Atrial natriuretic peptide prevents cancer metastasis through vascular endothelial cells

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Most patients suffering from cancer die of metastatic disease. Surgical removal of solid tumors is performed as an initial attempt to cure patients; however, surgery is often accompanied with trauma, which can promote early recurrence by provoking detachment of tumor cells into the blood stream or inducing systemic inflammation or both. We have previously reported that administration of atrial natriuretic peptide (ANP) during the perioperative period reduces inflammatory response and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery. Here we demonstrate that cancer recurrence after curative surgery was significantly lower in ANP-treated patients than in control patients (surgery alone). ANP is known to bind specifically to NPR1 [also called guanylyl cyclase-A (GC-A) receptor]. In mouse models, we found that metastasis of GC-A–nonexpressing tumor cells (i.e., B16 mouse melanoma cells) to the lung was increased in vascular endothelium-specific *GC-A* knockout mice and decreased in vascular endothelium-specific *GC-A* transgenic mice compared with control mice. We examined the effect of ANP on tumor metastasis in mice treated with lipopolysaccharide, which mimics systemic inflammation induced by surgical stress. ANP inhibited the adhesion of cancer cells to pulmonary arterial and micro-vascular endothelial cells by suppressing the E-selectin expression that is promoted by inflammation. These results suggest that ANP prevents cancer metastasis by inhibiting the adhesion of tumor cells to inflamed endothelial cells.

cardiac peptide | cancer metastasis | vascular endothelial cell | inflammation | surgery

The majority of cancer patients die from tumor metastasis. Despite substantial advances in our understanding of the mechanisms of tumor metastasis, effective prevention of metastasis has not been well established. Surgical removal of solid tumors is performed to cure patients if the primary tumor meets surgical indications; however, postoperative cancer recurrence is a major problem. Surgical trauma itself influences the development of early recurrence (1, 2). First, the procedure during tumor removal might provoke detachment of tumor cells; consistently, the number of circulating tumor cells is increased during primary tumor resection (3, 4). We previously reported that the presence of circulating tumor cells in pulmonary veins during lung cancer surgery could be a prognostic indicator for early cancer recurrence (4). Second, surgical trauma provokes a severe systemic inflammatory reaction. Emerging evidence suggests that systemic inflammation can accelerate the adhesion of circulating tumor cells to the vascular endothelium of distant organs, which is the first step of extravasation in hematogenous metastasis (5, 6).

We identified human atrial natriuretic peptide (ANP) as a diuretic, natriuretic, and vasodilating hormone from the human heart in 1984 (7). ANP binds specifically to the guanylyl cyclase-A (GC-A) receptor to exhibit biological functions, including promotion of diuresis, antifibrotic action, and inhibition of renin-angiotensin-aldosterone (8, 9). Thus, ANP has been used clinically for the treatment of heart failure since 1995 in Japan. We previously reported that administration of human ANP during the perioperative period reduces inflammatory responses and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery (10–12). In those studies,

Significance

Postoperative cancer recurrence is a major problem following curative cancer surgery. Perioperative systemic inflammation induces the adhesion of circulating tumor cells released from the primary tumor to the vascular endothelium of distant organs, which is the first step in hematogenous metastasis. We have previously reported that administration of atrial natriuretic peptide (ANP) during the perioperative period reduces inflammatory response and has a prophylactic effect on postoperative cardiopulmonary complications in lung cancer surgery. Here, we demonstrate that cancer recurrence after lung cancer surgery was significantly lower in ANP-treated patients than in control patients (surgery alone). We show that ANP prevents cancer metastasis by suppressing the inflammatory reaction of endothelial cells, thereby inhibiting cancer cell adhesion to vascular endothelial cells.

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ANP was used to promote diuresis during perioperative right-side heart failure caused by lung damage. Here, we further analyzed the effect of ANP on prevention of cancer recurrence after surgery and found that ANP might have antitumor metastatic activity. We explored the antimetastatic action of ANP by using tissue-specific *GC-A* transgenic and knockout mice of tumor metastasis models. Our results suggest that ANP could be useful as an antimetastasis peptide to prevent cancer recurrence after surgery.

Results

Clinical Impacts of ANP Therapy on Cancer Recurrence After Lung Cancer Surgery. We performed a retrospective study of the incidence of cancer recurrence in lung cancer patients after curative surgery, comparing patients who underwent perioperative ANP treatment with those who were subjected to surgery alone (control patients). The 2-y relapse-free survival (RFS) after surgery was significantly greater in ANP-treated patients than in control patients (91% vs. 75%, $P = 0.018$) (Fig. 1A). To eliminate bias, we reanalyzed the data by using propensity score matching. The 2-y RFS in the propensity score-matched analysis was also significantly greater in ANP-treated patients than in control patients (91% vs. 67%, $P = 0.0013$) (Fig. 1B and *SI Appendix*, Table S1). We hypothesized from these retrospective observations that ANP may prevent recurrence of lung cancer.

Antimetastatic Effects of ANP in Hematogenous Pulmonary Metastatic Models. Vascular inflammation is considered to render the endothelium adhesive to circulating tumor cells, thereby allowing the metastasis of tumor cells (5, 6). We previously reported that postoperative complications induced by inflammation are reduced by ANP (10–12). Therefore, to investigate whether ANP inhibits the metastasis of cancer cells to inflamed organs, we examined the effect of ANP on tumor metastases in mice injected with LPS, which mimics systemic inflammation induced by surgical stress (6, 13). The LPS-treated mice showed numerous hematogenous pulmonary metastases of intravenously injected A549 lung cancer cells expressing EGFP (A549-EGFP) cells (Fig. 2 A and B and *SI Appendix*, Fig. S1A). In contrast, the mice pretreated with ANP exhibited a large and significant reduction of LPS-induced pulmonary metastasis of A549-EGFP cells (Fig. 2 A and B and *SI Appendix*, Fig. S1A). ANP also significantly inhibited the pulmonary hematogenous metastasis of B16/F10 melanoma cells, which do not express GC-A (Fig. 2 C and D and *SI Appendix*, Fig. S1 B and C). Furthermore, we confirmed that ANP significantly inhibited the pulmonary hematogenous metastasis of A549-EGFP (*SI Appendix*, Fig. S2 A and B) and B16/F10 (*SI Appendix*, Fig. S2 C and D) cells even without LPS, suggesting that ANP inhibits tumor metastasis both under LPS-induced general inflammation and under non–LPS-induced massive inflammation. More importantly, these data indicate that ANP acts through GC-A expressed in nontumor mouse cells.

To eliminate the direct effect of ANP on tumor cell proliferation, we first examined the direct effects of ANP on the growth of cancer cells and found that GC-A was expressed in A549 and H460 human lung cancer cells (*SI Appendix*, Fig. S1B). Even though GC-A was expressed on A549 and H460 cells, ANP did not induce the proliferation of these tumor cells (*SI Appendix*, Fig. S3 A–C). Natriuretic peptide receptor-C, which is also known as a receptor of ANP, was expressed in A549, H460, and B16/F10 cells (*SI Appendix*, Fig. S3A); however, there were no significant effects of ANP on the growth of A549, H460, or B16/F10 cells (*SI Appendix*, Fig. S3 B–D). These results suggest that the inhibitory effect of ANP on tumor metastasis is dependent upon GC-A expressed on cells other than tumor cells.

We considered that GC-A expressed on endothelial cells might be responsible for the antimetastatic effect of ANP because cancer cell attachment to endothelial cells is the initial step in metastasis (5, 6). Vascular endothelial cells abundantly express GC-A, which exhibits a protective role in the cardiovascular system (8, 9). Therefore, to clearly show that the antimetastatic effect of ANP does not depend on GC-A expression in tumor cells, we examined the hematogenous pulmonary metastasis of B16/F10 cells in both endothelium-specific *GC-A* knockout mice (termed EC GC-A-KO mice) and *GC-A* transgenic mice (termed EC GC-A-Tg mice) (*SI Appendix*, Fig. S4). EC GC-A-KO mice exhibited a significant elevation of blood pressure and cardiac hypertrophy compared with *GC-A*^flox/flox^ mice. These phenotypic data were consistent with the previous report (14). The number of pulmonary metastases was significantly higher in EC GC-A-KO mice than in *GC-A*^flox/flox^ mice (Fig. 2 E and F). Furthermore, cardiac metastases were found in one-third of EC GC-A-KO mice, whereas no cardiac metastasis was found in *GC-A*^flox/flox^ mice (Fig. 2E). Overall survival was significantly shorter in EC GC-A-KO mice compared with *GC-A*^flox/flox^ mice (Fig. 2G). In contrast, the number of pulmonary metastases was significantly lower in EC GC-A-Tg mice than in WT mice (Fig. 2 H and I), and EC GC-A-Tg mice survived significantly longer than WT mice (Fig. 2J). Collectively, these data suggest that endothelial GC-A activated by ANP prevents hematogenous pulmonary metastasis of cancer cells in mice.

Mechanism of the Effect of ANP on Cancer Cell Adhesion to Vascular Endothelial Cells. We next attempted to uncover the molecular mechanism behind ANP-mediated inhibition of tumor metastasis through vascular endothelial cells. The attachment of A549-EGFP and H460 lung cancer cells expressing EGFP (H460-EGFP) cells to cultured human pulmonary artery endothelial cells (HPAECs) stimulated with LPS was dependent on the dose

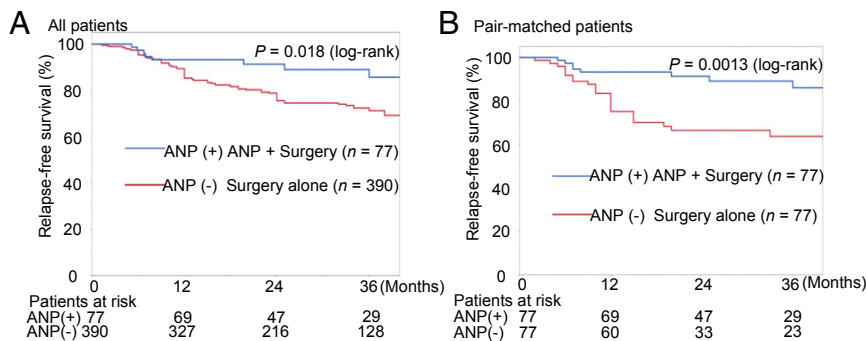**Fig. 1.** Effect of ANP treatment on RFS in patients with surgically resected nonsmall cell lung cancer. (A) Kaplan–Meier curves of the ANP group and control group (surgery alone) in all patients ($P = 0.018$, log-rank test). (B) Kaplan–Meier curves of the above groups in propensity score-matched patients ($P = 0.0013$, log-rank test). RFS was measured from the day of surgery to cancer recurrence.
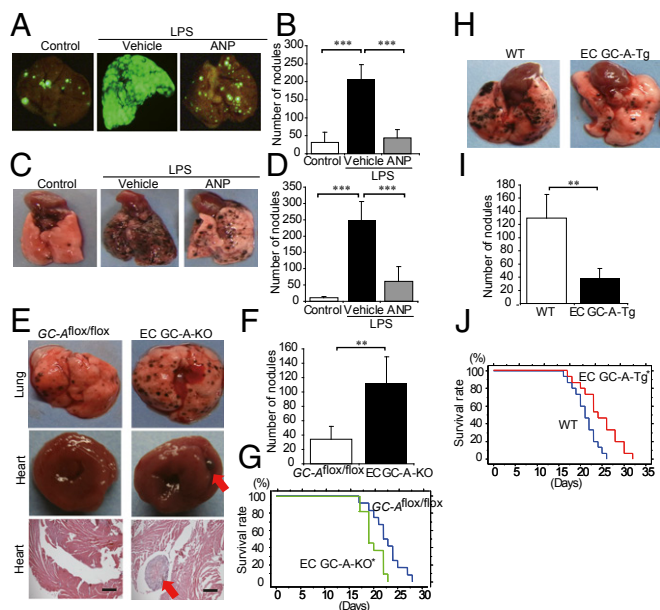
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Fig. 2. ANP inhibits the LPS-augmented metastasis of A549-EGFP lung cancer cells and B16/F10 mice melanoma cells to the lung. (*A*) Representative EGFP images of the lungs of mice that were pretreated with or without LPS and then injected with A549-EGFP cells (1×10^6 cells per mouse) and continuously treated with or without ANP for 4 wk. The mice were killed 6 wk after the injection of tumor cells. (*B*) Bar graph showing the number of nodules representing pulmonary metastasis of A549-EGFP cells in mice grouped as in *A*. Data are means ± SEM (*n* = 6, each group). ***P < 0.001, unpaired two-tailed t test. (*C*) Representative images of the lungs of mice that were pretreated with or without LPS and then injected with B16/F10 cells (2×10^5 cells per mouse) and continuously treated with or without ANP for 2 wk. The mice were killed 2 wk after the injection of the tumor cells. (*D*) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in *C*. Data are means ± SEM (*n* = 6, each group). ***P < 0.001, unpaired two-tailed t test. (*E*) Representative images of the lungs and hearts (*Top* and *Middle*, respectively) and histological cross-sections of the hearts (H&E staining, *Bottom*) of the GC-$A^{\text{flox/flox}}$ mice and EC GC-A-KO mice after injection of B16/F10 cells (2×10^5 cells per mouse). The mice were killed 2 wk after the injection of the tumor cells. (Scale bars, 500 μm.) Red arrows indicate metastasis in the heart. (*F*) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in *E*. Data are means ± SEM (*n* = 10, each group). **P < 0.01, unpaired two-tailed t test. (*G*) Kaplan–Meier curves comparing survival times between GC-$A^{\text{flox/flox}}$ and EC GC-A-KO mice after injection of B16/F10 cells (2×10^5 cells per mouse). *n* = 15 (each group), *P < 0.05, log-rank test. (*H*) Representative images of the lungs of WT and EC GC-A-Tg mice after injection of B16/F10 cells (5×10^5 cells per mouse). The mice were killed 2 wk after the injection of tumor cells. (*I*) Bar graph showing the number of nodules representing pulmonary metastasis of B16/F10 cells in mice grouped as in *H*. Data are means ± SEM (*n* = 10, each group). **P < 0.01, unpaired two-tailed t test. (*J*) Kaplan–Meier curves comparing survival times between WT and EC GC-A-Tg mice after injection of B16/F10 (5×10^5 cells per mouse). *n* = 15 (each group), *P < 0.05, log-rank test.

of LPS (Fig. 3*A*). ANP significantly inhibited LPS-induced tumor cell attachment to HPAECs (Fig. 3 *B* and *C*) and human lung microvascular endothelial cells (HMVEC-L) (*SI Appendix*, Fig. S5 *A*–*C*). LPS induces the expression of cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (15), which in turn promote the infiltration of inflammatory cells, thereby increasing inflammation. Among these cell adhesion molecules, E-selectin is considered to play a central role in hematogenous metastasis (16–18). We therefore examined the adhesion molecule-dependent attachment of tumor cells to vascular endothelial cells. The attachment of A549-EGFP cells to LPS-induced HPAECs

was significantly inhibited by knockdown of E-selectin, but not by knockdown of VCAM-1 or ICAM-1 (Fig. 3 *D* and *E* and *SI Appendix*, Fig. S6*A*).

To search for genes that could be responsible for the ANP-mediated inhibition of tumor cell attachment to vascular endothelial cells, we performed microarray analyses of human umbilical vein endothelial cells (HUVECs) stimulated by ANP. E-selectin expression was markedly reduced in ANP-treated HUVECs compared with those treated with vehicle alone (*SI Appendix*, Table S2). Consistently, the expression of E-selectin induced by LPS in HPAECs was inhibited by ANP, whereas that of neither VCAM-1 nor ICAM-1 was affected (Fig. 4*A*). ANP also significantly inhibited the expression of E-selectin induced by LPS in HMVEC-L (*SI Appendix*, Fig. S5*D*). Furthermore, ANP-mediated suppression of LPS-induced E-selectin expression was not observed in HPAECs depleted of *GC-A* (Fig. 4*B* and *SI Appendix*, Fig. S6*B*). These data indicate that ANP suppresses E-selectin expression through GC-A. Because LPS induces inflammation by inducing nuclear translocation of NF-κB, which in turn promotes E-selectin expression (19), we examined the effect of ANP on nuclear translocation induced by LPS. ANP significantly inhibited the accumulation of NF-κB in the nucleus of LPS-induced HPAECs (Fig. 4 *C* and *D*).

Finally, we performed an in vivo study to evaluate the effects on LPS-induced E-selectin expression in the lung. Five hours after injection of LPS, E-selectin expression in the lung increased dose-dependently, whereas pretreatment with ANP attenuated this increase at both the gene and protein levels (Fig. 4 *E* and *F*). Consistently, immunohistochemical analysis showed that pretreatment with ANP inhibited LPS-induced E-selectin expression in the CD31^{+} vascular endothelium (Fig. 4*G*). Furthermore, mice pretreated with E-selectin–neutralizing antibody exhibited a significant reduction of LPS-induced pulmonary metastasis of B16/F10 cells (*SI Appendix*, Fig. S7). Collectively, these data suggest that ANP inhibits E-selectin expression and reduces the E-selectin–mediated adhesion of tumor cells to vascular endothelium of the lung upon LPS-induced inflammation.
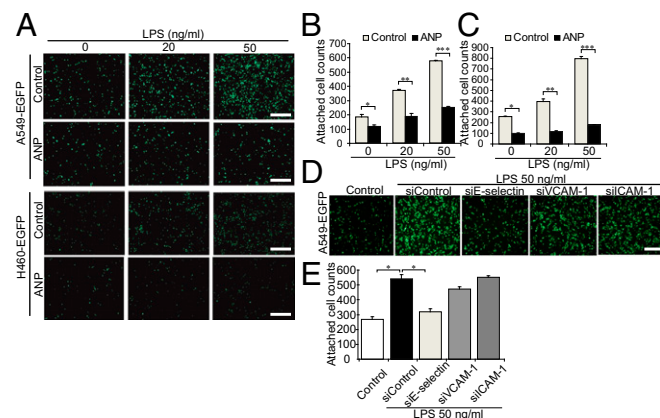
Fig. 3. ANP inhibits LPS-regulated E-selectin–dependent adhesion of cancer cells to vascular endothelial cells. (*A*) Representative images of the adhesion of tumor cells (A549-EGFP, *Upper*; H460-EGFP, *Lower*) to monolayer-cultured HPAECs pretreated with or without ANP. (*B* and *C*) Bar graphs showing the number of A549-EGFP cells (*B*) or H460-EGFP cells (*C*) attached to monolayer-cultured HPAECs pretreated with or without ANP. Data are means ± SEM (*n* = 5, each group). *P < 0.05, **P < 0.01, ***P < 0.001, unpaired two-tailed t test. (*D*) Representative images of A549-EGFP cells attached to HPAECs depleted of the indicated molecules by siRNA treatment and treated with LPS. (*E*) Bar graph showing the number of A549-EGFP cells attached to HPAECs treated as in (*D*). Data are means ± SEM (*n* = 5, each group). *P < 0.05, one-way ANOVA. (Scale bars, 500 μm.)
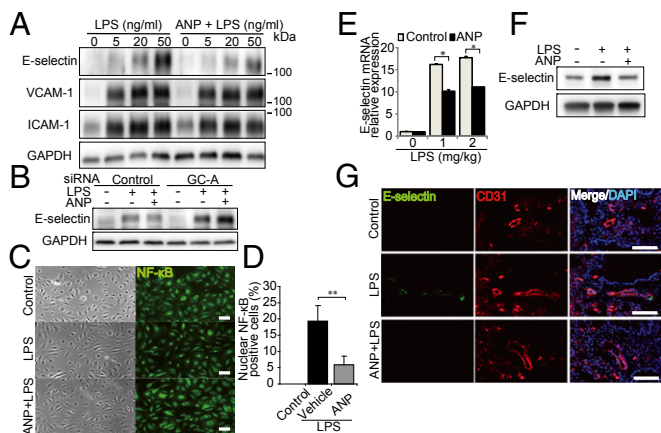
Fig. 4. ANP–GC-A signaling attenuates LPS-induced E-selectin expression. (*A*) Immunoblot analysis of the lysates of HPAECs pretreated with or without ANP followed by LPS stimulation; antibodies used are indicated on the left. Each blot is representative of six independent experiments. (*B*) E-selectin expression assessed by immunoblot analysis of the lysates of HPAECs transfected with the indicated siRNAs and stimulated with LPS. The result shown is representative of six independent experiments. (*C*) Bright field images (*Left*) and NF-κB immunofluorescence images (*Right*) of HPAECs that were unstimulated (control, *Top*), stimulated with LPS alone (*Middle*), or pretreated with ANP followed by LPS stimulation (*Bottom*). Each image is representative of five independent experiments. (Scale bars, 100 μm.) (*D*) Quantitative analyses of *C*. Each column shows the percentage of HPAECs with nuclear NF-κB expression in the indicated group. Data are means ± SEM ($n = 5$, each group); **$P < 0.01$, unpaired two-tailed t test. (*E*) Quantitative reverse transcriptase PCR analysis of E-selectin mRNA levels in the lungs of mice pretreated with ANP or vehicle (control) and treated with LPS. Data are normalized relative to 36B4 mRNA levels. Data are means ± SEM ($n = 6$, each group); *$P < 0.05$, unpaired two-tailed t test. (*F*) Immunoblot analysis of E-selectin levels in lung lysates of mice pretreated with or without ANP followed by LPS stimulation (1.0 mg/kg) for 5 h. Each blot is representative of six independent experiments. (*G*) E-selectin images (*Left*), CD31 images (*Center*), and merged images with DAPI staining (*Right*) of the lungs of mice pretreated with or without ANP followed by LPS stimulation (1.0 mg/kg) for 5 h. Each image is representative of six independent experiments. Nuclei are stained with DAPI (blue). (Scale bars, 100 μm.)

Discussion

Although many clinical trials aimed at preventing cancer recurrence during the perioperative period have been conducted, no prophylactic treatments have been established. The failure of these trials might be ascribed to the risk of surgery alone, and the side effects of the chemicals used in the trials (20, 21). We previously showed that ANP prevents the incidence of postoperative complications after lung cancer surgery (10–12). Here we demonstrate that cancer recurrence after curative surgery was significantly lower in ANP-treated patients than in control patients, suggesting that ANP could potentially be used to prevent cancer recurrence after surgery. We assumed two possibilities as to how ANP inhibited tumor metastases; one was that ANP directly inhibited the tumor cell proliferation and the other was that ANP indirectly inhibited tumor cell metastases by acting on nontumor cells. In previous studies of the direct effects of ANP on cancer cells, both inhibitory and stimulatory effects of ANP on the growth of cancer cells have been reported (22, 23); therefore, the direct effects of ANP on cancer cells remain controversial. In the present study, we focused on the possibility that ANP indirectly inhibits tumor cell metastases through effects on nontumor cells.

Our discovery that mice pretreated with ANP exhibited a dramatic reduction of LPS-induced pulmonary metastasis of introduced cancer cells provides direct evidence that ANP can prevent tumor metastasis in mice. This notion is supported by our finding that mice that specifically overexpress or lack expression of the receptor of ANP (i.e., GC-A) in the vascular endothelium have reduced or enhanced numbers of metastases, respectively, compared with the appropriate control mice. These results suggest that ANP prevents early relapse in patients at least in part by preventing metastasis through the vascular endothelium.

Surgical procedures induce postoperative complications and early recurrence after surgery by releasing inflammatory cytokines, such as IL-1β and TNF-α (1, 2). Recent studies indicate that postoperative complications, including severe inflammatory reaction and infection, after various types of cancer surgery are associated with poor cancer-specific survival (24–26). Endothelial cells that become inflamed during surgery are considered to be prone to adhering to circulating tumor cells, thereby allowing the initiation of metastasis (5, 6). Although most circulating tumor cells undergo rapid cell death by apoptosis (27, 28), it is possible that surgical inflammation promotes the adherence of residual cancer cells to inflamed endothelial cells (5, 6). ANP has anti-inflammatory and anti-infectious activity on endothelial cells. ANP pretreatment reduces serum TNF-α levels and NF-κB activation by inhibiting IκB-phosphorylation in mice injected with LPS (29) and has a protective role against LPS-induced lung injury and endothelial barrier dysfunction (30). Our finding that ANP has anti-inflammatory action (i.e., suppression of LPS-induced E-selectin) in vascular endothelial cells in mice is consistent with these studies. Taken together, our results suggest that ANP-mediated inhibition of metastasis occurs through inhibition of the inflammatory response.

Among the vascular adhesion molecules, E-selectin is essential for recruitment of inflammatory cells to damaged tissues (31), and it enables circulating tumor cells to roll and tether on the endothelium. Recent studies have shown that cross-talk between E-selectin and integrins could facilitate the movement of not only inflammatory cells but also tumor cells through the endothelium to inflammatory foci (16–18). In fact, tumor metastasis is increased in the lungs of E-selectin–overexpressing mice and reduced in E-selectin knockout mice (18). Therefore, E-selectin is considered to play a central role in hematogenous metastasis (16–18). In a clinical study, Gogali et al. reported that serum levels of soluble E-selectin in lung cancer patients were significantly elevated compared with those in control subjects (32). However, we assume that the antimetastasis activity of ANP does not solely depend upon the suppression of E-selectin, because extravasation of cancer cells in the metastatic process is regulated by many other steps. Recent experimental reports demonstrated that inflammatory chemokines including chemokine ligand (CCL) 2 and CCL5 contributed to not only leukocyte recruitment but also tumor cell homing to activated endothelial cells (33, 34). Because we focused on only E-selectin expressions in this study, further studies are necessary to elucidate the detailed mechanism and role of the ANP–GC-A system in cancer metastasis.

Because most current chemotherapeutic agents are cytotoxic and cause many side effects, chemotherapy cannot be used during surgical resection to prevent cancer recurrence. In contrast, ANP is an endogenous and physiological peptide and has been proved not to cause severe adverse effects when used in patients with heart failure (35). Because the target of ANP is considered to be vascular endothelium in all organs that express the GC-A receptor, including lung, liver, and brain, ANP might inhibit hematogenous cancer metastasis to all organs expressing GC-A receptor and could be used for all kinds of malignant tumors.

Materials and Methods

Clinical Study. We retrospectively evaluated 552 consecutive patients who underwent curative surgery for nonsmall cell lung cancer at Osaka University Hospital and National Hospital Organization Toneyama Hospital from August 2007 to December 2011. Patients with carcinoma in situ and those undergoing

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a limited resection, including wedge resection, were excluded. Patients with incomplete postoperative follow up ($n = 8$) were also excluded. Segmentectomy for curative surgery was not excluded. Finally, 467 patients who underwent curative surgery were included in the present study.

RFS, defined as the time from the day of surgery to cancer recurrence, was compared between patients who received ANP during the perioperative period and those that received surgery only. In the ANP group, the subjects received ANP intravenously at 0.025 μg·kg·min (Daiichi-Sankyo Pharmaceutical) without a bolus for 3 d continuously, starting just before the induction of general anesthesia. We previously reported that ANP has a prophylactic effect against postoperative cardiopulmonary complications for patients with elevated preoperative brain natriuretic peptide levels (10, 12, 36). Therefore, we performed a propensity score-matched analysis to reduce the treatment selection bias for each group. The propensity score was estimated by using a logistic regression model adjusted for age, sex, pathological staging [lung cancer tumor, node, metastasis (TMN) staging seventh edition], cancer histology, and preoperative brain natriuretic peptide levels. These variables were chosen for potential associations with the outcome of interest. An independent statistician selected the patients by matching propensity scores without access to clinical outcome information. Patient characteristics for the full and propensity score-matched cohorts are listed in SI Appendix, Table S1. In our matching algorithm, one patient who received ANP was matched to one patient who did not receive ANP by using nearest-neighbor matching without replacement. To measure covariate balance, we used the standardized difference. Estimation of propensity scores and matching were performed by using MATLAB r2011b software (Mathworks). Lung cancer-specific RFS was compared by using Kaplan–Meier estimates and the log-rank test for equality of survival curves. Calculations were conducted by using JMP statistical software (SAS Institute).

All patients received predefined treatment including chemotherapy according to the clinical guidelines for lung cancer in Japan. ANP treatment was performed just to prevent postoperative complications; therefore, there were no differences in the treatments including chemotherapy between control patients and ANP patients. We obtained complete pathological and follow-up data from all subjects. The study protocol was approved by the Institutional Review Boards of both institutions, and all patients gave written informed consent to participate in the study (Trial registration ID: JPRN-UMIN4880). The median follow-up duration was 36 mo (18–60 mo). All subjects underwent follow-up examinations at 3-mo intervals postoperatively: each evaluation included a physical examination, chest X-ray and blood tests including tumor markers. Thoraco-abdominal CT scans were generally performed at 6-mo intervals and additional bone scintigraphy and MRI of the brain for the detection of cancer recurrence were performed every year.

Cell Lines. The human lung cancer cell lines, A549-EGFP and H460-EGFP, were obtained from Wako and maintained in RPMI-1640 medium supplemented with 10% (vol/vol) FBS. The mice melanoma cell line, B16/F10, and the A549, H460, MCF-7, OVCAR3, CaCo2, GCIY, HepG2, ME-180, PANC1, and PC3 cell lines were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS at 37 °C under 5% CO_2. HPAECs, HMVEC-L, and HUVECs were purchased from Lonza, maintained in EGM-2 according to the manufacturer's instructions, and used within passages three to five.

Experimental Lung Metastasis Model. All animal experiments were performed according to the protocol approved by the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute. Six-week-old BALB/c nu/nu and C57BL/6 mice were purchased from Japan SLC. BALB/c nu/nu mice for A549-EGFP cells (1×10^6 cells per mouse) or C57BL/6 mice for B16/F10 cells ($2–5 \times 10^5$ cells per mouse) were used in this study. In the

experiments with LPS (Fig. 2 A–D), mice were divided into three groups: vehicle alone (control), vehicle/LPS, and ANP/LPS group with two kinds of cancer cells (either A549-EGFP or B16/F10 cells). ANP in 0.9% saline or vehicle was continuously injected by using osmotic pumps (Alzet Model 1002 or 2004, DURECT) implanted subcutaneously in the upper back of the mice 1 d before LPS injection. On the next day, the mice were intravenously injected with or without 1.0 mg/kg LPS (Wako). Five hours after LPS injection, cancer cells (either A549-EGFP or B16/F10 cells) were injected into the tail vein. In the experiments without LPS (SI Appendix, Fig. S2), mice were divided into two groups: vehicle and ANP groups with two kinds of cancer cells (either A549-EGFP or B16/F10 cells). ANP or vehicle alone was implanted 1 d before cancer cells injection. On the next day, cancer cells (either A549-EGFP or B16/F10 cells) were injected into the tail vein. To ascertain the efficiency of ANP administration, we confirmed that the blood levels of cGMP were elevated effectively when ANP (0.5 μg·kg·min) was infused subcutaneously in the mice (SI Appendix, Fig. S8). ANP (0.5 μg·kg·min) or vehicle infusion was started 1 d before the injection of cancer cells. At this dose, ANP did not change the blood pressure or heart rate of the mice (SI Appendix, Table S3). ANP or vehicle infusion was continued until the mice were euthanized. Six or 8 wk (A549-EGFP) or 2 wk (B16/F10) after tumor cell injection, the mice were killed for evaluation of pulmonary metastases. The number of nodules reflecting pulmonary metastasis of A549-EGFP or B16/F10 cells was counted by using images obtained with a fluorescent microscope (OV100, Olympus) or a camera (CX6, Ricoh).

Adhesion Assay. To quantify tumor cell adhesion to HPAECs or HMVEC-L, a standardized cell adhesion assay was performed by using a modification of the method of van Rossen et al. (37). Briefly, HPAEC or HMVEC-L monolayers were established in 35-mm collagen-coated dishes (IWAKI). Before coculture with tumor cells, HPAECs or HMVEC-L were either pretreated with 0.1 μM ANP for 15 min or untreated and subsequently treated with LPS (0–50 pg/mL) for 30 min, then washed three times with fresh M199 medium (Gibco; Invitrogen) containing 1% BSA (Sigma-Aldrich). A549-EGFP or H460-EGFP tumor cells (2×10^5 cells per dish) were added to the confluent monolayer-cultured HPAECs or HMVEC-L and cocultured for 3 h. The dishes were then washed three times with PBS to remove nonadherent tumor cells, and the cells were fixed with 4% (wt/vol) paraformaldehyde. The number of remaining EGFP$^+$ cells in the fixed dishes was counted by using images obtained with a fluorescence microscope (FSX100, Olympus) and a computer-aided manipulator program (Cell-sense, Olympus). In addition, the adhesion of the tumor cells to HPAECs depleted of either E-selectin, VCAM-1, or ICAM-1, and stimulated with LPS, was similarly analyzed.

Statistics. Data are presented as means ± SEM and were analyzed by using a two-tailed Student's t-test for paired samples or one-way ANOVA for multiple groups. P values less than 0.05 were considered statistically significant.

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