

Investigation of proNT/NMN secretion from small cell lung carcinoma cells using a mouse xenograft model

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Received March 6, 2012; Accepted May 4, 2012

DOI: 10.3892/or.2012.1926

Abstract. Proneurotensin/neuromedin N (proNT/NMN), the precursor of neurotensin (NT) and neuromedin N (NMN), is produced by cancer tissues derived from the pancreas and colon. NT stimulates tumor growth and proliferation through its receptors; however, little is known about the precursor molecule in cancer tissues. We previously demonstrated that proNT/NMN is secreted from small cell lung carcinoma (SCLC) cell lines in serum-free conditioned medium, but not from non-small cell lung carcinoma (NSCLC) cell lines. It was suggested that this precursor may serve as a tumor marker for SCLC. In this study, we established *in vivo* xenograft models to evaluate the possibility of proNT/NMN as a specific tumor marker. SBC3 cells, derived from human SCLC, were inoculated into mice, and the proNT/NMN levels in plasma and tumor tissues were detected using specific antibodies. In contrast to control mouse plasma, the proNT/NMN levels in tumor-bearing mice increased as the tumors grew, and the elevated plasma proNT/NMN levels were decreased by tumor resection. Moreover, proNT/NMN was expressed in SBC3 tumors, suggesting that proNT/NMN was secreted into blood from the tumor, and this secretion may be specific to SCLC.

Introduction

Proneurotensin/neuromedin N (proNT/NMN) is the common precursor of two neuropeptides, namely neurotensin (NT) and neuromedin N (NMN). It is expressed in the gut, brain, and adrenals (1), and in the cancer tissues derived from the pancreas (2) and colon (3). In these tissues, proNT/NMN undergoes differential processing, generating different patterns of maturation products such as NT, NMN, large NT, and large NMN (4-8). The biological and pharmacological behaviors of NT have been extensively studied, and NT is known to regulate digestive processes including gastrointestinal motilities and pancreatic and biliary secretions (9). In addition, NT stimulates the growth and proliferation of cancer cells derived from the gut, lungs, pancreas, and prostate through NT receptors (10-12). Friry *et al* (13) demonstrated that large forms of the processed products are more resistant to degradation than NT and NMN. Therefore, these products might function as long-lasting activators of NT receptors. However, little is known about the behavior of its precursor the proNT/NMN.

We previously used MS/MS proteomic analysis to identify secreted proteins produced by lung carcinoma cell lines in serum-free conditioned medium (14). In addition to the proteins previously found in serum-free conditioned media, proNT/NMN was identified in the media from small cell lung carcinoma (SCLC) cell lines, but not from non-small cell lung carcinoma (NSCLC) cell lines. These results suggest that proNT/NMN is specifically secreted from SCLC and might be a potential tumor marker.

To investigate the specific secretion of proNT/NMN from SCLC, we established *in vivo* xenograft models of SCLC. SBC3 cells, which secrete proNT/NMN mostly in SCLC cell lines, were inoculated into mice, and the plasma proNT/NMN levels of those mice were detected using specific antibodies. ProNT/NMN was detected in the plasma and tumor tissues of SBC3 tumor-bearing mice, while it was not detected in control mice. In addition, plasma proNT/NMN levels had a high correlation with tumor volume. These results suggest that SBC3-derived tumors produce and secrete proNT/NMN into blood.

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Abbreviations: aa, amino acids; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GRP, gastrin-releasing peptide; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; NMN, neuromedin N; NSCLC, non-small cell lung carcinoma; NT, neurotensin; PC, prohormone convertase; proNT/NMN, proneurotensin/neuromedin N; SCLC, small cell lung carcinoma

Key words: proneurotensin/neuromedin N, neurotensin, small cell lung carcinoma, tumor marker

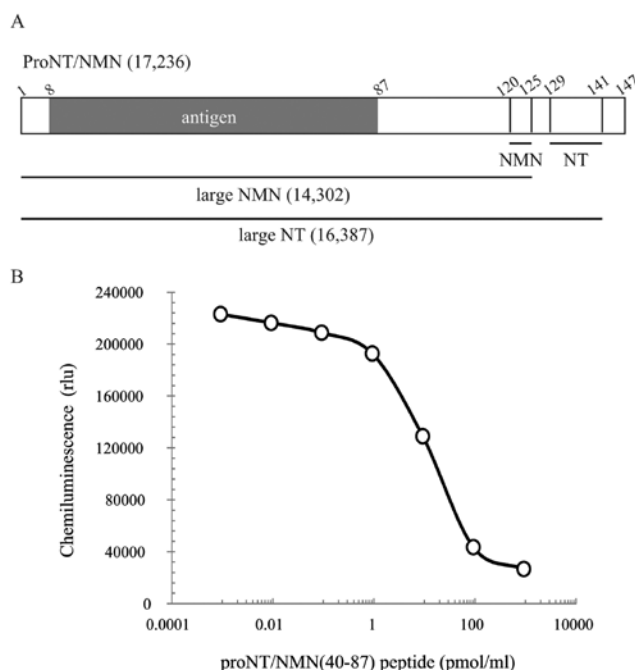


Figure 1. Schematic illustration of proNT/NMN (A) and a standard curve for the proNT/NMN ELISA using proNT/NMN (aa 40-87) (B).

Materials and methods

Antibodies. We established polyclonal antibodies against the peptide sequences of proNT/NMN (amino acids (aa) 8-87) shown in Fig. 1A. Chemically synthesized proNT/NMN (aa 8-87) was conjugated with keyhole limpet hemocyanin (KLH) and then purified. Antibodies were obtained by immunizing three SPF Japanese white rabbits with KLH-conjugated peptides.

Cell culture and preparation of serum-free conditioned medium. SBC3, a small cell lung carcinoma cell line, was purchased from the Japanese Cancer Research Bank. SBC3 cells were maintained in RPMI-1640 (Sigma, St. Louis, MO, USA) medium supplemented with 10% FBS (Gibco, Grand Island, NY, USA), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) in a humidified 5% CO₂ incubator. Serum-free conditioned media was prepared as previously described (14).

Animals. Six-week-old athymic nude mice (BALB/cAJcl-nu/nu) were purchased from CLEA Japan (Tokyo, Japan), and NRG mice (NOD.Cg-Rag1^{tm1Mom}/J2rg^{tm1Wjl}/SzJ) were purchased from Charles River (Wilmington, MA, USA). NRG mice were bred in the Experimental Animal Facility of Shizuoka Cancer Center Research Institute, and their offspring was used for experiments. All animal experiments were carried out in accordance with the general guidelines for animal experiments of Shizuoka Cancer Center Research Institute.

In vivo experiments. Nude mice were subcutaneously inoculated with SBC3 cells (5 × 10⁶ cells in 0.1 ml PBS/mouse) into the right forelimb, and blood samples were collected once a week. The tumor size was also measured before blood collection to

calculate the tumor volume. NRG mice were intradermally inoculated with SBC3 cells (5 × 10⁶ cells in 0.1 ml PBS/mouse) to examine the effect of tumor resection, and divided into two groups (n=5 per group). Four weeks after inoculation, tumors were surgically resected from one group and blood was collected from the non-resected and control mice after 24, 48 and 120 h. Blood samples were centrifuged, and plasma was collected. Plasma samples were stored at -80°C until use.

Preparation of mouse tissue lysates. Frozen tumor tissues were treated with lysis buffer containing 7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 50 mM Tris-HCl (pH 7.4), 2.5% N-octylglucoside, 6.25 mM Tris-carboxyethyl phosphine hydrochloride (TCEP), and 1.25 mM protease inhibitor, and homogenized by TissueLyser II (Qiagen K.K., Tokyo, Japan). Following rotation for 1 h, the samples were centrifuged at 14,000 × g for 30 min at 4°C, and the supernatants were collected. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well, Nunc C8 Maxisorp Immuno module; Nunc, Roskilde, Denmark) were coated with 100 μl of goat anti-rabbit IgG (Fc) solution (diluted 1:200 in 0.1 M NaHCO₃; MP Biomedicals, Irvine, CA, USA) and incubated for 24 h at 4°C. After incubation, the antibody solution was removed, and the wells were blocked overnight at 4°C by using block ace solution (DS Pharma Biomedical Co., Ltd., Osaka, Japan). Following three washes with 0.05% Tween-20 in PBS, the biotinylated antigen solution [20 ng/ml biotinyl-proNT/NMN (aa 40-87), prepared in PBS containing 0.1% BSA], the plasma samples, and the antibody solution [anti-proNT/NMN rabbit polyclonal antibody (105S), 1/3,000 dilution] were added and incubated with shaking for 3 h at room temperature. Plates were washed with PBS containing 0.05% Tween-20 four times before the addition of labeling solution (streptavidin, horseradish peroxidase conjugate; Merck, Darmstadt, Germany), and incubated for 2 h at room temperature. After four final washes, BM chemiluminescence ELISA substrate (POD) solution (Roche Applied Science, Mannheim, Germany) was added to each well and allowed to develop in the dark for 3 min. The chemiluminescence of BM ELISA was measured using Wallac 1420 ARVO MX (Perkin-Elmer Life Sciences, Waltham, MA, USA). A standard curve for proNT/NMN levels was obtained using proNT/NMN (aa 40-87) as the standard antigen.

Immunoblotting. Proteins were separated by SDS-PAGE on 14.25% polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using anti-proNT/NMN rabbit polyclonal antibodies (105S, 1:1,000 dilution), or anti-neurotensin goat polyclonal antibodies (N-17 and C-19, 1:1,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as the primary antibody, and anti-rabbit IgG-, or anti-goat IgG-horseradish peroxidase (HRP) conjugate (1:10,000 dilution; Jackson ImmunoResearch, West Grove, PA, USA) as the secondary antibody. HRP-dependent luminescence was developed using ECL Prime Western blotting detection reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) and detected in a LAS-3000 (Fuji Film, Tokyo, Japan).

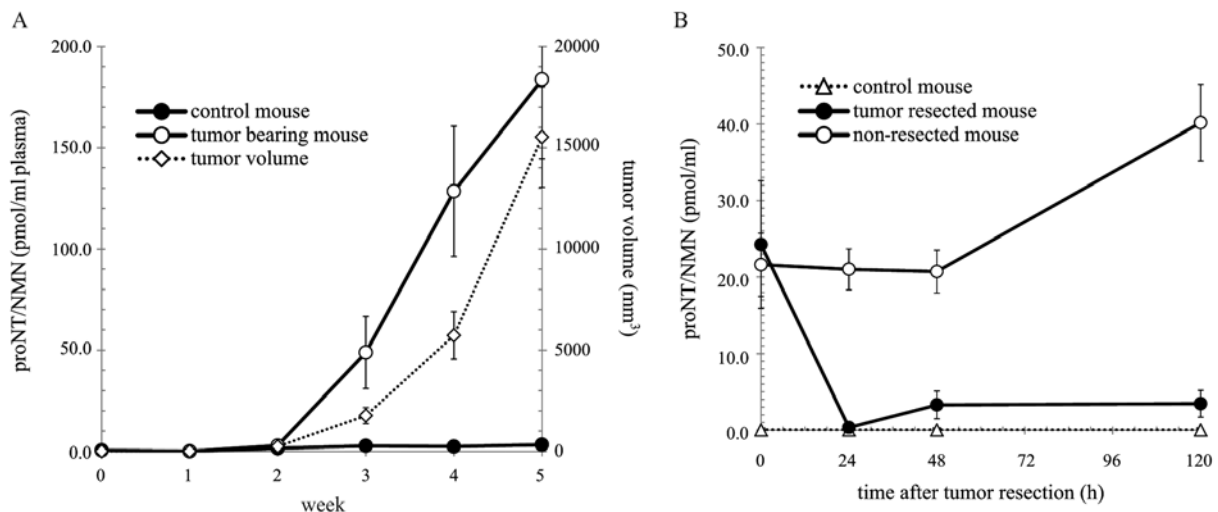


Figure 2. Time course of proNT/NMN levels in the plasma (A) and the effect of tumor resection on proNT/NMN levels (B). (A) Plasma was collected from nude mice at 0, 1, 2, 3, 4 and 5 weeks after inoculation. ProNT/NMN levels in the plasma [control mouse (●) and tumor-bearing mouse (○)] were measured by ELISA, as described in Materials and methods. The tumor volume (◇) was calculated using the formula: tumor volume (mm^3) = (length \times width²)/2. (B) Four weeks after inoculation, tumors were resected from the NRG mice, and plasma samples were collected from all groups of mice at 0, 24, 48 and 120 h after tumor resection. ProNT/NMN levels in the plasma [control mouse (Δ), tumor-resected mouse (●) and non-resected mouse (○)] were measured by ELISA, as described in Materials and methods. Data are expressed as the means \pm SE values in multi-replicate experiments (n=4-5). Statistical significance ($P < 0.05$ as compared with the group of non-resected mouse) was evaluated by Student's t-test.

Immunohistochemistry. Formalin-fixed tumor tissues were gradually dehydrated in ethanol and embedded in paraffin. Tissue sections (3- μm thick) were cut and subjected to immunohistochemistry. Sections were deparaffinized in xylene, and treated with 1% H_2O_2 in methanol for 30 min to block the endogenous peroxidase activity. After blocking, they were incubated with anti-proNT/NMN polyclonal antibody (1:200 dilution) or with the antibody in the presence of the antigen peptide for 30 min at room temperature. The sections were then washed with PBS and incubated with peroxidase labeled polymer (goat anti-rabbit IgG; Dako, Glostrup, Denmark) for 30 min. Following one wash with PBS, peroxidase activity was initiated with DAB substrate solution (Dako). The peroxidase reaction was completed, the sections were washed with PBS, counterstained with hematoxylin, dehydrated, cleared in xylene, and a coverslip was placed over the sections.

Statistical analysis. The results of the experiments are expressed as the mean \pm SE values. The statistical significance of differences was determined according to the Student's t-test.

Results

Evaluation of the proNT/NMN levels in tumor-bearing mice. We first established a rabbit polyclonal antibody against the proNT/NMN (aa 8-87) and developed a competitive ELISA system. Based on the results of the standard curve with proNT/NMN (aa 40-87) (Fig. 1B), the proNT/NMN levels in mouse plasma were measured by ELISA. Every week, plasma samples were collected from the control and SBC3-inoculated mice after inoculation. As shown in Fig. 2A, tumor volumes of SBC3-inoculated mouse gradually increased. Correlating with tumor volumes, the plasma proNT/NMN levels of the tumor-bearing mice were elevated, while the proNT/NMN levels in control mouse were not significantly detectable.

To further investigate proNT/NMN secretion, tumors were surgically resected from the NRG mice intradermally inoculated with SBC3 cells, and the plasma proNT/NMN levels were evaluated after resection. Compared to nude mice that were subcutaneously inoculated with SBC3 cells, the tumors of NRG mice grew slowly, and the plasma proNT/NMN levels were slightly elevated 4 weeks after inoculation (nude mice, 128.49 ± 32.18 pmol/ml plasma; NRG mice, 22.93 ± 4.67 pmol/ml plasma). The differences in the plasma proNT/NMN levels between the control and intradermally inoculated NRG mice were statistically significant. After surgical resection, the plasma proNT/NMN levels of the tumor-resected mice decreased until they reached the level identical to that of the control mice (Fig. 2B). Although the levels of the tumor-bearing mice (non-resected mice) were significantly elevated after 120 h, the levels of the tumor-resected mice remained the same as that of the control mice. These results clearly suggest that SBC3 tumors secreted proNT/NMN into blood.

Immunoblot detection of proNT/NMN in mouse plasma. Plasma proNT/NMN was also detected by immunoblotting using the same antibody as used in the ELISA. In SBC3 serum-free conditioned medium, the positive band was detected around 17 kDa (Fig. 3A left line, SBC3-CM). The medium contains proNT/NMN (14), and the theoretical molecular mass of proNT/NMN is about 17 kDa, suggesting that the band detected around 17 kDa is specific to proNT/NMN. In mouse plasma, several bands were detected around the molecular mass of 17 kDa. The band at 17 kDa, specific to full-length proNT/NMN, was only detected in tumor-bearing mouse plasma; however, the lower bands (~14-16 kDa) were detected in the plasma samples from both the tumor-bearing and control mice (Fig. 3A).

To gain more insight into whether the lower bands are derived from proNT/NMN, immunoblotting was performed

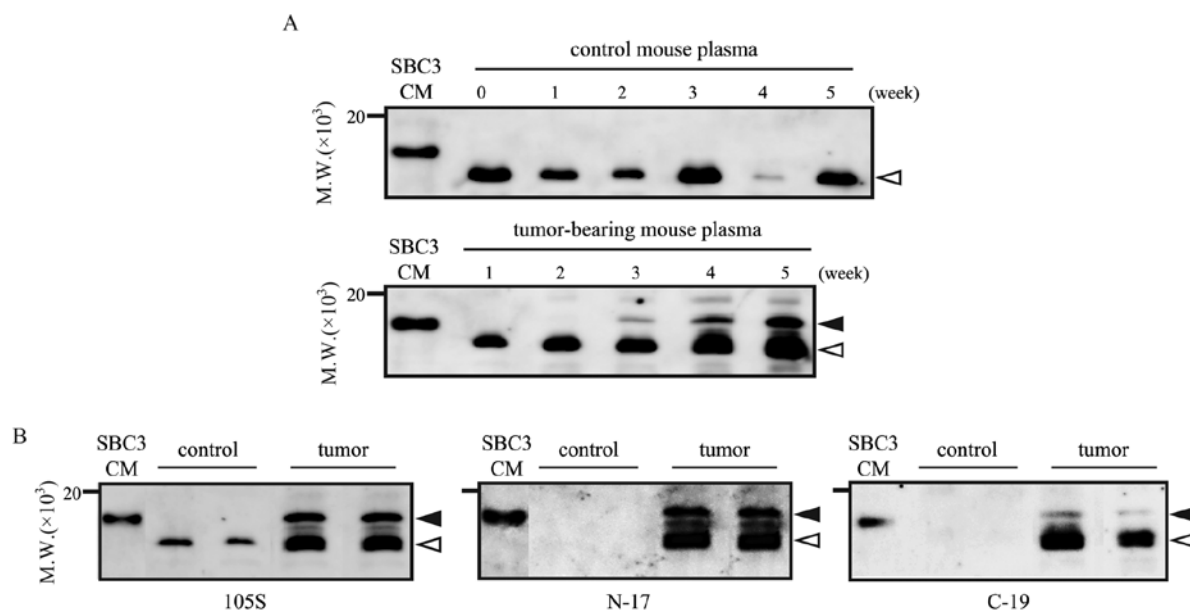


Figure 3. Immunoblot detection in mouse plasma. (A) ProNT/NMN levels in the mouse plasma (upper panel, control mouse; lower panel, tumor-bearing mouse) was determined by immunoblotting. SBC3 serum-free conditioned medium (0.25 μ l/lane, indicated as SBC3 CM) and plasma samples (1 μ l/lane) were separated on 14.25% polyacrylamide gels, and electrophoretically transferred onto PVDF membrane. ProNT/NMN was detected by anti-proNT/NMN rabbit polyclonal antibody, as described in Materials and methods. (B) SBC3 serum-free conditioned medium (SBC3 CM) and proNT/NMN in the mouse plasma (control, control mouse plasma; tumor, tumor-bearing mouse plasma) were also detected by immunoblotting using anti-NT antibodies (N-17, C-19), as described in Materials and methods.

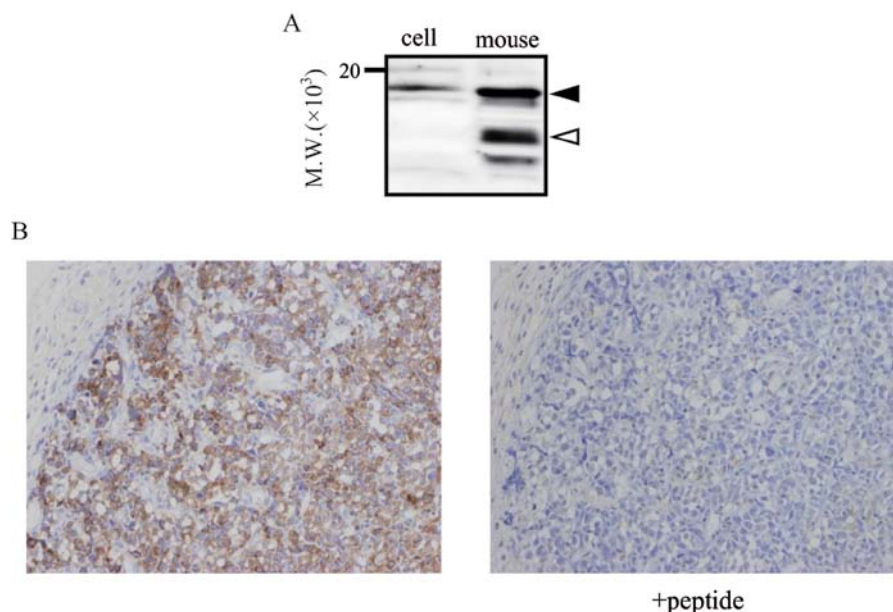


Figure 4. ProNT/NMN expression in mouse tumor tissues. (A) SBC3 cell lysates (20 μ g/lane, indicated as cell) and mouse tumor tissue lysates (10 μ g/lane, indicated as mouse) were used for immunoblotting, as described above. (B) Immunohistochemical analyses were performed in mouse tumor tissues, as described in Materials and methods. ProNT/NMN was stained with DAB (brown color), and the nuclei were counterstained with hematoxylin (purple color). Tissue sections were incubated with anti-proNT/NMN polyclonal antibody (left panel) or incubated with the antibody in the presence of proNT/NMN (aa 8-87) (right panel).

using anti-NT antibodies, N-17 and C-19. Both antibodies can recognize NT-containing products such as NT, large NT, proNT/NMN, and N-17 can also recognize large NMN. Using these two antibodies, the bands around 14–16 kDa were detected in the plasma of tumor-bearing mouse, but not in the plasma from the control mouse (Fig. 3B). These results suggest that the lower band detected in the control mouse plasma is not

derived from secreted proNT/NMN, but in the tumor-bearing mouse plasma, the processing products of proNT/NMN such as large NMN and large NT, were detected around 14–16 kDa.

ProNT/NMN expression in tumor tissues. ProNT/NMN expression in mouse tumor tissues was also determined by immunoblotting and immunohistochemistry. As with the

plasma samples, the positive bands derived from proNT/NMN were detected in the SBC3 tumor tissues (Fig. 4A). In addition, Fig. 4B shows that proNT/NMN was expressed particularly in the superficial layer of the tumor (left panel). Immunostaining was diminished when the tumor tissue section was treated with antibody in the presence of the antigen peptide (right panel). This peptide competition analysis confirmed the expression of proNT/NMN in the above-mentioned areas in the tumor tissue. ProNT/NMN expression in the tumor tissues indicated that this precursor was produced by SBC3 tumors and secreted into plasma as the tumors grew.

Discussion

Most peptide hormones including neuropeptides are derived from the processing of larger inactive polypeptide precursors by prohormone convertases (PCs). PC-mediated processing often generates different combinations of active peptides. NT is derived from the large precursor proNT/NMN and this precursor is mainly processed by PC1, PC2 and PC5 (8,15). Each PC cleaves at a different site of proNT/NMN; therefore, the pattern of processing products depends on the PC being expressed. Previous studies have demonstrated that the major products derived from proNT/NMN are NMN, NT, and large NT in the brain, large NMN in the gut, and NT, large NMN, and large NT in the adrenals (4,16-18). Moreover, proNT/NMN processing occurs in only ~50% of a subset of colon cancer cell lines, because PC1 and PC2, which are mainly related to the proNT/NMN processing, are not expressed in these cell lines (3). Most SCLC cell lines, including SBC3, do not concurrently express PC1, PC2 and PC5 (data not shown). Only the precursor was detected in the SBC3 cells and the conditioned medium, whereas the large forms of processing products were not detected (Fig. 3). On the other hand, not only proNT/NMN, but also the large processing products, was detected in the plasma of SBC3 tumor-bearing mice. These results suggest that proNT/NMN was the major product secreted from SCLC and the large forms of processing products were also produced in the SCLC tumor-bearing mice.

Neuropeptides are generally easy to metabolize, and therefore, have a short half-life in blood. Gastrin-releasing peptide (GRP) is one of the prototypical neuropeptide hormones. Although GRP is produced in high amounts and secreted into blood by SCLC cells, the unstable nature renders its detection in blood quite difficult. Therefore, the ProGRP (aa 31-98), which is also secreted into blood, was established as a useful tumor marker for SCLC (19-21). NT is also known to be more unstable than its precursor *in vivo* (22,23), as well as *in vitro* (13). While the production and secretion of NT, as well as the expression of NT receptors, were detected in many lung cancer cell lines including SCLC and NSCLC (24-26), it remains unclear whether NT or NT receptors are potential useful tumor markers. In fact, the plasma NT levels in the SBC3 tumor-bearing mice were slightly elevated compared with the control mice and were detected in the tumors. However, the NT levels were much lower than the proNT/NMN levels (data not shown). To our knowledge, this is the first report to investigate proNT/NMN secretion from SCLC tumors and to evaluate the plasma levels of proNT/NMN itself. Our findings suggest that proNT/NMN might

be a potential tumor marker for SCLC. To confirm this possibility, further analysis of the blood samples from SCLC patients is required.

Acknowledgements

This research was partially supported by the Ministry of Education, Science, Sports and Culture, grant-in-cooperation of the Regional Innovation Cluster Program 2010. The authors thank T. Ide for kindly providing technical assistance.

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