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Effect of carbohydrate structure on biological activity of artificially N-glycosylated eel calcitonin

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To reveal the function of the carbohydrate portion of glycopeptides and glycoproteins, we chemo-enzymatically synthesized artificially *N*-glycosylated derivatives of eel calcitonin and studied their three-dimensional structure and biological activity. The CD and NMR spectra in trifluoroethanol-H_2O solution showed that the glycosylation did not change the three-dimensional structure. All the derivatives retained the strong *in vivo* hypocalcemic activity of calcitonin. However, the relative activity was dependent on the structure of the attached carbohydrate. The single GlcNAc attachment best enhanced the activity, while larger carbohydrates decreased the activity. This relative activity order of compounds could be partly explained by their calcitonin-receptor binding affinity, though the affinity of the GlcNAc derivative did not exceed that of calcitonin. The enhanced hypocalcemic activity of the GlcNAc derivative was explained by its altered biodistribution. The GlcNAc attachment caused calcitonin to escape from the trap at the liver during the early circulation. Thus, the glycosylation was shown to modulate the biological activity of calcitonin depending on the carbohydrate structure without a change in the peptide backbone conformation.

Keywords: calcitonin, N-glycosylation, hypocalcemic activity, three-dimensional structure, receptor binding activity

Introduction

Oligosaccharides in glycoconjugates participate in many biological functions [1–3]. However, it is still difficult to derive specific rules for oligosaccharide functions, because the data concerning the carbohydrate functions are fragmental. Such a situation prevents practical application of oligosaccharides. One way to overcome this situation is a systematic structure and activity study with chemically pure and structurally defined samples. In that spirit, we investigated the function of oligosaccharides in glycopeptides using eel calcitonin (CT) as a model peptide.

CT is a 32-amino acid peptide hormone with hypocalcemic activity, and some of the derivatives are currently used as therapeutic drugs for hypercalcemia, Paget's disease and osteoporosis [4]. There are notable advantages in using CT as a model peptide: the chemo-enzymatic synthetic procedure has been established [5]; CT derivatives are known to assume helical three-dimensional structures in hydrophobic environments as elucidated by NMR [6,7] and CD spectroscopy [8]; a small amount of sample is sufficient to evaluate the hypocalcemic activity, the main pharmacological effect of CT [9].

We have reported the NMR structure of artificially *N*-glycosylated CT derivatives both in a SDS micelle solution and in a membrane-binding environment [10]. The glycosylation did not affect the helical three-dimensional structure of the CT peptide backbone. In this paper, we would like to report the biological activities of the *N*-glycosylated CT derivatives. The glycosylation modulated the biological activity in a carbohydrate structure-dependent manner [11].

Materials and methods

Sample preparation

The amino acid sequence of CT depends on the species from which CT is derived. Specifically, we employed the eel CT sequence. The preparation of CT, [Asn(GlcNAc)^3]-CT (CT-GN) and [Asn{(Man)$_6$(GlcNAc)$_2$}^{3}]-CT (CT-M6) was already described [5,10]. [Asn{(Man)$_5$(GlcNAc)$_2$}^{3}]-CT (CT-M5) and

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[Asn{(Man)$_1$(GlcNAc)$_2$}^{3}]-CT (CT-M1) were prepared by pruning the carbohydrate portion of the CT-M6 with α-mannosidases as follows.

CT-M6 (0.081 mg) was dissolved in water (11 μl). *Aspergillus saitoi* α-mannosidase solution (Oxford Glycosystem; 1 μU/μl; 5 μl) and 500 mM sodium-acetate buffer (pH 5.0; 4 μl) were added, and the solution was incubated at room temperature overnight. The reaction was stopped with the addition of 20% acetonitrile aqueous solution (100 μl). HPLC (Cosmosil 5C18-300, ϕ4.6 × 150 mm; Nacalai Tesque) purification (0.1% trifluoroacetic acid in 18–72% acetonitrile-H_2O 40 min gradient solution, flow rate 0.8 ml/min) yielded CT-M5 (retention time 22.3 min; 0.044 mg). MALDI-TOFMS: calculated 4632.0 ($C_{192}H_{317}O_{82}N_{45}S_2$); found 4632 ($[M+H]^+$).

Similarly, CT-M1 (retention time 23.4 min; 0.028 mg) was prepared from CT-M6 (0.081 mg) with *Jack bean* α-mannosidase solution (Oxford Glycosystem; 100 mU/μl; 5 μl). MALDI-TOFMS: calculated 3983.5 ($C_{168}H_{277}O_{62}N_{45}S_2$); found 3983 ($[M+H]^+$).

Sample concentration

The CT concentration was determined from its weight. The concentration of each glycosylated CT derivative was determined as the HPLC peak area, recorded at 220 nm, relative to that of CT.

CD measurement

CD spectra were obtained using a JASCO J-720 spectropolarimeter equipped with a thermostatted cell holder. Samples were dissolved in aqueous trifluoroethanol (TFE). The TFE concentration used was 0, 10, 20, 40 or 60% for CT and CT-GN, and only 40% for CT-M1, CT-M5 and CT-M6. The temperature dependency of the 40% TFE aqueous solution was measured at 25–60°C. The sample concentration range was 0.05–0.3 mM. The wavelength from 200 to 250 nm was recorded. The helical contents were estimated according to the method of Chen et al. [12].

NMR measurement

NMR experiments were performed at 500 MHz on a JEOL JNMA-500 spectrometer, and the temperature was set to 10°C. The sample concentration was 5.0 mM in a mixture of 50% H_2O, 10% D_2O and 40% TFE-d2. DQF-COSY, HOHAHA and NOESY spectra were obtained, and the sequence-specific assignment of the proton resonance from each residue was done using the standard procedure [13]. Due to the sample amount, only CT, CT-GN and CT-M6 were measured.

Hypocalcemic activity

The hypocalcemic activity of the CT derivatives was measured by the reported method [9]. A 0.2 ml aliquot of 1.8 (low dose) or 3.6 (high dose) pmol/ml sample solution was injected into the tail vein of male Sprague-Dawley rats, 4 to 5 weeks old, and the blood sample was taken 60 min after the injection. The serum calcium concentration was determined with atomic absorption. The blank value is the serum calcium concentration of the untreated animal, and the standard value is that of the animal injected with elcatonin, a synthetic analogue of eel CT [4]. Each value represents the average of 10 animals except the blank value (the average of 5 animals).

Receptor binding assay

Osteoclast-like cells were prepared by co-culture of osteoblastic cells and bone marrow cells as previously described [14,15]. Primary osteoblastic cells prepared from newborn ddY mouse calvaria, and bone marrow cells from 6-week-old male ddY mice were co-cultured on dishes coated with collagen gel matrix (Nitta Gelatin Co., Osaka, Japan) in αMEM containing 10% FCS in the presence of 10 nM 1α,25-dihydroxyvitamin D3 for 7 days. At the end of the culture, dishes were treated with collagenase (0.2%). Equal aliquots of the cells were inoculated into 48-well plates, and the cells were allowed to settle for 24 hours before further experiments in the medium without 1α,25-dihydroxyvitamin D3.

^{125}I-elcatonin binding experiments were done as previously described [14]. Elcatonin is known to have comparable binding activity to CT [4]. The osteoclast-like cell preparations were placed in 48-well plates as described above, then incubated for 30 min at 25°C in the binding buffer, αMEM containing 0.1% crystallized BSA and 0.1% bacitracin. For competition experiments, incubations were carried out for 2 hours at 25°C in the binding buffer containing 0.5 nM of ^{125}I-elcatonin (Amersham Pharmacia Biotech, Buckinghamshire, England) and various concentrations of cold competitors. At the end of the incubations, the cells were washed twice in ice-cold PBS, solubilized in 0.2 N NaOH, and radioactive binding to the cells was assessed with a gamma counter.

Biodistribution

Samples were labeled with ^{125}I by a modified chloramine-T method similarly to the reported method [16]. Male ddY mice (20–25 g body weight) were weighed and injected with an ^{125}I-sample (10 kBq in 0.1 ml saline) through the tail vein. Animals were killed with ether anesthesia at 5, 15, 30, 45, 60 or 120 min after the injection. Blood samples were collected by heart puncture, and the animals were dissected. Organs of interest were weighed and the radioactivity was measured with a well-type scintillation counter (ARC300, Aloka, Japan). Tissue accumulation was calculated as a differential absorption ratio (DAR).

$$\text{DAR} = 100 \times (\text{body weight} \times \text{sample count}) / (\text{tissue weight} \times \text{injected count})$$

Each value represents the average (with 1 s. d.) of 5 animals.

Results

We had already prepared CT, CT-GN and CT-M6 [10], but we thought it better to add a variety in the carbohydrate structure. Using CT-M6 as a starting material, we enzymatically truncated the carbohydrate portion to produce CT-M5 and CT-M1. The chemical structures of all compounds used in this study are summarized in Figure 1.

We compared the three-dimensional structures of all compounds with CD spectra. As shown in Figure 2, CT assumed a random coil structure in aqueous solution and gradually accumulated the helical content as the TFE concentration increased. At the 40% TFE concentration, the helical content almost reached saturation with *ca*. 60%. Similar TFE concentration dependency was observed for CT-GN. All of the glycosylated CT derivatives showed CD spectra quite similar to that of CT in 40% TFE solution. Sample concentration ranging from 0.05 to 0.3 mM did not affect the CD spectra.

The NMR structure of eel CT in TFE aqueous solution had already been reported [17], and it was shown to be similar to the structure that we observed in SDS micelle solution [10]. Thus, we double-checked the three-dimensional structure of CT-GN and CT-M6 in the 40% TFE aqueous solution with NMR, and confirmed the results of the CD spectra (data not shown). Due to the sample requirement, we obtained NMR spectra of only CT, CT-GN and CT-M6.

We also examined the temperature dependence of the CD spectra with the 40% TFE aqueous solution to determine the structural stability. The helical contents of all the compounds decreased linearly as the temperature increased, but there was no remarkable difference among them as shown in Figure 3.

The best known physiological activity of CT is a hypocalcemic effect, and the activity in rats has been adapted as the standard pharmaceutical unit of CT derivatives [4]. The

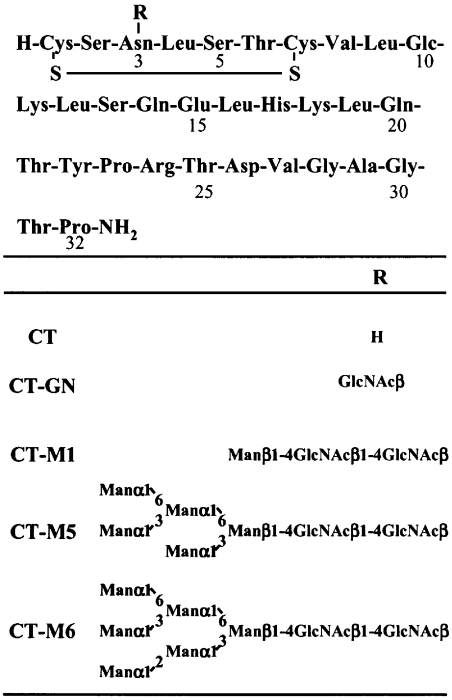

Figure 1. Amino acid sequence of eel CT and carbohydrate structures of glycosylated CT derivatives. Each carbohydrate is attached to Asn3.

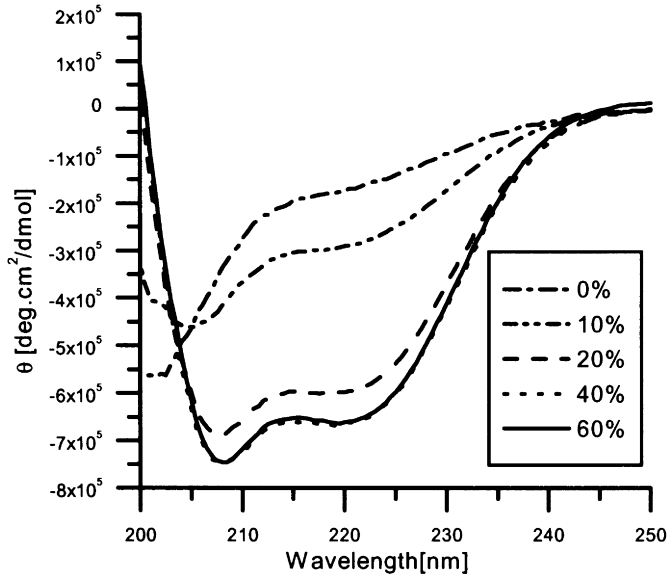

Figure 2. TFE concentration-dependent CD spectrum change in CT recorded at 0% (– · – · –), 10% (– · · –), 20% (– – ––), 40% (·····) and 60% (———).

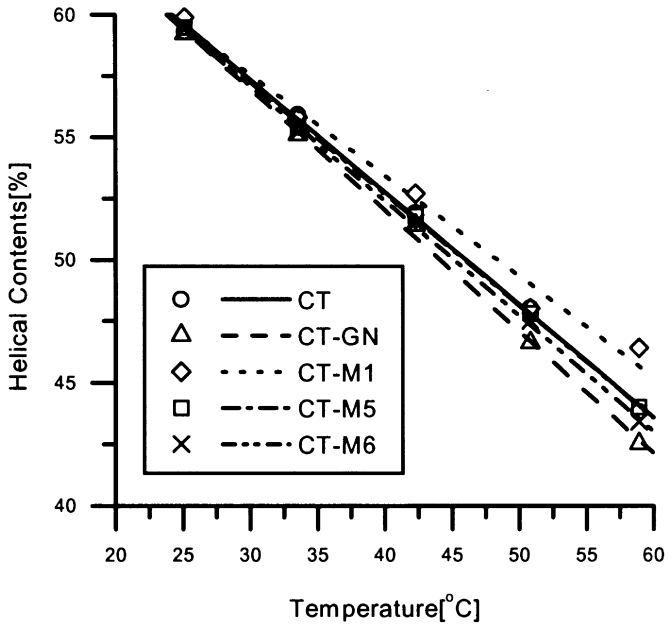

Figure 3. Temperature-dependent helical content change in CT (○), CT-GN (△), CT-M1 (◇), CT-M5 (□) and CT-M6 (×). The helical content is estimated from the CD spectrum according to the method of Chen et al. [12].

hypocalcemic effect of the compounds is summarized in Table 1. The broken line in the table shows that two sets of samples were measured on different days, and the corresponding blank and elcatonin (standard) values are accompanied by each sample set. We performed this assay twice, and the relative activity changed slightly due to the *in vivo* nature of the assay. CT binds to the receptors on the osteoclast cells, and inhibits their absorption of the bone and the accompanying release of calcium ions. Therefore, the lower calcium concentration indicates the stronger activity. The activity decreased roughly in the order of CT-GN, CT-M1, CT, CT-M5 and CT-M6, and the activity enhancement of CT-GN was unambiguous.

CT receptor (CT-R) is a member of the G-protein coupled seven transmembrane receptor family [18] and is abundant on the osteoclast cells. The osteoclast-like cell expresses CT-R abundantly, and can be employed as a source of active receptors. Using the osteoclast-like cells, a CT-R binding assay had been developed [14,15]. We measured the receptor binding activity with a direct competition assay. The result of the competitive CT-R binding assay of the glycosylated CT samples is shown in Figure 4. The binding curve of CT-GN almost overlapped with that of CT, and that of CT-M5 did that of CT-M6. The large carbohydrate structures tended to produce more weakly binding derivatives. Among the compounds, CT-M1 was the weakest binder to CT-R.

Table 1. Hypocalcemic activity (two trials) shown in serum calcium concentration (mg/dL)

	Low dose	*High dose*
First trial		
Blank	9.32 ± 0.13	
Elcatonin	8.37 ± 0.35	7.39 ± 0.50
CT-M6	8.63 ± 0.33	8.11 ± 0.49
CT-GN	7.92 ± 0.41	6.84 ± 0.23
Blank	9.75 ± 0.19	
Elcatonin	8.32 ± 0.34	7.23 ± 0.33
CT	8.52 ± 0.49	8.05 ± 0.34
CT-M1	8.53 ± 0.34	6.95 ± 0.37
CT-M5	8.64 ± 0.35	8.01 ± 0.45
Second trial		
Blank	10.23 ± 0.09	
Elcatonin	9.03 ± 0.30	7.97 ± 0.25
CT	9.04 ± 0.32	8.22 ± 0.31
CT-GN	9.22 ± 0.22	7.67 ± 0.39
Blank	9.43 ± 0.11	
Elcatonin	8.82 ± 0.48	7.82 ± 0.71
CT-M1	9.26 ± 0.31	7.89 ± 0.32
CT-M5	9.34 ± 0.56	8.35 ± 0.66
CT-M6	9.45 ± 0.48	9.12 ± 0.47

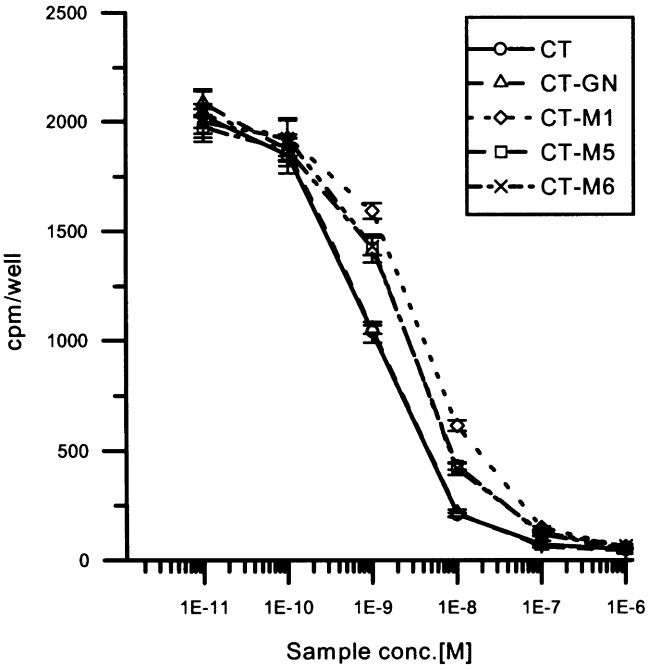

Figure 4. Calcitonin receptor binding activity of CT (○), CT-GN (△), CT-M1 (◇), CT-M5 (□) and CT-M6 (×). Each error bar corresponds to single standard deviation. CT-GN almost overlaps with CT, and CT-M5 does CT-M6.

CT has only one tyrosine residue in the sequence, and the tyrosine can be labelled with ^{125}I by the standard procedure. Using the radio-labelled compounds, their distribution in an animal was studied. The biodistribution of CT and CT-GN is compared in Figure 5. The concentration of CT-GN was higher in the blood and the bone and lower in the liver than that of CT. The difference in the kidney and other organ distribution is small (data not shown). It is desirable to employ the same species for both hypocalcemic and biodistribution experiments. We observed similar enhancement in the blood concentration with rat experiments (data not shown), but we chose the mouse experiments for the detailed analysis due to the ease of experimentation.

Discussion

The CT amino acid sequence contains the consensus sequence for *N*-glycosylation site at Asn3, and that portion of the sequence is conserved among species. Although high mannose-type oligosaccharides are reported to attach to Asn3 as minor components in a rat thyroid carcinoma cell line [19], the major natural component of CT is not glycosylated. Thus, we chemo-enzymatically synthesized the artificially *N*-glycosylated derivatives of eel CT, and studied their three-dimensional structures and biological activities with chemically pure and structurally defined samples.

In the previous NMR study [10], we employed only CT-GN and CT-M6 as the glycosylated derivatives. It is possible,

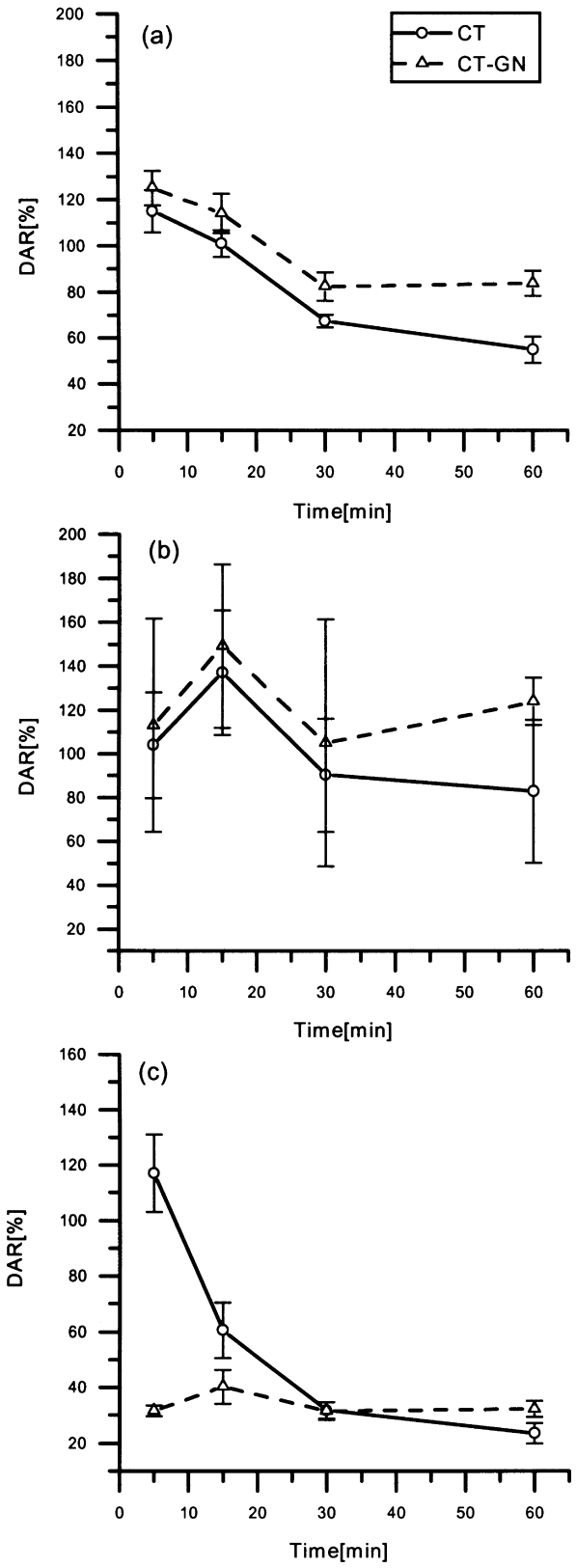

Figure 5. Biodistribution of CT (○) and CT-GN (△) in blood (a), bone (b) and liver (c). DAR = 100 × (body weight × sample count)/(tissue weight × injected count). Each error bar corresponds to single standard deviation.

however, that the effect of glycosylation is more significant to the biological activity than to the three-dimensional structure. Even a single amino acid change can cause a drastic effect on the activity of peptides. A mono-saccharide residue is comparable to or even larger than an amino acid residue, and there is seven residue difference between CT-GN (one sugar residue) and CT-M6 (eight residues). Therefore, we thought it desirable to interpolate them by adding a carbohydrate chain variety. We prepared two more carbohydrate structures, CT-M5 (seven residues) and CT-M1 (three residues), by pruning the carbohydrate portion of CT-M6 with α-mannosidases. It is an advantage of the carbohydrate chain transfer reaction that a large carbohydrate structure is attached at once and modified by subsequent enzyme digestions.

The strong hypocalcemic activity of CT allowed us the *in vivo* hypocalcemic assay even for the small amount of samples of CT-M5 and CT-M1. Although there were some deviations, we obtained fairly reproducible results in two trials as shown in Table 1. All of the samples retained the strong hypocalcemic activity of CT. When we examined the result more precisely, the activity decreased roughly in the order of CT-GN, CT-M1, CT, CT-M5 and CT-M6. It is very interesting that the activity was dependent on the carbohydrate structure. How did the carbohydrate moiety modulated the activity of CT?

One possible answer is the structural change caused by the glycosylation. We reported the solution and the membrane-bound NMR structures of CT-GN and CT-M6, but the peptide backbone structures were almost identical to that of CT [10]. Because the sample amount did not permit the NMR analysis of CT-M1 and CT-M5, we employed CD spectroscopy to determine their structure. All of the compounds showed quite similar CD spectra at 40% TFE concentration assuming *ca.* 60% helical structure. Thus, we confirmed that there was no structural change at least in the peptide backbone even for the newly synthesized CT-M1 and CT-M5.

The structural stability can also be affected by the glycosylation, and may modify the biological activity of CT. Though we examined the temperature dependence of the CD spectra with the 40% TFE aqueous solution, there was no remarkable difference among CT and the glycosylated derivatives. In the NMR paper [10], we proposed a possibility that the glycosylation stabilized the helical structure of CT. The discrepancy may have arisen from the difference in experimental conditions, or the CD experiment we employed is not sufficiently sensitive for determining a subtle stability change. However, even if there is some stabilization effect, we think it must be too small to explain the difference in the hypocalcemic activity.

Because the difference in the three-dimensional structure or the structural stability was ruled out as the activation mechanism, we then examined the change in the receptor binding activity. The result in Figure 4 shows that the binding activity of the glycosylated CT derivatives is comparable with that of CT. It reveals some binding interference by the carbohydrate attachment probably due to the steric hindrance,

and explains some part of the hypocalcemic activity change in that the larger carbohydrate structure results in a less active derivative, though the relative order of CT-M1, CT-M5 and CT-M6 is different in the two assays. The receptor binding activity alone cannot explain the hypocalcemic activity enhancement by CT-GN, the affinity of which was almost identical to that of CT.

Another possible mechanism is the modulation of biodistribution by the glycosylation. Such a function of a carbohydrate is thought to be applicable to a drug delivery system. It is also interesting in that respect, if the glycosylation is found to modify the biodistribution. We compared the distribution of CT and CT-GN, the most active derivative, in mice and found a difference as shown in Figure 5. The blood concentration of CT-GN was higher than that of CT, resulting in an enhanced distribution in the target tissue, bone in this case. The higher blood level seemed to come from the escape from the liver trapping during the early circulation. At present, we think this is the main mechanism that enhanced the hypocalcemic activity of CT-GN. Though the scarce sample amount of CT-M1, CT-M5 and CT-M6 after other experiments did not allow their biodistribution experiment, the discrepancy in their relative order of activity between the hypocalcemic and receptor binding assays may also be ascribed partly to the change in their biodistribution, because mannose-binding proteins are reported to be expressed in the liver [20].

Though the effect of GlcNAc was surprising to us, related observations have been reported in the literature once with a derivative of renin inhibitor peptides [21] and quite recently with a derivative of cyclic RGD peptides [22]. Those reported structures are more artificial than ours, but they contain a GlcNAc structure, and are reported to have the effect of reducing the peptide uptake in the liver. Thus, the effect of GlcNAc may be generally applicable to the modification of other biologically important peptides.

Currently, we are investigating the three-dimensional structures and biological activities of other glycosylated CT derivatives, as well as the generalization of the present result. However, even from the data discussed in this report, we can conclude that the glycosylation affects biological activities depending on the carbohydrate structure, even if the three-dimensional structure as can be measured by CD and NMR does not change.

Acknowledgments

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