Neo-kyotorphin, an analgesic peptide isolated from human lung carcinoma

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A pentapeptide with analgesic activity has been isolated from human lung squamous cell carcinoma and from three other types of propagated tumors of human lung small-cell carcinoma (SCC), adenoma (AD) and large-cell carcinoma (LCC) in nude mice. The amino acid sequence of the peptide has been revealed to be H-Thr-Ser-Lys-Tyr-Arg-OH, which is exactly the same as that of neo-kyotorphin, an analgesic peptide originally isolated from bovine brain [(1982) Life Sci. 31, 1733]. No neo-kyotorphin could be isolated from normal lung tissue using the same procedures as those used for carcinomas. The results suggest that the presence of neo-kyotorphin in the lung carcinoma may represent the ectopic expression of peptide hormone. Our findings constitute the first example of a human lung carcinoma producing analgesic peptide.

(Human lung) *Carcinoma* *Neo-kyotorphin* *Analgesic peptide* *Analgesic activity* *Peptide isolation* *Peptide synthesis*

1. INTRODUCTION

Human lung small-cell carcinoma (SCC), squamous cell carcinoma (SQCC), adenoma (AD) and large-cell carcinoma (LCC) cover most types of the lung carcinoma identified in the clinic. Among them SCC amounts to nearly 20% of the identifiable human lung carcinoma [1]. These neoplasms are frequently a source for ectopic hormone production [1–7]. Most notable among the peptide hormones detected in extracts of lung carcinoma and at elevated levels in the plasma of patients with these tumors were ACTH [2,9], prolactin [2], oxytocin [2], calcitonin [9], bombesin [10], physalamin [11], neurotensin [12], etc. It has been suggested and confirmed that many of the clinical symptoms manifested by patients with lung carcinoma can be attributed to increased peptide hormones [5–7].

Searching for presently undiscovered peptide hormones that may cause some kinds of syndrome or serve as a potential peptide marker in the screening of patients with lung carcinoma, we have investigated most of the human lung carcinomas and found that an analgesic pentapeptide, neo-kyotorphin, is richly presented in four types of different human lung carcinomas. Here we report the isolation, sequence determination, synthesis and analgesic activity of the peptide.

2. MATERIALS AND METHODS

2.1. *Tumors*

Human lung SQCC was resected directly during operation from a 40-year old man with apparent endocrinopathies. SCC from a 69-year old man, AD from a 72-year old man and LCC from a 57-year old man were separately propagated in nude mice and resected after 45, 21 and 14 days, respectively.

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2.2. *Extraction and isolation*

Four types of lung carcinoma obtained were separately homogenized and extracted in 5 vols (v/w) of 1 M acetic acid containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1.5 μM pepstatin for 24 h at 4°C. After centrifugation at 20000 × g for 20 min, the supernatants were lyophilized to give the crude extracts.

The isolation details described below for human SQCC, the procedures and results of isolation and purification for the other three types of propagated carcinoma were very similar and not shown here.

The crude extract of SQCC was then deposited on a Sephadex G-50 (fig.1) column (2.3 × 90 cm) and eluted with 0.5 M acetic acid. Six fractions thus obtained were collected and freeze-dried. The fraction E that possessed a stimulating activity on a guinea pig ileum was rechromatographed on a Sephadex G-25 (superfine) column (2.3 × 90 cm) and eluted with the same solvent as used in the first gel-filtration. The active fraction D from second gel-filtration was subsequently purified by reverse-phase high performance liquid chromatography (RP-HPLC). The experimental conditions used for HPLC are given in the legend to fig.2.

2.3. *Amino acid sequence determination*

2.3.1. Automatic Edman degradation

2 nM of the sample (the peak denoted NK in fig.2B) was sequenced using a 470 A gas-phase sequencer (Applied Biosystems Inc.) with an automatic PTH-amino acid converter. The phenylthiohydantoin amino acids (PTH-amino acids) were identified by an on-line RP-HPLC.

2.3.2. Fast atom bombardment mass spectrometry (FAB-MS)

FAB-MS was carried out using a Varian MAT 711 mass spectrometer equipped with a fast atom gun. The peptide (5 nM) in 2 μl glycerol was loaded onto a probe tip. Spectra were recorded at 300 s per decade in mass.

2.3.3. Manual sequence determination

Manual sequence determination of the peptide was performed by using a colored Edman degradation method, where DABITC was used instead of PITC [15]. DABITC-amino acids obtained during the sequencing were identified in thin-layer sheets.

2.3.4. Carboxypeptidase Y digestion

For the sequence determination of the COOH-terminal portion of the peptide, 10 nM of the sample was dissolved in 200 μl of 0.1 M ammonium acetate (pH 5.7). Carboxypeptidase Y (1 μg, from Boehringer Mannheim) was then added and the digestion was performed at 37°C. Aliquots of reaction mixture were taken out at the time intervals of 0, 30 and 240 min. The lyophilized materials were then passed on the amino acid analyzer (LKB-4400). As a control, the peptide was incubated under the same conditions without adding the enzyme.

2.4. *Synthesis*

Neo-kyotorphin was synthesized by the manual conventional liquid method. Protected serine, BOC-Ser(OBZ)-OH, was first coupled with H-Lys(Z)-OCH_3 in the presence of DCCI to afford BOC-Ser(OBZ)-Lys(Z)-OCH_3, which, after removal of the protecting group by TFA, was further reacted with BOC-Thr(OBZ)-OH by employing the DCCI method and then saponified in the KOH solution to obtain a protected tripeptide, BOC-Thr(OBZ)-Ser(OBZ)-Lys(Z)-OH (**I**).

BOC-Tyr(OBZ)-OH and H-Arg(NO_2)-OCH_3 was condensed by the DCCI method and then treated by TFA to form a protected dipeptide, H-Tyr(OBZ)-Arg(NO_2)-OCH_3 (**II**).

I and **II** were condensed in the presence of *t*-butylchloroformate and *N*-methylmorpholine to give BOC-Thr(OBZ)-Ser(OBZ)-Lys(Z)-Tyr(OBZ)-Arg(NO_2)-OCH_3 (**III**).

After converting **III** to corresponding acid by KOH, the final deprotection of the protected pentapeptide was successively carried out with catalytic hydrogenation (H_2-Pd/C) and hydrolyzed by TFA to afford a crude product. The crude product thus obtained was purified by means of gel filtration on a Sephadex G-10 column to form a pure pentapeptide, neo-kyotorphin (**IV**).

2.5. *Analgesic activity of neo-kyotorphin*

The analgesic activity of synthetic neo-kyotorphin was determined by the tail-pinch method on eighteen assayed mice. Pain responses were recorded during 10–40 min after administration of the sample.

3. RESULTS

3.1. *Purification procedures*

Fig.1A shows the initial fractionation of the crude human SQCC extract. Second gel filtration of active fraction E from fig.1A on Sephadex G-25 is presented in fig.1B. The active fraction D from fig.1B was then repurified twice by RP-HPLC (shown in fig.2) and provided a pure peptide (denoted NK), on which amino acid analysis and sequencing were performed.

3.2. *Amino acid analysis*

In table 1, the results of amino acid composition of the purified peptide are depicted. It can be seen that the peptide has a very good amino acid integral and the length of the peptide is presumed to be five amino acids.

3.3. *Sequencing*

Sequence determination of the purified peptide on a nanomole scale proceeded successfully to the carboxyl end in both automatic and manual Edman degradation procedures. The results obtained (fig.3) suggest that the peptide has the amino acid sequence of H-Thr-Ser-Lys-Tyr-Arg-OH. Digestion of the peptide by carboxypeptidase Y released Arg (8.4 nM) and Tyr (2.9 nM). The COOH-terminal sequence thus revealed is in good agreement with that determined by the Edman degradation.

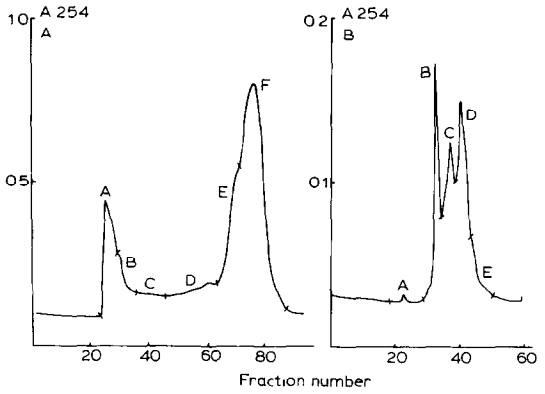

Fig.1. Gel-permeation chromatography of crude extract of human AD on the Sephadex G-50 column (A) and second gel-permeation chromatography of fraction D from (A) on the Sephadex G-25 column (B). Flow rate: 20 ml/h, column size and elution solvents are given in the text.

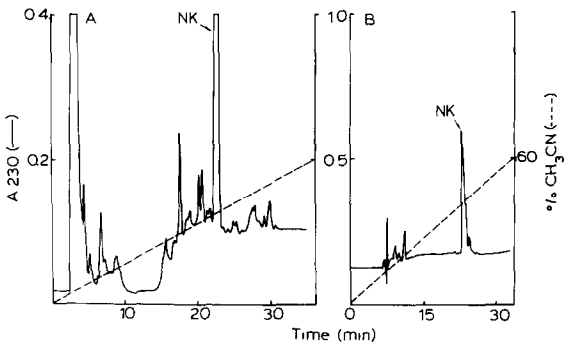

Fig.2. Reverse-phase HPLC purification of neo-kyotorphin. (A) The initial purification of fraction E from fig.1B on an analytical μBondapak C_{18} column (Waters 39 300 mm). The peak denoted NK was collected for further purification. (B) Repurification of NK by second RP-HPLC. The peak denoted NK in (B) provided a pure neo-kyotorphin. The RP-HPLC apparatus consisted of two Waters 6000 A pumps, a model 460 solvent programmer, a model 450 variable wavelength detector and a model 730 data module. Elutions were made using a linear gradient of 0–60% of acetonitrile (containing 0.01 M HFBA, v/v) at a flow rate of 0.6 ml/min.

The results of sequence determination of the peptide by FAB-MS are shown in fig.4. The types of peptide fragmentation by FAB are deduced according to Schufer [16]. As can be seen, M + H 654 confirmed the amino acid composition and the COOH-terminal amino acid. Ions *m/e* 74, 189, 317 and 480 indicate the presence of amino-carboxonium ions (ACO), while ions *m/e* 553, 466, 338 and 175 show the cleavage of amino-carboxy ions (AC) with charge on the -COOH

Table 1

Amino acid composition of neo-kyotorphin

Amino acid	Residues per molecule	Nearest integer
Thr	1.06	1
Ser	0.99	1
Tyr	0.94	1
Lys	1.01	1
Arg	0.99	1

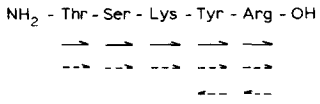

Fig.3. Determined sequence of neo-kyotorphin: (—→) determined by automatic Edman degradation; (---→) determined by manual Edman degradation; (←---) C-terminal sequence deduced by carboxypeptidase Y digestion.

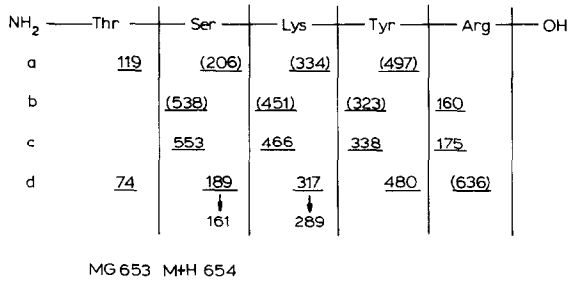

Fig.4. Data from fast atom bombardment mass spectrometry of neo-kyotorphin. (a–d) Different forms of cleavages of neo-kyotorphin: (a) cleavage of the amino-alkyl bond to form an amino-amide ion (A.A); (b) cleavage of the same bond to form an alkyl-carboxyl ion (ALKC); (c) cleavage of the amide bond to form an amino-carboxyl ion (AC); (d) cleavage of the amide bond to form an amino-carboxonium ion (ACO). The data of peak *m/e* show the peaks formed in the chromatogram, while the data listed in parentheses show the peaks not formed in the chromatogram.

moiety. Ion *m/e* 553 suggests Thr at the NH_2-terminus of the peptide (*m/e* 553 = 654 (M + H) − 101 (Thr)). Accepting Thr to be the NH_2-terminal amino acid, one can easily add Ser (553–466), Lys (466–338), Tyr (338–175) and Arg successively.

Based on these data, it is unambiguously concluded that the amino acid sequence of the peptide is H-Thr-Ser-Lys-Tyr-Arg-OH.

3.4. *Synthesis of neo-kyotorphin*

Fig.5 shows the scheme of synthesis for neo-kyotorphin. The total yield of the final purified peptide was 15% (w/w). Both the amino acid composition and behaviour on RP-HPLC of the synthetic sample were identical to the authentic peptide (not shown).

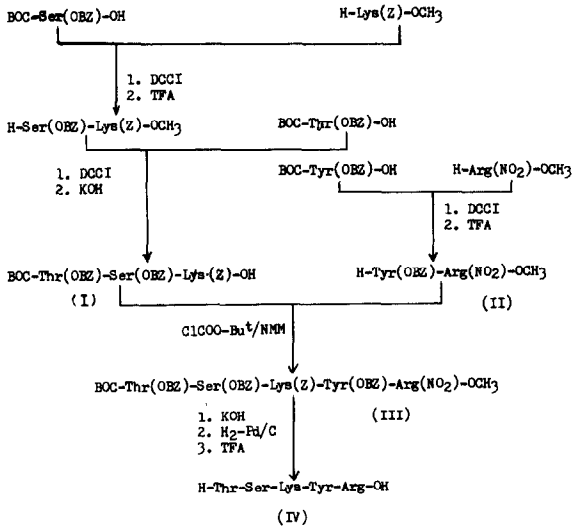

Fig.5. The synthetic scheme of neo-kyotorphin by the conventional liquid method. *t*-BOC, *tert*-butyloxycarbonyl; Bz, benzyl; Z, benzylcarbonyl; NO_2, nitro; DCCI, *N,N'*-dicyclohexylcarbodiimide; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; ClCOOBut, *t*-butylchloroformate.

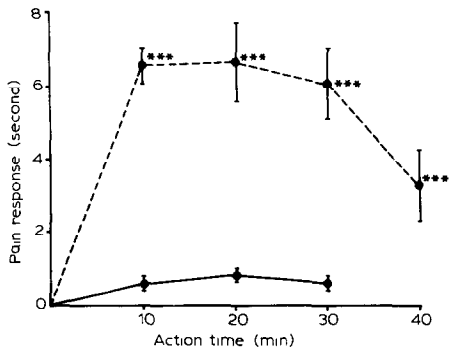

Fig.6. Analgesic activity of synthetic neo-kyotorphin on mice. (– – –) The pain response on tested mice after intraventricular injection of the sample (50 μg). (——) The pain response of control group of mice. *** indicates that the *p*-value of the results obtained was < 0.01.

3.5. *Analgesic activity of neo-kyotorphin*

Synthetic neo-kyotorphin showed a dose-dependent effect on mice after intraventricular injection. A dose of 100 μg (152 nM) of synthetic neo-kyotorphin caused strongly spasmodic action on the tested mice, while 50 μg (76 nM) of the sam-

ple exhibited profound analgesic activity (fig.6). The analgesic activity initiated from 10 min after administration and could last for about 30 min.

4. DISCUSSION

The present results draw an interesting conclusion that high levels of neo-kyotorphin are present in four kinds of human carcinoma. Neo-kyotorphin has been proved to be an analgesic peptide by Takagi et al. [17] and by this work. Our findings also constitute the first example of a human lung carcinoma producing analgesic peptide.

It is still unknown whether the tumor-derived hormone production is a simple reflection of derepression of genetic material consequent to dedifferentiation or whether the hormones exert a positive influence on the tumor growth of the tumor cell [18]. However, the finding of peptide hormones which specifically occur in the tumor cell, but not in the normal cell, suggests that the probability for success in using the plasma concentration of the peptide as a biochemical marker for this disorder is high.

The results of a computer search for possible sequence homology of neo-kyotorphin indicated that the amino acid sequence of neo-kyotorphin is identical to that of the five C-terminal amino acid residues of hemoglobin A-chain (Thr-137 to Arg-141). As the amino acid residue preceding Thr-137 is Leu, it seems likely that neo-kyotorphin is an enzymatically cleaved fragment of hemoglobin A-chain, by an endogenous chymotrypsin-like enzyme. This possibility needs to be proved by some further work.

It is still too early to evaluate the physiological importance of neo-kyotorphin. We hope that the further work including the studies on the comprehensive biological activity, the regional distribution, the structure-activity as well as the establishment of antiserum against neo-kyotorphin and the radioimmunoassay system will throw some new light on the peptide.

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