Size heterogeneity of immunoreactive prolactin in patients with prolactinoma

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Abstract. We investigated the chromatographic pattern of serum prolactin in 41 patients with prolactinoma and correlated the distribution of immunoreactive prolactin with the clinical variables sex, tumour size, age, and response to bromocriptine therapy. In addition, the effect of long-term storage and repeated freezing and thawing on the different molecular weight forms of prolactin was evaluated. Gel chromatography (column 100 cm × 1.5 cm) was performed in 0.1 mol/l phosphate buffer, pH 7.5, using Ultrogel ACA 54 (LKB).

No correlation of age or the response to drug therapy to the elution pattern of prolactin was found. Females showed a higher percentage of big prolactin than males (10.4 ± 1.2% vs 6.8 ± 0.7%, x ± sem, P < 0.05) and patients with microprolactinomas too had a higher percentage of big prolactin than those with macroprolactinomas (11.3 ± 1.8% vs 7.7 ± 0.7%, P < 0.05).

Serum samples kept frozen for more than 2 years showed a higher percentage of bigbig prolactin (P < 0.01) than samples stored for less than 12 months suggesting formation in vitro. However, examination of fresh samples prior to freezing also demonstrated bigbig prolactin, indicating that bigbig prolactin circulates in vivo.

Repeated freezing and thawing of bigbig prolactin led to almost complete interconversion to little prolactin without any increase in immunoreactivity. This finding supports the concept that bigbig prolactin represents little prolactin loosely associated to a carrier molecule.

Size heterogeneity of immunoreactive prolactin in human serum has been recognized for more than ten years (von Werder & Clemm 1974; Suh & Frantz 1974; Rogol & Rosen 1974). At least three discrete immunoreactive molecular weight variants have been described for circulating human prolactin: 'little' prolactin (molecular weight 23 000 daltons) corresponding to the monomeric hormone, 'big' prolactin (molecular weight between 40 000 and 62 000 daltons), and 'bigbig' prolactin (molecular weight in excess of 100 000 daltons), the structure of which remains unclear (Guyda 1975; Garnier et al. 1978; Kiefer & Malarkey 1978; Andersen et al. 1982; Whitaker et al. 1984).

Size heterogeneity of human prolactin has been demonstrated in sera from normal subjects (Kiefer & Malarkey 1978; Faroukh et al. 1979), patients with functional hyperprolactinaemia (Suh & Frantz 1974; Fang & Kim 1975), and pituitary tumours (Guyda 1976; Whitaker et al. 1984). However, the physiological significance of serum prolactin heterogeneity is still not understood. There are several reports on patients with hyperprolactinaemia owing to a large molecular weight variant of prolactin with distinct clinical features (Jackson et al. 1983, 1985; Whitaker et al. 1981; Soong et al. 1982). No studies have been performed in patients with prolactinoma trying to link the chromatographic pattern of serum prolactin with clinical variables like tumour size, response to drug therapy, and sex. We, therefore, analysed the distribution of the different molecular weight variants of prolactin by gel chromatography in a large sample of patients with prolactinoma. In addition, the stability of the different molecular weight variants of immunoreactive prolactin was investigated.
Material and Methods

Subjects

Sera from 41 patients (17 males, 24 females, aged 19–61 years) were studied (see Table 1). All patients had elevated baseline serum prolactin concentrations (range 86–5200 µg/l) with a blunted response to TRH (prolactin increase less than 100%). Patients with a normal fossa on skull X-rays and a serum prolactin below 250 µg/l were classified as having a microprolactinoma (3 males, 10 females). Patients were divided into two groups according to their response to bromocriptine therapy. If a daily dose of 20 mg of bromocriptine or less was sufficient to induce normoprolactinaemia, the patients were classified as ‘low dose responders’ (N = 25), whereas the remaining patients were termed ‘high dose responders’ (N = 16). In two patients even 60 mg failed to suppress serum prolactin into the normal range, although a pronounced fall of serum prolactin occurred. Seven patients were studied prior to drug therapy and again after a fall in serum prolactin of at least 50% had been achieved with bromocriptine. Prior to drug therapy 13 patients had undergone surgery.

Samples

Samples of serum were stored at −20°C until chromatography. To exclude artifacts by freezing, fresh serum was chromatographed immediately after centrifugation in some instances. To analyze the effect of long-term storage we compared the chromatographic pattern of sera stored for more than two years at −20°C with the profile of samples kept frozen for less than 1 year.

Gel chromatography

Samples were fractionated at 4°C on a column (100 x 1.5 cm) of Ultrogel ACA 54 (LKB) equilibrated with 0.05 mol/l phosphate buffer, pH 7.5 containing bovine serum albumin (BSA) (0.5% w/v). The eluant was 0.1 mol/l phosphate buffer, pH 7.5, with 0.1% BSA.

The column was calibrated with blue dextran, myoglobin, monomeric prolactin, 125I-prolactin, and 125I.

The flow was 4.5 ml/h and fractions of 2 ml were collected. A sample volume of 0.5–3.0 ml was applied to the column. Recovery of serum prolactin from the column ranged from 74–96%. The column was recalibrated at regular intervals. Aliquots of 100–200 µl were taken for radioimmunoassay. In some instances, fractions of the elution profile were rechromatographed after six cycles of freezing and thawing. The distribution of serum prolactin immunoreactivity was calculated as per cent of total prolactin immunoreactivity. The mean percentages of distribution of the prolactin peaks from different groups were compared using Student’s t-test, where a P-value of less than 0.05 was considered significant.

Prolactin radioimmunoassay

We used reagents from a commercially available serum kit (Serono, Freiburg, FRG).

To adapt the sensitivity to the low concentrations in some of the fractions we reduced the amount of labelled prolactin (1:4) and first antibody (1:4) per tube and increased the sample volume to 200 µl. The second antibody was also diluted in assay buffer (1:2). The detection limit of the modified assay was 0.15 µg/l, and we found an intra-assay variance of 5.8% (N = 15) and an inter-assay variance of 6.4% (N = 10). All samples from the same chromatographic profile were measured in the same assay.

Results

Gel filtration

Calibration of the column allowed the identification of three immunoreactive peaks as the bigbig,
big and little forms of prolactin described by others (Fig. 1). Taking all patients together (N = 41), the mean percentage of prolactin in peak I, near the void volume, was 3.4 ± 0.8% (X ± SEM) (range 0–23.4%), peak II comprised 8.8 ± 0.7% (range 0.4–28.0%), and peak III was 87.9 ± 1.2% (range 52.8–99.6%) of the total immunoreactive prolactin. The prolactin standard eluted as a single peak of little prolactin. All three fractions of immunoreactive prolactin produced parallel displacement of the tracer in the radioimmunoassay.

When the effect of long-term storage was evaluated, a significantly higher percentage (P < 0.01) of big big prolactin was detected in samples stored for more than 2 years, suggesting formation in vitro (Table 1). This increase was reflected by a decrease in little prolactin. Thus for all patient groups, the subgroup of samples consisting of fresh sera only was evaluated separately.

Although in all patients the majority of immunoreactive prolactin eluted as little prolactin (peak III), considerable differences in the elution pattern were present (Fig. 2). Analysis of samples from individual patients taken at different time points showed stability of the characteristic elution pattern in all cases (N = 6).

No influence of age or previous surgery on prolactin profiles in our patients with hyperprolactinaemia was found. Similarly, we were unable to demonstrate any difference in the chromatographic pattern of 'low dose responders' (N = 25) compared with 'high dose responders' (N = 16): peak I 3.7 ± 0.9% vs 3.3 ± 1.4%, peak II 8.4 ± 1.0% vs 8.5 ± 1.1%, peak III 88.3 ± 1.8% vs 88.2 ± 2.0%. This held true also when only fresh samples were considered. Moreover, the two patients in whom no normoprolactinaemia was achieved even by a dose of 60 mg/day, showed an average distribution of immunoreactive prolactin. On the other hand, patients with an unusual chromatographic pattern (i.e. high percentages of big big and/or big prolactin) exhibited no unusual response to drug therapy. In the seven patients who were studied prior to and during drug therapy, serum prolactin was decreased by bromocriptine from 2825 ± 1187 to 129 ± 29 µg/l. However, no preferential suppression of any of the three forms of immunoreactive prolactin was observed: peak I 5.5 ± 2.3% vs 3.5 ± 3.3%, peak II 2.4 ± 1.0% vs 1.2% ± 1.3%, peak III 84.7 ± 3.7% vs 88.7 ± 2.8%.

![Fig. 2. Gel chromatography of sera from two patients with a macroprolactinoma illustrating the variability of the elution pattern in different patients.](image)
Table 1.
Distribution of serum prolactin immunoreactivity in relation to age, tumour size, sex, previous surgery, response to drug therapy, and time of sample storage in 41 patients with prolactinoma (mean ± SEM).

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>N</th>
<th>Prolactin (% of total immunoreactivity)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Bigbig</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50 years</td>
<td>31</td>
<td>3.8 ± 1.9</td>
</tr>
<tr>
<td>&gt; 50 years</td>
<td>10</td>
<td>2.9 ± 1.0</td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microprolactinoma</td>
<td>13</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>Macroprolactinoma</td>
<td>28</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>24</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>Males</td>
<td>17</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td>Response to bromocriptine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Low dose responders'1</td>
<td>25</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>'High dose non-responders'</td>
<td>16</td>
<td>3.3 ± 1.4</td>
</tr>
<tr>
<td>Previous surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13</td>
<td>3.9 ± 1.8</td>
</tr>
<tr>
<td>No</td>
<td>28</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>Duration of sample storage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 2 years</td>
<td>18</td>
<td>6.2 ± 1.0**</td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>30</td>
<td>1.7 ± 1.0</td>
</tr>
</tbody>
</table>

1 Bromocriptine dose to induce normoprolactinaemia ≤ 20 mg/day.
* P < 0.05, ** P < 0.01.

Table 2.
Percentage of big prolactin (X ± SEM) in samples stored for less than 12 months in relation to tumour size and sex (N = 30).

<table>
<thead>
<tr>
<th>Tumour size</th>
<th>N</th>
<th>Big prolactin (% of total immunoreactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microprolactinoma</td>
<td>10</td>
<td>12.0 ± 2.7*</td>
</tr>
<tr>
<td>Macroprolactinoma</td>
<td>20</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>18</td>
<td>10.6 ± 1.7*</td>
</tr>
<tr>
<td>Males</td>
<td>12</td>
<td>5.8 ± 1.0</td>
</tr>
<tr>
<td>Macroprolactinomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>9.0 ± 1.3 n.s.</td>
</tr>
<tr>
<td>Males</td>
<td>9</td>
<td>6.5 ± 0.9</td>
</tr>
</tbody>
</table>

* P < 0.05; n.s.: not significant.

II 8.9 ± 1.1% vs 11.2 ± 2.3%, peak III 85.6 ± 1.8% vs 85.3 ± 4.4%. Females showed a significantly higher percentage of big prolactin than males (P < 0.05) and patients with a microprolactinoma also had a higher percentage of big prolactin than those with macroprolactinomas (P < 0.05) (Table 1). This remained true when only fresh sera were evaluated (see Table 2). As in this group microprolactinomas were unevenly distributed among males (N = 3) and females (N = 7), the influence of sex was separately analysed for patients with macroprolactinomas. Although here females again showed a higher percentage of big prolactin, the difference did not reach the level of significance (Table 2).

Stability studies
As the results of long-term storage suggested a...
significant in vitro formation of bigbig prolactin, several studies on the nature of the large molecular weight fractions were performed. Chromatography of fresh serum from patients with high concentrations of big and bigbig prolactin without previous freezing gave the same elution pattern that had been demonstrated in samples kept frozen for several months. Repeated freezing and thawing of little prolactin did not lead to any shift of immunoreactivity to high molecular weight forms of prolactin. In contrast, repeated freezing and thawing of big prolactin induced a considerable alteration in the elution pattern with almost 50% eluting as peak III (N = 3). When bigbig prolactin...
prolactin was rechromatographed (N = 3) after freezing and thawing, almost all immunoreactivity eluted as 'little' prolactin (peak III) (Fig. 3). No increase in prolactin immunoreactivity was observed after rechromatography of big or big big prolactin.

Discussion

Prolonged treatment of prolactinomas with bromocriptine may lead to perivascular fibrosis of the tumour rendering selective extirpation more difficult and affecting the surgical outcome (Landolt et al. 1982; Landolt & Osterwalder 1984). On the other hand, we found persisting normoprolactinaemia after withdrawal of bromocriptine in a substantial subgroup of patients with prolactinoma after treatment with bromocriptine for more than four years (Winkelmann et al. 1985). Thus, to select the most appropriate therapy, methods are needed which allow to predict the outcome of long-term drug therapy as early as possible. In this study we evaluated the prognostic potential of the chromatographic pattern of serum prolactin immunoreactivity in patients with prolactinomas. However, no correlation of the chromatographic distribution of prolactin and the response to drug therapy was found. 'Low dose responders' showed the same elution pattern as 'high dose responders'. Patients with an atypical distribution of immunoreactive prolactin exhibited an average response to bromocriptine and two patients who were resistant to drug therapy had an average chromatographic profile of serum prolactin. In addition, no preferential suppression of any fraction of immunoreactive prolactin by bromocriptine was found.

Similarly, neither age nor previous surgery had any influence on the chromatographic pattern. The distribution of immunoreactive prolactin among big big prolactin (peak I), big prolactin (peak II), and 'little' prolactin was in good agreement with the results of previous studies in smaller groups of patients with prolactinoma (Suh & Frantz 1974; Garnier et al. 1978; Whitaker et al. 1984). In all our patients little prolactin was predominant, and hyperprolactinaemia exclusively owing to large molecular weight forms of prolactin (Whitaker et al. 1981; Andersen et al. 1982; Rogol & Rosen 1974; Soong et al. 1982; Jackson et al. 1985) was not observed in our series.

Interestingly, patients with microprolactinomas and females had a higher percentage of big prolactin than patients with macroprolactinomas or males. Whitaker et al. (1984) found a higher percentage of big prolactin in normals compared with patients with prolactinoma and the highest percentage of big prolactin has been observed during pregnancy (Suh & Frantz 1974). Thus one may speculate that the higher percentage of big prolactin in female patients with prolactinoma possibly reflects hormonal influences, whereas the higher percentage of big prolactin in microprolactinomas may be related to the more benign nature of this tumour type.

The physiological significance and the biochemical nature of the higher molecular weight variants of prolactin are not fully understood. In agreement with the results of Suh & Frantz (1974), our study suggests that big big prolactin may be formed in vitro after prolonged freezing. Nyberg et al. (1981) reported a higher yield of big big prolactin from extracts of long-term stored pituitaries compared with fresher material and raised the question, whether big big prolactin is an artifact. However, in our study no direct interconversion of little prolactin to big big prolactin was demonstrated in vitro and high amounts of big big prolactin were found in freshly collected samples indicating that big big prolactin circulates in vivo (Jackson et al. 1985).

It is not clear whether big big prolactin is produced through binding of smaller molecular weight forms of prolactin to themselves or to a binding protein in the periphery or represents a true secretory product of the pituitary (Whitaker et al. 1981). We have here shown that breaking noncovalent bonds by repeated freezing and thawing produced a clear shift in the elution profile from big big prolactin to little prolactin. As no increase in immunoreactivity was observed, it is unlikely that big big prolactin is an aggregated form of little prolactin. Thus, our findings support the concept of big big prolactin consisting of little prolactin noncovalently bound to a carrier molecule. In contrast to our results in tumour patients, no interconversion of big big prolactin has been found in patients with idiopathic hyperprolactinaemia after treatment with 6 mol/l of guanidine, whereas mercaptoethanol led to a shift in the gel filtration profile with the presence of big and little prolactin (Jackson et al. 1985). This may indicate that big big prolactin in idiopathic prolactinomas...
hyperprolactinaemia is structurally different from bigbig prolactin in patients with prolactinoma. This could also explain the different results with regard to the bioactivity of bigbig prolactin (Whitaker et al. 1984; Jackson et al. 1985). In our study big prolactin too showed interconversion to little prolactin after repeated freezing and thawing, although about 50% continued to elute as big prolactin. Benveniste et al. (1979) reported that reduction with mercaptoethanol leads to almost complete interconversion of big prolactin to little prolactin suggesting that big prolactin consists of dimers of little prolactin-linked disulphide bonds. However, there is evidence that a carbohydrate-containing immunoreactive prolactin may represent a part of big prolactin (Shoupe et al. 1983) indicating that big prolactin is not a homogeneous entity. In fact, recent reports have shown that little prolactin also consists of different fractions (Sinha et al. 1984; Lewis 1984).

In conclusion, at present the chromatographic analysis of serum prolactin is of little value in the management of patients with hyperprolactinemia. In the future, a more refined analysis of the structure of the different molecular weight forms of prolactin together with an understanding of their physiological role may offer new possibilities.

Acknowledgments

We are indebted to Mrs H. Hofmann, Mrs G. Rollbach, Mrs K. Wehner and Miss D. Vollmar for skilful technical assistance, and to Mrs G. Hermeling-Mayer for preparing the manuscript.

This work was supported by Landesamt für Forschung NRW, Düsseldorf, FRG.

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Received March 10th, 1986.
Accepted November 10th, 1986.

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