Showcasing research by Dr. Norimichi Nagano, Mr. Yuki Ichihashi, Dr. Toru Komatsu and Prof. Yasuteru Urano et al., Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan. The illustration was created by Dr. Ryo Tachibana.

Development of fluorogenic substrates for colorectal tumor-related neuropeptidases for activity-based diagnosis

A fluorogenic substrate probe was developed to detect the activity of the colorectal tumor-related peptidase, neurolysin. This probe was used to monitor activity alterations in colorectal tumor tissues and blood samples obtained from patients with colorectal tumor. The study suggests that single-molecule neurolysin activity analysis has the potential to be a promising blood biomarker for the activity-based diagnosis of colorectal tumor.

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Development of fluorogetic substrates for colorectal tumor-related neuropeptidases for activity-based diagnosis†

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The M3 metalloproteases, neurolysin and THOP1, are neuropeptidases that are expressed in various tissues and metabolize neuropeptides, such as neurotensin. The biological roles of these enzymes are not well characterized, partially because the chemical tools to analyse their activities are not well developed. Here, we developed a fluorogetic substrate probe for neurolysin and thimet oligopeptidase 1 (THOP1), which enabled the analysis of enzymatic activity changes in tissue and plasma samples. In particular, the probe was useful for studying enzyme activities in a single-molecule enzyme assay platform, which can detect enzyme activity with high sensitivity. We detected the activity of neurolysin in plasma samples and revealed higher enzyme activity in the blood samples of patients with colorectal tumor. The result indicated that single-molecule neurolysin activity is a promising candidate for a blood biomarker for colorectal cancer diagnosis.

Introduction

In living systems, there are more than 5000 enzymatic reactions and alterations in enzymatic activity are often tightly connected to pathogenesis. Analysing these disease-related alterations is a powerful source for the detection, understanding, and treatment of diseases. 1,2 Fluorogetic substrate probes are molecules that act as substrates for targeted enzymes and report their activities through fluorescence signal changes. 3 They are useful tools for studying enzyme activities in physiology and pathology. Especially when they are used in single-molecule enzyme activity analysis, they offer a highly sensitive enzyme analysis methodology to study disease biomarker enzyme activities for diagnosis. 4

Currently, many enzymes lack selective fluorogetic substrate probes to aid in reporting their activities in complex biological systems. The M3 metalloproteases, neurolysin (EC 3.4.24.16) and thimet oligopeptidase 1 (THOP1, EC 3.4.24.15), are two of those enzymes that do not have specific fluorogetic substrates. 5 We have recently identified their altered activity levels in colorectal tumor tissues; therefore, detecting their activity in tissue and blood samples will offer a useful source for activity-based disease diagnosis. 6,7 Neurolysin is expressed in various tissues and is responsible for the metabolism of neurotensin, bradykinin, and substance P. 8 The biological role of THOP1 is not well characterized; however, it has more than 50% sequence homology with neurolysin. 9 Both peptidases are thought to act as neuropeptidases that channel neuropeptide signalling by metabolizing them.

Endopeptidase probes are designed by the fluorescence resonance energy transfer (FRET) mechanism, in which the fluorophore and quencher are attached to both sides of the substrate peptide; thus, cleavage of the linker peptides leads to fluorescence activation. Currently, fluorogetic substrates available for the M3 metalloproteases are limited to those based on the 2-aminobenzoic acid (Abz) fluorophore.
Results and discussion

We previously identified the altered activity of neurolysin and THOP1 in colorectal tumor tissues via the elevated metabolism of the LVVSTQTALA peptide (Fig. 1B), and this was because the peptide serves as a good substrate for these neuropeptidases (Fig. 1C, D and S1). We considered its potential as an ideal neurolysin substrate probe for the M3 metalloproteases lies in the unique substrate specificity of the enzyme; substrate recognition is not based on specific amino acid side chains (such as the P1 position) as in most endopeptidases, but on the overall molecular structure. This is due to the presence of a long and deep substrate binding cleft (Fig. 1A). It is known that unstructured short peptides less than 20 amino acids long are preferable, but this knowledge was not sufficient to predict or design good substrates.

N-terminus was relatively open to modification. However, the modification of hydroxycoumarin, a structurally small fluorophore, at the N-terminus reduced the activity, and change of the fluorophore to fluorescein, a visible light activatable fluorophore, showed an almost complete loss in the activity. We then added a fluorescence quencher Dabcyl (in the form of a linker to be incorporated into the Fmoc solid phase peptide synthesis, named aminopropyl Dabcyl (Apd)) to the N-terminus. However, the Apd fluorophore, at the N-terminus reduced the activity, and change of the fluorophore to fluorescein, a visible light activatable fluorophore, showed an almost complete loss in the activity. We then added a fluorescence quencher Dabcyl (in the form of a linker to be incorporated into the Fmoc solid phase peptide synthesis, named aminopropyl Dabcyl (Apd)) to the N-terminus.

To optimize the C-terminal structures (Fig. 2B and S3), we used the YVVSTQTALA peptide as the initial peptide for structural modification. Amidation of C-terminal Ala or the extension of glycine linkers after it significantly reduced the activity; therefore, it seemed inappropriate to modify the main

(A) Optimization of the N-terminal sequences. (B) Optimization of C-terminal sequences. (C) Structure of Apd−STQTALK(NBD) and expected cleavage site. (D) Michaelis–Menten analysis of Apd−STQTALK(NBD) metabolism by recombinant neurolysin (NLN) or THOP1.
chain of peptides at the C-terminal; hence, we tried to modify the side chain of the C-terminal amino acid. Changing the C-terminal Ala to other amino acids, such as Lys, Asp, Glu, and Lys(Cbz), was accepted, and modifying the C-terminal amino acid to Lys(NBD) enhanced its activity. In the same position, modification of fluorescein was not accepted; therefore, the use of an appropriate fluorophore was indispensable for the design.

NBD is a fluorophore that exhibits visible-light absorbance and fluorescence. It was combined with the N-terminal Apd modification, and the overall peptide, NH$_2$–Apd–STQTALK(NBD)–OH, was established as the best substrate, with the expectation that visible light fluorescence activation occurs upon cleavage of the peptide (Fig. 2C). The probe was sufficiently soluble in aqueous media up to 50 μM (Fig. S4†).

In this concentration range, Michaelis–Menten analysis was performed, and we have determined the kinetic values by non-linear regression; $k_{cat}/K_m$ was calculated to be $5.4 \times 10^3$ (M$^{-1}$ s$^{-1}$) for neurolysin and $8.0 \times 10^3$ (M$^{-1}$ s$^{-1}$) for THOP1 (Fig. 2D and S5†). The peptide probe reacted with recombinant neurolysin to exhibit a fluorescence increase of more than 15 times (Fig. 3A), and we confirmed that the cleavage site was as designed, between Gln and Thr, for both neurolysin and THOP1 (Fig. 3B).

We utilized a probe to detect neuropeptidase activity in colorectal tumor tissues. As reported previously, the activities of neurolysin and THOP1 were highly elevated in colorectal tumor tissues compared with that in non-tumor tissues, and we were able to detect changes in the tissue lysates from surgical specimens using a fluorometric multiwell plate reader (Fig. 3C and S6†) or by the naked eye (Fig. 3D) by fluorescence activation.

As we have successfully detected the activity of neuropeptidases in tumor tissues, we next tried to detect their activities in blood samples. It is often more challenging to detect activity of enzymes in blood samples than in tissues, because of the lower number of molecules present, and the activity can be modified by endogenous enzyme inhibitors in circulation. We have recently reported that single-molecule enzyme assays are useful for analysing enzyme activities in blood samples because of their high sensitivity and the possibility to discriminately detect the varied activities of enzyme molecules originating from enzyme subtypes and...
proteoforms. We applied our probe for single-molecule analysis of neuropeptidase activities in blood samples. The single-molecule enzyme activity analysis is performed by preparing a microdevice that has 10^5 to 10^6 reaction chambers with volumes of 10–100 fL and loading the diluted enzyme solution such that theoretically 0 or 1 target enzyme molecule is included in each chamber (Fig. 4A). All chambers are loaded with the fluorogenic probes and are sealed with oil. If the target single-molecule enzyme is included in the chamber, the turnover of the enzyme generates the fluorescent product, which quickly accumulates to reach the detectable concentration in the chamber. Currently, only limited classes of enzymes, phosphatases and glycosidases, have been analysed in this platform. We considered that our probe, with visible light excitation/emission and good reactivity with neuropeptidases, was suitable for the assay platform.

The assay was performed by loading the probe mixed with the diluted enzyme in the microdevice, and after sealing with oil and incubation, the fluorescence images were taken to detect and count the wells with the signals. We first optimized the assay conditions using the probe and the microdevice and determined that the addition of sufficient concentrations of detergents (i.e. >3 mM Triton X-100) was required for the reliable assay (Fig. S7†). Using the optimized assay conditions, the calculated detection limit of the active enzyme was 1.49 × 10^{-2} M (Fig. S8†). We then tested if neuropeptidase activities were detectable in the blood samples. In the conventional fluorometric assay using a microplate reader, the activity was barely detectable (Fig. S9 and S10†), presumably because of the low concentration of active enzymes in blood. However, in single-molecule analysis, the activity spots were observed, with the fluorescence intensities comparable to that of recombinant neurolysin (Fig. S11†). Based on the knowledge that, at the tissue level, neuropeptidase activity was elevated in patients of colorectal cancer, we expected that enzyme activity in blood might also show the elevation and could be useful as a biomarker for the detection of colorectal tumors. We analysed the single-molecule activities of neuropeptidases in plasma samples from 30 healthy subjects and 28 patients with colorectal cancer (stages I–IV, Table S1†). The study was approved by the ethical committee of Nippon Medical School. As a result, we discovered that some patients showed elevated counts of the enzymes in blood samples (Fig. 4B–D and S12–S14, Table S3†), and this change was not observed in patients with other cancers (pancreatic and gastric cancer, Steel test P < 0.01). An area under the curve (AUC) of 0.75 (sensitivity = 0.82, specificity = 0.70, Table S5†) was obtained in discriminating patients with colorectal cancer from healthy subjects (Fig. 4E). The result was validated by preparing a different set of samples from 30 healthy subjects and 27 colorectal tumor patients who are treated by the National Cancer Center (Tokyo, Japan) and repeating the experiments under double-blind conditions (P < 0.05, Mann–Whitney U test, AUC = 0.65, Fig. S15 and Tables S2, S4 and S6†).

While neurolysin is known to function in potentiating immune responses, the contribution of neurolysin to tumor pathogenesis remains unknown. These results provide useful leads for further research on their involvement in colorectal tumors, such as immunologic activities around tumor areas and in circulation.

Currently, the methods for early detection of colorectal tumors have not been sufficiently developed. The screening test based on a faecal occult has low specificity, and the blood biomarker test using the representative tumor markers CA19-9 and CEA is reported to have limited AUC (AUC = 0.62 for CA19-9 and 0.669 for CEA). Endoscopic analysis is more reliable, but it is more invasive and costly and is not easily performed. In some cases, colorectal tumors are detected at an advanced stage, which cannot be completely cured. Here, the development of novel biomarkers for colorectal tumors is highly coveted. Therefore, the blood neurolysin activity discovered in the study has promising potential for diagnosis, especially since neuropeptidase activity can be detected in plasma from a small amount of blood sample (sample requirement was less than 1 μL).

Conclusions

In summary, our study outlines the development of a fluorogenic probe for the M3 metalloproteases, neurolysin and THOP1, which enabled the detection of neuropeptidase activity in tissue lysates and blood samples. In particular, the study indicated that the activity of neurolysin was elevated in the plasma samples of patients with colorectal tumors, thus highlighting the potential of single-molecule neurolysin activity analysis as a promising candidate for a colorectal tumor biomarker.

Author contributions

T. K. and Y. U. conceived the project and designed the experiments. N. N., Y. I., Y. M. and T. K. performed the experiments and data analyses. H. M., K. H., Y. K. and H. O. collected human samples. Y. I. and Y. M. synthesized the compounds. R. W. prepared microdevices. M. S., S. S., Y. K., A. K., K. T., and R. W. directed and supported the experiments. N. N. and T. K. wrote the manuscript.

Conflicts of interest

S. S. and Y. K. are employees and shareholders of Cosomil, Inc. M. S. is an employee of Cosomil, Inc. K. H., R. W. and T. Komatsu are advisors and shareholders of Cosomil, Inc.

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