Nitric Oxide in Breast Cancer: Induction of Vascular Endothelial Growth Factor-C and Correlation with Metastasis and Poor Prognosis

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Abstract

Purpose: Metastasis to regional lymph nodes through the lymphatic vessels is a common step in the progression of cancer. Recent evidence suggests that tumor production of vascular endothelial growth factor-C (VEGF-C) promotes lymphagiogenesis, which in turn promotes lymphatic metastasis. Nitric oxide (NO) may also increase metastatic ability in human cancers.

Experimental Design: Nitrite/nitrate levels and VEGF-C production were assessed in MDA-MB-231 breast cancer cells after induction and/or inhibition of NO synthesis. Formation of nitrotyrosine, a biomarker for peroxynitrate formation from NO *in vivo*, was analyzed in primary human breast carcinoma with long-term follow-up. The relationship between nitrotyrosine levels and lymph node status, VEGF-C immunoreactivity, and other established clinicopathologic variables, as well as prognosis, was analyzed.

Results: Production of nitrite/nitrate and VEGF-C in MDA-MB-231 cells was increased by treatment with the NO donor DETA NONOate. The NO synthase inhibitor N^G-nitro-L-arginine methyl ester eliminated this increase. High-grade nitrotyrosine staining was observed in 57.5% (65 of 113) of the invasive breast carcinomas. Nitrotyrosine levels were significantly correlated with VEGF-C immunoreactivity and lymph node metastasis. Survival curves determined by the Kaplan-Meier method showed that high nitrotyrosine levels were associated with reduced disease-free and overall survival. In multivariate analysis, high nitrotyrosine levels emerged as a significant independent predictor for overall survival.

Conclusions: Our data showed a role for NO in stimulating VEGF-C expression *in vitro*. Formation of its biomarker nitrotyrosine was also correlated with VEGF-C expression and lymph node metastasis. Furthermore, high nitrotyrosine levels may serve as a significant prognostic factor for long-term survival in breast cancer.

Nitric oxide (NO) is a pleiotropic regulator, critical to numerous biological processes, including vasodilation, neurotransmission, and macrophage-mediated immunity (1). It also has both genotoxic and angiogenic properties. Increased NO generation in cancer cells may contribute to tumor angiogenesis by up-regulating vascular endothelial growth factor (VEGF), and VEGF-induced neovascularization may increase the tumors' metastatic ability (2). The effects of NO are mediated in part by its metabolites, such as peroxynitrite. Peroxynitrite can oxidize and nitrate DNA and can also nitrate tyrosine in proteins to produce nitrotyrosine (3). Thus, the presence of nitrotyrosine in tissues has been used as a biomarker for peroxynitrite formation *in vivo* from NO.

Metastasis to the regional lymph nodes via the lymphatic vessels is a common step in the progression of cancer, an important prognostic indicator in many types of cancers, and the basis for surgical and radiation treatment of local lymph nodes. Recent evidence suggests that tumor lymphangiogenesis (i.e., the growth of tumor-associated lymphatic vessels) promotes lymphatic metastasis (4–10). VEGF-C, a novel VEGF member, has been found to induce not only hemangiogenesis but also lymphangiogenesis via VEGF receptor-2 and VEGF receptor-3 (also known as Flt-4; ref. 11). Because VEGF receptor-3 has been shown to be expressed predominantly in the lymphatic endothelium and thus is considered to be a major regulator of lymphangiogenesis (12, 13), VEGF-C seems to be an important lymphangiogenic factor. Previous studies have shown that up-regulated VEGF-C expression in human breast cancer is strongly correlated with lymph node metastasis and unfavorable prognosis (14).

In this study, we show that incubation of MDA-MB-231 cells with an NO donor results in induction of VEGF-C expression. This induction is significantly inhibited by addition of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). We then examine how nitrotyrosine formation relates to

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lymph node metastasis and VEGF-C expression in human breast cancer tissues and further investigate whether nitrotyrosine formation has any value or relevance for predicting disease outcome.

Materials and Methods

Cell culture. The MDA-MB-231 breast carcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained at 37°C in 5% CO_2, as monolayers in tissue culture dishes containing DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (HyClone, South Logan, UT). For the experiments, 6-cm tissue culture plates (Corning, Corning, NY) were seeded with 3×10^5 cells in 3 mL of phenol red–free DMEM + 10% FCS. Medium was changed (day 3), and when the cells were subconfluent (day 5), 5 mmol/L L-NAME (Sigma-Aldrich, Tokyo, Japan), if administered, was added 2 hours before 1 mmol/L DETA NONOate (Cayman Chemical, Ann Arbor, MI). These concentrations of L-NAME or DETA NONOate had no effect on cell viability as measured by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI; data not shown).

Measurement of nitrate/nitrite production. After DETA NONOate administration, cells were incubated for 4, 6, 8, 12, 16, and 24 hours. The supernatants were collected and centrifuged to remove cell debris. The amount of nitrate/nitrite was determined using Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical; ref. 15). Cellular nitrate/nitrite production was quantitated by subtracting the level of nitrate/nitrite present in the media with or without 1 mmol/L DETA NONOate (in the absence of cells). Determinations were done in triplicate. The amount of nitrate/nitrite in the supernatants collected from control cells, to which neither DETA NONOate nor L-NAME was administered, was arbitrarily set as 1.

Determination of VEGF-C mRNA production. After DETA NONOate administration, cells were incubated for 4, 6, 8, 12, 16, and 24 hours. For total RNA extraction, the cells were washed with PBS, scraped from the plates, and stored at −70°C if not processed immediately. Total RNA was extracted using the Trizol method (Invitrogen) according to the protocol provided by the manufacturer. After DNase treatment using DNA-free (Ambion, Austin, TX), mRNA was reverse-transcribed for single-strand cDNA using Oligo-$(dT)_{20}$ primer and Thermoscript (Invitrogen). The reverse transcription reaction was done at 55°C for 60 minutes followed by heating at 85°C for 5 minutes. VEGF-C transcription was measured by quantitative real-time PCR of the resulting cDNA, using universal Taqman PCR reagents, and an ABI Prism 7000 sequence detector equipped with a 96-well thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). cDNA templates (50 ng) were subjected to a 5-minute initial denaturation at 95°C followed by 40 cycles of PCR (95°C for 15 seconds and 60°C for 1 minute, per cycle). The following oligonucleotides were used for real-time reverse transcription-PCR: forward VEGF-C primer, 5′-CCCACCAATTACATGTGGAATAATC-3′; reverse VEGF-C primer, 5′-TCTCCAGCATCCGAGGAAAA-3′; VEGF-C probe, 5′-FAM-TCTGCAGATGCCTGGCTCAGGAAGATCTTCCTGAGCCAGGCATCTGCAGATAMRA-3′. The primer and probe mixture for glyceraldehyde-3-phosphate dehydrogenase was purchased from Perkin-Elmer Applied Biosystems, and PCR was carried out according to the manufacturer's protocol. VEGF-C mRNA expression was quantitated relative to control cells (treated with neither DETA NONOate or L-NAME) based on a real-time PCR standard curve constructed with serially diluted solutions of a VEGF-C cDNA-containing plasmid as templates. All experiments were done in triplicate, although amplification conditions have been shown to be very stable and tube-to-tube variability to be very low. Mean values were used for statistical testing. The glyceraldehyde-3-phosphate dehydrogenase transcript levels of each sample were also monitored; tube-to-tube variability was very low for these amplifications as well (data not shown).

Determination of VEGF-C protein production. For the determination of VEGF-C protein production, cells were incubated for 12 and 24 hours after DETA NONOate administration and harvested as described above. Cell lysates were prepared using T-PER Tissue Protein Extraction kit (Pierce, Rockford, IL) containing Halt Protease Inhibitor Cocktail (Pierce). Protein concentration was measured using a Coomassie Plus Protein Assay Reagent kit (Pierce). Total conditioned media from cells were concentrated 100-fold, using Amicon Ultra-4 columns (Millipore, Billerica, MA). For Western blot analysis of VEGF-C, 40-μg samples of whole-cell lysate or concentrated culture media were separated by electrophoresis on 10% to 20% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (SureBlot F1 System, Astellas Pharma, Inc., Tokyo, Japan) by electroblotting. The membrane was blocked with 5% skim milk in PBS for 1 hour at room temperature, incubated overnight with anti-human VEGF-C rabbit antibody (IBL, Gunma, Japan), rinsed with PBS, and labeled with peroxidase-conjugated anti-rabbit secondary antibody (DakoCytomation, Copenhagen, Denmark) for 1 hour at room temperature. The signals were visualized using the LumiGLO Reserve chemiluminescence substrate kit (KPL, Inc., Gaithersburg, MD) and recorded by luminocapture (ATTO, Tokyo, Japan). Anti-β_2-microglobulin antibody (DakoCytomation) was used for the internal control. To compare levels of proteins, the density of each band was measured by densitometry.

Determination of nitrotyrosine formation. Western blot analysis for nitrotyrosine was conducted as described above using 50 μg of whole-cell lysate from cells incubated for 24 hours after DETA NONOate administration, anti-human nitrotyrosine mouse antibody (Hyclut Biotechnology, Uden, the Netherlands), peroxidase-conjugated anti-mouse secondary antibody (Amersham Biosciences, Piscataway, NJ), and enhanced chemiluminescence detection system (Amersham Biosciences). The signals were visualized and quantitated as described above.

Patients and tumor samples. Paraffin-embedded archival specimens from 113 patients with invasive breast cancer, who were diagnosed and treated in the Osaka Police Hospital, Japan between 1981 and 1992, were studied using immunohistochemistry.

None of these cases had a family history of breast cancer or malignancy in first-degree relatives as judged by questioning at the time of admission for surgery. The patients had received mastectomy with axillary lymph node dissection. All women were apparently free of distant metastasis. All cases received postoperative adjuvant therapy consisting of combination chemotherapy and hormone treatment. The results of immunostaining for VEGF-C, estrogen receptor (ER), progesterone receptor, c-erbB-2, and p53 were obtained from our pathologic data files (14). As described previously, these markers were evaluated immunohistochemically using anti-VEGF-C antibody (AF752, R&D Systems, Minneapolis, MN), anti-ER antibody (ER1D5, IMMUNOTECH, Marseilles, France), anti–progesterone receptor antibody (PgR636, DakoCytomation), anti-c-erbB-1 antibody (CB-11, Novocastra, Newcastle upon Tyne, United Kingdom), and anti-p53 antibody (CM-1, Novocastra), respectively, followed by incubation with Histofine Simple Stain MAX PO (NICHIREI, Tokyo, Japan).

The size of the primary tumor was determined from the surgical specimen. Lymph node metastasis was determined by counting the number of axillary lymph nodes with histologic evidence of metastatic breast carcinoma. Histologic typing and histologic grading were done according to the WHO classification (16) and the Nottingham method (Bloom Richardson; ref. 17). Patient and tumor characteristics are shown in Table 1.

Immunohistochemistry. For immunostaining, paraffin sections of 4-μm thickness were deparaffinized, placed in a solution of 97% methanol and 3% hydrogen peroxide for 5 minutes, then autoclaved for antigen retrieval. After washing in PBS, the slides were treated for 20 minutes with Protein Block Serum-free (DakoCytomation, Carpinteria, CA). This was followed by an overnight incubation at 4°C in a humidified chamber with a 1:100 diluted anti-human nitrotyrosine

Table 1. Relationship between nitrotyrosine and other variables

Factor	Nitrotyrosine formation		*P*
	Low	High	
Age (y)			0.2485
<50	31 (48%)	34 (52%)	
51	17 (35%)	31 (65%)	
Histology			0.0354
Ductal	41 (39%)	63 (61%)	
Other	7 (78%)	2 (22%)	
Tumor size			0.3102
pT1	18 (50%)	18 (50%)	
pT2-4	30 (39%)	47 (61%)	
Lymph node metastasis			<0.001
Negative	38 (70%)	16 (30%)	
Positive	10 (17%)	49 (83%)	
ER			0.2489
Negative	16 (36%)	29 (64%)	
Positive	32 (47%)	36 (53%)	
PR			0.3432
Negative	19 (46%)	32 (54%)	
Positive	29 (47%)	33 (53%)	
c-erbB-2			0.0600
Negative	39 (48%)	42 (52%)	
Positive	9 (28%)	23 (72%)	
p53			0.3198
Negative	34 (47%)	39 (53%)	
Positive	14 (35%)	26 (65%)	
Histologic grade			0.1819
I and II	32 (48%)	35 (52%)	
III	16 (35%)	30 (65%)	
VEGF-C			<0.001
Negative	18 (90%)	2 (10%)	
Positive	30 (32%)	63 (68%)	

Abbreviation: PR, progesterone receptor.

mouse antibody (Hyclut Biotechnology). After the overnight treatment, to avoid the nonspecific biotin reaction, we used Histofine Simple Stain MAX PO (NICHIREI) as the second antibody for 60 minutes according to the manufacturer's instructions. Color was developed using diaminobenzidine with 0.01% hydrogen peroxide. Hematoxylin was used as a counterstain. For the negative control, all reagents except for the primary antibody were used.

The immunohistochemical scoring was done blindly by three pathologists (Y.N., H.Y., and M.T.) who had no clinical knowledge of the patients. The intensity of nitrotyrosine immunostainings was evaluated by dividing the cytoplasmic staining reaction into four groups: 1, weak; 2, moderate; 3, strong; and 4, very strong. The quantity of immunostainined cells was evaluated as follows: 1, <25%; 2, 25% to 50%; 3, 50% to 75%; and 4, >75% of tumor cells showing cytoplasmic positivity. A combined score for nitrotyrosine immunostainings was generated by adding the qualitative and quantitative scores. These summed scores were then divided into two groups as low grade (2–4) and high grade (5–8) for statistical testing.

Statistics. The effects of drug treatment were analyzed by ANOVA. Fisher's exact test was used to examine the association of nitrotyrosine levels with age, histologic type, tumor size, lymph node metastasis, ER, progesterone receptor, c-erbB-2, p53, histologic grade, and VEGF-C immunoreactivity. Overall survival curves and disease-free survival (DFS) curves were obtained using the Kaplan-Meier method and compared using the log-rank test. A multivariate model using the Cox

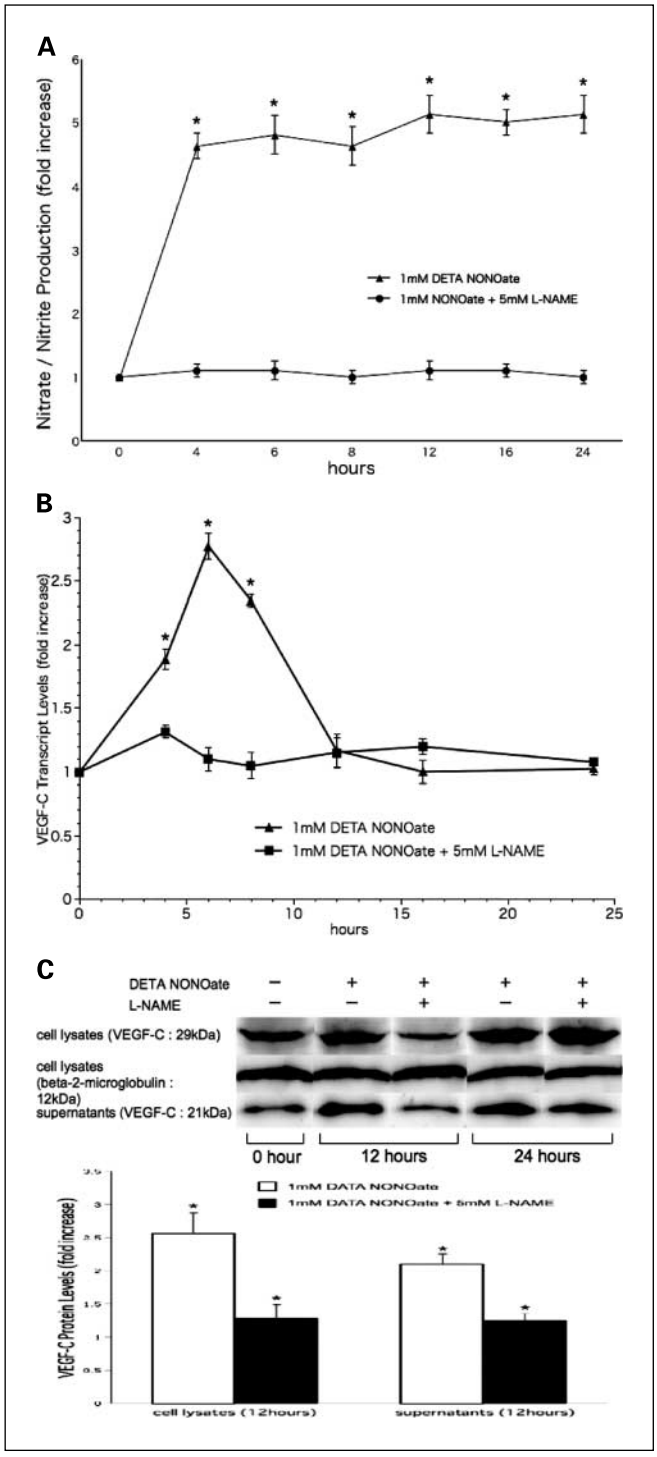

Fig. 1. Effects of DETA NONOate in the presence or absence of L-NAME on (*A*) nitrate/nitrite production, (*B*) VEGF-C mRNA expression, and (*C*) VEGF-C protein expression. MDA-MB-231 cells were treated with 1 mmol/L DETA NONOate in the presence or absence of 5 mmol/L L-NAME for various time periods and prepared for (*A*) measurement of nitrate/nitrite production, (*B*) real-time reverse transcription-PCR analysis, and (*C*) Western blot analysis, as described in Materials and Methods. Determinations were done in triplicate and expressed as mean of three experiments ± SE. Fold increase relative to control (untreated) cells. *, $P < 0.05$, significant difference from control and/or L-NAME – treated cells.

stepwise regression analysis was used to evaluate the statistical strength of independent association between covariates and DFS and/or overall survival. $P < 0.05$ was considered significant. A software package (JMP IN 5.1.1, SAS Institute, Cary, NC) was used for all statistical testing and management of the database.

Results

Effects of NO on VEGF-C expression. To examine the effect of NO on VEGF-C induction, MDA-MB-231 cells were treated with the NO donor DETA NONOate. A significant increase in nitrate/nitrite production in the supernatants after stimulation with DETA NONOate was observed (Fig. 1A). Pretreatment of the cells with the NO synthase inhibitor L-NAME completely inhibited this increase. As shown in Fig. 1B and C, a significant increase in VEGF-C mRNA and protein expression was observed after stimulation with DETA NONOate. A significant increase in VEGF-C protein expression in both cell lysates and supernatants was observed. The VEGF-C detected in the lysates was the immature 29-kDa form, whereas the supernatants contained the proteolytically processed and secreted 21-kDa form. Both fractions showed increased levels of VEGF-C protein after DETA NONOate treatment. Pretreatment with L-NAME significantly inhibited all these effects of DETA NONOate on VEGF-C expression.

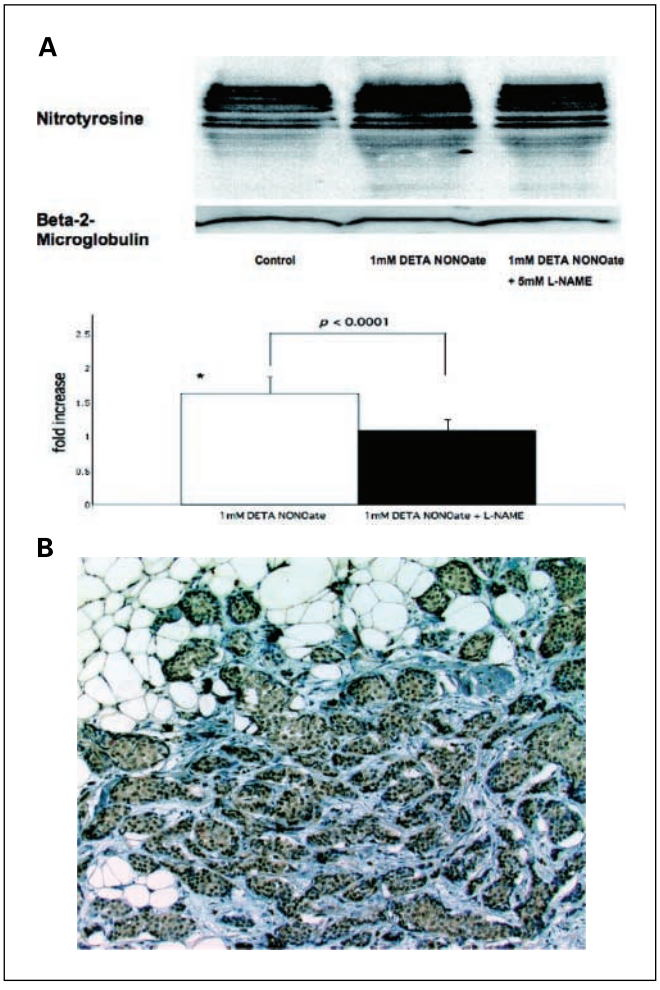

Fig. 2. Nitrotyrosine formation in breast cancer cell line and human breast cancer tissue. *A*, effects of DETA NONOate in the presence or absence of L-NAME on nitrotyrosine formation. MDA-MB-231 cells were treated with 1 mmol/L DETA NONOate in the presence or absence of 5 mmol/L L-NAME for 24 hours and prepared for Western blot analysis. Control indicates cells with no treatment. Fold increase normalized to control. *B*, immunohistochemical analysis for nitrotyrosine in breast cancer. Most tumor cells show diffuse cytoplasmic staining for nitrotyrosine. Focal nuclear staining is also visible.

Nitrotyrosine formation in a breast cancer cell line. To examine and confirm the effect of NO on nitrotyrosine formation, MDA-MB-231 cells were treated with the NO donor DETA NONOate. A significant increase in nitrotyrosine formation after stimulation with DETA NONOate was observed (Fig. 2A). Pretreatment of the cells with the NO synthase inhibitor L-NAME significantly inhibited this increase.

Demographics and clinical data. The median age at diagnosis for the 113 patients was 51 years (range, 24-87 years). Fifty-eight percent of the patients were younger than 50 years ($n = 65$), and 52% ($n = 59$) of the patients had lymph node metastasis at the time of surgery (Table 1). Median follow-up time for the 113 subjects was 116 months (range, 10-230 months). Forty-four subjects had relapsed by the time of last follow-up. Nineteen patients had bone metastasis, 16 had visceral metastasis (including lung and liver metastasis), 6 had brain metastasis, and 25 had soft tissue recurrence. Thirty-six patients died of breast carcinoma.

Nitrotyrosine formation in breast cancer tissue. Nitrotyrosine was detected by immunohistochemistry in all invasive breast carcinomas. Nitrotyrosine was observed mainly in the cytoplasm and focally in the nucleus (Fig. 2B). The nitrotyrosine staining was heterogeneous. Strong staining could be seen in stromal macrophages and neutrophils, and weak or no staining could be seen in stromal lymphocytes or fibroblasts. For statistical analysis, nitrotyrosine antibody–stained sections were grouped as low grade (2–4) and high grade (5–8). According to these criteria, high-grade nitrotyrosine staining was observed in 57.5% (65 of 113) of the invasive breast carcinomas.

Nitrotyrosine levels are correlated with VEGF-C expression and lymph node metastasis. We have previously reported that VEGF-C protein was expressed as diffuse cytoplasmic staining in breast cancer cells (9, 14). Evaluation of cytoplasmic VEGF-C immunoreactivity of tumor cells was classified as either negative (if no staining or positive staining was present in <10% of tumor cells) or positive (if >10% of tumor cells stained positively). As shown in Table 1, VEGF-C protein expression was positive in 82% (93 of 113) of the breast cancer patients, and its expression was significantly correlated with lymph node metastasis ($P = 0.001$). High-grade nitrotyrosine staining was correlated with VEGF-C immunoreactivity ($P < 0.001$) and lymph node metastasis ($P < 0.001$). High-grade nitrotyrosine staining was also significantly associated with histologic type ($P = 0.0354$). There was no significant correlation between nitrotyrosine levels and the age of the patients, tumor size, ER, progesterone receptor, c-erbB-2, p53 status, or histologic grading.

Nitrotyrosine formation is correlated with patients' survival. The survival analysis was done on 113 patients and examined the following variables: nitrotyrosine formation, patient's age, histologic type, tumor size, lymph node metastasis, hormonal status, c-erbB-2, p53, histologic grade, and VEGF-C immunoreactivity. Univariate survival analysis showed that tumor size, lymph node metastasis, ER status, c-erbB-2, VEGF-C

Table 2. Univariate analysis of DFS and overall survival by various clinicopathologic factors

Factor	DFS		OS	
	No.	*P*	No.	*P*
Age (y)		0.5382		0.2603
<50	25/65 (38.5%)		18/65 (27.7%)	
51	19/48 (39.6%)		18/48 (37.5%)	
Histology		0.2733		0.2060
Ductal	42/104 (40.4%)		35/104 (33.7%)	
Other	2/9 (22.2%)		1/9 (11.1%)	
Tumor size (cm)		0.0003		0.1002
<2	5/36 (13.9%)		8/36 (22.2%)	
2	39/77 (50.6%)		28/77 (36.4%)	
Lymph node metastasis		<0.0001		<0.001
Negative	6/54 (11.1%)		8/54 (14.8%)	
Positive	38/59 (64.4%)		28/59 (47.5%)	
ER		0.0271		0.5131
Negative	23/45 (51.1%)		15/45 (33.3%)	
Positive	21/68 (30.9%)		21/68(30.9%)	
PR		0.1331		0.1557
Negative	23/51 (45.1%)		19/51 (37.3%)	
Positive	21/62 (33.9%)		17/62 (27.4%)	
c-erbB-2		0.0012		0.1556
Negative	23/81 (28.4%)		23/81 (28.4%)	
Positive	20/32 (62.5%)		13/32 (40.6%)	
p53		0.5951		0.6081
Negative	27/73 (37.0%)		25/73 (34.2%)	
Positive	17/40 (42.5%)		11/40 (27.5%)	
Histologic grade		0.4566		0.2393
I and II	29/67(43.3%)		25/67 (37.3%)	
III	15/46 (32.6%)		11/46 (23.9%)	
VEGF-C		0.0026		0.0212
Negative	2/20 (10.0%)		2/20 (10.0%)	
Positive	42/93 (45.2%)		34/93 (36.6%)	
Nitrotyrosine formation		<0.001		<0.001
Low	8/48 (16.7%)		5/48 (10.4%)	
High	36/65 (55.4%)		31/65 (47.7%)	

Abbreviations: PR, progesterone receptor; OS, overall survival.

immunoreactivity, and high-grade nitrotyrosine staining were of significant prognostic value for DFS (Table 2; Fig. 3A). Lymph node metastasis, VEGF-C immunoreactivity, and high-grade nitrotyrosine staining were of significant prognostic value for overall survival (Table 2; Fig. 3B). As shown in Table 3, multivariate Cox regression analysis of all covariates focusing on overall survival identified the following as independent significant prognostic factors: high-grade nitrotyrosine staining, $P = 0.0235$; lymph node metastasis, $P = 0.0111$; and histologic grade, $P = 0.0429$. The odds ratio for high-grade nitrotyrosine staining was 3.69. Based on multivariate Cox regression analysis, lymph node metastasis and tumor size were identified as an independent prognostic factor for DFS (lymph node metastasis, $P = 0.0007$; tumor size, $P = 0.0230$). High-grade nitrotyrosine staining was not identified as an independent prognostic factor for DFS ($P = 0.1792$). Survival analysis was then done separately on the 54 node-negative patients and the 59 node-positive patients, examining the variables described above. Based on univariate analysis, high-grade nitrotyrosine staining was identified as prognostic factor for overall survival in the node-negative patients (DFS, $P = 0.7649$; overall survival, $P = 0.0020$) and for DFS in the node-positive patients (DFS, $P = 0.025$; overall survival, $P = 0.171$). By multivariate analysis, high-grade nitrotyrosine staining was not identified as an independent prognostic factor for DFS in the node-negative patients ($P = 0.2318$) or overall survival in the node-positive patients ($P = 0.1910$).

Discussion

NO, an inorganic free radical gas, is an important signaling molecule and bioactive agent that mediates vasodilation and various other actions, such as neurotransmission and host defense (1). NO produced by macrophages can mediate antibacterial and antitumor functions; however, chronic

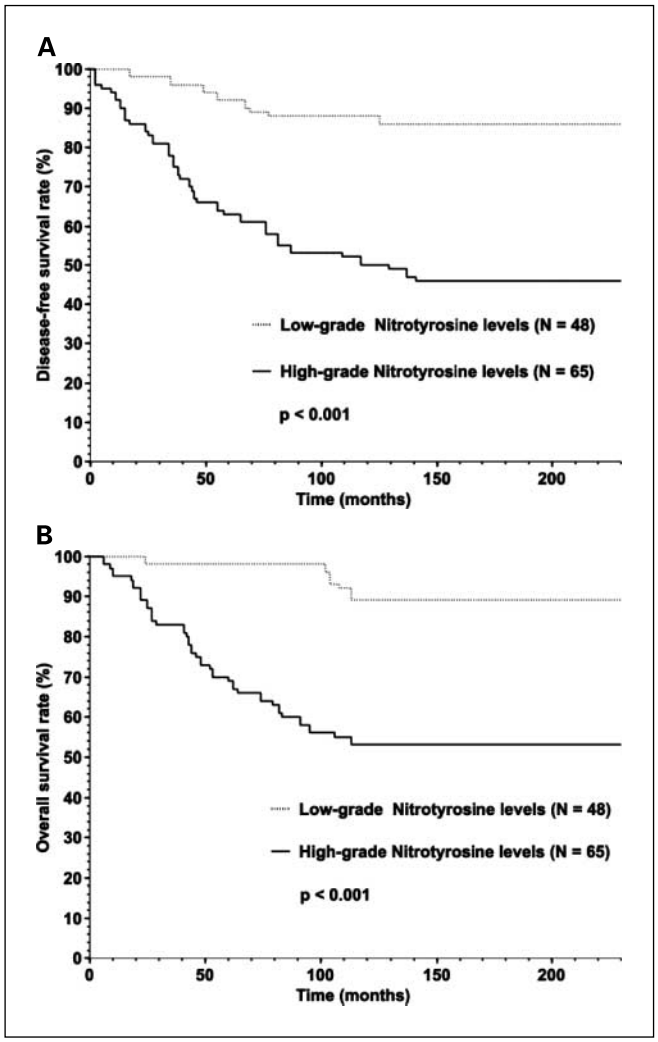

Fig. 3. Association of nitrotyrosine formation with patients' prognosis in breast cancer (Kaplan-Meier method and log-rank test). High-grade nitrotyrosine staining was significantly related to recurrence (*A*, $P < 0.001$) and death (*B*, $P < 0.001$).

induction of NO and NO synthase may contribute to many pathologic processes, including inflammation and cancer (18, 19). In experimental tumor models, a contributory role of NO in tumor metastasis has been also shown (2, 20).

Tumor metastasis may depend on the capacity of tumor cells to induce hemangiogenesis and/or lymphangiogenesis. For tumors to spread to regional lymph nodes, cancer cells must first invade the lymphatic system. The discovery of VEGF-C changed the landscape for lymphatic study, and VEGF-C has been associated with the normal development of lymphatic vessels (21) as well as the promotion of tumor lymphangiogenesis (7, 9).

In this study, the NO donor DETA NONOate induced VEGF-C mRNA and protein expression in the MDA-MB-231 breast cancer cell line. A significant increase in VEGF-C protein expression in both cell lysates and supernatants was observed after treatment with DETA NONOate. In the supernatants, a significant increase in mature form of VEGF-C was observed, indicating that DETA NONOate stimulated secretion. All these increases were significantly inhibited in the presence of the NO synthase inhibitor L-NAME. A significant increase in nitrate/nitrite production in the supernatants after stimulation with DETA NONOate was also observed, and treatment of cells with L-NAME completely inhibited this increase as well. Our results suggest that VEGF-C expression may be regulated by NO in breast cancer cells.

In addition, immunohistochemistry revealed that nitrotyrosine levels were significantly correlated with VEGF-C immunoreactivity and lymph node metastasis in human breast cancer. Our findings suggest that nitrotyrosine formation in human breast cancer may be associated with VEGF-C expression and lymph node metastasis. However, Samoszuk et al. were unable to find a significant relationship between nitrotyrosine levels and lymph node metastasis (22). The discrepancy between our findings and those reported by Samoszuk et al. may be due to use of different antibodies or may be because of case selection bias. Consistent with up-regulation of VEGF-C expression by NO, 90% of the VEGF-C–negative patients showed low nitrotyrosine staining.

Table 3. Results of multivariate Cox regression analysis of overall survival in 113 breast cancer cases

Variable	Odds ratio (95% confidence interval)	*P*
Age (y)		0.3820
<50	1.0 (reference)	
51	1.373 (0.675-2.794)	
Histology		0.7982
Ductal	1.0 (reference)	
Other	1.337 (0.144-12.378)	
Tumor size		0.4633
pT1	1.0 (reference)	
pT2-4	1.393 (0.574-3.377)	
Lymph node metastasis		0.0111
Negative	1.0 (reference)	
Positive	3.391 (1.321-8.702)	
ER		0.4879
Positive	1.0 (reference)	
Negative	1.383 (0.553-3.458)	
PR		0.1176
Positive	1.0 (reference)	
Negative	1.991 (0.840-4.719)	
c-erbB-2		0.5760
Negative	1.0 (reference)	
Positive	1.256 (0.565-2.791)	
p53		0.3975
Negative	1.0 (reference)	
Positive	1.435 (0.622-3.309)	
Histologic grade		0.0429
I and II	1.0 (reference)	
III	2.212 (1.026-4.770)	
VEGF-C		0.7218
Negative	1.0 (reference)	
Positive	1.367 (0.244-7.658)	
Nitrotyrosine formation		0.0235
Low	1.0 (reference)	
High	3.687 (1.192-11.404)	

Abbreviations: PR, progesterone receptor; OS, overall survival.

However, 32% of the VEGF-C–positive patients also showed low nitrotyrosine staining. Although this is lower than the overall percentage showing low nitrotyrosine (42%), it indicates that other factors regulate VEGF-C expression in these patients. As it has been reported previously that VEGF-C expression is induced by tumor necrosis factor-α (23), interleukin-1β (23), heregulin-β 1 (24), or cyclooxygenase-2 (25), these factors may induce VEGF-C expression in the cases with low levels of nitrotyrosine.

Another question of practical importance was whether measurement of nitrotyrosine formation has any value or relevance with respect to predicting the disease course in breast carcinomas. In our results, survival curves determined by the Kaplan-Meier method and univariate analysis showed that high-grade nitrotyrosine staining was negatively associated with both DFS and overall survival. Furthermore, multivariate analysis using the Cox stepwise regression analysis showed that high-grade nitrotyrosine staining was still related with poor overall survival after consideration of other prognostic factors. Therefore, nitrotyrosine formation seems to be a reliable prognostic biomarker. Although nitrotyrosine formation was not identified as an independent prognostic factor for DFS in the whole study population, it may be influenced by other factors, such as tumor size, ER, c-erbB-2, which are identified as poor prognostic factors for DFS but not overall survival. In addition, survival curves determined by the univariate analysis showed that high-grade nitrotyrosine staining was negatively associated with overall survival but not DFS in 54 node-negative patients. This may be due to possible lymph node micrometastases that could not be detected by routine histopathologic examination.

In conclusion, nitric oxide induces VEGF-C expression *in vitro* and *in vivo*, and nitrotyrosine levels are significantly associated with lymph node metastasis and VEGF-C expression and may play an important role in lymph node metastasis in breast cancer. Furthermore, high nitrotyrosine levels may serve as a significant prognostic factor for long-term survival in breast cancer.

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