ORIGINAL ARTICLE

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INDUCIBLE NITRIC OXIDE SYNTHASE: CORRELATION WITH EXTRACAPSULAR SPREAD AND ENHANCEMENT OF TUMOR CELL INVASION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract: *Background.* Extracapsular nodal spread is a major prognostic indicator in head and neck cancer. Nitric oxide (NO), primarily produced by the enzyme inducible NO synthase (iNOS), has a large number of actions in cancer biology, but no studies have investigated its possible role in extracapsular spread or tumor invasion.

Methods. Immunochemistry was used to study iNOS expression in 48 patients with either extracapsular or encapsulated metastasis. In vitro invasion assays were performed using H357 (an oral squamous cell carinoma cell line) using the iNOS inhibitor drug, 1400 W.

Results. iNOS expression was significantly associated with extracapsular spread, with 22/27 cases showing positive iNOS expression compared with 8/21 cases in the encapsulated group ($p = .01$). Invasion of H357 cells was inhibited by 1400 W at concentrations of 100 μM and 1 m*M* ($p = .002$, $p = .003$).

Conclusion. iNOS protein seems to be associated with extracapsular spread and invasion in head and neck cancer. Further studies are required to understand this role more fully. ©2007 Wiley Periodicals, Inc. *Head Neck* **30:** 208–214, 2008

Keywords: lymph node; extracapsular; head and neck; nitric oxide; invasion

The prognosis of patients with mucosal associated head and neck squamous cell carcinoma (HNSCC) falls significantly with the involvement of metastatic cervical lymph nodes.[1] The risk of recurrent disease and death is increased further in patients who are found to have extracapsular spread.[2–5] In particular, macroscopic extracapsular spread is an independent adverse prognostic factor for survival.[2] Extracapsular spread is noted

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in the majority of lymph nodes greater than 3 cm and in a significant number of nodes less than 2 cm. It has even been demonstrated in lymph nodes measuring less than 1 cm.[6,7] Following arrival of the metastatic cells in the lymph node, they must produce angiogenic factors at an early point if they are to survive.[8] Furthermore, they have to secrete various enzymes that degrade the extracellular matrix.[9] There are many proteolytic enzymes involved in this process, including several isoforms of the matrix metalloproteinases family (MMP).[10]

There has been a great deal of interest in the role that the signaling molecule, nitric oxide (NO) plays in tumor biology. NO, produced by the enzyme inducible NO synthase (iNOS), has been shown to assist both angiogenesis and tumor growth.[11,12] Once induced by various cytokines, including tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κ B), interleukins 1, 10, and 12, iNOS produces much greater NO concentrations than the constitutively expressed endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS), which are dependent on raised local calcium concentrations for activity.[13] In head and neck cancer, iNOS has been found in primary tumors to have an adverse affect on tumor biology.[14,15] It has also been found that iNOS status correlates with lymph node metastasis in oral squamous cell carcinoma.[16] NO, produced by iNOS, also influences the expression of MMP in head and neck cancer. Acting via a p53 mutation, it is likely that raised NO results in increased angiogenesis and invasiveness via this pathway, ultimately contributing to tumor progression.[17]

To date, there are no studies that have investigated the expression of iNOS in lymph node metastasis (both encapsulated and extracapsular) in patients with HNSCC. Furthermore, to our knowledge no articles have been published on the possible role of iNOS in tumor cell invasion in HNSCC. In this study, we investigated iNOS expression using immunohistochemistry in patients with either encapsulated or extracapsular positive nodal disease. We also performed in vitro invasion assays using the cell line H357, derived from an oral squamous cell carcinoma, to assess the effect of iNOS inhibition on tumor cell invasion.

MATERIALS AND METHODS

Immunohistochemistry. Patients were identified from our database those who had undergone neck dissection with positive nodal disease for known primary HNSCC, and were initially grouped into encapsulated disease and extracapsular spread. Nodal metastasis greater than 2 cm was excluded together with patients who had lymph nodes containing both encapsulated disease and extracapsular spread. To obtain comparable study groups, the primary tumor sites were matched as closely as possible, and a small number of unknown primary tumor cases were included for completeness.

Representative lymph nodes were chosen following staining of sections with hematoxylin and eosin. These were then stained using a monoclonal antibody against iNOS (BD Transduction Laboratories, Oxford, UK). We have previously shown that this antibody is specific for the iNOS isoform.[16]

Sections were placed onto positively charged slides, heated to 50°C for 40 minutes, dewaxed in xylene for 10 minutes, and then rehydrated in alcohol solutions and placed in 5% hydrogen peroxide in absolute alcohol (to block endogenous peroxidase activity) and finally water to complete rehydration. For antigen retrieval, the slides were boiled in a pressure cooker for 2 minutes using freshly prepared 0.05*M* citrate buffer (pH 6.0). Sections were treated with normal goat serum at a dilution of 1/10 with 1 drop/mL of avidin block for 20 minutes and incubated at room temperature for 1 hour with iNOS monoclonal antibody at a dilution of 1/100. The sections were then rinsed with Tris-buffered saline (TBS) and incubated with biotinylated anti-mouse immunoglobulin (Dako, Glostrup, Germany) at a dilution of 1/200 for 30 minutes. After rinsing, the slides were treated with avidin–biotin-peroxidase complex (Dako) for 30 minutes. Immunostaining was developed using diaminobenzidine (DAB), and the slides were rinsed with distilled water and counterstained with Mayer's hematoxylin.

Controls. An oral squamous cell carcinoma known to have high iNOS expression and activity was used as a positive control.[16] Negative controls were treated in the same manner, but with the omission of the primary iNOS antibody to check for nonspecific secondary antibody staining. Additionally, an irrelevant p53 antibody (clone DO-7, BD, Oxford, UK) with the same isotype as iNOS (IgG2a) was used at the same concentration to check for nonspecific primary antibody staining. Samples from 5 patients with no evidence of metastatic nodal disease following neck dissection were also stained for iNOS to check for nonspecific staining.

Scoring. Each patient was scored on a scale that related to the approximate percentage of cells that stained and measured as follows: 0 = no staining in any field; 1+ = less than 25% of tissue stained; 2+ = between 25% and 50% stained; 3+ = between 50% and 75% stained, and 4+ = more than 75% stained. Using a conference microscope, the entire section was visualized by 2 observers (P.A.B. and S.D.), and 4 random areas (magnification 100×) were chosen, giving a possible score range of 0 to 16. The intensity of staining was documented by comparing the samples at high power (200×) with the positive control sections, according to the following scale: 0 = none; 1+ = weak staining; 2+ = moderate staining; 3+ = intense staining. As staining intensity is known to vary between batches of samples, and is dependant on many variables, the intensity of staining was used for descriptive purposes only and scores were not included in the statistical analysis.

Cell Culture. H357 cell line, derived from a squamous cell carcinoma of the tongue, was used in this study.[18] Cells were grown in keratinocyte growth medium (KGM) as described previously.[19]

Invasion Assays. Cell invasion assays were performed using Matrigel-coated polycarbonate filters (8-μm pore size, Transwell, Beckton Dickinson) as previously described (Thomas et al, 2001).[19] Matrigel (70 μL; 1:2 dilution in α-MEM) was added to the upper membrane and allowed to gel for 1 hour at 37°C. To act as a chemotactant, 500 μL of KGM was placed in the lower chamber. H357 cells were plated in the upper chamber of quadruplicate wells at a density of 5×10^4 in 200 μL of α-MEM. For blocking experiments, the iNOS inhibitor, 1400 W (at concentrations of 100 μ*M* or 1 m*M*, supplied as a gift from Glaxo Welcome Laboratories, Stevenage, UK), was added to cells 30 minutes prior to plating and was also present in the KGM in the lower chamber throughout the experiment. After 72 hours, the cells in the lower chamber (including those attached to the undersurface of the membrane) were trypsinized and counted on a Casy 1 counter (Sharfe System GmbH, Germany). H357 cells without 1400 W added to the culture medium were used as controls. The effect of 1400 W on tumor cell invasion was recorded as a percentage relative to the control group.

Growth Assays. Cell growth assays were performed in 24-well plates. 1×10^4 cells were plated in KGM containing the iNOS inhibitor, 1400 W, at concentrations of 100 μ*M* and 1 m*M*. At various time points, the cells from triplicate wells were trypsinized and counted on a Casy 1 counter (Sharfe System GmbH, Germany).

Table 1. Group demographics.

Characteristic	Extracapsular (n = 27)	Encapsulated (n = 21)
Mean age, y	65	63
Sex (M/F)	18:9	12:9
Primary site		
Lip and oral cavity	12	9
Larynx	6	3
Pharynx	5	6
Unknown	4	3

Note. Values represent number of cases, except as otherwise stated.

Statistical Analysis. The difference in iNOS expression between extracapsular and encapsulated disease was assessed using chi-square test for categorical data (positive or negative for iNOS expression) and Mann Whitney U test for nonparametric ordinal data (total score for iNOS expression 0–16). The level of significance for the tests was chosen to be $p < .01$.

Invasion and growth data are expressed as the mean (± standard deviation) of a given number of observations. In which ever appropriate, one way analysis of variance (ANOVA) was used to compare multiple groups. Comparisons between groups were by Fisher's PLSD. A p value of < .05 was considered to be significant.

RESULTS

Lymph nodes were taken from patients with an age range of 36 to 90 years (mean age, 64 years). The primary tumor sites and demographic data between the extracapsular and encapsulated groups are shown in Table 1. The immunostaining was of high quality and easy to interpret (Figures 1 and 2). Positive control slides showed constant intense staining in each batch ensuring staining reproducibility. There was no nonspecific primary or secondary antibody binding, and control slides of normal lymph nodes had no iNOS expression.

iNOS expression was found in 22/27 cases of extracapsular nodal disease and in 8/21 cases of encapsulated disease. It was predominantly located to tumor cell cytoplasm and was found consistently at the periphery of tumor cell nests and at the invading tumor front (Figure 1). Using chi-square analysis, a significant correlation was found between iNOS expression and extracapsular spread ($\chi^2 = 9.5, p < .01$) (Table 2).

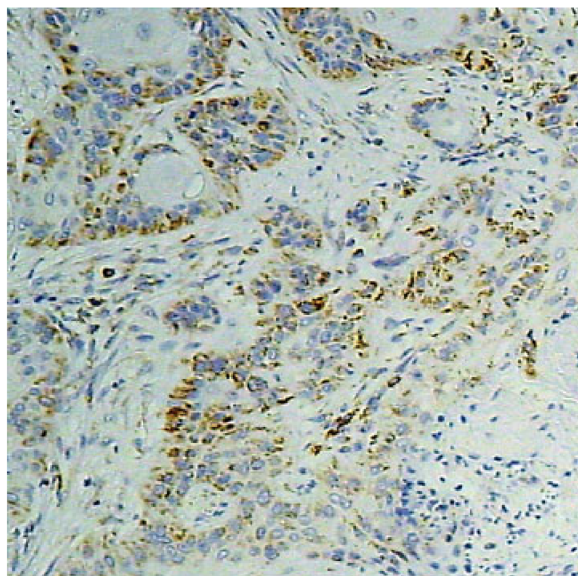

FIGURE 1. Inducible nitric oxide synthase expression, predominantly localized to the invading tumor margin in a lymph node with extracapsular spread (original magnification × 100). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Using Mann Whitney U test, the total iNOS scores was significantly higher in the greater extracapsular group (median = 8) compared with the encapsulated nodes (median = 4) ($p = .002$) (Figure 3).

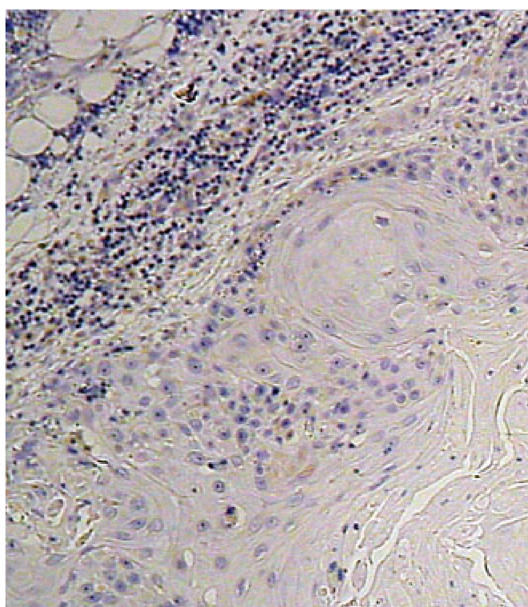

FIGURE 2. Encapsulated lymph node metastasis showing no inducible nitric oxide synthase expression (original magnification × 100). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 2. Nodal metastasis (extracapsular or encapsulated) versus presence or absence of iNOS expression.

Disease type	Extracapsular	Encapsulated	Total
iNOS expression			
Positive	22	8	30
Negative	5	13	18
Total	27	21	48

Abbreviation: iNOS, inducible nitric oxide synthase.
Note: Significantly higher iNOS expression was found in the extracapsular group ($p < .01$). Values represent number of cases.

To determine whether NO is involved in HNSCC invasion, we carried out Transwell invasion assays using the tongue SCC cell line, H357. This cell line produces basal NO levels (measured as nitrite) of 0.3 ± 0.05 nmol nitrite per 5×10^6 cells.[14] Figure 4 shows that H357 cell invasion was significantly inhibited at both 100 μ*M* and 1 m*M* concentrations of 1400 W tested relative to the control group ($p = .002$, $p = .0003$, respectively). At a concentration of 1 m*M*, invasion was reduced by nearly 70% when compared with the control group (no 1400 W added). However, at both concentrations, 1400 W did not inhibit the growth rate of the H357 cells (Figure 5), suggesting that the observed effects were invasion specific.

DISCUSSION

It is well known that extracapsular spread is associated with poor prognosis in patients with

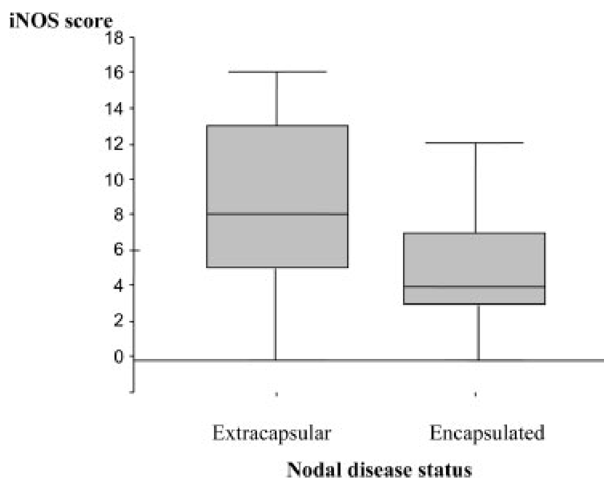

FIGURE 3. Box plot (with range) of inducible nitric oxide synthase immunohistochemical scores (0–16) for the extracapsular and encapsulated groups (medians = 8 and 4, respectively). The inducible nitric oxide synthase score was significantly greater in the extracapsular group ($p = .002$).

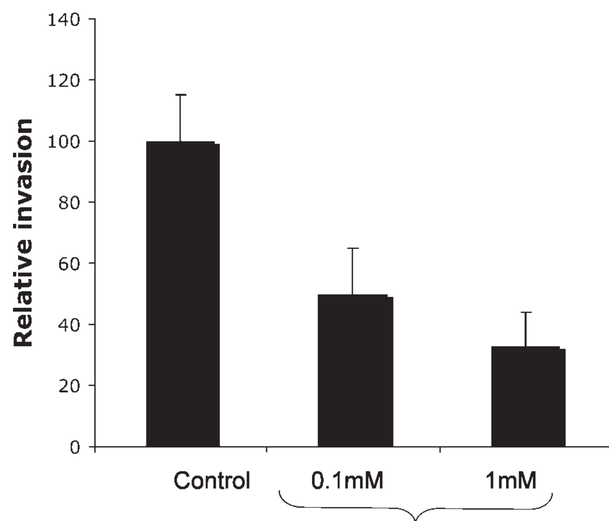

FIGURE 4. Invasion assays with the addition of the specific inducible nitric oxide synthase inhibitor, 1400 W. Assays are shown in percentages relative to the results obtained without the addition of 1400 W (control group).

HNSCC,[2–5] although there are no studies to date that have investigated the role of iNOS and NO in this process. An in vivo study using a spontaneously arising breast carcinoma (S13) found that tumor-draining lymph node cells (TDLN) from mice inoculated with this tumor had greater NOS activity than controls, and that tumor angiogenesis and growth could be reduced using NOS inhibitor drugs.[20]

NO plays a crucial role in angiogenesis. Inhibition of NOS in experimental animals results in large areas of tumor necrosis, and considerable reduction in tumor vessel density when compared with untreated controls.[11] Similar findings have also been found in HNSCC.[12] On the basis of these findings and the current results showing increased iNOS expression, in nodes with extracapsular spread, particularly at the invading tumor margin, it is likely that iNOS has a role to play in this process, possibly by facilitating tumor cell invasion. However, as 5/27 cases with extracapsular spread did not have iNOS expression, it is not pathognomonic for this process. Similar results to those found in the current study on the reduction or inhibition of tumor cell invasion have been found using nonspecific NOS inhibitor drugs such as L-NAME in colorectal and breast cancer cell lines.[21,22] Several mechanisms have been suggested by which NO facilitates tumor cell invasion, including up-regulation of MMP-2 and the involvement of other biochemical pathways,[21–23] but further research is required to understand the role of NOS in tumor cell invasion more fully in HNSCC.

There is great debate about whether cancer cells can metastasize by expansion and invasion of preexisting peritumoral lymphatics or by the formation and invasion of new lymphatics within tumors (lymphangiogenesis). This area has recently received considerable attention, and there is increasing evidence that it is relevant for metastasis to lymph nodes in HNSCC.[24–26] A study was recently published on the correlation between iNOS activity and lymphangiogenesis in a series of 60 patients with HNSCC.[26] iNOS activity measured in specimens from the tumor periphery correlated strongly with both lymphatic vessel density and lymphatic vessel area. In addition, the lymphangiogenic factor vascular endothelial growth factor C (VEGF-C) mRNA expression was significantly elevated in tumors with high iNOS activity, and VEGF-C expression correlated positively with the presence of lymph node metastases. The results suggested that that iNOS activity may promote lymphangiogenesis and spread to lymph nodes in HNSCC, with the possible involvement of VEGF-C.

iNOS expression in primary tumors is known to correlate with tumor metastasis in HNSCC.[16,26] As a result of our earlier study,[16] we identified 22 patients from the current study who had also had iNOS staining performed of the primary tumor. Sixteen of these patients had similar iNOS scores in both primary tumor and metastasis, and 4 patients had no iNOS expression in either site. This suggests that iNOS expression is not up-regulated in the nodal metastasis when compared with the primary tumor.

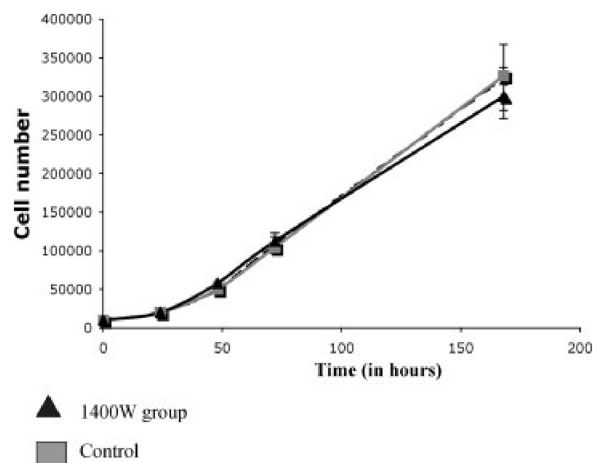

FIGURE 5. H357 cell growth in standard culture medium (control) and following addition of 1400 W at a concentration of 1 m*M*.

We found that the addition of 1400 W to the cell culture medium did not affect tumor cell growth. NO itself can have a positive or negative effect on cell growth, which is both concentration dependent, and a result of its interaction with other proteins such as hypoxia inducible factor-1 and extracellular signal-regulated kinase.[14,27] In vivo, inhibition of iNOS using 1400 W results in reduced tumor growth.[28] This is probably a result of reduced angiogenesis rather than a direct effect on cell growth.[11] High NO concentrations produced by iNOS in certain animals results in apoptosis, but it is unlikely that these concentrations are ever produced in human cancers, probably due to reduced calmodulin binding by iNOS in humans compared with lower species.[29]

The current study used the results of immunochemistry from tumor metastasis and a cell line to study the effects of iNOS on tumor cell invasion. Unfortunately, it is not readily possible to culture tumor cells from each individual patient to assess and correlate the behavior of these cells in vitro with the immunochemistry findings. Growth of cells from patients can also lead to fibroblast overgrowth and the hazards associated with possible viral transmission to laboratory staff. Therefore, the majority of studies use recognized cell lines for in vitro experiments, which has the advantage that it is reproducible.

The future therapeutic manipulation of iNOS in the clinic to reduce tumor growth may be possible,[30] although many of the current experimental NOS inhibitor drugs, including 1400 W have toxic side effects.

In conclusion, we have found that iNOS expression correlates with extracapsular spread in HNSCC. Furthermore, inhibition of iNOS results in reduced tumor cell invasion in vitro. Both of these findings suggest that iNOS may have a role to play in the process of extracapsular spread. Further research is required to understand this process more fully.

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