Immunochemical and biological characterization of calcitonin originating from transplanted medullary thyroid carcinoma in rats

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Abstract. The immunoreactive and biological activities of calcitonin (CT) produced by transplanted rat medullary thyroid carcinomas (MCT) have been studied. Immunoreactive CT (iCT) in serum and in MCT tissues of rats carrying tumours of generations 5–8 was characterized by gel filtration on Sephadex G-100 followed by radioimmunological measurements using region specific antiserum. The hypocalcaemic effect of sera and tumour extracts was tested in a rat bioassay. Rats with transplanted MCT of the 5th and 6th generations had mainly (70–84%) circulating iCT species of molecular size comparable to intact hormone. However, in rats with tumours of the 7th and 8th generations corresponding circulating iCT forms comprised less than 52% of total immunoreactivity while 32–38% eluted earlier. In comparison, iCT corresponding to intact hormone represented 30–50% of total immunoreactivity in the tumour extracts and no differences were observed between the generations. Subcutaneous injections of sera from MCT rats and of tumour extracts reduced the serum levels of ionized calcium in test rats. The sera containing mainly intact iCT showed the strongest biological potency. We conclude that rat MCT transplanted under the kidney capsule is able to secrete biologically active CT. However, the heterogeneity of circulating iCT increases in rats with transplanted tumours of older generations, approaching the heterogeneity of stored hormone in the gland.

Medullary thyroid carcinoma (MCT) is a rare disorder in man representing less than 10% of all thyroid carcinomas (Hazard et al. 1959; Williams 1966; Steiner et al. 1968). The disease is characterized and diagnosed by high serum concentrations of calcitonin (CT) which is produced by the tumour cells (Tashjian & Melvin 1968; Melvin & Tashjian 1968; Cuncliff et al. 1968).

Several reports have shown that circulating immunoreactive CT (iCT) in patients with overt MCT is not homogenous (Neher et al. 1968; Singer & Habener 1974; Deftos et al. 1975; Sizemore & Heath III 1975; Snider et al. 1977; Myhre & Gautvik 1979). Moreover the heterogeneity of circulating iCT differs from one patient to another and both larger and smaller molecular forms of CT exist (Myhre & Gautvik 1979).

MCT occurs spontaneously in 10–40% of old rats (Boorman et al. 1972; Gilbert & Gillman 1958; Lindsay et al. 1968; Triggs et al. 1975). The ultrastructure of rat MCT resembles that of the human tumour (Boorman et al. 1972; De Lellis et al. 1979). Like human MCT the corresponding tumour in rats has retained the ability to secrete CT (Boorman et al. 1974; Bollman & Pearse 1974; Triggs et al. 1975) and to react to physiological secretagogous (Normann et al. 1977).

Since MCT in rats can be propagated by serial transplantation, an animal model for the study of various aspects of the tumour disease has been established (Boorman et al. 1974; Boorman & Hollander 1976). The studies of this rat model have mainly been concentrated on morphological investigations of the tumour cells, and their ability...
to secrete CT after stimulation (Lindsay et al. 1968; Normann et al. 1977; Triggs et al. 1975). In the present study, the molecular forms of iCT in blood and in tumour tissue of MCT rats have been characterized using gel filtration and immunochemical methods in comparison to a bioassay.

Material and Methods

Animals
Eleven inbred Wistar derived, male Wag/Rij rats were used. When the rats were 6–7 weeks old, a specimen from a rat MCT was transplanted beneath the left kidney capsule under ether anesthesia, as described by Boorman et al. (1974). The original tumour, which was found in the thyroid of old rats, had been serially transplanted beneath the kidney capsule of host rats for 8 generations. The tumour specimens used for transplantations represented different generations as indicated.

The rats were fed an ordinary stock diet and water ad libitum.

Blood and tumour sampling
Following the transplantation blood was sampled from the tail of the rats at regular intervals and assayed for CT. When serum iCT values exceeded 5 μg/l (more than 10 times the physiological concentrations), the rats were sacrificed by aortic exsanguination under ether anesthesia. All blood samples were centrifuged and the sera stored at −20°C for further analysis. Tumour samples were dissected free, weighed and stored at −20°C until they were homogenized with 0.1 mol/l HCl for CT extraction (Tashjian et al. 1970).

The animals were routinely examined for metastases, which were never observed.

Determination of CT
CT in sera and tumour extracts were determined radioimmunologically using a heterologous assay employing 125I-labelled synthetic human CT (Calcitomin M, Ciba) and rabbit antisera against synthetic human CT (Gautvik et al. 1976) with affinities against both the N- and C-terminal part of the molecule (Myhre & Gautvik 1979). Scatchard plot analysis (Scatchard 1949) which was used to examine the characteristics of the antisera, showed the presence of two independent binding sites and suggests the presence of two populations of immunoglobulins. The affinity constants (Kd) were calculated to be 2.1 × 10^−10 mol/l and 1.3 × 10^−12 mol/l, respectively. At the dilutions of the antiserum used (1:75000–1:100000), 20–30% of the immunoglobulins reacted with the N-terminal sequences.

Serial dilution of rat sera and tumour extract gave corresponding decrease in measured iCT suggesting a close immunological relationship between human and rat CT (Normann et al. 1977; Sand et al. 1981). The lower limit of detection of the rat and human hormone is the same and the dilution curves are superimposable, suggesting a good immunological cross-reactivity for rat CT in this assay (Sand et al. 1981).

Gel filtration
Sera from rats with transplanted MCT were concentrated, by lyophilization, 2–3 times and aliquots of 1 ml were applied on Sephadex G-100 column (1.2 × 40 cm) for gel filtration. Tumour extracts were gel filtered without further treatment. The elution buffer was 0.1 mol/l Tris-HCl (Sigma), pH 7.5, containing 0.1% bovine serum albumin (Sigma, grade V). The column was calibrated with three radioactively labelled reference proteins: parathyroid hormone (molecular weight about 8500, Wilson & Co., USA), calcitonin (molecular weight 3500, Ciba-Geigy, Switzerland) and the carboxyterminal calcitonin fragment (11–32 amino acid residues) (molecular weight 2000, Ciba-Geigy, Switzerland). Immuno-reactive CT was determined in 1 ml fractions.

Bioassay
The hypocalcaemic effects of sera and tumour extracts were tested in a rat bioassay. Serum and tumour samples were injected sc in young Wistar rats (80–100 g) of both sexes, using two or several doses. One hour later the blood was collected from aorta using vacutainer. The blood was centrifuged and serum ionized calcium was analyzed with a Radiometer Calcium Ion Selectrode® (Myhre 1980) within 2 h in the anaerobically handled samples.

The calcium lowering effect of test samples were compared with the hypocalcemia caused by synthetic human CT (Cibacalcin, Ciba-Geigy), corresponding volumes of buffer solution containing no CT and control rat serum. Three to 9 rats were injected with the same test sample. The per cent reduction in serum ionized calcium 1 h after injection was calculated. The results are presented as mean ± SE. The bioassay had a detection limit of 0.01 μg CT when using the human calcitonin standard (Cibacalcin).

Results

Gel filtration
Gel filtration of 8 sera from rats with a CT secreting tumour gave two different elution profiles as presented in Figs. 1 and 2. Fig. 1 shows one representative elution profile for sera from rats with tumour of the 5th or the 6th generation. The major immunoreactive form of iCT (70–84%) had molecular size corresponding to the intact
Fig. 1.
Representative elution profile from gel filtration on Sephadex G-100 column of sera from rats with a transplanted calcitonin secreting tumour (MCT) of 5th and 6th generation. Immunoreactive CT (iCT) was measured in 1 ml fractions and the following reference proteins were used: parathyroid hormone, molecular weight 8500 (a), intact human CT, molecular weight 3500 (b) and aminoterminal CT fragment, molecular weight 2000 (c).

Fig. 2.
Representative elution profile from Sephadex G-100 column of serum from a rat with transplanted MCT of 8th generation. See Fig. 1 for details.

Table 1.
Elution pattern of immunoreactive calcitonin (iCT) from sera and tumour extracts after gel filtration on Sephadex G-100 columns, followed by radioimmunological determination of iCT using region specific antisera. S = serum. T = tumour extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tumour generation</th>
<th>Percentage distribution of calcitonin immunoreactivity according to molecular size</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>≥ 9500</td>
</tr>
<tr>
<td>S 274</td>
<td>V</td>
<td>9.4</td>
</tr>
<tr>
<td>S 285</td>
<td>V</td>
<td>12.4</td>
</tr>
<tr>
<td>S 252</td>
<td>VI</td>
<td>6.3</td>
</tr>
<tr>
<td>S 545</td>
<td>VII</td>
<td>35.4</td>
</tr>
<tr>
<td>S 562</td>
<td>VII</td>
<td>32.4</td>
</tr>
<tr>
<td>S 572</td>
<td>VII</td>
<td>32.5</td>
</tr>
<tr>
<td>S 8</td>
<td>VIII</td>
<td>33.4</td>
</tr>
<tr>
<td>S 633</td>
<td>VIII</td>
<td>38.7</td>
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<tr>
<td>T 219</td>
<td>V</td>
<td>13.1</td>
</tr>
<tr>
<td>T 220</td>
<td>V</td>
<td>35.4</td>
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<tr>
<td>T 8</td>
<td>VIII</td>
<td>24.5</td>
</tr>
<tr>
<td>T 639</td>
<td>VIII</td>
<td>39.9</td>
</tr>
</tbody>
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hormone and only a minor shoulder could be recognized eluting before the major peak.

Fig. 2 shows a corresponding elution profile from the serum of a rat bearing a MCT-tumour of
the 8th generation. Also in this serum the major part of the immunoreactivity corresponded to intact CT, but a greater part was distributed as peptides of molecular size greater and smaller than the intact hormone. Similar elution patterns of iCT were found in sera from rats bearing a tumour of the 7th generation (Table 1).

Two extracts of tumours from the 5th and two from the 8th generations were submitted to gel filtration. Their elution profiles were similar showing the presence of different molecular forms of iCT where 30–50% eluted as the intact hormone, (Fig. 3 and Table 1). The heterogeneity of iCT from tumour extracts resembled the elution profile from serum iCT of the 7th and 8th generations.

Bioassay
All sera and tumour extracts from MCT rats were tested for hypocalcaemic activity in rats in comparison with synthetic human CT. Fig. 4 shows the effect of two different sera from rats with CT secreting tumour of the 5th and of the 6th generation 1 h after injections. The total iCT concentrations of the rat sera had been analyzed by radioimmunoassay before injection. The injected MCT sera, but not similar volumes of control serum, produced a significant hypocalcaemia, which, however, did not give a dose-response curve parallel to the CT standard curve at low hormone concentrations.

Fig. 5 shows the hypocalcaemic activity of 3 tumour extracts in test rats 1 h after the sc injection. The volume of injected tumour extracts had

![Figure 3](image_url)

**Fig. 3.**
Representative elution profile obtained by gel filtration on Sephadex G-100 of iCT extracted from a rat MCT. See Fig. 1 for details.

![Figure 4](image_url)

**Fig. 4.**
Hypocalcaemic responses in young Wistar rats 1 h after sc injection of synthetic human CT (hCT), control rat serum (iCT < 0.5 µg/l) and two rat sera containing 6.55 µg/l and 5.25 µg/l of iCT. The concentrations of synthetic hCT are given on the abscissa as is the rat iCT concentrations and the corresponding volumes. Each point represents mean ± se for 3 to 9 rats.
Hypocalcaemic responses in young Wistar rats 1 h after sc injection of synthetic human CT (hCT) or tumour extracts with different iCT concentrations. The volumes of injected extracts are calculated after measurements of total iCT concentrations. The tumour extracts contained 84 ng/ml, 131 ng/ml and 324 ng/ml. Each point represents mean ± se for 3 to 9 rats.

been calculated from the radioimmunological measurements of total iCT present. The degree of hypocalcaemia after injection of tumour extracts was of the same magnitude and gave parallel dose-response curves to that obtained with synthetic human CT. Injection of extracts from a prolactin producing tumour in same volumes and containing similar amounts of protein did not cause hypocalcaemia in test rats (unpublished data).

Discussion

Unlike circulating iCT in patients with MCT (Neher et al. 1968; Sizemore & Heath III 1975; Snider et al. 1977; Myhre & Gautvik 1979) rats with transplanted MCT up to the 6th tumour generation showed a uniform elution pattern of serum iCT corresponding to that of the intact hormone. Tumours of older generations gave rise to heterogeneous circulating forms of iCT resembling that of the tumour iCT profile which did not differ from one tumour generation to another. The existence of iCT heterogeneity in tumour tissue is probably important to realize in order to understand hormone synthesis and secretion in neoplastic C-cells. In MCT rats carrying earlier generations of the tumour, hormone heterogeneity was more pronounced in tumour extracts than in sera, and this suggested the presence of cellular CT forms which are not secreted. However, in tumours of older generations the molecular forms of serum iCT reflected the profile obtained from the tumour tissue indicating that the pattern of CT secretion from MCT cells may vary. In vitro synthesis of precursor(s) of CT has been demonstrated in human MCT tissues (van der Donk et al. 1978) and in the rat tumour (van der Donk et al. 1978; Amara et al. 1980). Recently it has been shown that some of the precursors are high molecular weight glycoprotein forms of iCT (O’Neil et al. 1981). The existence of CT precursors in the present rat MCT tumours are indicated by the data in Fig. 3. Whether tumour cells show a defect in the synthesis and/or processing of the prehormone is presently unknown.

Alteration in the ability to secrete CT by time from transplanta-l rat MCT have been reported previously (Boorman et al. 1974; Roos et al. 1979). Therefore, the described change in serum iCT profile resides probably in different forms of CT being secreted rather than in alteration in the peripheral metabolism of the hormone. Established functional cell lines from MCT would be helpful to delineate this question.

Tumour bearing rats with hypercalcitoninaemia have reduced serum ionized calcium as an indica-
tion of biologically active circulating hormone (Ekeland et al. 1980; Myhre et al. 1981). In agreement with these findings (Ekeland et al. 1980; Myhre et al. 1981) the rat sera tested in bioassay caused hypocalcaemia. However, the dose-response curves obtained using serum were not parallel to those obtained by synthetic human CT. Becker et al. (1978) compared radioimmunoassay and radioreceptor assay values for CT in fractions of gel filtered human MCT sera. They found that the fractions co-eluting with intact CT were nearly twice as active in the radioreceptor assay as in the radioimmunoassay. In contrast, the fractions that eluted later than the intact hormone were non-reactive in the radioreceptor assay. Goltzman & Tischler (1978) used a bioassay for CT where the increase in adenyl cyclase in membranes from rat kidney was measured after hormone stimulation. They found that fraction of gel filtered MCT-culture medium corresponding to the human CT monomer could be demonstrated to stimulate adenyl cyclase. Greater or smaller fragments of iCT were not found to be biological active. This may explain why the hypocalcaemic responses to our rat MCT sera were more pronounced than tumour extracts of equal iCT concentrations. The MCT sera tested in bioassay contain mainly intact CT, while intact hormone in the tested tumour extracts comprises only half or less of total iCT.

Unfortunately, the fractions which contained the different iCT forms after gel filtration of sera and tumour extracts did not contain sufficient amounts of iCT for testing in the bioassay. However, serum from MCT rats may contain other biologically active substances which are not present in normal rats and which could cause or contribute to development of hypocalcaemia.

The presence of substances other than CT, with hypocalcaemic effect may also explain why the biological activity of serum was apparently higher than that of synthetic human CT. The possibility also exists that damage of synthetic human CT may cause small and non-detectable immunochemical changes, but lead to a substantial reduction in the biological activity. In agreement with this suggestion, tumour extracts, which were shown after gel filtration to contain a maximum of 50% of intact CT, gave parallel dose-response curves to synthetic human CT. Also species differences in biological responsiveness to human and rat CT may be of importance (Otani et al. 1978). Non-specific effects of tumour material in the bioassay-rats were less likely to occur since equivalent amounts of protein from a rat prolactin producing tumour did not induce hypocalcaemia.

The present study shows that biological active CT exists in sera and in tumour extracts from MCT-rats. The molecular forms and the biological potency of circulating iCT vary, however, and seem to depend on the generation of the transplanted tumour.

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