CLINICAL STUDY

Circulating procalcitonin and cleavage products in septicemia compared with medullary thyroid carcinoma

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Abstract

Objective: Raised plasma levels of procalcitonin (proCT) represent an early marker for septicemia. They are related to disease severity and inversely to outcome and response to treatment. ProCT is presumably synthesised in tissues other than the thyroid C-cells which are the source of calcitonin (CT) in normal physiology. This study compares proCT and its cleavage products in the serum of patients with septicemia with those in medullary thyroid carcinoma (MTC).

Methods: Immunoreactive proCT and its cleavage products were extracted from the serum of patients with septicemia or MTC using octadecylsilyl silica columns and characterised by reversed phase HPLC and Western blot analysis. ProCT, CT(1–32) and the flanking peptides PAS-57 and PDN-21 were recognised with antibodies specific for the individual peptides.

Results: ProCT and a 10 kDa polypeptide were recognised with antibodies to PAS-57, CT(1–32) and PDN-21. An 8 kDa proCT fragment was detected with antibodies to CT and PDN-21. However, intact CT(1–32), PAS-57 and PDN-21, found in the serum of MTC patients, were undetectable. The results indicate partial cleavage of proCT in septicemia different from that in MTC patients.

Conclusions: ProCT and 10 and 8 kDa proCT fragments were recognised in the circulation of septic patients. They were different from the known proCT-processing products PAS-57, CT(1–32) and PDN-21 identified in the serum of normal subjects and of MTC patients. Distinct cleavage of proCT may contribute to the symptoms of septicemia.

European Journal of Endocrinology 147 727–731

Introduction

Raised immunoreactive procalcitonin (proCT) in the circulation is a useful diagnostic marker for septicemia (1–4). The serum levels of immunoreactive proCT are related to the severity of bacterial infection and are used to monitor outcome and therapy (5–7). The 13 kDa proCT(1–116) has been identified in a cell line derived from human medullary thyroid carcinoma (MTC) (8). In thyroid C-cells, proCT is processed into the hormone calcitonin (CT)(1–32) which inhibits osteolytic bone resorption, and into the N- and C-terminal flanking peptides PAS-57 and PDN-21 with unknown biological functions (9). All three cleavage products are secreted in a calcium-dependent manner into the circulation of normal subjects and MTC patients, together with proCT (10–12). Moreover, serum levels of immunoreactive CT represent a diagnostic marker of MTC.

In septicemia, the thyroid C-cells are not considered as an important source of elevated levels of circulating proCT, since immunoreactive proCT was recognised in thyroidectomised patients (1, 13). Expression of CT gene transcripts in several non-thyroidal tissues has been reported (14). Moreover, immunoreactive proCT(3–116) has been identified in the circulation of septic patients (15). Cleavage of proCT(1–116) to proCT(3–116) was thought to involve dipeptidyl peptidase IV (16). Interestingly, intraperitoneal injections of an antiserum to CT lowered the mortality of septic hamsters (17). Nevertheless, the pathophysiological roles of proCT(3–116) and of individual less well-characterised cleavage products of proCT remain to be clarified in septic patients.

Here, we have characterised circulating forms of proCT and of its cleavage products in patients with septicemia and with MTC. Serum extracts were analysed by HPLC and on Western blots with antisera specific for proCT, PAS-57, CT(1–32) and PDN-21. The molecular

weight of proCT in septicaemia was smaller than that in MTC patients. Ten and eight kDa proCT fragments were recognised in the serum of septic patients. They were different from the classical proCT processing products PAS-57, CT(1–32) and PDN-21 identified in MTC patients and normal subjects.

Subjects and methods

Subjects

Patients with septicaemia and with MTC have been investigated. Two female and seven male patients fulfilled the criteria of a systemic inflammatory response syndrome. Eight of the nine patients had positive bacterial blood cultures. Two female and two male patients presented histologically verified MTC. The clinical data of all the patients are summarised in Table 1. The collection of blood samples from septic patients was approved by the Ethical Committee of the University of Ulm (Germany), whereas the collection of the serum from MTC patients for diagnostic purposes predated the establishment of an Ethical Committee of the Zurich University Hospitals (Switzerland).

Materials

Salmon (s) CT was donated by S Guttman and human (h) CT and PAS-57 by H Rink (Novartis, Basel, Switzerland). Human α -calcitonin gene-related peptide (α CGRP) was purchased from Bachem (Bubendorf, Switzerland) and the carboxyl-terminal cleavage peptide of proCT (PDN-21) from Peninsula Laboratories, Inc. (Belmont, CA, USA). Acetonitrile, heptafluorobutyric acid and β -mercaptoethanol were obtained from Fluka Chemic AG (Buchs, Switzerland). Other chemicals and reagents were purchased from Sigma

Chemicals Co. (St Louis, MO, USA) and E Merck (Darmstadt, Germany) at the best purity available.

Immunoassays

Serum levels of proCT were measured in a bioluminescence immunoassay (B.R.A.H.M.S.; Diagnostica, Berlin, Germany) according to the manufacturer's instructions. Immunoreactive CT in serum and HPLC fractions, and PAS-57 and PDN-21 in HPLC fractions were identified by homologous and specific radioimmunoassays (RIA) in several dilutions as previously described (11, 12).

Peptide extraction

Serum samples collected from septic and MTC patients were kept frozen at -20°C until used. The samples of individual MTC patients collected on different days were pooled prior to peptide extraction. The serum samples were then diluted with equal volumes of 0.1% trifluoroacetic acid and kept for 30 min at room temperature. Subsequently, they were cleared by centrifugation at $10\,000\text{ g}$ at 4°C for 10 min. The supernatants were applied to Sep-Pak C18 cartridges (Waters Associates, Milford, MA, USA) activated with 10 ml methanol and equilibrated with 10 ml 0.1% trifluoroacetic acid. The cartridges were washed with 10 ml 0.1% trifluoroacetic acid and the peptides eluted with 3 ml 80% acetonitrile in 0.1% trifluoroacetic acid. The extracts were lyophilised and redissolved in 1 ml 0.1 M acetic acid for further analysis. The overall recovery of trace amounts of $[^{125}\text{I}]\text{sCT}$ added to individual serum samples prior to the extractions was $85.5 \pm 7.5\%$ (means $\pm$ s.d.).

Table 1 Patients and laboratory parameters.

Patient	Age (years)	Sex	Body temperature ($^{\circ}\text{C}$)	C-reactive protein (mg/l)	Leukocytes ($10^9/\text{l}$)	Blood culture	Calcium (mmol/l)	ProCT (ng eq/ml)	CT (ng eq/ml)
Septicaemia									
1	52	F	39.1	142	4.8	Positive	2.25	4.6	n.d.
2	49	M	40.0	6	9.9	Positive	1.53	14.5	2.7
3	72	M	38.8	300	8.2	Positive	2.33	7.3	1.5
4	64	F	42.0	294	16.7	Negative	2.15	4.2	n.d.
5	73	M	38.7	241	36.9	Positive	2.12	8.2	n.d.
6	60	M	40.0	259	15.7	Positive	n.d.	54.3	4.9
7	62	M	39.0	359	13.4	Positive	n.d.	2.4	n.d.
8	69	M	39.0	289	12.1	Positive	2.22	9.9	2.7
9	47	M	40.1	150	2.3	Positive	2.27	20.6	3.7
MTC									
10	57	F	n.d.	n.d.	n.d.	n.d.	2.20	n.d.	187.0
11	72	F	n.d.	n.d.	n.d.	n.d.	2.45	n.d.	3.9
12	50	M	n.d.	n.d.	n.d.	n.d.	2.20	n.d.	43.0
13	35	M	n.d.	n.d.	n.d.	n.d.	2.46	n.d.	69.0

n.d. = not done. Normal range: C-reactive protein $< 5\text{ mg/l}$; leukocytes $5\text{--}10 \times 10^9/\text{l}$; calcium $2.2\text{--}2.6\text{ mmol/l}$; ProCT $< 0.5\text{ ng eq/ml}$; CT $< 0.05\text{ ng eq/ml}$.

HPLC analysis

Serum extracts were subjected to reversed phase HPLC as described (12). Trace amounts of [125 I]sCT and [125 I]h α CGRP, not interfering in the RIA, were used for calibration of retention times in individual chromatograms. Fractions (1 ml) were collected for analysis by RIA. Aliquots (100 μ l) of the individual fractions were lyophilised in the presence of 100 μ l charcoal-treated serum and reconstituted in 1 ml RIA buffer. Fractions analysed on Western blots were lyophilised and reconstituted and kept at 95 °C for 5 min in 10 μ l protein gel loading buffer.

Western blot analysis

Proteins in HPLC fractions were separated by SDS-PAGE and electro-transferred at 25 V and 4 °C overnight to Hybond ECL nitrocellulose membranes in a Trans-Blot cell (BioRad Laboratories, Richmond, CA, USA). Kaleidoscope polypeptide size markers (3.5–37.6 kDa; BioRad Laboratories) were used.

Immunoblots were blocked with 10% low-fat milk in 20 mM Tris–HCl, pH 7.6, 137 mM NaCl (TBS) at room temperature for 1 h and washed five times for 10 min with TBS containing 0.1% Tween 20. Immunoreactive PAS-57 and CT components were sequentially visualised by enhanced chemiluminescence (ECL) with goat antibodies to synthetic PAS-57 (1:1000) (12) and secondary horseradish peroxidase (HRP)-labelled mouse antibodies to goat immunoglobulins (1:10 000) (Amersham Bioscience, Little Chalfont, Bucks, UK), and with rabbit antibodies to CT (1:1000) (Chemicon, Temecula, CA, USA) and secondary biotin-labelled antibodies to rabbit immunoglobulins (1:10 000) and HRP-labelled streptavidin (Amersham Bioscience). The blots were exposed to X-ray films at room temperature for 1 h in the presence of ECL Western blotting detection reagent (Amersham Bioscience). Antibodies to PAS-57 were removed from filters by incubation on a horizontal shaker in 100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7, at 50 °C for 30 min. Protein immunostaining with preimmune serum from goats was considered as non-specific.

X-ray films were scanned with a high resolution digital camera (PIXERA, Los Gatos, CA, USA) and non-specifically stained proteins on digital images were subtracted by computer-based inverse overlay of specific and non-specific images.

Results

Immunoreactive proCT and CT in the serum of septic patients ranged from 2.4 to 54.3 ngeq/ml and from 1.5 to 4.9 ngeq/ml respectively (Table 1). In the serum of MTC patients, CT levels varied between 3.9 and 187.0 ngeq/ml.

Immunoreactive CT, PAS-57 and PDN-21 in serum extracts from septic and MTC patients were characterised by reversed phase HPLC (Fig. 1) and subsequent Western blot analysis of individual HPLC fractions (Fig. 2). The quality of the PDN-21 antiserum was insufficient for Western blot analysis of the HPLC fractions containing immunoreactive PDN-21.

In septic patients, circulating CT and PDN-21 consisted of three major forms with retention times of 61, 73 and 77 min, different from those of synthetic CT(1–32) and PDN-21 (Fig. 1A). The components with the retention times of 73 and 77 min were also recognised with antibodies to PAS-57, but were separated from mature PAS-57. On Western blot analysis, the CT immunoreactive component with the retention time of 61 min, corresponding to HPLC fractions 62–64 (Fig. 2A), had an apparent size of 8 kDa. It was undetectable with antibodies to PAS-57. The PAS-57, CT- and PDN-21 immunoreactive components with retention times on HPLC of 73 min and 77 min, corresponding to fractions 72 and fractions 75–77 respectively in Fig. 2A and B, had apparent sizes of 10 and

Figure 1 Representative reversed phase HPLC profiles of immunoreactive CT (Δ), PDN-21 ($\blacktriangle$) and PAS-57 ($\square$) in extracts of 10 ml serum from septic (A) and MTC (B) patients. Serum from patients (six septic and eleven MTC) (Table 1) was extracted as described in Subjects and methods. Columns of Nucleosil 100-7C18 (Waters, Milford, MA, USA) were eluted with a gradient of acetonitrile and heptafluorobutyric acid (broken line). Elution positions of synthetic PDN-21 (I), hCT (II) and PAS-57 (V) and of [125 I]sCT (III) and [125 I]h α CGRP (IV) are indicated by arrows.

Figure 2 Representative combined HPLC and Western blot analysis of immunoreactive CT (A and C) and PAS-57 (B and D) components in HPLC fractions obtained from the serum of the septic patients (six) (A and B) and from a pool of MTC patients (C and D). Extracts of 12 ml serum were analysed. Immunoreactive PAS-57 and CT components were undetectable in HPLC fractions 1–54 and 65–67 (A and B) and in fractions 1–54 and 63–65 (C and D) (not shown).

12 kDa. The size of the 12 kDa component was slightly smaller than the calculated molecular weight of proCT.

In serum extracts of patients with MTC, predominant forms of immunoreactive CT and PAS-57 had the retention times and sizes of synthetic CT(1–32) and PAS-57 (Figs 1B and 2C and D). A PDN-21 component, eluting on HPLC with the retention time of synthetic PDN-21 was only recognised by antibodies to the peptide (Fig. 1B). A CT/PDN-21 immunoreactive polypeptide with a retention time of 58 min and a size slightly smaller than 6 kDa (fractions 60–62; Fig. 2C) was separated from the individual mature peptides (Fig. 1B). Another predominant polypeptide recognised by antibodies to PAS-57, CT(1–32) and PDN-21 eluted between 69 and 76 min and had the size of proCT (Figs 1B and 2C and D). A co-eluting 10 kDa polypeptide recognised with antibodies to CT and PAS-57 remains to be identified.

Taken together, CT, PDN-21 and PAS-57 components in serum extracts of septic and MTC patients differed in retention times on HPLC and in size on Western blot

analysis. In septic patients, proCT was not cleaved into the classical processing products CT(1–32), PDN-21 and PAS-57 identified in the serum of MTC patients.

Discussion

ProCT(3–116), rather than intact proCT, has been identified as the predominant protein product of the CT gene in the circulation of patients with septicaemia (15, 16). In the present study, major circulating cleavage products of proCT in septic patients were characterised by combined HPLC and Western blot analysis and compared with proCT processing products recognised in the serum of MTC patients (Fig. 3).

ProCT of approximately 12 kDa in serum extracts of septic patients was confirmed to be slightly smaller in size than that observed in MTC patients. It likely represents proCT(3–116) identified by Weglöhner *et al.* (15). Here, two novel 10 and 8 kDa proCT fragments have been identified with antibodies to CT(1–32) and PDN-21 as predominant components in the circulation of septic patients. The 10 kDa proCT fragment was also recognised with a PAS-57 antiserum. Proteolytic cleavage of proCT(3–116) into this 10 kDa fragment presumably occurred through N-terminal truncation, leaving the PAS-57 epitope intact. The 8 kDa polypeptide was not recognised by antibodies to PAS-57, but was larger than a 6 kDa proCT fragment observed in MTC patients that likely consisted of CT and PDN-21. The cleavage therefore occurred within the N-terminal PAS-57 sequence of proCT thereby removing the PAS-57 epitope. Additional proteolytic truncation in the C-terminal PDN-21 portion of the 10 and 8 kDa proCT fragments, leaving the epitope recognised by the PDN-21 antibodies intact, cannot be ruled out. With the 10 and 8 kDa proCT fragments identified here it seems unlikely that the processing of proCT in septic patients is that observed in MTC patients and in normal subjects as implied by Snider *et al.* (10).

In conclusion, proCT(3–116) and 10 and 8 kDa proteolytic cleavage products in the circulation of septic patients, different from CT(1–32), PAS-57 and PDN-21 identified in normal subjects and in MTC patients, may contribute to the symptoms of septicaemia.

Figure 3 Schematic illustration of proCT cleavage pathways in patients with septicaemia and MTC.

Acknowledgements

This study was supported by the Swiss National Science Foundation, the Kanton of Zurich and the Schweizerischer Verein Balgrist.

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Received 23 May 2002

Accepted 16 September 2002