Heparanase, a Potential Marker for Premalignant Oral Cavity Cancer

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Abstract. *Background/Aim: In the past we have shown that the heparanase gene expression significantly correlates to oral cancer patient survival. Our aim was to study heparanase expression in all stages of carcinogenesis. Materials and Methods: Heparanase expression (mRNA and protein), as well as its enzymatic activity were studied separately in the nucleus and cytoplasm of both normal and cancerous cells using an in vivo oral cancer mouse model. Results: Heparanase nuclear expression was associated with normal tissue; at the time carcinogenesis is initiated heparanase translocates to the cytoplasm and increases protein expression and enzymatic activity, as the cancer progresses. Heparanase overall expression is increased in cancer formation from premalignant to invasive squamous cell carcinoma (SCC). Conclusion: Heparanase is suggested to be a prognostic and diagnostic marker for oral premalignant lesions which could have a major impact on future prognosis and diagnosis of SCC of the oral cavity.*

Oral cancer accounts for 2.3% of malignancies in the United States and has one of the lowest five-year survival rates. The overall prognosis for oral cancer patients is poor, with 5- and 10-year survival rates of 60% and 48%, respectively (1). The majority of oral cancers (~90%) are SCC - malignant epithelium transformation (2). Cancers of the oral cavity and pharynx have been etiologically-linked to the individual's exposure to known carcinogens in tobacco and alcoholic beverages, which are estimated to account for 75% of all cancers of the oral cavity and pharynx in the United States (3). Since cancer formation is a multi-step process and due to

possible constrains in availability of large amounts of human tissues from multiple stages of oral carcinogenesis, including normal tissues, alternative studies on *in vitro* models are being widely used. Alternative models for investigation of cancer include *in vitro* models, *in vivo* models or both. A disadvantage of *in vitro* studies is the difference between physiological processes *in vivo* and processes *in vitro*, thus giving incomplete or misleading results. 4-nitroquinoline-1-oxide (4NQO) is a water soluble carcinogen. When applied to animal oral tissue it exerts potent intracellular oxidative stress and its metabolic products bind to DNA, predominantly at guanine residues. The results of this insult appear similar to damage imposed by tobacco, which is a major risk factor for oral cancer formation. In addition, 4NQO exhibits similar histological, as well as molecular changes observed in human oral carcinogenesis (4). When comparing the 4NQO model to other carcinogenesis models such as cell lines, nude mice models and to the 7,12-dimethlbenz(a)anthrance (DMBA) model, the main advantage of the 4NQO model is its similarity to the physiological process. The drawback of the nude mice model is the lack of an immunocompetent component. The disadvantages of the DMBA model are dissimilarity of the tumors with the human equivalent (4) and an inflammatory response and necrosis, making it difficult to study early squamous lesions (4, 5). As opposed to DMBA, 4NQO does not induce an inflammatory or necrosis response (6). In the 4NQO model, both the cell line and the nude mice models are less time-consuming and cell line experiments are not as expensive and readily available. The sequential stages of carcinogenesis - hyperplasia, dysplasia, severe dysplasia, *in situ* carcinoma and SCC - are all induced by 4NQO. This sequence of stages is similar both histologically and molecularly to human oral carcinogenesis (4, 5, 7). Heparan sulfate proteoglycans (HSPGs) are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of tissues (8, 9). HSPGs play a critical role in development (10), cytoskeleton organization, cell-to-cell and cell-to-ECM interactions (11-13). Over the past few decades it was hypothesized that HSPGs are involved in inhibition of invasion of tumors due to their

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promotion of cell-to-cell and cell-to-ECM adhesions (14). This hypothesis is supported by the reduction in expression of heparan sulfate (HS) in transformed cells (15-18), a reduction that correlates with increased metastatic capability (19-21). Heparanase is an endo-β-glucuronidase that specifically cleaves HS side chains of HSPGs (22-24). Enzymatic degradation of HS leads to disintegration of the ECM and is, therefore, associated with tissue remodeling and cell migration that includes inflammation, angiogenesis and metastasis (23, 25, 26). Moreover, the heparanase protein exerts non-enzymatic activities that further promote tumor angiogenesis, growth, survival and dissemination (27). Recently, we have demonstrated heparanase overexpression in human oral carcinomas. This overexpression was associated with tumor differentiation level and inversely-correlated with patient survival. We also showed that while cytoplasmic localization of heparanase was associated with high-grade carcinomas and poor prognosis of the patients, nuclear localization of the enzyme was found primarily in low-grade, well-differentiated tumors which exert good patient prognosis (9). In the current study we established an *in vivo* oral cancer mouse model, that exhibits all the different stages of cancer formation, including hyperkeratosis, early dysplasia, moderate and severe dysplasia, *in situ* carcinoma and invasive SCC. The aim of the present study was to investigate the expression and enzymatic activity of heparanase in the different stages of oral carcinogenesis and to further establish its role in cancer formation and prognosis.

Materials and Methods

Animals and treatment. Male wild-type C57BL/6J mice (8 weeks old) were housed 5 per cage in our animal facility in a 12-h light-dark cycle. Animals were allowed free access to drink (tap water or 4NQO water (50 µg/ml, Sigma, St. Louis, MO, USA)). All procedures involving the use of mice were in accordance with the Institutional Animal Use and Care Committee (Technion, Israel). Mice were divided into four groups after one week of acclimation. Group 1 (n=10) was given either normal drinking water or 4NQO water for 6 weeks (five served as control and five were given the carcinogenic water). Group 2 (n=10) was given either normal drinking water or 4NQO water for 12 weeks. Group 3 (n=10) was given either normal drinking water or 4NQO water for 18 weeks. Finally, group 4 (n=10) was given either normal drinking water or 4NQO water for 30 weeks. The overall experimental period was 30 weeks. 4NQO treatment of the experimental groups was given at a concentration of 50µg/ml to all the age groups. The body weights were monitored bi-weekly and health status was examined daily until the end of the experiment.

Tissue processing and histopathological analyses. At each time frame (6, 12, 18, 30 weeks) 10 mice were sacrificed using the cervical dislocation method; the tongue was harvested and examined for the presence of macroscopic alterations (Figure 1), then split 3 ways: one third of the tongue was snap frozen in liquid nitrogen for protein extraction and nuclear and cytosolic separation, another third was immersed in Tri-Reagent® and processed for mRNA extraction

according to the manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH, USA) and the last third was fixed overnight in 10% neutral-buffered formalin, transferred to 70% ethanol, processed and embedded in paraffin for histopathology.

Heparanase immunostaining. Staining of formalin-fixed, paraffin-embedded 5-micron sections for heparanase was performed essentially as described (9). Briefly, slides were de-paraffinized with xylene, rehydrated and endogenous peroxidase was quenched by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling in citrate buffer, pH 6, blocked with 10% normal goat serum and incubated with anti-heparanase monoclonal MIAO antibodies diluted 1:100-200 in blocking solution (Vlodavsky, Haifa, Israel). Slides were then extensively washed and incubated with a secondary reagent (Envision kit) according to the manufacturer's (Dako, city, CA, USA) instructions. Following additional washes, color was developed with the 3-amino-9-ethylcarbazole (AEC) reagent (Sigma-Aldrich), sections were counterstained with hematoxylin and mounted, as described (9). Slides were scored as 0-negligible staining, 1-weak staining and 2-strong staining, while the specific intracellular localization was recorded.

Real time quantitative-polymerase chain reaction (PCR). Total RNA was extracted using Tri-Reagent® and according to the manufacturer's protocol. The total RNA was subjected to reverse transcription according to the manufacturer's protocol (superscript RNase H- Reverse Transcriptase kit, Invitrogene, Carlsbad, CA, USA). For quantification, heparanase PCR standards were prepared. Standards were diluted to yield serial dilutions in order to produce an appropriate standard curve. The fluorescent signal was plotted in real time and the relative quantification of the heparanase mRNA was established.

Heparanase enzymatic activity assay. Preparation of sulfate labeled ECM: To evaluate the enzymatic activity of heparanase in the nucleus and the cytoplasm of the mice tongue's cells we used a 35S-labeled ECM. Bovine corneal endothelial cells were plated into 35-mm tissue culture dishes and cultured in Fischer's medium (sulfate low) DMEM supplemented with 10% FCS and 5% newborn calf serum (Beit-Haemeck, Israel). Also 4% dextran T-40 was included in the growth medium (28, 29). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. Nearly 80% of the radioactivity is usually incorporated into HSPGs (28, 29).

Enzymatic activity: Cell fractionation was carried out utilizing NE-PER® nuclear and cytoplasmic extraction reagents according to the manufacturer's (Pierce, Rockford, IL, USA) instructions. Cytosolic and nuclear fractions were derived from the different stages of mouse tongue cancer and also normal tongue tissue prepared from the control groups. The different fractions and matched control cells were incubated (15 h, 37°C, pH 5.8) with sulfate-labeled ECM, as described (28, 29). The cytosolic and nuclear fractions were incubated with the ECM for 15 h in order to allow the enzymatic reaction to proceed beyond the linear phase. Under these conditions, more than 80% of the total labeled substrate is degraded, precluding quantitative estimation of the actual amount of cytosolic vs. nuclear heparanase. To evaluate the occurrence of proteoglycan degradation, the incubation medium is collected and applied for gel filtration on
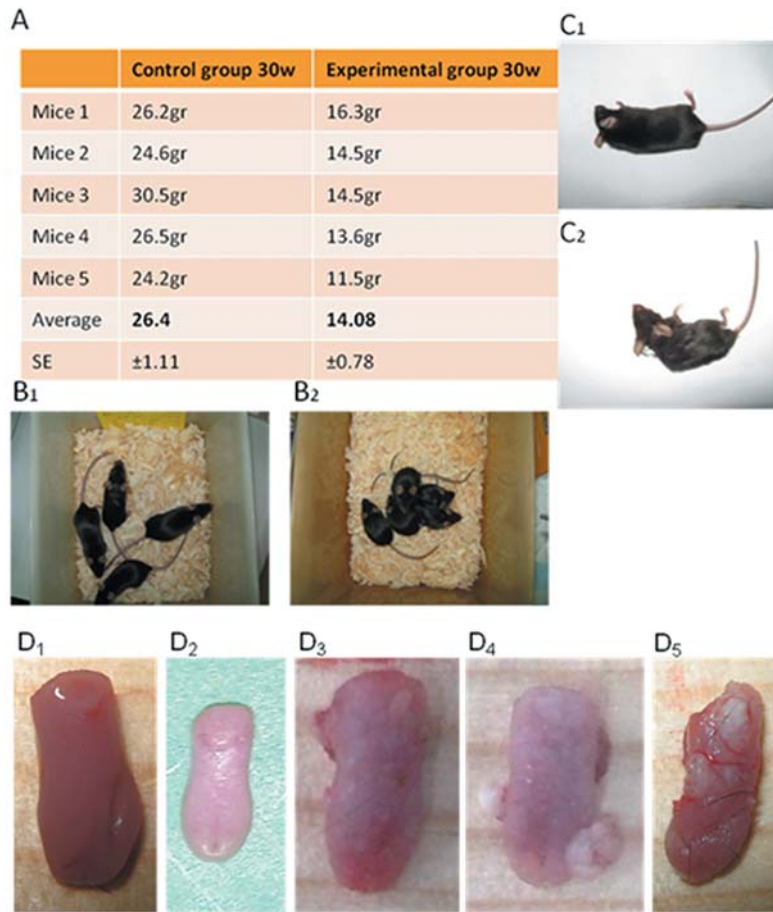
Figure 1. *Establishing the 4NQO model. A, Weight of mice from the control and experimental groups 30 weeks post-4NQO treatment; B_1, Group dynamic of mice from control group; B_2, Group dynamic from experimental group 30 weeks post 4NQO treatment. C_1, external features of the control group; C_2, external features of experimental group 30 weeks post 4NQO treatment; D_1 Normal tongue; D_2, 6 weeks; D_3, 12 weeks, D_4, 18 weeks; D_5, 30 weeks post 4NQO treatment.*
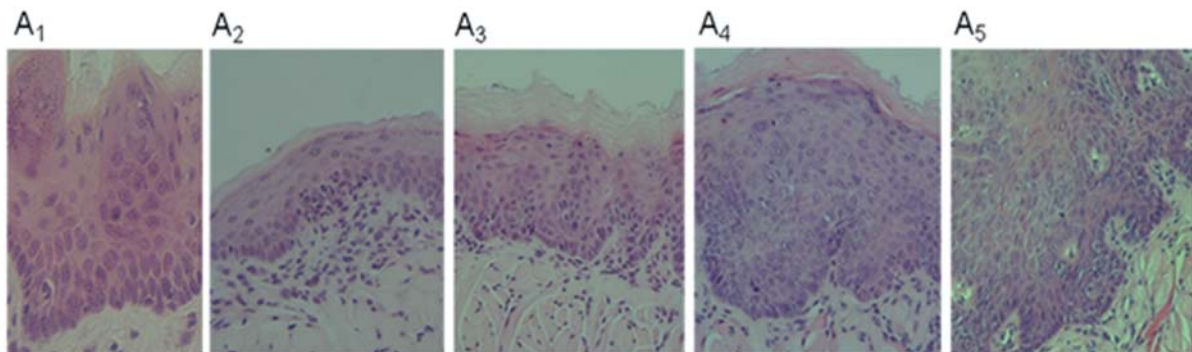

Figure 2. *Histology of the different tongue cancer stages as created in the model. A_1, Normal; A_2, Mild dysplasia; A_3, Moderate dysplasia; A4, Carcinoma in situ; A5, Squamous cell carcinoma.*

Sepharose 6B columns (0.9×30 cm). Fractions (0.2 ml) are eluted with PBS at a flow rate of 5ml/h and counted for radioactivity in a β-scintillation counter (Phosphor Imager, FLA 7000, GE, New York, USA). Degradation fragments of HS side chains are eluted from Sepharose 6B at 0.5<Kav<0.8 (peak II). Nearly intact HSPGs is eluted at Kav<0.2 (peak I) (29, 30).
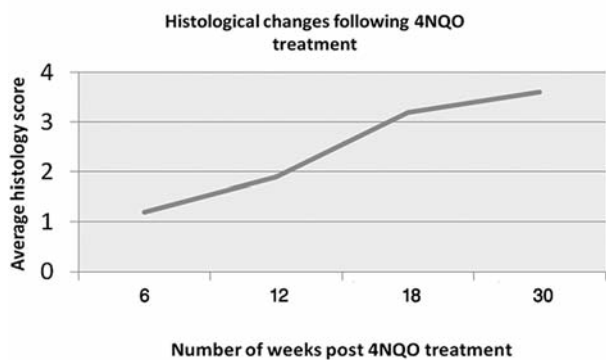
Figure 3. *Histology score of samples from the different experimental groups. Average of the histology score given to each sample. 0, Normal; 1, Mild dysplasia; 2, Moderate dysplasia; 3, Severe dysplasia/Carcinoma in situ; 4, Early squamous cell carcinoma invasion; 5, Squamous cell carcinoma.*

Figure 4. *Pathology score representing the extent of heparanase expression in samples from the different experimental groups. Average of the heparanase extent expression score given to each sample. 0, No expression; 1, Mild expression; 2, Moderate expression; 3, High expression.*

Statistical analyses. The student's *t*-test for unpaired observations was used (Microsoft® office Excel). Values are given as mean±S.E. *p*-Values less than 0.05 were considered as statistically significant. In the real time PCR evaluation, in order to overcome normal gross fluctuations in gene expression levels observed between individual animals, heparanase expression levels were normalized to the expression of a reference gene (*Rplp0*) and presented as fold increase over control.

Results

4NQO-induced oral carcinogenesis. Mice were treated as described in 'Materials and Methods' Section. When examining the features of the mice there are big differences in appearance and weight, as described in Figure 1. The mice in the experimental group, 30 weeks post-transition to 4NQO containing drinking water, appear to have abnormal hair orientation, they are underweight and have a tendency to huddle together. The average weight of the control group was 26.4 g as compared to 14.08 g in the experimental group. Different stages of cancer can be noticed macroscopically (Figure 1D) as well as histologically (Figure 2), thus further establishing this model as an appropriate tool for cancer research. Upon histological examination, each sample was given a score in a scale of 0 to 5, 0 representing normal and 5 representing SCC. The average results for each group were compared to the rest of the experimental groups (Figure 3). As expected, from 6 weeks of 4NQO addition to drinking water the epithelial changes gradually worsened until carcinoma was dominant in the entire experimental mice group at 30 weeks of 4NQO treatment.

Heparanase protein expression. Using a monoclonal as well as polyclonal antibodies in an indirect immunohisto-chemistry staining, the expression extent of the heparanase protein in each
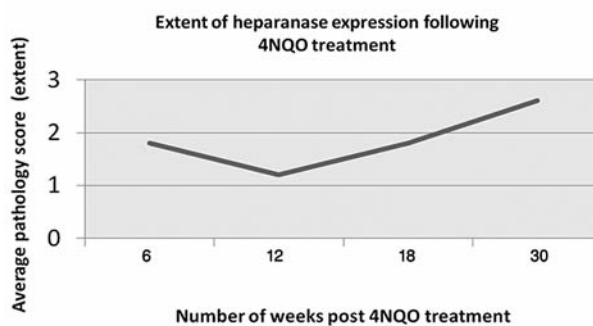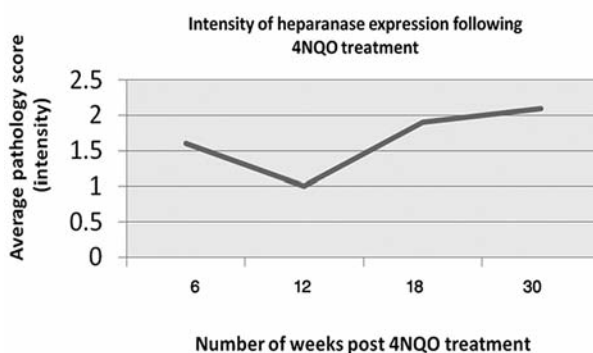

Figure 5. *Pathology score representing the intensity of heparanase expression in samples from the different experimental groups. Average of the heparanase intensity expression score given to each sample. 0, No expression; 1, Mild expression; 2, Moderate expression; 3, High expression.*

of the samples was evaluated and each sample was given a score in a scale of 0 to 3, 0 representing negligible-no expression and 3 representing high expression (extent as well as intensity). The average scores in each group were compared to the rest of the experimental groups (Figure 4). Using the same assay, the intensity of the heparanase protein expression was evaluated. The same score scale of 0 to 3 was used (Figure 5). Heparanase protein expression in the different experimental groups showed a dynamic expression histologically (Figure 6). In early dysplastic changes, heparanase appears to be expressed both in the cytoplasm and nucleolus, as opposed to later stages, where heparanase expression is more dominant in the cytoplasm of the cells (Figures 6 and 7). The control mice were also stained for heparanase protein expression (Figure 8). In this group, when observing the tongue epithelium, all age groups demonstrated expression which appeared dominantly in the nucleus and to a lesser extent in the cytoplasm.
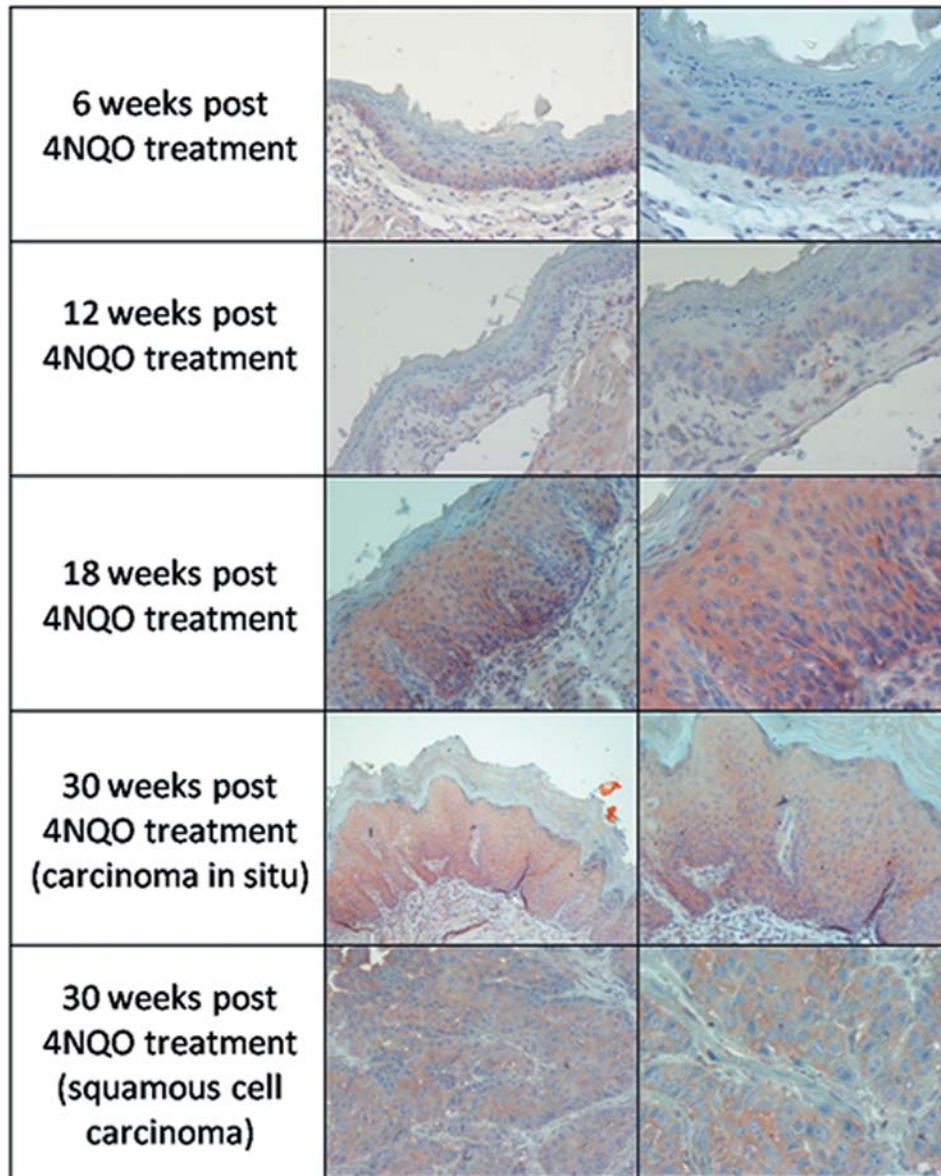
Figure 6. *Indirect immunohistochemistry exhibits heparanase protein expression in the different experimental groups. The right column is a magnification (×300) of the middle column (×100). Red staining represents heparanase expression.*

Heparanase enzymatic activity. After establishment of the expression of heparanase in the different stages of carcinoma development, the enzymatic activity of the protein was analyzed both in the control groups (Figure 9) and the experimental groups (Figure 10). Due to the different protein expression patterns in the nucleus and cytoplasm in different stages of carcinoma, the activity of heparanase protein was then measured in each of them separately (Figure 9B, Figure 10B). Following separation of nuclear and cytoplasmic total proteins (NE-PER®), heparanase enzymatic activity was evaluated by using a 35S-labeled ECM. The incubation medium (1 ml) containing sulfate-labeled degradation fragments were subjected to gel filtration on Sepharose CL-6B columns and the radioactivity of the different fractions was counted in a β-scintillation counter. Heparanase activity in the control groups did not change dramatically, the pattern of activity showed a decrease from 6 weeks to 12 and 18 weeks and increased from 18 weeks to 30 weeks from the start of the experiment. In addition, heparanase activity was higher in the nucleus throughout the experiment. In the experimental groups, there was a decrease in activity between 6 weeks and 12 weeks (as was seen in the intensity and extent of the protein expression;

Figures 4 and 5) and an increase between 12 weeks to 18 and 30 weeks. As noted in the immunohistochemical results, heparanase activity was higher in the cytoplasm when compared to the nucleus in the experimental groups.

Quantification of heparanase mRNA. Heparanase mRNA expression was quantified during the different stages of carcinogenesis using real-time PCR analysis (Figure 11). Heparanase mRNA expression was found to be significantly higher in the experimental 4NQO group compared to the control groups throughout the experimental at 12-, 18- and 30-week time points ($p<0.008$, $p<0.02$, $p<0.001$, respectively). The mRNA quantification did not show a significant increase from 6 weeks till 30 weeks of 4NQO treatment.

Discussion

HS glycosaminoglycan is one of the most important components of the ECM, basement membranes and cell surface molecules. Various molecules bind to the sulfated
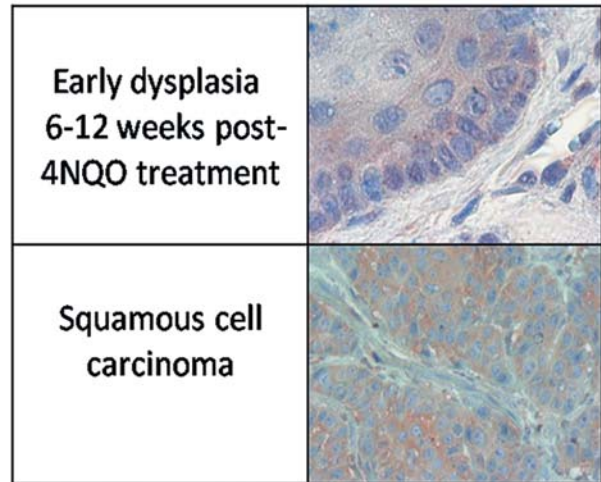

Figure 7. *Indirect immunohistochemistry exhibits heparanase protein expression in early dysplasia and squamous cell carcinoma. Red staining represents heparanase expression; notice that heparanase is predominantly expressed in the cytoplasm in the stages of squamous cell carcinoma formation.*
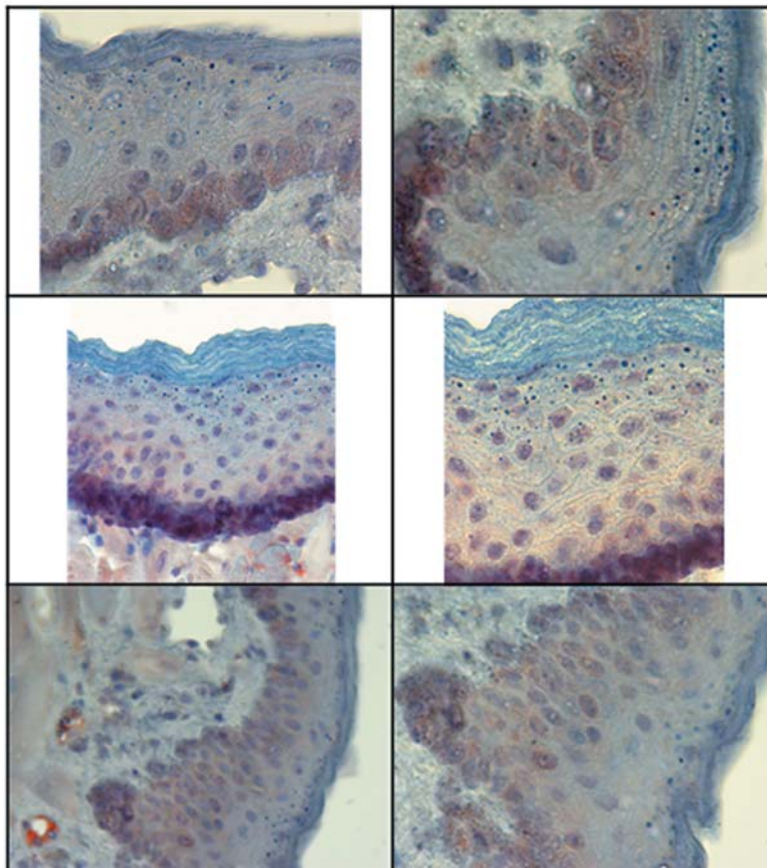

Figure 8. *Indirect immunohistochemistry exhibits heparanase protein expression in control groups. The right column is a magnification (×300) of the left column (×300). Red staining represents heparanase expression; notice heparanase is predominantly expressed in the nucleus of the normal tongues.*
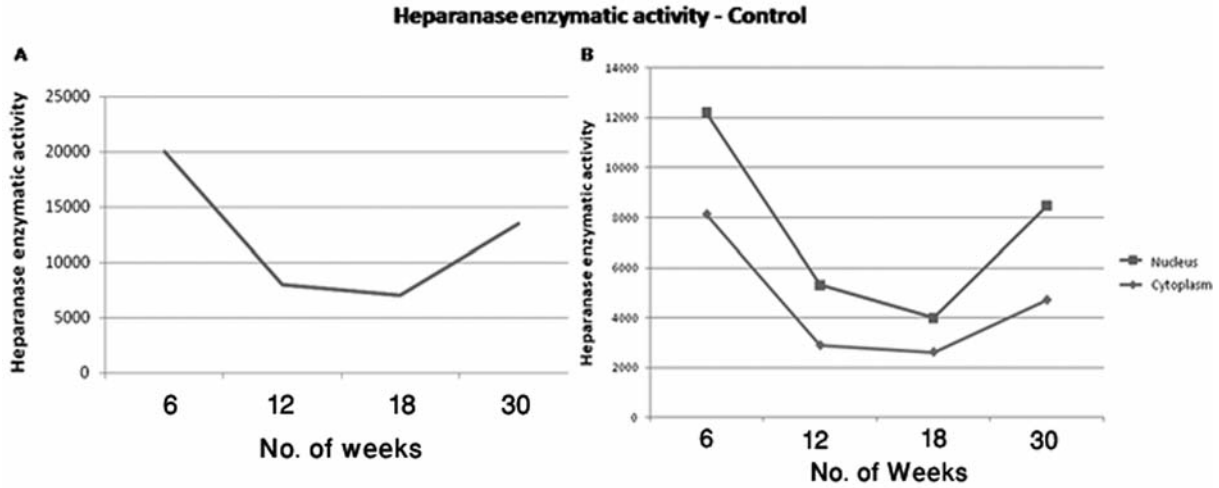
Heparanase enzymatic activity - Control

Figure 9. *Heparanase protein enzymatic activity in the control groups. A, Total heparanase enzymatic activity; B, cytoplasmic vs. nuclear distribution of heparanase activity to the nucleus and cytoplasm; notice that heparanase enzymatic activity is higher in the nucleus compared to the cytoplasm of the control group.*
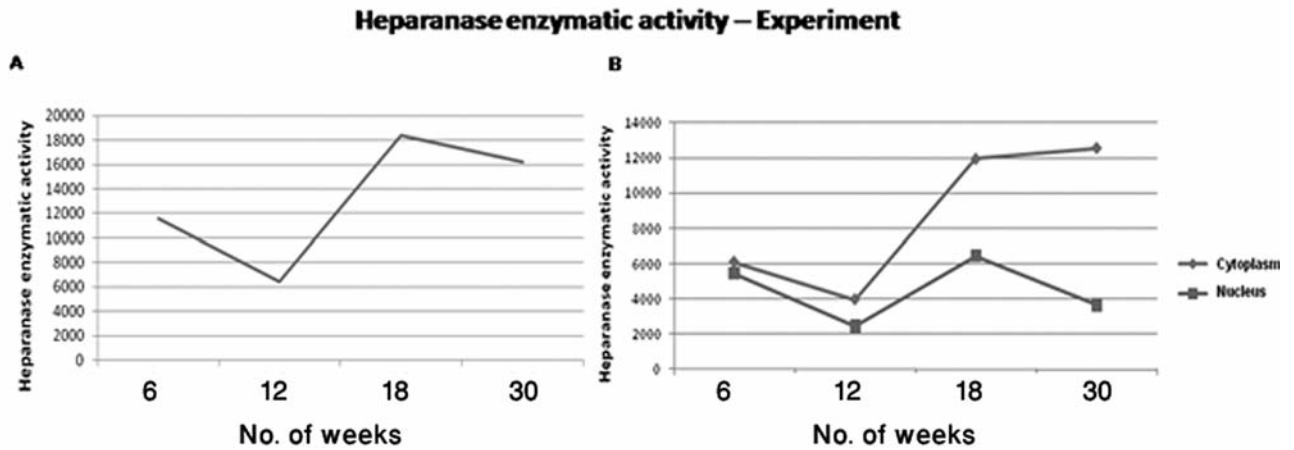
Heparanase enzymatic activity – Experiment

Figure 10. *Heparanase protein enzymatic activity in tongue squamous cell carcinoma. A, Total heparanase enzymatic activity; B, cytoplasmic vs. nuclear distribution of heparanase activity to the nucleus and cytoplasm; notice that the enzymatic activity of heparanase is higher in the cytoplasm as cancer progresses.*

saccharide domains and thus to the ECM. Heparanase is the sole HS degrading endoglycosidase. Remodeling of the ECM is important for several processes, among them morphogenesis, tissue repair, inflammation, vascularization and cancer metastasis (11, 31-33). During tumor development, modification of the ECM is essential. In the present study we have further established 4NQO as a suitable carcinogen, when added to drinking water, for oral cancer study model. We have shown the induction and development of SCC of the tongue and all its different stages, both macroscopically and histologically. The histological changes have similar appearance to those seen

in human squamous carcinomas, as previously described (7). In our study normal tongue tissue showed heparanase expression dominantly in the nucleus, while in carcinoma stages heparanase is dominantly found in the cytoplasm of the cells; these results further establish our previous observations that the expression of heparanase in the nucleus is associated with good prognosis of oral cancer patients and cytoplasmic translocation is associated with poor cancer patient prognosis (9). The behavioral changes and weight reduction observed in the experimental group are expected in late stages of the disease (34, 35). When examining the extent and intensity of heparanase protein

Quantification of Heparanase mRNA Expression in The Different Stages of Carcinoma genesis, Compared to the Control Group
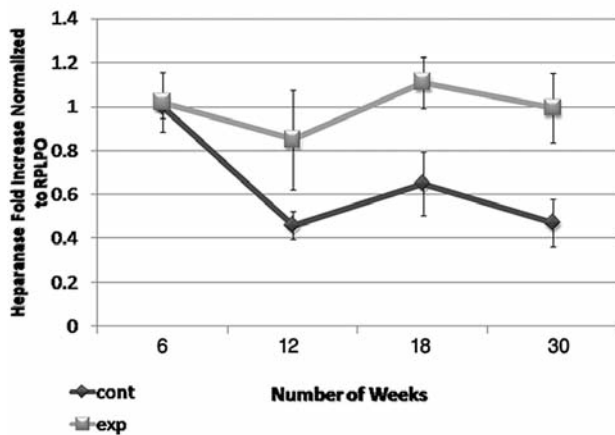

Figure 11. *Real-time PCR analysis of mRNA showing the expression of heparanase mRNA. mRNA levels were measured in the nucleus and in the cytoplasm at 6, 12, 18 and 30 weeks post-4NQO treatment. The results were normalized to large ribosomal protein (RPLPO). Notice that the overall mRNA expression in the experimental carcinogenesis group is significantly higher (p<0.05) compared to the control group.*

expression, as well as its activity, we can notice a decrease followed by an increase in expression during the late carcinogenesis stages (severe dysplasia, carcinoma *in situ* and oral SCC). Different oncogenes can be expressed at different stages of carcinogenesis. This finding is interesting and one might hypothesize that heparanase belongs to the late-stage expressed oncogenes; further studies are needed to establish this hypothesis. Histologically, heparanase protein expression was dynamic in the different experimental groups. The expression appears to shift from the nucleus to the cytoplasm with the advancement in carcinogenesis. When measuring the heparanase mRNA levels, as was predicted, heparanase overall expression is significantly higher ($p<0.05$) in the carcinogenesis (experimental) group compared to the control group in all the different mice group ages, yet there was no significant changes between the group ages in the mRNA level implying that, as shown in our previous studies, heparanase is an important molecular marker for oral cancer formation and the proteins' translocation to the cytoplasm as well as its increase in enzymatic activity is found, in the present study, to be an ominous sign of cancer progression.

To summarize, the expression and activity of heparanase is slightly diminished during the early stages of carcinogenisis (hyperkeratosis and hyperplasia) and elevated during the late stages (dysplasia, carcinoma *in situ* and invasive SCC), which could imply its possible role as a late

oncogene. Heparanase enzymatic activity and expression was shown predominantly in the nucleous of normal tongue tissue and, as carcinogenesis progresses, heparanase expression and enzymatic activity are dominantly found in the cytoplasm of these cancerous cells. These findings, combined with those of our previous study (9), indicate that heparanase cellular localization and enzymatic activity has an important role in cancer patients' prognosis from premalignant to malignant SCC. Heparanase might have the potential to be used as a prognostic and diagnostic marker for oral premalignant lesions which would have a major impact on the future prognosis and diagnosis of SCC of the oral cavity.

Acknowledgements

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