).[36]

Immunohistochemistry

Immunohistochemistry was used to localize MnSOD and CuZnSOD in non-inflamed normal oral mucosa, inflamed oral mucosa, dysplastic oral mucosa and oral SCC samples. The formalin fixation times of the dysplastic samples were comparable to the formalin fixation times of the other samples. Tissues were fixed in 4% formalin in PBS and embedded in paraffin. Deparaffinized tissue sections (4 um thick) were treated with 3.0% H_2O_2 and then underwent antigen retrieval in a tris-urea solution. The tissue sections were then incubated overnight with either anti-CuZnSOD 1:100 primary antibody from StressGen or anti-MnSOD 1:500 primary antibody from StressGen. LSAB + kit (Dako) was applied to the tissue sections and then the tissues were stained with DAB solution (DAKO). Ammonia water was used to blue the tissue sections, which were then dehydrated and mounted.

Percent Epithelium

The percent epithelium was determined for the tissue samples in order to normalize the results of the functional assays, since the immunohistochemical studies revealed the CuZnSOD and MnSOD were located almost exclusively in the epithelial tissues. The percent of epithelium of each tissue sample was estimated by microscopic examination (by the senior author) of the H&E stained slides and the results of the functional assays were then adjusted to reflect the epithelial content of each sample.

Histological Diagnosis

The H&E slides of each tissue sample was examined by the senior author in order to confirm the diagnosis and categorize the tissue samples into one of the following: non-inflamed normal, inflamed normal, dysplastic oral mucosa or SCC. Due to processing errors during the functional assaying and immunohistochemical staining phases the final sample sizes were: non-inflamed normal = 13, inflamed normal = 6, SCC = 11, and dysplasia = 10.

CHAPTER 4

RESULTS

Results for the SOD functional assays are summarized in Table 4.1. All of the tissue types show the highest proportion of SOD activity located within the mitochondria. Both the inflamed normal and the SCC tissues contain a heavy influx of host inflammatory cells - clinical data that corresponds with increases in mitochondrial SOD distributions. Although the findings are not statistically significant, the overall data trends show a SCC > inflamed normal > non-inflamed normal tissue distribution in both total and MnSOD activities (Figures 4.1, 4.2). The CuZn activities are graphed in Figure 4.3. Table 4.2 Comparison of Functional Activities compares the functional activities of oral mucosa found in this study with those reported by Westman and Marklund in 1981[37] for liver, heart and lung. Oral mucosal tissues are one of the highest sites or SOD functional activity in the human body. The results of the functional assays of individual samples can be found in Tables 4.3-4.5.

The immunohistochemical findings show a markedly greater SOD protein distribution in epithelial tissues (either of surface or salivary gland origin) relative to fibrovascular connective tissues. Only scattered cells within the connective tissues (inclusive of endothelial cells and phagocytes) show SOD staining positivity. CuZnSOD staining characteristics for each type of tissue are as follows. The non-inflamed normal

tissues (Plate 4.1) demonstrate full epithelial thickness staining distribution, with the most intense staining in the granular layer epithelial cells. The inflamed normal tissues (Plate 4.2) show the same full thickness staining distribution, with increased intensity at the granular layer. The dysplastic tissues (Plate 4.3), even though staining intensity remained greatest at the granular layer, have an overall reduction in CuZnSOD protein expression. The SCC tissues (Plate 4.4) have a highly variable CuZnSOD protein distribution within lesional SCC cells. Foci of intense staining are noted within occasional well-differentiated SCC tumor cells. When present, overlying surface epithelium show higher staining intensity relative to SCC tumor cells.

MnSOD staining characteristics are notably different than the CuZnSOD staining characteristics. In non-inflamed normal tissues (Plate 4.1), intense staining occurs at the basilar layer keratinocytes, and in a granular, perinuclear distribution in the granular layer epithelial cells. In inflamed normal tissues (Plate 4.2) the intense staining was not restricted to basilar layer keratinocytes. A more uniform epithelial full-thickness, perinuclear granular distribution is observed. The dysplastic tissues (Plate 4.3) have an overall dramatic reduction in Mn staining, with foci of positivity noted in scattered granular layer epithelial cells. Relative to the overlying dysplastic tissues, dramatically reduced Mn staining intensity in SCC tumor cells (Plate 4.4) is noted. Scattered foci of positive tumor cells are observed within the more well-differentiated tumor cells e.g., those showing keratin pearl formation. The results of the immunohistochemical staining of individual samples are summarized in Table 4.6-4.13.

The results from determining the percent epithelium of each sample and adjusting the functional activities did not significantly alter the findings.

In regards to the Null Hypotheses:

H_o1: There is no difference between the SOD (Total, CuZn, Mn) functional activities of the three human oral mucosal tissue types (non-inflamed normal, inflamed normal, SCC). This cannot be rejected since the differences were not statistically significant. Therefore, only a trend could be noted: non-inflamed normal<inflamed normal<SCC.

H_o2: There is no difference in the cellular tissue specific localization of SOD (epithelial verses mesenchymal) in the four human oral mucosal tissue types (non-inflamed normal, inflamed normal, dysplasia, SCC). This can be rejected based upon qualitative differences. There is a difference (epithelial>mesenchymal) in the cellular tissue specific localization of SOD in the four human oral mucosal tissue types (non-inflamed normal, inflamed normal, dysplasia, SCC).

H_o3: There is no difference in the immunohistochemical staining intensity for SOD (Total, CuZn, Mn) in the four human oral mucosal tissue types (non-inflamed normal, inflamed normal, dysplastic, SCC). This cannot be rejected, only a trend can be noted: inflamed normal>non-inflamed normal>dysplasia for MnSOD.

Tissue Type	Total SOD	%CuZn	%Mn
Non-inflamed Normal (n=6)	31.4 ±7.7	21.4 ±9.4	78.6 ±9.4
Inflamed Normal (n=13)	41.6 ±5.6	17.9 ±4.8	82.1 ±4.3
Oral SCC (n=11)	54.3 ±17.8	6.6 ±2.7	93.4 ±9.0

Table 4.1: Results of SOD Functional Assays reported in units of activity/mg protein (Means ±SEM).

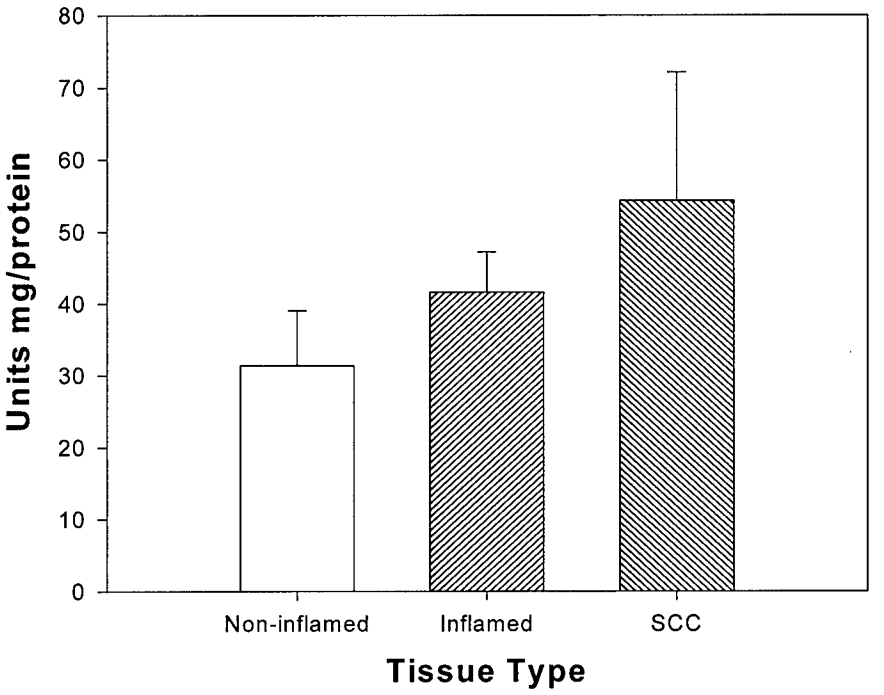

Figure 4.1: Functional Assays for Total SOD

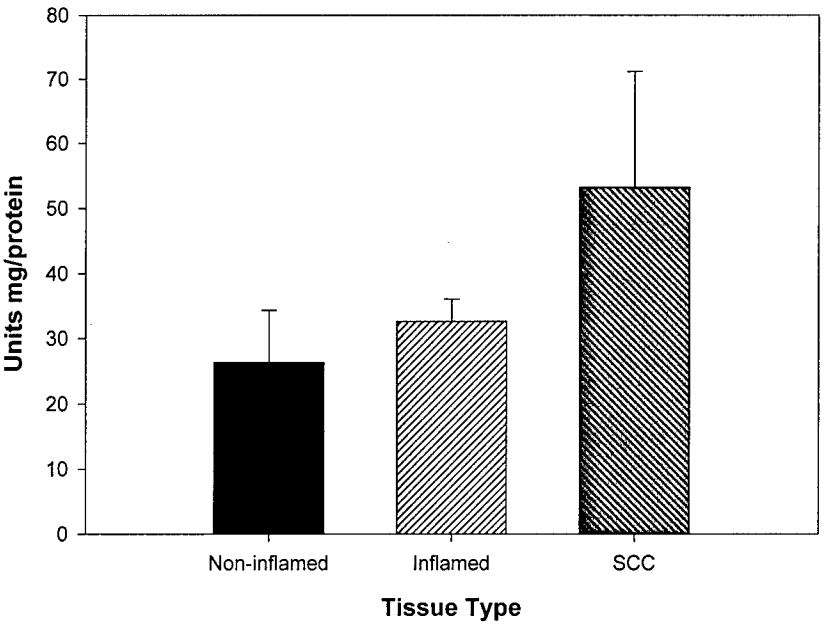

Figure 4.2: Functional Assays for MnSOD

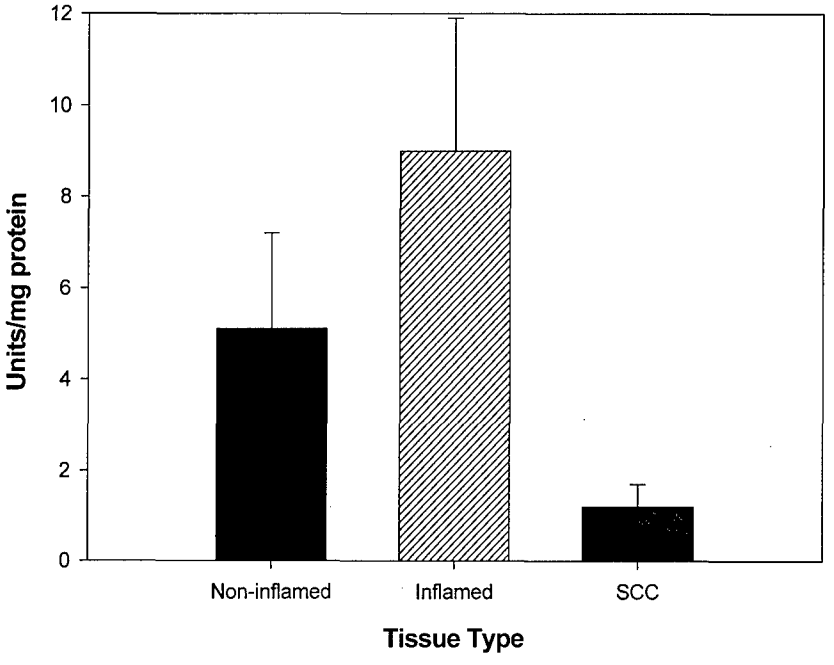

Figure 4.3: Functional Assays for CuZn

Tissue Type	Total	% CuZn	% Mn
Non-inflamed Normal	31.4	21.4	78.6
Inflamed Normal	41.6	17.9	82.1
Oral SCC	54.3	6.6	93.4
Liver*	40.3	80.3	19.7
Heart*	19.2	44.8	55.2
Lung*	5.9	68.3	31.7

Table 4.2: Comparison of Functional Activities reported in units of activity/mg protein *Data converted from Westman and Marklund, 1981

I.D. No.	Total	CuZn	% CuZn	Mn	% Mn
H-300	16.17	7.59	46.94	8.58	53.06
H-500	24.97	13.04	52.22	11.93	47.78
H-700	63.19	7.13	11.28	56.06	88.72
H-900	15.47	2.77	17.91	12.70	82.09
H-1700	44.38	0	0	44.38	100
H-2300	24.20	0	0	24.20	100
Mean	31.40	5.09	21.39	26.31	78.61
+/- sem	±7.65	±2.09	±9.37	±8.00	±9.37

Table 4.3: Functional Activities of Non-inflamed Normals. Functional activities reported in units of activity/mg protein. CuZn values determined by subtracting Mn values from Total values. In the cases where the Mn value was greater than the Total value, the Mn value was adjusted to equal the Total value.

I.D. No.	Total	CuZn	% CuZn	Mn	% Mn
H-200	24.22	5.64	23.29	18.58	76.71
H-400	44.97	5.09	11.32	39.88	88.68
H-600	25.61	0	0	25.61	100
H-1000	19.49	0.35	1.80	19.14	98.20
H-1100	25.72	2.52	9.80	23.20	90.20
H-1200	39.20	4.73	12.07	34.47	87.93
H-1300	60.53	24.20	39.98	36.33	60.02
H-1400	24.88	10.78	43.33	14.10	56.67
H-1500	43.74	4.46	10.20	39.28	89.80
H-1800	68.78	16.46	23.93	52.32	76.07
H-2000	84.47	35.11	41.57	49.36	58.43
H-2100	54.29	8.40	15.47	45.89	84.53
H-2500	25.44	0	0	25.44	100
Mean	41.64	9.04	17.90	32.58	82.10
+/- sem	±5.63	±2.91	±4.28	±3.46	±4.28

Table 4.4: Functional Activities of Inflamed Normals. Functional activities reported in units of activity/mg protein. CuZN values determined by subtracting Mn values from Total values. In the cases where the Mn value was greater than the Total value, the Mn value was adjusted to equal the Total value.

I.D. No.	Total	CuZn	% CuZn	Mn	% Mn
SCC-1	14.82	2.84	19.16	11.98	80.84
SCC-3	17.16	3.40	19.81	13.76	80.19
SCC-4	6.32	0.80	1.27	6.24	98.73
SCC-4T	24.05	4.50	18.71	19.55	81.13
SCC-5	158.04	0.49	0.31	157.55	99.69
SCC-6	6.92	0.94	13.58	5.98	86.42
SCC-8	57.40	0	0	57.40	100
SCC-11	74.80	0	0	74.80	100
SCC-14	23.27	0	0	23.27	100
SCC-16	41.97	0	0	41.97	100
SCC-17	172.99	0	0	172.99	100
Mean	54.34	1.18	6.62	53.23	93.36
+/- sem	±17.77	±0.49	±2.72	±17.96	±9.04

Table 4.5: Functional Activities of SCC Samples. Functional activity reported in units of activity/mg protein. CuZn values determined by subtracting Mn values from Total values. In the cases where the Mn value was greater than the Total value, the Mn value was adjusted to equal the Total value.

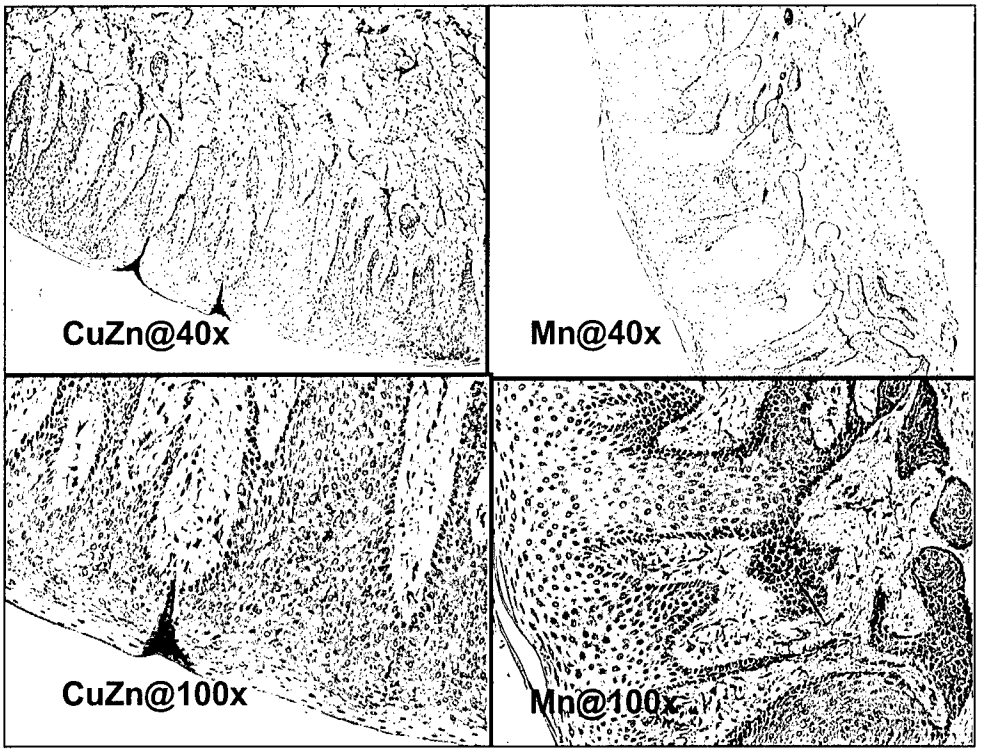

Plate 4.1: Representative histologic sample of non-inflamed normal tissue

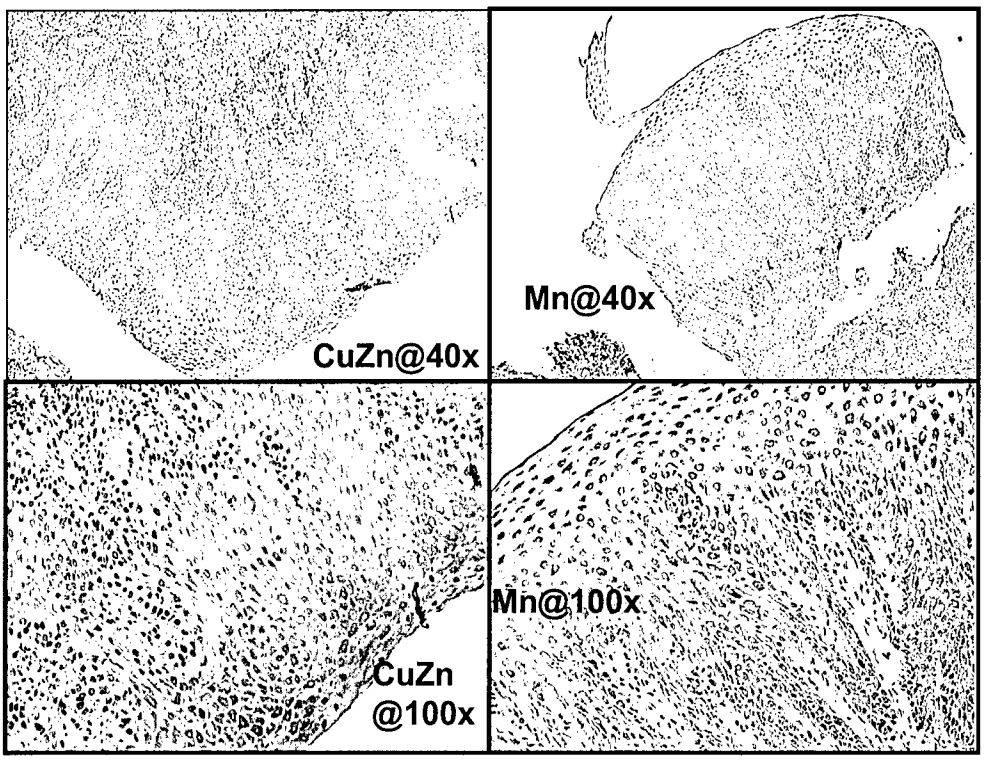

Plate 4.2: Representative histological sample of inflamed normal

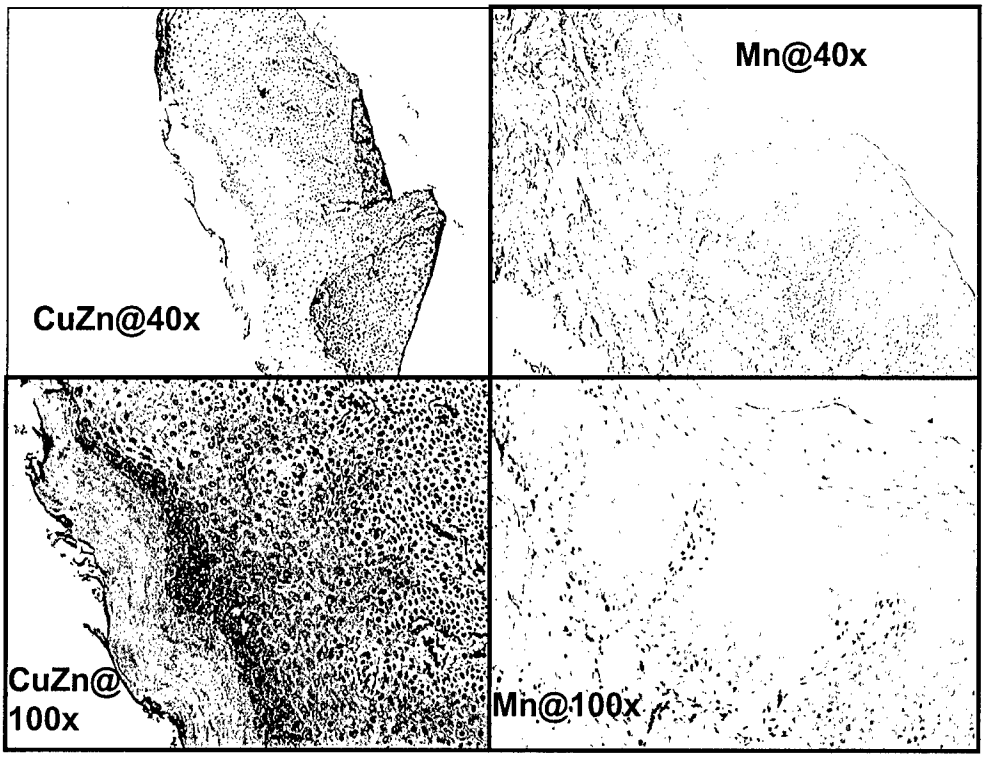

Plate 4.3: Two representative histological samples of oral dysplasia

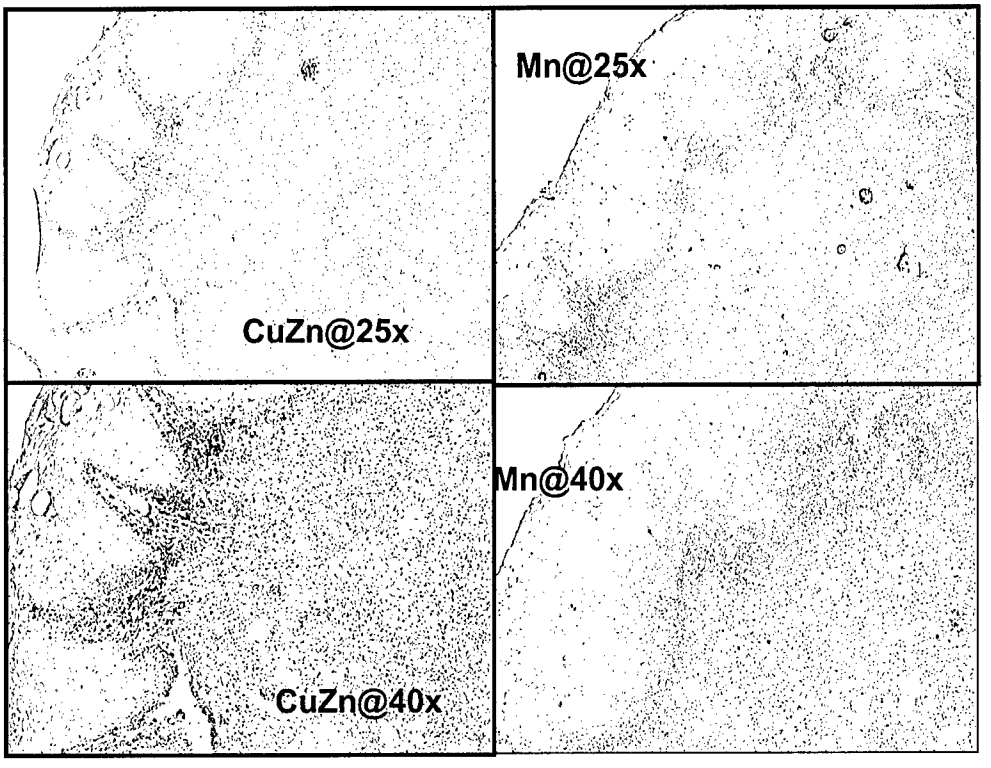

Plate 4.4: Representative histological sample of SCC

I.D. No.	CuZn Activity	Staining Characteristics
H300	16.17	Uniform blush in granular layer, some nuclear stain in basilar layer, epith>c.t.
H500	13.04	Uniform blush in granular layer, some nuclear in basilar layer, epith>c.t.
H700	7.13	Uniform blush in granular layer, epith> c.t.
H900	2.77	Uniform blush in basilar, granular, and stratum layers
H1700	0	Uniform cytosol blush, epith>c.t.
H2300	0	Uniform blush except in corneal layer, epith>c.t.

Table 4.6: Immunohistochemistry Results For CuZnSOD in Non-Inflamed Normals

I.D. No.	CuZn Activity	Staining Characteristics
H200	5.64	Uniform perinuclear punctate staining in granular layer, inflammatory cells in c.t.
H400	5.09	Uniform blush in granular layer, eptih>c.t.
H600	0	N/A
H1000	0.35	Uniform blush throughout epith, epith>c.t.
H1100	2.52	N/A
H1200	4.73	Intense uniform stain except corneal layer, heterogeneity, epith>c.t.
H1300	24.20	Uniform cytosol blush, cellular heterogeneity, epith>c.t.
H1400	10.78	Uniform cytosol blush
H1500	4.46	Uniform cytosol blush, epith>c.t.
H1800	16.46	Intense uniform stain in basilar, granular, and corneal layers, >spinous layers, epith>c.t.
H2000	35.11	Intense in granular layer, epth>c.t., cellular heterogeneity
H2100	8.40	Intense uniform staining in epith
H2500	0	N/A

Table 4.7: Immunohistochemistry Results For CuZnSOD in Inflamed Normals

I.D. No.	Straining Characteristics
66A	Intense granular layer stain with nuclear stain
149	Light blush with nuclear>cytosol, heterogeneity
232	Uniform cytosol blush, greater in granular layer, focal nuclear staining
381	Uniform blush in spinous & granular layers, some nuclear staining
429	Cytosol blush>in spinous, nuclear stain in granular layer
694C	Light cytosol stain, focal nuclear stain, blue granules in cytoplasm & brown nuclei in granular layer
694A	Very light cytosol blush, no granular staining, focal nuclear stain, heterogeneity
694B	Very light cytosol blush, focal nuclear staining
694D	Very faint nuclear staining, muscle stains well, muscle>epith & c.t.
751A	Cytosol blush throughout, blue nuclei

Table 4.8: Immunohistochemistry Results for CuZnSOD in Dysplastic Samples

I.D. No.	CuZn Activity	Staining characteristics
SCC1	2.84	Higher differentiated blush>poorly differentiated blush, heterogeneity surf epith darker
SCC3	3.40	Uniform epith blush
SCC4	0.80	Fat, no epith
SCC4 T	4.50	Intense band of stain in basilar layer, perinuclear & intranuclear stain in tumor cells, heterogeneity
SCC5	0.49	Normal epith
SCC6	0.94	N/A (tissue lost)
SCC8	0	Tumor epith<c.t. due to inflam cells in c.t.
SCC11	0	Very little cytosol stain, some nuclear stain, >stain in inflam & dysplastic cells
SCC14	0	Uniform blush in tumor>c.t., muscle>c.t., tumor=neural
SCC16	0	Uniform cytosolic blush, c.t.=epith
SCC17	0	Light blush throughout

Table 4.9: Immunohistochemistry Results for CuZnSOD in SCC Samples

I.D. No.	MnSOD Activity	Staining Characteristics
H300	8.58	N/A. tissue lost
H500	11.93	Perinuclear punctate, epith>c.t.
H700	56.06	Perinuclear punctate in granular layer, increased in area of inflammation
H900	12.70	Perinuclear punctate in granular layer, epith>c.t.
H1700	44.38	Light blush w/ focal punctated areas, cellular heterogeneity
H2300	24.20	Intense perinuclear punctate full thickness staining

Figure 4.10: Immunohistochemistry Results for MnSOD in Non-inflamed Normals

I.D. No.	MnSOD Activity	Staining characteristics
H200	18.58	Perinuclear punctate in granular layer, some nuclear, >in areas of inflammation, epith>c.t.
H400	39.88	Increased in areas of increased inflammation
H600	25.61	Very little epith, c.t. no staining
H1000	19.14	Perinuclear punctate > in granular layer, epith>c.t .
H1100	23.20	Perinuclear punctate in granular
H1200	34.47	Perinuclear punctate in granular
H1300	36.33	Perinuclear punctate in granular, epith>c.t .
H1400	14.10	Intense perinuclear punctate, epith>c.t.
H1500	39.28	Very light staining
H1800	52.32	Punctated full thickness in inflamed area, light perinuclear in non-inflamed area
H2000	49.36	Perinuclear punctate in granular layer
H2100	45.89	Intense perinuclear punctate full thickness staining
H2500	25.44	N/A

Table 4.11: Immunohistochemical Results for MnSOD in Inflamed Normal

I.D. No.	Staining Characteristics
66A	Light focal perinuclear punctate with no nuclear staining
149	Perinuclear punctate staining, cellular heterogeneity
232	Light focal perinuclear punctate staining, epith>c.t.
381	Focal perinuclear punctate staining, associated with increased exocytosis, salivary ducts stained positive
429	Very light, no nuclear or perinuclear staining
694C	Very little staining
694A	Very light, no perinuclear stain, punctate staining in cytoplasm
694B	Very little stain of epith or c.t., scattered focal punctate staining, muscle stained positive
694D	Focal perinuclear punctate staining in spinous layer, muscle stained positive
751A	Very faint blush throughout epith, scattered peinuclear punctate staining in spinous layer
751B	Punctated blush throughout epith & c.t., focal perinuclear staining in granular layer
857	Light perinuclear staining in granular layer, faint cytosolic blush, muscle stained positive
919	Very faint staining

Table 4.12: Immunihistochemistry Results for MnSOD in Dysplastic Samples

I.D. No.	MnSOD Activity	Staining Characteristics
SCC1	11.98	Normal tissue stains>tumor (light blush), better differentiated stains better, some punctated staining
SCC3	13.76	Very light stain, epith=c.t.
SCC4	6.24	Fat, no stain
SCC4T	19.55	Some focal perinuclear punctated staining, some cells with no stain
SCC5	157.55	N/A
SCC6	5.98	Perinuclear punctated
SCC8	57.40	Stain in granular layer only, lighter than CuZn
SCC11	74.80	Blush in dysplastic normal, very little stain in tumor, most stain in areas of inflammation
SCC14	23.27	Tumor=c.t., muscle>tumor, nerve very weakly stained
SCC16	41.97	c.t.>tumor
SCC17	172.99	Blush throughout, c.t.=tumor, isolated faint perinuclear, cellular heterogeneity

Table 4.13: Immunohistochemistry Results for MnSOD in SCC Samples

CHAPTER 5

DISCUSSION

The functional SOD assay finding that all the tissue (non-inflamed normal, inflamed normal and SCC) samples show the highest proportion of total SOD activity being contributed by MnSOD (which is located within the mitochondria) is consistent with the oral mucosa representing a site of high oxidative mitochondrial metabolism. Mitochondria are exposed to a high risk of free radical damage due to the high percentage of oxidative metabolism (e.g. electron transport system). Therefore MnSOD that is expressed within mitochondria is assumed to play an important role in the protection of mitochondria from free radical damage.[38] Because an increase in MnSOD activity is reflective of ongoing oxidative challenge,[39] the proportionately high mitochondrial distributions in oral tissues, suggests a state of ongoing oxidative stress in the oral cavity. Tissues with high metabolism, e.g., liver and kidney, appear to possess more SOD than do others.[37] The finding that more than 90% of oxygen is consumed in mitochondria and 1% of oxygen is converted to superoxide anions[40] further substantiates that oral mucosal tissues are an important site of oxidative stress. Both the inflamed normal and SCC tissues contain a heavy influx of host inflammatory cells. This corresponds with increases in mitochondrial SOD distributions.

The results of the immunohistochemical studies revealed a generalized increase in intensity of staining for SOD as the amount of inflammation increased. This is in agreement with other studies, which have found a positive correlation between the reactivity for MnSOD in human pituitary lesions and the intensity of histological inflammatory reactions.[34] Several studies have reported that the levels of SOD activity indicate the intensity of the antioxidant defense system in cancer tissue.[28,29,30] It has been reported that the number of mitochondria is often decreased in malignant cells, and mitochondria from malignant cells have been reported to be devoid of MnSOD.[41] Although we found a dramatic reduction in MnSOD protein expression in dysplastic tissues and reduced Mn staining intensity in SCC tumor cells, the SCC samples had the highest SOD functional activities. A possible explanation for the SCC cells decreased staining, yet the SCC tissues having increased functional activity may be the cellular make up of the tumors. The SCC samples were composed of almost entirely of epithelial cells and inflammatory cells. Therefore, although individual cells may possess less SOD, because of the large number of cells and decreased connective tissue, the cumulative effect is an increase in functional activity. Another possibility for the higher MnSOD functional activity in the SCC tissues may be due to the development of a functional epitope. This epitope is not recognized by the antibody and therefore does not stain well.[42] The decreased MnSOD staining in the dysplastic samples may be due to proteins acquiring new antigenic properties because of the appearance of new specific epitopes on the polypeptide chains resulting from oxidative stress.[43]

Our results of immunohistochemical staining were similar to that found by other investigators. CuZn staining occurred as a cytosolic blush in our samples (Plate 4.1). Ojika et al.[27] reported CuZnSOD stained diffusely in the cytoplasm of tumor cells of lung surface epithelial cells and the stromal cells in the endometrium and stained diffusely in the cytoplasm. Mn staining in our sample of tissues had a granular, perinuclear distribution in the granular layer epithelial cells (Plate 4.1). Ojika et al.[27] described a granular pattern of Mn SOD staining in lung SCC tumor cells and Ota et al.[44] reported the Mn enzyme stained with a coarse texture in the cytoplasm of glandular and surface epithelial cells.

The immunohistochemical studies revealed epithelial tissues (either of surface or salivary gland origin) demonstrated markedly greater SOD staining relative to fibrovascular connective tissues. Only scattered cells within the connective tissues (inclusive of endothelial and phagocytes) showed SOD staining positivity (Plate 4.1). By histological examination it was noted that there was a great variability amongst the samples [some SCC samples (Plate 4.4) were entirely comprised of epithelial cells] in regard to the amount of epithelial cells present. Westman and Marklund[37] observed when comparing tissues and corresponding tumors that tumors in general are derived from one cell type, whereas the tissues are composed of several cell types. Muse et al.[45] found large variations in the levels of antioxidant enzymes in different cell types. Again this could help explain the increased SOD activities noted in the SCC samples, which were composed almost entirely of epithelial cells and inflammatory cells, cells known to have high SOD activities.

MacMillan-Crow et al.[39] reported in 1996 on a process (tyrosine nitration) that inactivates MnSOD and may be a general cytotoxic mechanism in many disease states (to include the development of SCC)[32] that are associated with sustained inflammation and nitric oxide synthase expression. This could explain the dramatic decrease in MnSOD staining that occurred in the dysplastic samples. Due to the inactivation of MnSOD by peroxynitrite, the intramitochondrial levels of superoxide increases. This may result in additional cytotoxic effects, and possibly the development of dysplasia.

CHAPTER 6

CONCLUSIONS

In human oral mucosal tissue samples, the total and MnSOD functional activity increased from non-flamed normal to inflamed normal to oral SCC, probably due to increased inflammatory cells with associated increase in ROI. By far, the majority of functional activity was found in the mitochondria (MnSOD). Oral mucosal tissue was found to be one of the highest sites for SOD functional activity. CuZnSOD is found primarily in the cytoplasm while MnSOD is located predominately in the mitochondria. Both forms of SOD are found in the epithelium almost exclusively, with very little found in the connective tissue except for in endothelial cells and phagocytes.

Immunohistochemistry revealed markedly reduced staining for SOD in dysplastic tissues and a mildly reduced staining in SCC when compared to non-inflamed or inflamed normal oral mucosa, revealing a decreased ability to cope with the superoxide radical and its deleterious effects.

Although the results from this study were not statistically significant due to the small sample sizes and the known heterogeneity of SOD levels in human cells, trends were identified that may be useful in helping to develop chemopreventive agents against oral SCC and dysplasia. Since the oral mucosa is high in SOD activity and the epithelium is the primary site of action, delivery of a chemopreventive agent by use of a

bio-adhesive patch, oral troche or through the saliva could be beneficial in preventing dysplastic oral mucosa from developing into oral SCC.

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