Overexpression of iNOS Gene Suppresses the Tumorigenicity and Metastasis of Oral Cancer Cells

KOJI HARADA[1], SUPRIATNO[1], SHIN-ICHI KAWAGUCHI[2],
ONOUE TOMITARO[1], HIDEO YOSHIDA[1] and MITSUNOBU SATO[1]

[1]Second Department of Oral and Maxillofacial Surgery and [2]Department of Oral and Maxillofacial Radiology,
University of Tokushima, School of Dentistry, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan

Abstract. *Background: The inducible nitric oxide synthase (iNOS) is associated with inflammatory processes and cancer formation through production of nitric oxide (NO). However, the clinical importance of the expression of iNOS in oral cancer remains unclear. In the present study, we examined whether up-regulation of the iNOS gene can affect growth and metastasis of an oral cancer cell line (B88t cell) in vitro and in vivo. Materials and Methods: We constructed an expression vector containing sense-oriented murine iNOS cDNA with pcDNA3.1. We transfected B88t cells with the sense expression vector to up-regulate the expression of the iNOS gene in the sense transfectants. Results: The expression of iNOS protein was up-regulated in the sense transfectants and that up-regulation of the iNOS gene exerted a growth inhibitory effect on B88t cells in vitro and in vivo. Moreover, up-regulation of the iNOS gene markedly inhibited the migration of cancer cells in a Boyden chamber. Furthermore, up-regulation of the iNOS gene dramatically inhibited metastases to the cervical lymph node in vivo. Conclusion: These findings suggest that up-regulation of the iNOS gene may suppress the tumorigenicity and metastasis of oral cancer cells.*

Nitric oxide (NO), a free-radical gas, is a short-living molecule which is involved in a multitude of biological processes including inflammation and cancer (1). NO can react with other radicals to form cytotoxic compounds, such as peroxynitrite, which can cause DNA damage and protein modifications (2). NO can also react directly with a variety of enzymes and other proteins to either activate or inhibit

Correspondence to: Dr Koji Harada, Second Department of Oral and Maxillofacial Surgery, University of Tokushima, School of Dentistry, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan. Tel: +81 88 633 7354, Fax: +81 88 633 7462, e-mail: harako@dent.tokushima-u.ac.jp

their functions (3). NO is generated by NO synthases (NOS) that convert L-arginine to L-citrulline in tumor cells, macrophages and fibroblasts (4). NOS exists in three different isoforms; endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively produced, while inducible NOS (iNOS) is modulated in response to inflammatory cytokines and bacterial endotoxins (5). Only iNOS is principally involved in inflammatory processes and cancer formation because it is capable of producing sustained NO concentrations in the micromolar range (6,7).

It has been reported that an enhanced expression of iNOS was seen in oral squamous cell carcinomas (8), in the stroma of breast cancer (9) and in gynecological malignancies (10). It has also been reported that overexpression of iNOS was correlated with cervical lymph node metastasis (11) and angiogenesis (12). From these reports, iNOS seems to play an important role in tumor growth, metastasis and angiogenesis. On the other hand, it has been reported that overexpression of the iNOS gene can suppress tumor growth and metastasis of melanoma and renal carcinoma cell (13,14). Moreover, it has also been reported that NO can induce apoptosis in tumor cells by suppressing NF-ÎB activity (15), or caspase (16). It seems that iNOS may be related to the antitumor effect as well as to carcinogenesis.

In the present study, we examined whether up-regulation of the iNOS gene can affect the growth and metastasis of oral cancer cells, B88t, *in vitro* and *in vivo* by transfecting an expression vector containing sense-oriented murine iNOS cDNA to B88t cells.

Materials and Methods

Cell and cell culture. B88 cells were isolated from an oral squamous cell carcinoma patient in our laboratory (17). The original tumor of the B88 cells was a moderately-differentiated squamous cell carcinoma of the tongue and was not invasive into the muscle layer. The B88 cells were established from a cervical lymph node metastasis. The B88t cells were recovered from a subcutaneous tumor formed on a nude mouse. The B88t cells showed a higher

tumorigenicity and metastatic potential on the nude mouse orthotopic inoculation model than the B88 cells (data not shown). The cells were cultured on plastic Petri dishes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) with Dulbecco's modified Eagle medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Moregate BioTech, Bulimba, Australia), 100 μg/ml streptomycin and 100 units/ml penicillin (Gibco). They were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and were passaged to new dishes before confluence. In addition, selected clones obtained after transfection were maintained in the same medium supplemented with Geneticin (800 μg/ml G418, Sigma).

Construction of a mammalian expression vector. The mammalian expression vector pcDNA3.1-iNOS containing sense-oriented murine iNOS cDNA was constructed as follows: pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) is a mammalian expression vector containing a CMV promoter. pcDNA3.1 (+) was digested with Hind III (Takara Biomedicals, Kusatsu, Japan) and Eco RV (Takara Biomedicals) and dephosphorylated by calf intestinal alkaline phosphatase (Roche Diagnostics, Mannheim, Germany). The murine iNOS cDNA fragment (3.9kb Hind III and Eco RV fragment) containing the murine iNOS open reading frame was purchased from Oxford Biomedical Research (NS 05, Oxford, MI, USA) and cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen) at the Hind III and Eco RV restriction sites. The murine iNOS cDNA fragment (3.9kb Hind III and Eco RV fragment) containing the murine iNOS open reading frame was ligated to the prepared cloning site of pcDNA3.1 (+) by T4 DNA ligase (Takara Biomedicals). The direction of the ligated fragment was confirmed by sequencing analysis. The DNA sequence was determined by the dideoxy chain termination method, using fluorescein-labeled primers and a Thermo Sequenase Cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Electrophoresis and scanning were performed with a Shimadzu DSQ-500 DNA sequencer (Shimadzu, Kyoto, Japan).

Transfection. Cells (5 x 10⁵ cells/dish) were seeded in 100-mm culture dishes (Falcon) in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 5 μg of pcDNA3.1-iNOS or pcDNA3.1 without insert (empty vector) by use of the Superfect reagent (Qiagen, Hilden, Germany). The cells were incubated for 24 hours in DMEM containing 10% FCS, then trypsinized and seeded at a 1 : 5 ratio in 100-mm culture dishes in DMEM medium containing 10% FCS. Forty-eight hours later, the cells were switched to a selective medium containing Geneticin (800 μg/ml G418, Sigma). After 14 days of culture in the selective medium, ten representative G418-resistant clones were isolated and expanded in a 24-well cluster dish (Falcon).

Western blotting. Cell lysates were prepared from the transfectants as follows: cells were cultured to subconfluence, washed three times with 100 mM phosphate-buffered saline (PBS) and lysed with 50 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid, HEPES (pH7.5) containing 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM NaF, 100 mM p-nitrophenyl phosphate, 5 U/ml aprotinin and 1 mM phenyl-methylsulfonyl fluoride. The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Fifty

micrograms of protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to PVDF membrane (Bio-Rad). Next, the membrane was incubated with a 1:500 dilution of the rabbit polyclonal antibody against iNOS (N-20; Santa Cruz Biotech. Inc., CA, USA) as the primary antibody and an Amersham ECL kit (Amersham Pharmacia Biotech.). Also, anti-α-tubulin monoclonal antibody (TU-02; Santa Cruz) was used for normalization of Western blot analysis.

MTT assay. Cells were seeded on 96-well plates (Falcon) at 5 x 10³ cells per well in DMEM containing 10% FCS with/without Geneticin. After 2, 4 and 6 days, the number of cells was quantitated by an assay in which MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)] was used (18).

Migration assay with Boyden chamber. Chemotaxis (directed migration) was evaluated in the Boyden chamber apparatus (Neuro-probe,Inc., Cabin John, MD, USA). Briefly, subconfluent cells were starved for 24 hours and harvested with 0.05% trypsin containing 0.02% EDTA, washed twice with PBS and resuspended to a final density of 5x10⁵/ml in serum-free medium with 0.1% fraction V bovine serum albumin (BSA). PVP filters (Nuclepore Corp, Palo Alto, CA, USA) of 8-μm pore size were precoated with gelatin (0.1 μg/ml), rinsed in sterile water and used for the assay. The bottom wells of the chamber were filled with 25 μl of 10% FCS DMEM per well and covered with a gelatin-coated membrane and then 50 μl of cells, yielding 500 cells/μl, was added to the top wells. After 24 hours of incubation, the membranes were stained with Giemsa solution. Cells on the upper surface of the filter were carefully removed with a cotton swab and the cells that had migrated through the membrane to the lower surface were counted in 12 different fields, under a light microscope at x 400 magnification. Each experiment was performed in triplicate wells and repeated 3 times.

In vivo tumorigenesis and metastasis assay. The tumorigenicity of tumor cells were examined in the nude mouse with Balb/cA Jcl-nu genetic background (CLEA Japan, Inc. Tokyo, Japan). Tumor cells (1x10⁶) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of 5-week-old nude mice using a 27-gauge needle. The size of the tumors were determined by first measuring length (L) and width (W) and then calculating the volume (0.4 x L x W²) every 2 days. The body weight of the mice was also measured every 2 days. The mice were sacrificed 21 days after inoculation. In the same way, the invasiveness and metastatic potential of tumor cells were examined in the nude mouse orthotopic inoculation model. In short, tumor cells (1x10⁶) were suspended in 0.05 ml of serum-free medium and injected into the tongue of nude mice. The mice were sacrificed 60 days after inoculation. Then, the primary tumor, regional (cervical, or axillary and inguinal) lymph nodes, lung, liver, spleen and kidney were also examined.

Immunohistochemical staining. Two serial sections of 4 μm were cut from formalin-fixed, paraffin-embedded tissues and mounted on poly-L-lysine-coated slides. Sections were dewaxed in xylene and rehydrated in graded ethanols according to standard procedures. A serial section from each specimen was stained with hematoxylin and eosin for histological evaluation. Other sections were microwaved in a citrate buffer, pH 6.0, two times for 5 min, cooled
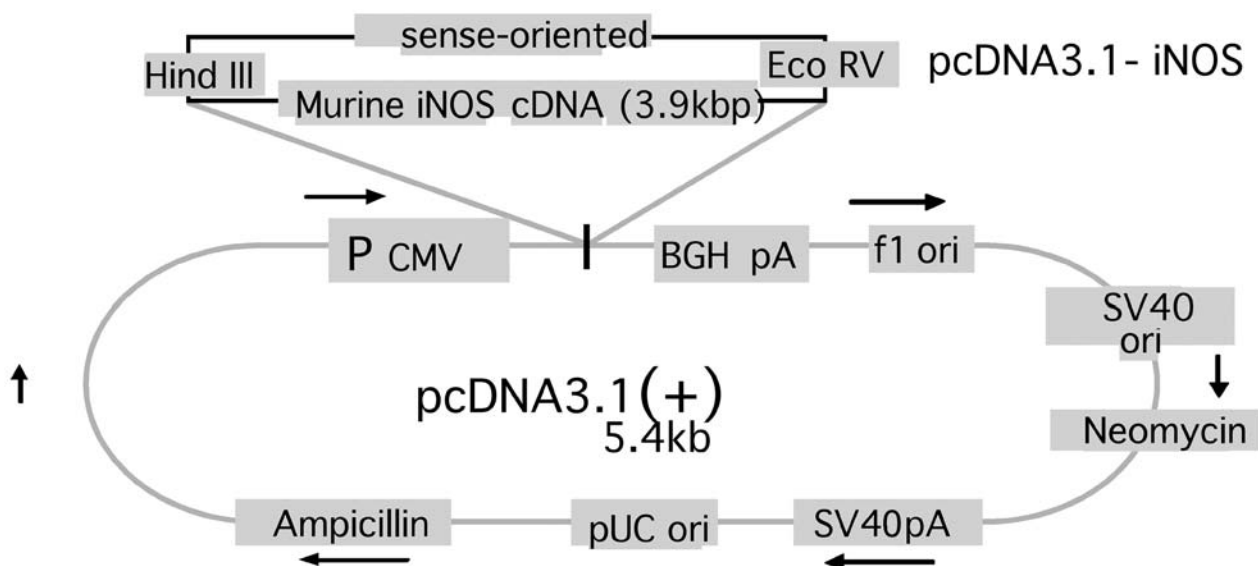
Figure 1. *iNOS plasmid construction. pcDNA3.1-iNOS contains the sense-oriented 3.9kb murine iNOS cDNA under the transcriptional control of the cytomegalovirus (CMV) promoter. The murine iNOS cDNA fragment (3.9kb Hind III and Eco RV fragment), containing the murine iNOS open reading frame, was cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen) at the Hind III and Eco RV restriction sites.*

to room temperature gradually and then rinsed in distilled water. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 30 min and sections were rinsed in distilled water and in PBS at room temperature. A 10% normal goat serum was applied to the sections for 30 min as a blocking reagent to reduce nonspecific binding. A 1:500 dilution of the rabbit polyclonal antibody against iNOS (N-20; Santa Cruz Biotech. Inc.) was used as the primary antibody. Sections were incubated at 37°C for 90 min. They were rinsed in PBS and incubated with biotinylated secondary antibody for 30 min, followed by incubation with streptavidin peroxidase reagents (Vector, CA, USA) for 30 min. After being washed in PBS, they were incubated in diaminobenzidine solution with H_2O_2 for 3 min. Finally, the sections were counterstained with hematoxylin for 30 sec, washed in water, dehydrated and mounted, according to standard procedures. Negative controls for each material were processed in the same manner, using PBS instead of the primary antibody.

TUNEL method. Apoptosis was analyzed *in situ* by the terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique using ApopTag (Oncor, Inc., Gaithersburg, MD, USA), labeling 3'-OH DNA ends generated by DNA fragmentation (19). Nude mice tumors were fixed in 4% formaldehyde in PBS(-). Dewaxed paraffin sections were treated with 20 µg/ml proteinase K in PBS (-) for 10 min to digest protein, then treated with 3% H_2O_2 in PBS (-) for 5 min to quench endogenous peroxidase activity and equilibrated. Terminal deoxynucleotidyl transferase (TdT) enzyme was applied to the cells or sections which were incubated at 37°C for 1 h, treated with stop/wash buffer, then exposed to anti-digoxigenin peroxidase, which was developed by DAB and counterstained with hematoxylin. After each step, the sections were rinsed with PBS(-).

Results

Expression of iNOS protein in the transfectants. After transfection with pcDNA3.1-iNOS (Figure 1), or pcDNA3.1, we obtained more than 100 G418-resistant colonies in sense transfectants and isolated 10 representative G418-resistant clones. They were screened for the expression of iNOS protein by Western blot analysis. Approximately 90% of the representative G418-resistant clones in sense transfectants (B88t-iNOS) expressed the up-regulation of iNOS protein (data not shown). As shown in Figure 2, we detected the up-regulation of iNOS protein in B88t-iNOS when compared with that in parental cells (B88t) or control cells which were transfected with pcDNA3.1 without insert (B88t-neo). The expression of α-tubulin as an internal control was approximately the same in all of the cells.

Growth of cells in vitro. The relative cell number was evaluated by comparing the absorbance in each cell at days 2, 4 and 6. No significant difference in the cell number was seen among the cells on day 2. However, the cell number of sense transfectants was significantly decreased on day 4 and day 6 when compared with that of control cells ($p<0.01$; one-way ANOVA) (Figure 3).

In vitro migration assay. Cell migration is an essential process involved in tumor invasion and metastasis. B88t-iNOS showed a low ability to migrate when compared with that of control cells by one-way ANOVA. Briefly, up-
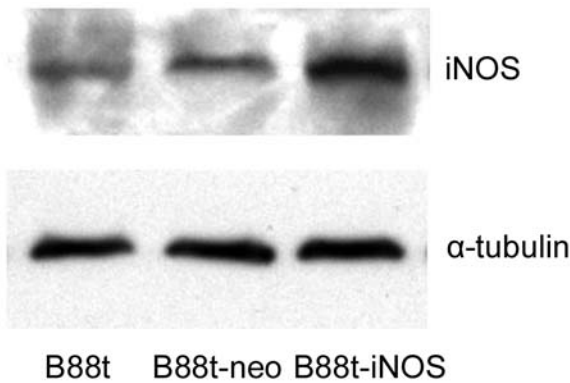
Figure 2. *Expression of iNOS protein in the transfectants. A Western blot analysis. Cell lysates were prepared from parental cells (B88t), control cells (B88t-neo) and the sense transfectants (B88t-iNOS). Protein samples (50 μg/ml) were electrophoresed on SDS-polyacrylamide gel, transferred to PVDF membrane, and incubated with anti-iNOS polyclonal antibody and anti-α-tubulin monoclonal antibody.*
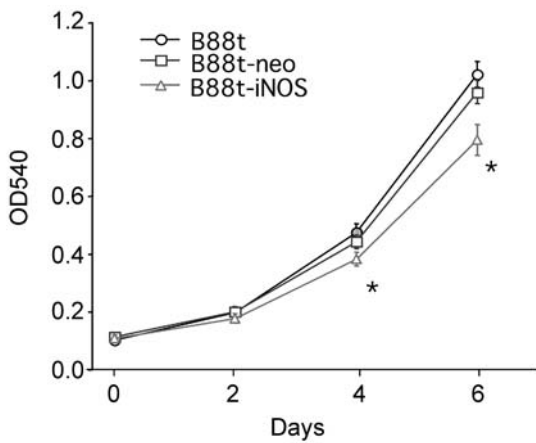

Figure 3. *Growth of transfectants in vitro. Relative cell number was evaluated by comparing the absorbance in each cell on days 2, 4 and 6. The values shown are the mean of six determinations; bars, SD. *, $p<0.01$ when compared with that of control cells by one-way ANOVA.*

regulation of the iNOS gene inhibited the migration of B88t cells (Figure 4).

In vivo tumorigenicity and metastasis assay of the transfectants. First, we inoculated cancer cells subcutaneously into the backs of nude mice. Each of the 5 mice that received control cells (B88t-neo) developed moderate-sized tumors. Each of the 5 mice that received sense transfectants (B88t-iNOS) developed small tumors. As shown in Figure 5, the tumors induced by iNOS-up-regulated transfectants (B88t-iNOS) were much smaller than those of control cells (B88t-neo). During the experimental period, no loss of body weight was observed in either transfectant group.
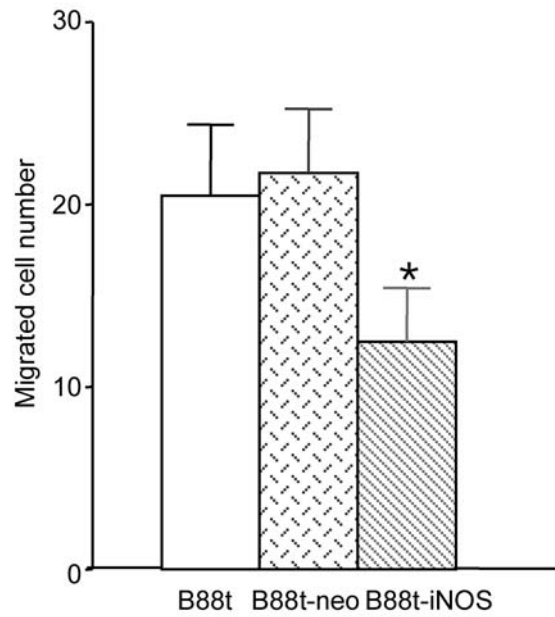

Figure 4. *Cell migration was evaluated by the use of a 48-well modified Boyden chamber. After 24 hours of incubation, the migrated cells in each transfectant were counted in 12 different fields under a light microscope. *, $p<0.01$ when compared with that of control cells by one-way ANOVA.*

Next, we tried to inoculate cancer cells orthotopically. All of the 5 mice that received control cells (B88t-neo) developed moderate-sized tumors. Of these 5 mice, 4 demonstrated metastases at the cervical lymph node, but there were no distant metastases in the control cells group. All of the 5 mice that received sense transfectants (B88t-iNOS) developed small tumors. No mice demonstrated metastases at the cervical lymph node or distant metastases in iNOS-up-regulated transfectants group (Table I).

In vivo evaluation of iNOS expression and apoptosis. We investigated the iNOS expression in nude mice tumor immunohistochemically and the internucleosomal DNA fragmentation using the TUNEL method. As shown in Figure 6, a high expression of iNOS was observed in tumors produced by the sense transfectants (B88t-iNOS), but low expression of iNOS was observed in tumors produced by control cells (B88t-neo). Moreover, the TUNEL-positive cells were significantly increased in tumors produced by the sense transfectants compared to tumors produced by control cells.

Discussion

NO and NOS have been recognized as having important roles in many and diverse biological processes, including inflammation and carcinogenesis (6). However, it seems that NO and iNOS are related to the antitumor effect as well as tumor progression. For example, it has been reported that
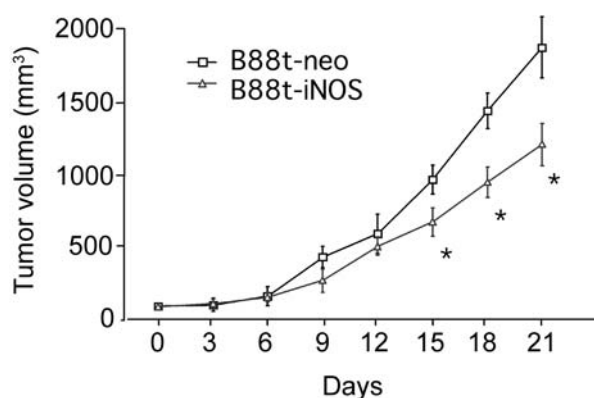
Figure 5. *Growth of tumors formed by transfectants. Tumor cells (1^1x10^6) were suspended in 0.1 ml of serum-free medium and injected into the subcutaneous tissue of nude mice. Sizes of the tumors were determined by first measuring length (L) and width (W) and then calculating the volume ($0.4xLxW^2$). Each group had 5 mice. The values shown are the mean of four tumors (mm^3). bars, SD. *, $p<0.01$ when compared with that of control cells by one-way ANOVA.*

Table I. *Tumorigenicity, invasive and metastatic potential of each transfectant in nude mice.*

Transfectants	Subcutaneous injection ($1x10^6$)			Tongue injection ($1x10^6$)		
	Tumorigenicity	Metastases		Tumorigenicity	Metastases	
		Lymph (neck)	Lung		Lymph (neck)	Lung
B88t-neo	5/5	0/5	0/5	5/5	4/5	0/5
B88t-iNOS	5/5	0/5	0/5	5/5	0/5	0/5

NO production in breast cancer cells correlates with tumor grade and metastasis (20). In addition, it is thought that NO may enhance mammary tumor development and metastases by increasing DNA damage, angiogenesis and migration of breast cancer cells (21,22). On the other hand, an inverse correlation has been reported between iNOS expression and breast cancer progression and metastasis (23). Moreover, the induction of a high concentration of NO in some types of cancer cells may cause apoptosis (13), whereas excessive NO production resulted in rapid tumor growth in a rat solid tumor (24). Furthermore, Fidler *et al.* have reported that overexpression of the iNOS gene can suppress tumor growth and metastasis of melanoma and renal carcinoma cells (13,14). Although iNOS expression and activity have been demonstrated even in head and neck cancer (25,26), the results are still controversial. Therefore, we tried to examine whether up-regulation of the iNOS gene can affect the growth and metastasis of oral cancer cells, B88t *in vitro* and *in vivo*. We constructed an expression vector containing sense-oriented murine iNOS cDNA with pcDNA3.1. We transfected B88t cells with the sense expression vector to up-regulate the expression of the iNOS gene in sense transfectants.

In this investigation, we detected the up-regulation of iNOS protein in sense transfectants when compared with that in parental cells or control cells which were transfected with pcDNA3.1 without insert. In addition, we demonstrated that up-regulation of the iNOS gene may suppress cell growth and migration *in vitro* and tumorigenicity and metastasis *in vivo*. Briefly, consistent with a report of Fidler *et al.* in melanoma and renal

carcinoma cells (13,14), overexpression of the iNOS gene could suppress the tumorigenicity and metastasis of oral cancer cells. Interestingly, we could not detect the growth inhibitory effect in sense transfectants until day 2, which was considered to be in the logarithmic growth phase in the case of *in vitro* assay. However, the cell number of sense transfectant was significantly decreased on days 4 and 6, which was semi-confluenct when compared to that of control cells. In the case of the *in vivo* assay, up-regulation of the iNOS gene exerted a dramatic growth inhibitory effect. When the tumor cells were inoculated into nude mice, the cells were tightly packed in the limited space similar to a contact-inhibition *in vitro*. Briefly, the growth inhibitory effect was marked in sense transfectant when the cell density was high.

These effects may be the result of high NO production by up-regulation of the iNOS gene in tumor cells. It is thought that low concentrations of NO (20 pmol per min per mg of protein) promote tumor growth and angiogenesis whereas at high concentrations (1 to 2 orders of magnitude higher) NO has anti-tumor activity by inducing cytotoxicity (27) and apoptosis (13). High concentrations of NO might exsit around sense transfectants when the cell density is high. Also, the effects could not be attributed to nonspecific effects of the vector since B88t-neo did not result in any growth inhibitory effect.

We then examined whether iNOS overexpression is related to the induction of apoptosis. We could detect a marked enhancement of apoptosis in B88t-iNOS tumor when compared with that of control cells *in vivo*. We have already observed that apoptosis was significantly increased with high concentrations of exogenous NO, delivered by the NO donor, NOC-18 (data not shown). Taken together, these observations indicate that iNOS overexpression in oral cancer cells leads to high NO production that enhances apoptosis.

A significant suppression of tumor growth of sense transfectants *in vivo* would be worthy of note. In addition, transfectants did not affect the body weight of the nude mice (data not shown). These findings may suggest that up-
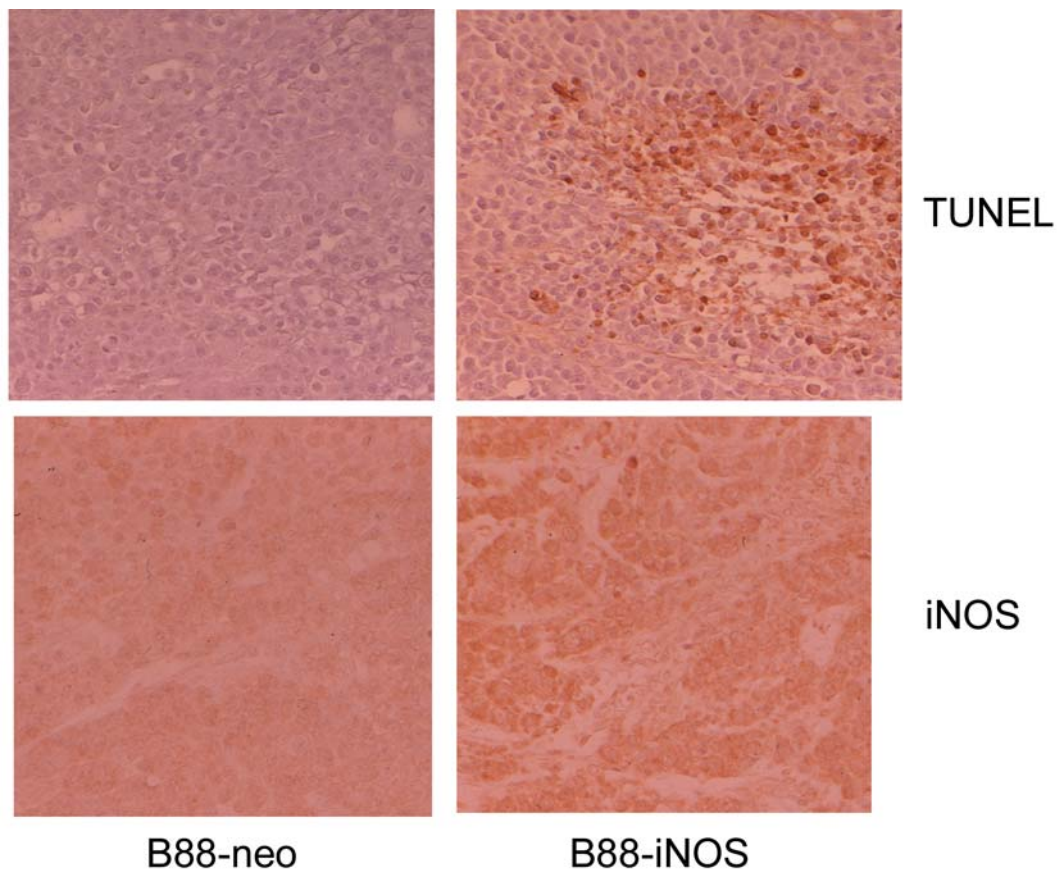
Figure 6. *Internucleosomal DNA fragmentation in nude mice tumor was investigated by the TUNEL method and iNOS expression was investigated by immunohistochemical staining with anti-iNOS polyclonal antibody.*

regulation of the iNOS gene in tumor cells will be safe for the body and that the iNOS gene can be used as molecular target for gene therapy if its precise function can be clarified. Recently, extensive investigations have been performed to elucidate the role of iNOS in normal and neoplastic cells of the oral cavity (28-30), but conclusions have not been drawn about its function. In fact, iNOS must be seen as a double-edged sword for cancer therapy. However, it may be a very useful strategy for cancer treatment to up-regulate the iNOS gene in cancer cells to produce high concentrations of NO around them.

Acknowledgements

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