Nitric Oxide Accelerates Interleukin-13 Cytotoxin-Mediated Regression in Head and Neck Cancer Animal Model

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ABSTRACT
Receptors for interleukin-13 (IL-13R) are overexpressed on several types of solid cancers including glioblastoma, renal cell carcinoma, AIDS Kaposi’s sarcoma, and head and neck cancer. Recombinant fusion proteins IL-13 cytotoxin (IL13-PE38QQR or IL13-PE38) have been developed to directly target IL-13R-expressing cancer cells. Although it has been found that IL-13 cytotoxin has a direct potent antitumor activity in vivo in nude mouse models of human cancers, the involvement of indirect antitumor effector molecules such as nitric oxide (NO) is unknown. To address this issue, we assessed the effect of NO inhibitor [N\textsuperscript{*}-monomethyl-L-arginine on IL-13 cytotoxin-mediated cytotoxicity and NO2/NO3 production in HN12 head and neck cancer cells. In addition, antitumor effects and NO levels in HN12 and KCCT873 head and neck tumors xenografted s.c. in nude mice when treated with IL-13 cytotoxin were evaluated by tumor measurement, Western blot, and immunohistochemistry analyses. Finally, macrophage depletion and NO inhibition experiments were performed to confirm whether NO accelerates antitumor effect of IL-13 cytotoxin on head and neck tumor cells.

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INTRODUCTION
Receptors for a Th2-cell-derived pleiotropic immune regulatory cytokine interleukin-13 (IL-13R) were found to be overexpressed on solid tumor cells including glioblastoma (1–5), renal cell carcinoma (6), AIDS Kaposi’s sarcoma (7), and cancers of prostate (8), ovary (9), and head and neck (10, 11). Although the significance of IL-13R expression in tumor cells is still unknown, IL-13Rα2 chain, a premier IL-13 binding/internalization component in the IL-13R complex (12–14), may play a role in tumorigenicity and cancer progression (11, 15–17). To directly target IL-13R on tumor cells, chimeric fusion proteins composed of IL-13 and a truncated form of Pseudomonas exotoxin, IL-13 cytotoxins (IL13-PE38 and IL13-PE38QQR), have been developed (10, 18). These molecules are found to be highly cytotoxic to tumor cells expressing IL-13R in vitro (1, 7, 8, 10, 18) and exhibit a potent antitumor activity in vivo in athymic nude mouse models of human cancers (19–22). IL-13 cytotoxin mediated apoptotic and necrotic cell death in head and neck tumor in vitro and in vivo (23, 24). Based on these preclinical results, Phase I/II clinical trials are currently ongoing to evaluate its safety, tolerability, and efficacy in patients with recurrent glioblastoma multiforme (25–27).

Nitric oxide (NO) is an important signal transduction molecule and has been implicated in a variety of functions such as vasodilatation, neurotransmission, host defense, and apoptosis (28, 29). In tumor cells, NO can cause cell death by either necrosis or apoptosis. NO is endogenously produced by NO synthase (NOS). Inducible isoenzyme (iNOS or NOS2) was originally isolated from mouse macrophages, but later it was identified in a variety of cell types including human cell types. NOS expression in macrophages is controlled by cytokines and microbial products, primarily by transcriptional induction (30, 31). NO induces both cytotoxic effect and anti-apoptotic effects on tumor cells (30, 32–36). The role of NO is also investigated in tumor therapy including IL-2 immunotherapy, hyaluronan, and protein A of Staphylococcus aureus (30, 37, 38). We have previously demonstrated that IL-13 cytotoxin can induce NO production by head and neck cancer cells (23). However, no documented report is available as to whether NO contributes to the antitumor mechanisms of IL-13 cytotoxin and what is the mechanism of action.

To address these issues, in this study, we assessed the effect of NO inhibitor [N\textsuperscript{*}-monomethyl-L-arginine on IL-13 cytotoxin-mediated cytotoxicity and NO2/NO3 production in HN12 head and neck cancer cells. In addition, NO levels in HN12 and KCCT873 head and neck tumors xenografted s.c. in nude mice, when treated with IL-13 cytotoxin, were evaluated by Western blot and immunohistochemistry analyses. Finally, in vivo macrophage depletion and NO inhibition experiments were performed to confirm whether NO accelerates antitumor effect of IL-13 cytotoxin on head and neck tumor cells.
MATERIALS AND METHODS

Cell Culture and Cytotoxin. Human head and neck cancer cell line WSU-HN12 (termed HN12) was a kind gift from Dr. Andrew Yeudall (National Dental and Craniofacial Research Institute, NIH, Bethesda, MD; Ref. 23). KCCT873 cell line was established at Research Institute, Kanagawa Cancer Center (Yokohama, Japan; Ref. 39). Cells were cultured in Eagle’s modified essential medium (HN12) or RPMI 1640 (KCCT873) with 10% fetal bovine serum, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM l-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Biosource International, Inc., Camarillo, CA). Recombinant IL13-PE38 was generated following the procedure described previously (10, 18) and diluted in PBS containing 0.2% human serum albumin for all studies.

Athymic Nude Mouse Models of Human Head and Neck Cancer. Four-week-old (about 20 g in body weight) athymic nude mice were obtained from the Frederick Cancer Center Animal Facilities (National Cancer Institute, Frederick, MD). Animal care was in accordance with the guidelines of the NIH Animal Research Advisory Committee. Human head and neck tumor models were established in the nude mice by s.c. injection of HN12 or KCCT873 tumor cells (5 × 10⁶) in 150 μl of PBS into the flank. Palpable tumors developed within 3–4 weeks. Tumors were measured by Vernier calipers. Five to seven mice were used for each group.

Western Blot Analysis. Tumor lysates were mixed with SDS sample buffer and heated for 5 min at 97°C. Proteins (20 μg/lane) were separated by 4–12% SDS/PAGE and transferred onto a polyvinyldene difluoride membrane (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 h in Tris-buffered saline tween-20 [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Tween 20] and blocking buffer. After blocking, membranes were incubated for 1 h with rabbit antimouse iNOS polyclonal antibody (M19; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated for 18 h at 4°C or isotype control for 18 h at 4°C. Slides were then developed using 3,3′-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich, Inc.).

For immunofluorescent assays, frozen sections were costained with antibodies for macrophages (Mac-3; BD PharMingen, San Diego, CA) and iNOS. Slides were fixed in acetone at −20°C for 5 min and briefly dried. Nonspecific binding was blocked by treatment with 10% serum for 1 h followed by incubation with antibodies or isotype control. Sections were subsequently incubated for 1 h with secondary antibodies that had either tetramethylrhodamine isothiocyanate or FITC tags. After three washes with PBS, slides were dried and layered with Vectashield antifluorescence fading mounting medium (Vector Laboratories) and a coverslip. The slides were viewed in an Olympus IX70 fluorescence microscope using appropriate filters (Olympus Optical Co., Tokyo, Japan). Images were compiled from sets of three consecutive single optical sections using SPOT INSIGHT V 3.2 software (Diagnostic Instruments, Sterling Heights, MI). Immunohistochemical assays were performed two to three times independently with similar results.

Isolation of Murine Macrophages and in Vitro Nitric Oxide Inhibition Assay. Athymic nude mice received i.p. injections of thioglycolate (40 mg/mouse). Five days after injection, mice were sacrificed, and peritoneal exudate cells were collected by washing peritoneum with cold PBS. Cells were incubated with ACK lysis buffer (Biowhittaker, Inc., Walkersville, MD) and washed with cold PBS, and 1 × 10⁶ cells were seeded in 6-well plates with DMEM. For NO inhibition experiments, 0.5 mM Nω-monomethyl-γ-arginine (Sigma-Aldrich, Inc.) was added to HN12 cells cocultured with murine macrophages (30).

Nitric Oxide Measurement. Concentration of nitrite and nitrate in cell culture or blood serum taken from nude mice was measured with a Griess assay reagent (NO2/NO3 detection kit; Dajindo, Kumamoto, Japan) as described previously (23). In brief, supernatant of the cultured medium without phenol serum was collected and then reagent with a Griess reagent. The azo coupling between diazonium species, which are produced from sulfanilamide with NO2, and 1-naphthylethylenediamine was measured at 540 nm with an MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA).

Macrophage Depletion in Vivo. Macrophage depletion in animals was performed as described previously (40, 41). Carrageenan (type II; Sigma-Aldrich) was dissolved in sterile PBS at 5 mg/ml. The solution was heated to 56°C to ensure complete solubilization. Mice were treated by i.p. injection of 200 μl (1 mg) of carrageenan on days 3, 7, and 14 after the tumor implantation. Control mice received 200 μl of sterile PBS.

Nitric Oxide Inhibition in Vivo. NO inhibition was performed by loading a mini-osmotic Alzet pump model 1007D (total of 100 μl/7 days infusion; Durect Co., Cupertino, CA) with 1.6 M Nω-monomethyl-γ-arginine (30). The pump was surgically implanted i.p. on day 3 after tumor implantation. In brief, nude mice were anesthetized with ketamine and xylazine and placed in the supine position. An upper midline abdominal incision was made, and pumps were inserted from the top of the device (22).

Statistical Analysis. Tumor size was calculated by multiplying length and width of tumor on a given day. The statistical significance of tumor regression was calculated by Student’s t test. All statistical tests were two-sided.

RESULTS

Nitric Oxide Synthase Expression in Head and Neck Tumors That Received Injections of Intratumoral Interleukin-13 Cytotoxin. First, we assessed the NOS expression in head and neck tumors treated with IL-13 cytotoxin. HN12 or
KCCT873 s.c. tumors in nude mice received one intratumoral injection of IL13-PE38 (2 μg) 4 days after tumor implantation. Tumors were harvested 0.5–72 h post injection, and then Western blot analysis was performed to analyze NOS expression in tumor lysates. As shown in Fig. 1A, NOS was detectable as early as 0.5 h post injection in both HN12 and KCCT873 tumors. High levels of NOS were induced in HN12 tumors, which could be detected up to 72 h. In contrast, NOS expression was modest in KCCT873 tumors. HN12 tumors also received injections of IL13-PE (2 μg) on alternate days on days 4, 6, and 8 post tumor implantation. Tumors were harvested 6 h to 15 days after the first injection (day 4), and Western blot analysis was performed. As shown in Fig. 1B, stronger intensity of NOS expression was observed on the days of injection (6, 48, and 96 h), and NOS was detectable at slightly higher than the baseline even after 15 days. Blood serum samples were also collected from HN12 tumor-bearing animals receiving one intratumoral or i.p. injection of IL13-PE38 (2 μg), at 0.5–6 h post injection. Nitrite levels in blood serum were measured by Griess assay. As shown in Fig. 1C, neither intratumoral nor i.p. IL-13 cytotoxin injection affected serum NO level in treated mice. These results suggest that NOS detected in tumor samples were not originated from blood circulation or host reaction to IL-13 cytotoxin.

**Macrophages at Treated Tumor Site Produce Nitric Oxide.** To determine which cell type produces NO in the IL-13 cytotoxin-injected tumors, HN12 tumors receiving intratumoral IL13-PE38 (2 μg) were harvested 24 h post injection and fixed in formalin or snap frozen. Paraffin-embedded samples were assessed for NOS expression by immunohistochemistry. As shown in Fig. 2A, a majority of NOS-positive cells in the IL-13 cytotoxin-injected dying tumors were identified to be monocytes. No strong staining was observed in control tumors.

![Fig. 1](image1.png)

**Fig. 1** NOS expression in head and neck tumors treated with IL-13 cytotoxin. HN12 or KCCT873 tumors xenografted in nude mice (day 0) were treated with 2 μg of intratumoral IL13-PE38—one injection on day 4 (A) and three injections on days 4, 6, and 8 (B)—and harvested at indicated time periods. Protein extracts (pooled from two each animals) were assessed for NOS expression by Western blot analysis. Actin staining served as an internal control. C, HN12 tumor-bearing mice received one i.p. (IP) or intratumoral (IT) injection of IL13-PE38 (2 μg). Blood serum samples were taken at indicated time periods post injection, and nitrite levels were measured by Griess assay. Bars indicate SD in samples (n = 3). Experiments were repeated three times.

![Fig. 2](image2.png)

**Fig. 2** Immunohistochemistry in head and neck tumors treated with intratumoral IL-13 cytotoxin. HN12 (A and B) or KCCT873 (B) tumors xenografted in nude mice (day 0) were treated with one intratumoral IL13-PE38 (2 μg) on day 4. Twenty-four h post injection, tumors were harvested, fixed, and stained with antibody to NOS (A) or co-stained with antibodies to NOS and macrophage (B). NOS and Mac-3 stained pictures were superimposed. B, original magnification, ×600.
We then costained tumor sections with antibodies to macrophages (Mac-3) and NOS (M19) and assessed by immunofluorescent microscopy. As shown in Fig. 2B, NOS2-positive cells and Mac-3-positive cells were observed in tumors treated with intratumoral IL-13 cytotoxin. Certain Mac-3-positive cells were also positive for NOS, as assessed by superimposing two images stained with Mac-3 and M19 antibodies (Fig. 2B, bottom panel). In contrast, positively stained cells were not found in control tumors (data not shown). These results suggest that NO at IL-13 cytotoxin-treated tumor site was mainly produced from infiltrating macrophages.

Nitric Oxide Released from Macrophages Helps Mediate Cytotoxicity of Interleukin-13 Cytotoxin in Tumor Cells. Because we found NO-positive macrophages in tumors receiving IL-13 cytotoxin, the impact of NO released from the active macrophages on cytotoxicity of IL13-PE38 was then evaluated using HN12 cells. HN12 cells were cocultured with murine macrophages. Cocultured cells were incubated with IL13-PE38 (0–100 ng/ml) for 48 h with or without NO inhibitor (N\textsubscript{\text{\textregistered}}-monomethyl-L-arginine), and then viable HN12 cells were counted by trypan blue staining. As shown in Fig. 3A, macrophage showed modest but significant effect on IL-13 cytotoxin-mediated cell death (*, \(P < 0.05\)). The presence of NO inhibitor reversed this cytotoxicity (#, \(P < 0.05\)), and the number of viable HN12 cells reached baseline comparable with only NO inhibitor-treated group.

Using cell culture supernatant, we also measured NO levels in each group. As shown in Fig. 3B, both nitrite and nitrate levels were found augmented in groups cocultured with macrophage. NO levels were significantly higher (+, \(P < 0.05\) by NOS2 measurement) in HN12 cells cocultured with macrophages and IL13-PE38, compared with no treatment groups. As expected, NO inhibitor reversed NO levels in all groups. These results suggest that NO is released from macrophages and partially contributes to cytotoxicity mediated by IL-13 cytotoxin in head and neck cancer cells.

Macrophage Depletion Reverses the Effect of Interleukin-13 Cytotoxin on Tumor Growth. To assess the definite role of macrophages in the tumor regression mechanism mediated by IL-13 cytotoxin, mice received injections of HN12 tumor cells s.c. (day 0) and then received injections of carrageenan (type II) on days 3, 7, and 14. Subsequently, the treatment with three intratumoral IL13-PE38 (2 \(\mu\)g/injection on days 4, 6, and 8) was performed. As shown in Fig. 4, established tumors treated with IL13-PE38 showed regression in both macrophage-depleted and nondepleted groups. The extent of tumor regression in macrophage-depleted mice was less pronounced compared with nondepleted mice. Due to some complete responder animals in nondepleted group, SDs were large, however, mean

![Fig. 3](https://example.com/fig3.png) Effect of NO inhibitor on IL-13 cytotoxin-mediated cancer cell death. HN12 cells (2 \(\times\) 10\(^5\)/well) were cocultured with or without murine macrophages (2 \(\times\) 10\(^6\)/well), 0.5 mM N\textsubscript{\text{\textregistered}}-monomethyl-L-arginine (MLA), and IL13-PE38 (0–100 ng/ml) in 6-well culture plates for 48 h. A, viable cancer cells were counted by trypan blue exclusion. Data represent percent viability with SD (\(n = 3\)). *\(P < 0.05\); and #, \(P < 0.05\) assessed by two-sided Student’s t test. B, nitrite and nitrate levels in the cultured medium (phenol red-free) were measured by Griess assay. Cocultured cells were treated with IL13-PE38 (100 ng/ml). Bars represent SD (\(n = 2\)). *\(P < 0.05\) assessed by two-sided Student’s t test. Experiments were repeated two times.
tumor size in macrophage-depleted mice was significantly larger (94.7 ± 35.7 mm²) compared with mice without macrophage depletion (44.6 ± 45.4 mm²) by the end of the experiment (day 35; \( P < 0.05 \)). These results confirm that macrophages infiltrating into regressing tumors followed by IL-13 cytotoxin therapy play some role in the antitumor mechanism.

**Effect of Nitric Oxide Inhibitor on Interleukin-13 Cytotoxin-Mediated Tumor Regression in Vivo.** To analyze the role of NO in the tumor regression mechanism induced by IL-13 cytotoxin, mice were surgically i.p. implanted with mini-osmotic pump loaded with NO inhibitor (N\(^{\text{e}}\)-monomethyl-L-arginine). Pumps were implanted on day 3 post HN12 tumor cell injection, and NO inhibitor was administered in mice for 7 days by the osmotic pressure (42). Mice received three intratumoral IL13-PE38 (2 µg) on days 4, 6, and 8. As shown in Fig. 5, IL13-PE38 showed less antitumor activity in animals treated with NO inhibitor even though they were treated with identical dose and schedule (days 4–8). After day 14, tumors in mice receiving NO inhibitor and IL13-PE38 started growing. However, tumor growth in mice receiving IL13-PE38 remained suppressed. Mean tumor size in mice receiving NO inhibitor and IL13-PE38 was significantly larger (99.3 ± 17.9 mm²) compared with mice receiving IL13-PE38 alone (31.5 ± 18.2 mm²) by the end of the experiment (day 30; \( P < 0.05 \)).

**DISCUSSION**

We demonstrated that NO accelerates IL-13 cytotoxin-mediated cell death in vitro and antitumor effects in vivo. These conclusions were drawn based on several lines of experimental evidence: (a) NOS expression was detected in xenografted head and neck tumors as early as 0.5 h post intratumoral IL13-PE38 administration; (b) NO was mostly found to be released from macrophages infiltrating tumors; (c) in vitro inhibition of macrophage-derived NO by N\(^{\text{e}}\)-monomethyl-L-arginine inhibited IL-13 cytotoxin-mediated cytotoxicity of head and neck tumor cells; (d) in vivo depletion of macrophage reduced antitumor effects of IL-13 cytotoxin; and (e) treatment of head and neck tumor bearing mice with NO inhibitor N\(^{\text{e}}\)-monomethyl-L-arginine significantly reduced the efficacy of IL-13 cytotoxin.

We have previously demonstrated that NO is expressed in regressing tumors treated with IL-13 cytotoxin (43), however, its significance and contribution of NO in the IL-13 cytotoxin-mediated antitumor mechanism was unknown. Here, we show that NO plays a modest role in in vitro cytotoxic effect of IL-13 cytotoxin to cancer cells. In contrast, the effect of NO was significant in vivo, because NO inhibitor sharply reversed the antitumor effect of IL-13 cytotoxin. Although it is possible that NO-producing macrophages were recruited by dying tumor cells, our study indicates that these cells mount an impact on additional tumor regression by IL-13 cytotoxin and the commitment of NO is important in this mechanism.
It is of interest that in vivo NO inhibition experiments clearly exhibited the contribution of NO in tumor regression induced by IL-13 cytotoxin, whereas macrophage depletion did not prominently alter the antitumor activity of the agent. This could be explained by production of NO by other cellular components besides macrophages, which release a majority of NO in IL-13 cytotoxin-treated tumors. Thus macrophage depletion might not have been enough to shut down all NO in tumor. Alternatively, because it is impossible to deplete all macrophages in the mouse body, remaining macrophages and other cellular components were capable of releasing cytotoxic chemokine(s) or substrate(s) including NO, which helped in mediating the antitumor effect of IL-13 cytotoxin.

NO can modulate both tumor growth and antitumor immune responses (28, 34, 44). Chen et al. (45) demonstrated that human head and neck tumor specimens express detectable levels of NOS. Previously, we have also demonstrated that HN12 head and neck tumors produce basal level of NO (23). It is further hypothesized that NOS expression correlates with cervical lymph node metastasis from oral squamous cell carcinoma (45). The role of basal NO in head and neck tumors is still unknown. There have been a number of reports which document the involvement of NO in tumor immunotherapy (30, 34, 37, 38, 44). However, the role of NO in cancer immunotoxin/cytotoxin therapy has been rarely investigated. Our current study is therefore valuable as it demonstrates the involvement of NO in cancer therapy with a targeted agent.

It is not clear why macrophages and NO enhance the effect of IL-13 cytotoxin. The effect of IL-13 cytotoxin is mediated by Pseudomonas exotoxin (PE) portion of IL13-PE38 molecule. After binding to IL-13R, IL13-PE38 is internalized inside the cell, and then through a series of biochemical events, domain III of PE molecule inhibits protein synthesis by ADP ribosylation of elongation factor 2 (17). Thus, a primary mechanism of IL-13 cytotoxin-mediated cell death is by direct cytotoxicity. It is possible that dying tumor cells attract macrophages as a part of activation of innate immune response, which combine with IL-13 cytotoxin in mediating cell death. Alternatively, it is possible that activated immune response kills tumor cells that do not express high levels of IL-13R. Later hypothesis is perhaps supported by heterogeneity of IL-13R expression in head and neck tumor cells. Our recent study has demonstrated that head and neck tumor samples do not uniformly express all three chains of IL-13R involved in the formation of IL-13R complex (11). The involvement of innate immune system in killing IL-13R-negative or low-IL-13R-expressing cells is further supported by our recent findings that gene transfer of IL-13Rα2 cDNA in tumors enhances the cytotoxic effect of IL-13 cytotoxin in vitro and in vivo (21, 41, 46). Additional studies involving experimental tumors induced by mixing of IL-13R-positive and IL-13R-negative tumor cells followed by IL-13 cytotoxin administration will further elucidate the role of NO in IL-13 cytotoxin-induced tumor regression.

Three Phase I/II clinical trials are currently ongoing to evaluate the tolerability and safety as well as antitumor effect of IL-13 cytotoxin in patients with recurrent glioblastoma. These studies employ a novel route of drug infusion termed convection-enhanced delivery. Convection-enhanced delivery is a local delivery method in which pressure gradient or bulk flow is used to control an infusate through the extracellular fluid compartment (47). Thus far, this route of IL-13 cytotoxin administration appears to be very well tolerated with no neurotoxicity (25–27). In addition, preclinical studies employing intratumoral IL-13Rα2 gene transfer and IL-13 cytotoxin exhibit a dramatic antitumor response in several animal models of human cancers (17, 21, 41, 46). Therefore, information acquired from our current study is critically important for the additional development of IL-13 cytotoxin and application of this agent in a variety of cancer types such as head and neck cancer.

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