Demonstration and characterization of immunoreactive methionine-enkephalin, leucine-enkephalin, methionine-enkephalin-Arg⁶-Gly⁷-Leu⁸ and methionine-enkephalin-Arg⁶-Phe⁷ in human phaeochromocytoma

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Abstract. To elucidate whether or not human phaeochromocytoma contains methionine-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-enkephalin-Arg-Gly-Leu) and methionine-enkephalin-Arg⁶-Phe⁷ (Met-enkephalin-Arg-Phe) together with methionine-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin), all of which are known to exist in the same precursor molecule (preproenkephalin A), we examined extracts from 16 phaeochromocytomas using high performance liquid chromatography (HPLC) and gel exclusion chromatography coupled with radioimmunoassays (RIAs) for these four opioid peptides. Met-enkephalin-Arg-Gly-Leu-like immunoreactivity (LI) and Met-enkephalin-Arg-Phe-LI existed together with Met-enkephalin-LI and Leu-enkephalin-LI in 16 phaeochromocytomas. There was a wide variation in contents of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI. Significant positive correlations were observed among the contents of these four opioid peptides in 16 phaeochromocytomas. HPLC and gel exclusion chromatography followed by the RIAs showed the presence of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe together with their high molecular weight forms, which existed in variable amounts. Molar ratios of the contents of these four opioid peptides determined after HPLC varies from case to case. These results indicate the co-existence of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe in human phaeochromocytomas, suggesting the preservation of amino acid sequences of these four opioid peptides even in neoplastic tissues. Evidence for some abnormalities in the posttranslational processing of preproenkephalin A to these four opioid peptides was obtained in certain phaeochromocytomas.

Methionine-enkephalin (Met-enkephalin) and leucine-enkephalin (Leu-enkephalin) have been demonstrated to exist in human phaeochromocytomas by immunohistochemistry, radioreceptor assays and radioimmunoassays (RIAs) (Sullivan et al. 1978; Lundberg et al. 1979; Wilson et al. 1981; Yoshimasa et al. 1982). We have shown in the previous study that a marked difference exists in Met-enkephalin and Leu-enkephalin contents between two adrenal and two extra-adrenal phaeochromocytomas (Yoshimasa et al. 1982). Furthermore, our recent study has revealed that Met-
enkephalin and Leu-enkephalin are secreted into
circulation from certain phaeochromocytomas
(Yoshimasa et al. 1983).

Recently, Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (Met-
enkephalin-Arg-Gly-Leu) and Met-enkephalin-
Arg⁶-Phe⁷ (Met-enkephalin-Arg-Phe) were proved
to be derived from the same precursor molecule as
that of Met-enkephalin and Leu-enkephalin (pre-
proenkephalin A) by analyzing the nucleotide se-
quence of cloned DNA complementary to mRNA
from the bovine adrenal medulla and a human
phaeochromocytoma (Noda et al. 1982a; Gubler et
al. 1982; Comb et al. 1982). We have shown the
occurrence of Met-enkephalin-Arg-Gly-Leu and
Met-enkephalin-Arg-Phe together with Met-enke-
phalin and Leu-enkephalin in the adrenal medulla,
brain and gut by high performance liquid chroma-
tography (HPLC) and gel exclusion chromatog-
raphy followed by specific RIAs for these four opioid
peptides (Nakao et al. 1982; Ikeda et al. 1982;
Sakamoto et al. 1983). However, it is not known
whether or not Met-enkephalin-Arg-Gly-Leu and
Met-enkephalin-Arg-Phe exist in human phaeo-
chromocytomas.

In the present study, we examined extracts from
16 phaeochromocytomas using RIAs for Met-enke-
phalin-Arg-Gly-Leu. Met-enkephalin-Arg-Phe,
Met-enkephalin and Leu-enkephalin to dem-
strate the production of these peptides by the
tumours and characterized these immunoreactive
materials by HPLC and gel filtration.

Materials and Methods

Tumour samples and extraction
Phaeochromocytomas were obtained from 16 patients.
Pertinent data on these patients are listed in Table 1. Of
16 phaeochromocytomas 11 originated from the adrenal
medulla (cases 1–9, 12 and 14) and the remaining 5 were
of extra-adrenal origin (cases 10, 11, 13, 15 and 16). Two
phaeochromocytomas were considered to be malignant
cases 7 and 14). All tumours were obtained at surgery,
and the tissues were immediately cut into cubic slices of
about 5 mm in diameter and stored at −70°C until
extraction. Two slices of each tumour were subjected to
subsequent extraction.

Tissue extraction was performed by acidified methanol
according to the procedure described previously (Nakao

Table 1.
Contents of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI
and Met-enkephalin-Arg-Phe-LI in 16 phaeochromocytomas (ng/g wet weight).

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Origin</th>
<th>Met-enkephalin-LI</th>
<th>Leu-enkephalin-LI</th>
<th>Met-enkephalin-Arg-Gly-Leu-LI</th>
<th>Met-enkephalin-Arg-Phe-LI</th>
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<tr>
<td>1</td>
<td>56</td>
<td>F</td>
<td>Adrenal</td>
<td>94 600</td>
<td>33 000</td>
<td>78 700</td>
<td>72 800</td>
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<tr>
<td>2</td>
<td>46</td>
<td>F</td>
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<td>79 500</td>
<td>27 600</td>
<td>27 300</td>
<td>20 200</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>M</td>
<td>Adrenal</td>
<td>74 500</td>
<td>44 200</td>
<td>51 400</td>
<td>32 700</td>
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<td>4</td>
<td>61</td>
<td>F</td>
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<td>13 300</td>
<td>40 500</td>
<td>23 300</td>
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<td>5</td>
<td>58</td>
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<td>35 600</td>
<td>24 700</td>
<td>16 600</td>
<td>15 500</td>
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<td>6</td>
<td>50</td>
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<td>51</td>
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<td>657</td>
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<td>8</td>
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<td>264</td>
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<td>10</td>
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<td>184</td>
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<td>99.7</td>
<td>112</td>
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<tr>
<td>11</td>
<td>52</td>
<td>F</td>
<td>Paraaortic</td>
<td>179</td>
<td>61.5</td>
<td>77.2</td>
<td>117</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>M</td>
<td>Adrenal</td>
<td>66.6</td>
<td>15.5</td>
<td>44.7</td>
<td>46.6</td>
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<tr>
<td>13</td>
<td>48</td>
<td>F</td>
<td>Paraaortic</td>
<td>63.6</td>
<td>49.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>14</td>
<td>70</td>
<td>M</td>
<td>Adrenal</td>
<td>7.6</td>
<td>5.8</td>
<td>8.6</td>
<td>5.8</td>
</tr>
<tr>
<td>15</td>
<td>43</td>
<td>F</td>
<td>Paraaortic</td>
<td>6.2</td>
<td>2.7</td>
<td>1.5</td>
<td>5.6</td>
</tr>
<tr>
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<td>15</td>
<td>M</td>
<td>Mediastinal</td>
<td>3.3</td>
<td>0.6</td>
<td>2.1</td>
<td>0.6</td>
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</table>
et al. 1981). In brief, tissues were weighed and homogenized in 10 vol of acidified methanol consisting of equal parts of methanol and 0.1 N HCl. The homogenate was centrifuged at 50,000 g for 30 min at 4°C. and the supernatant was stored at -20°C.

The extracted samples were neutralized with 0.1 N NaOH immediately before RIA.

**RIAs**

RIAs for Met-enkephalin and Leu-enkephalin were performed as previously described, using dextran-coated charcoal to separate bound and free ligands. (Yoshimasa et al. 1982, 1983). The antiserum for Met-enkephalin (MC3-1210) showed cross-reactivities of 10.3% with Leu-enkephalin, 1.7% with Met-enkephalin-Arg-Gly-L-leu and 4.8% with Met-enkephalin-Arg-Phe. The antiserum for Leu-enkephalin (LC1-226) cross-reacted with Met-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe at the levels of 0.7, 0.02 and 0.09%, respectively.

RIAs for Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe were performed according to the method described previously, using dextran-coated charcoal to separate bound from free fraction (Ikeda et al. 1982). The Met-enkephalin-Arg-Gly-Leu antiserum (NE2-206) used showed no significant cross-reactivity with Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg-Phe (less than 0.01% on a molar basis). The Met-enkephalin-Arg-Phe antiserum (AP3-311) did not cross-react significantly with Met-enkephalin. Leu-enkephalin and Met-enkephalin-Arg-Gly-Leu (less than 0.01% on a molar basis). These antiserum showed no significant cross-reactivity with α-, β-neo-endorphin, dynorphin, human β-endorphin and ACTH (less than 0.01% on a molar basis).

**HPLC**

The apparatus used for HPLC consisted of Shimadzu model LC-4A liquid chromatograph equipped with a SIL-1A injector and variable wave length UV detector SPD-2A (Shimadzu Co., Kyoto, Japan). Reverse phase HPLC was carried out on an Ultrasphere ODS (Altex Scientific Inc., USA) column (4.6 x 150 mm). The acid-methanol extracts were directly applied to the column and eluted with 42% methanol in 10 mM ammonium acetate, pH 4.2, as a solvent. The flow rate was 1.0 ml/min and the fraction volume was 1 ml. The retention times of synthetic Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe monitored by UV absorption and RIAs were 4, 8, 15 and 20 min, respectively. Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe contents of each fraction were measured by the RIAs. Recoveries of authentic Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe applied on the column were 93, 96, 72 and 74%, respectively.

**Gel exclusion chromatography**

The tumour extracts were applied on a 0.7 x 48 cm column of Sephadex G-50 equilibrated and eluted with 0.1 M acetic acid at a flow rate of 8.7 ml/h at 4°C. The fraction volume was 0.60 ml. The column was calibrated with blue dextran (for void volume), human β-endorphin (for a molecular weight of about 3400), Met-enkephalin and 125I (for salt peak). The contents of these four opioid peptides of each fraction were measured by the RIAs. Recoveries of authentic Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe applied on the column were 90-100%.

**Results**

**Tumour contents**

The dilution curves of extracts of 16 phaeochromocytomas were parallel with the standard curves of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe in respective RIAs. The contents of Met-enkephalin-like immunoreactivity (-LI), Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI in 16 phaeochromocytomas are shown in Table 1. Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI were detected together with Met-enkephalin-LI and Leu-enkephalin-LI in all of the tumours studied. There were remarkable variations in tumour contents of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI, that ranged from approximately 1 ng to 100 µg per g tissue. When Met-enkephalin-LI content was compared with Leu-enkephalin-LI content, a significant positive correlation (r = 0.99, P < 0.001) existed between Met-enkephalin-LI and Leu-enkephalin-LI contents. Comparisons between Met-enkephalin-LI and Met-enkephalin-Arg-Phe-LI contents or between Met-enkephalin-LI and Met-enkephalin-Arg-Gly-Leu-LI contents also showed significant positive correlations (r = 0.95, P < 0.001 and r = 0.95, P < 0.001, respectively).

**HPLC**

As depicted in Fig. 1, HPLC analyses of the tumour extracts from cases 1, 2 and 3 revealed one peak each of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe immunoreactivity eluting with the same reten-
Fig. 1.
High performance liquid chromatographic profiles on an Ultra sphere ODS column (4.6 x 150 mm) of extracts from 3 phaeochromocytomas. A: case 1, B: case 2, C: case 3. Arrows indicate the retention times of synthetic Met-enkephalin (I), Leu-enkephalin (II), Met-enkephalin-Arg-Gly-Leu (III) and Met-enkephalin-Arg-Phe (IV).

Discussion

We have shown previously that a marked difference in contents of Met-enkephalin-LI and Leu-enkephalin-LI existed in 4 phaeochromocytomas (Yoshimasa et al. 1982). The present study further extended our previous observation, demonstrating that Met-enkephalin-LI and Leu-enkephalin-LI existed in amounts varying five orders of magnitude in 16 phaeochromocytomas. Furthermore, the occurrence of Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI together with Met-enkephalin-Arg-Gly-Leu existed in an amount comparable to that of Leu-enkephalin.

Table 2.
Contents of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe in 3 phaeochromocytomas (nmole/g wet weight).

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-enkephalin</td>
<td>131</td>
<td>87</td>
<td>53</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>41</td>
<td>51</td>
<td>27</td>
</tr>
<tr>
<td>Met-enkephalin-Arg-Gly-Leu</td>
<td>22</td>
<td>2.8</td>
<td>18</td>
</tr>
<tr>
<td>Met-enkephalin-Arg-Phe</td>
<td>39</td>
<td>40</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Fig. 2.

Gel filtration patterns on a Sephadex G-50 column (0.7 x 48 cm) of extracts from 3 pheochromocytomas. A: case 1, B: case 2, C: case 3. Arrows indicate the elution positions of markers. I: blue dextran for Vo, II: human ß-endorphin, III: Met-enkephalin, IV: iodine for Vt.

Met-enkephalin-LI and Leu-enkephalin-LI was shown in all of the 16 tumours. A wide variation also existed in Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI contents in these tumours. In spite of the wide variation in contents of these four opioid peptides, significant positive correlations were observed among contents of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI. Co-existence of these four opioid peptides in pheochromocytomas is compatible with recent findings that these four opioid peptides are contained in the same precursor molecule, preproenkephalin A, in the bovine adrenal medulla (Noda et al. 1982a; Gubler et al. 1982) and a human pheochromocytoma (Comb et al. 1982). Currently, the structure of human preproenkephalin A deduced from the nucleotide sequence of cloned cDNA for mRNA from a human pheochromocytoma (Comb et al. 1982) proved to be the same as that determined from nucleotide sequence of the cloned human preproenkephalin A gene (Noda et al. 1982b). Even so, it is possible that there exists either deletion or substitution of amino acids in the preproenkephalin A molecule in some human neoplastic sympathoadrenal tissues. However, the observation that there were highly positive correlations among the contents of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI in 16 pheochromocytomas strongly suggests the preservation of amino acid sequences of these four opioid peptides in the precursor molecule even in neoplastic tissues. This is on the line of evidence the same as the one seen in ACTH synthesized by ectopic ACTH producing tumours, in which the presence of structurally abnormal ACTH has not yet been demonstrated (Imura 1980).

The ratios of concentrations of Met-enkephalin-LI, Leu-enkephalin-LI, Met-enkephalin-Arg-Gly-Leu-LI and Met-enkephalin-Arg-Phe-LI were variable in the 16 pheochromocytomas studied. This suggests that the preproenkephalin A molecules are processed in a different manner from tumour to tumour, giving rise to Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu, Met-
enkephalin-Arg-Phe and their high molecular weight forms in variable ratios. In fact, gel filtration chromatography and HPLC of extracts from three tumours (cases 1, 2 and 3) have shown that high molecular weight forms having immunoreactivities of Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe were present in variable amounts and the molar ratios of contents of Met-enkephalin, Leu-enkephalin, Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe varied in tumours. These results suggest that the preproenkephalin A molecule is processed in a different way in some tumours. In other words, there are possible abnormalities in the posttranslational processing of preproenkephalin A to these four opioid peptides in certain phaeochromocytomas.

It has been shown that dynorphin exists in human phaeochromocytomas in amounts comparable to those in normal human adrenal medulla (Yoshimasa et al. 1981; Suda et al. 1983). Recently the primary structure of the common precursor of neo-endorphin and dynorphin (preproenkephalin B) has been deduced from the nucleotide sequence of cloned cDNA for mRNA from the porcine hypothalamus (Kakidani et al. 1982). This precursor protein contains three Leu-enkephalin sequences flanked by paired basic amino acid residues. However, it remains to be clarified whether or not this precursor molecule gives rise to Leu-enkephalin. In human phaeochromocytomas, good correlations existed among contents of Met-enkephalin-L.I, Leu-enkephalin-L.I, Met-enkephalin-Arg-Gly-Leu-L.I and Met-enkephalin-Arg-Phe-L.I, and the ratios of concentrations of these four opioid peptides were almost comparable with those of these four peptides contained in the preproenkephalin A molecule. Moreover, the amounts of preproenkephalin B-derived peptides in phaeochromocytomas were far lower than those of preproenkephalin A-derived peptides. It appears, therefore, that little Leu-enkephalin, if any, is derived from preproenkephalin B in phaeochromocytomas.

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