Early Spontaneous Deficiency of Calcitonin Renal Binding Sites in Rats with a High Incidence of Calcitonin-secreting Tumors (WAG/Rij)


Abstract

Old rats of the WAG/Rij strain have a high incidence (50%) of medullary thyroid carcinoma, a calcitonin (CT)-secreting tumor. We have characterized and quantified the topographical distribution of [125I]salmon calcitonin (sCT) binding sites in the kidneys of this strain, as compared to Wistar CF rats (2% incidence of spontaneous medullary thyroid carcinoma). We report here that, up to 15 days of postnatal development, the distribution of CT-binding sites in the kidney of the WAG/Rij strain was quite similar to that found in developing and adult Wistar CF rats. However, from the age of 1 month, sCT-binding sites were dramatically reduced in both the medulla and the inner part of the kidney cortex, though plasma CT levels were not significantly different in both strains. Adult WAG/Rij rats bearing a transplanted tumor for 12 weeks had a high level of plasma calcitonin and exhibited an even greater reduction of both medullary and cortical sCT-binding sites. These results suggest that the modification in the CT-binding sites in WAG/Rij rats is not a consequence of a possible down regulation due to elevated circulating hormonal level but could be inherited and possibly associated with the later development of the tumor in this strain.

Introduction

Human MTC (1) exists in two forms, a sporadic and a familial one. In both cases the tumor is characterized by an abnormal hypersecretion of CT (2, 3), the hypocalcemic hypophosphatemic hormone (4, 5). However, patients suffering from this disease do not show abnormal variations in plasma calcium or phosphate levels, although the hormone in the circulation and in the tumor tissue is biologically active (2). This phenomenon could be due to the development, in these patients, of a CT resistance as a consequence of alterations in CT receptor sensitivity.

The WAG/Rij rat (a Wistar-derived strain) shows a high incidence (50%) of MTC in old animals (6) and thus represents a good animal model to study the early development of medullary thyroid carcinoma as well as the eventual modifications of hormonal receptors. We have used a modification of a recently developed in vitro autoradiographic method to localize and quantitate CT-binding sites in the rat kidney (7). We report here for the first time, the topographical distribution and biochemical parameters of renal binding sites for the hormone with respect to its plasma levels in WAG/Rij rats. The results show that, in the first 4 weeks of postnatal development, the CT-binding sites normally present in the inner cortex and in the medulla of the kidney, dramatically decrease in WAG/Rij rats, suggesting that a genetic defect might be one of the factors involved in the susceptibility of protracting MTC.

Materials and Methods

Biological Material. Adult male Wistar CF rats weighing 200–250 g (10–12 weeks old), and young rats, 1, 2, and 4 weeks old (weighing respectively 13–17, 30–34, and 79–82 g) were obtained from the Centre Animalier, CHU Saint-Antoine, Paris, France.

Adult WAG/Rij rats, 12–16 weeks old, weighing 200–250 g, and young rats, 1, 2, and 4 weeks old, weighing respectively 10–14, 21–26, and 40–58 g, were raised in our laboratory (Université Paris-Sud, Orsay, France). The breeding stock was obtained from the TNO (Rijswijk, The Netherlands).

Tumor transplantation was carried out according to the method described by Zeytinoglu et al. (8): in brief, a tumoral cell suspension in sterile isotonic saline was injected s.c. to young, 4-week-old WAG/Rij rats; a small tumor usually appeared 3 weeks later. The rats weighing 130–150 g were killed 12 weeks after the tumor transplantation; their bilateral tumors weighed approximately 2 g each.

Hormones and Chemicals. sCT, biological activity MRC 4000 U/mg; batch No. 20051), kindly supplied by Sandoz (Basel, Switzerland) was iodinated by the chloramine T method (9) to a specific activity of 400 Ci/g. [125I]Na (17 Ci/ng; 1 Ci = 3.7 × 10^10 Bq) was obtained from New England Nuclear. Denatured human serum albumin was obtained from Centre National de Transfusion Sanguine, Paris, and the selective enzyme inhibitor Antagolan (Aprotinin, 2500 U antiplasmin corresponding to 1,000,000 KIU/10 ml) from Hoescht, France. We previously reported that [125I]sCT retains all the biological activity of the unlabeled sCT (10).

In Vitro Incubations and Quantitative Autoradiography. Adult Wistar CF and WAG/Rij rats were killed by a sharp blow on the head. The kidneys were removed from batches of five to seven rats and frozen. Serial 20-μm sections were cut with a cryostat (Bright) at −18°C, mounted on gelatin-coated slides, stored overnight at −20°C, and then at −80°C until processed. The slides were washed at room temperature and incubated for 75 min in 50 mm Tris-HCl buffer, pH 7.6, containing 1% denatured human albumin and 10% Antagolan, in the presence of 0.07 nM [125I]sCT with or without increasing concentrations of unlabeled sCT ranging from 10^-6 to 10^-11 M. After incubation, the sections were washed four times with 40 mm Tris-HCl buffer, pH 7.6, at 4°C for 2 min each. This washing procedure gave the best ratio between total and nonspecific binding (7).

Batches of four sections were removed from the slides with Whatman GF/B filter paper and placed in plastic tubes for counting (counting efficiency, 60%) in a Multiprias Packard γ Counter. Routinely, nonspecific binding was calculated from the amount of [125I]sCT bound in the presence of an excess unlabeled sCT (10^-7 M) and accounted for 15–20% of the total binding. The protein content was estimated according to the method of Bradford (11) on scraped adjacent kidney sections.

Mean values of several experiments were compared according to Student’s t test and differences were considered significant at P < 0.05. A Scatchard analysis (12) of the binding parameters which were calculated by the least squares method (13) was performed.

In Situ Autoradiography. Some slides were incubated and washed as previously described, dipped in distilled water, and dried with cold air. Autoradiographs were prepared using a modification of the procedure developed by Rostène et al. (14) in order to facilitate quantitative absorbance measurements. The slides were placed in contact with (hydrogen-3) Ulrotifilm (LKl) in Kodak X-ray film holders for 12 days at room temperature. The film was then developed with Kodak D-19 and the density of the enlarged autoradiographic images was measured.

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3 The abbreviations used are: MTC, medullary thyroid carcinoma; CT, calcitonin; sCT, salmon calcitonin.
with a densitometer that converted the amount of light into millivolts. Results are expressed as means ± SE. The number of [125I]sCT-binding sites was measured by means of iodine-125 standards (15) and expressed as fmol/mg protein.

Radioimmunoassays. Blood samples (average volume 1 ml) were obtained by retro-orbital sinus puncture, collected in heparinized tubes, centrifuged immediately, and stored at -20°C until assayed. CT was measured using a previously described assay for human CT that cross-reacts completely with murine CT (16); in brief, an anti-human CT serum (As 732) at a final dilution of 1/200,000 was incubated with 125I-labeled sCT in the presence or absence of increasing amounts of synthetic human CT or samples (50 µl of plasma). The incubation in 0.2 M phosphate buffer containing 0.1% sodium azide and 1% human albumin was carried out at 4°C for 4 days. Free and bound tracer were separated by dextran-coated charcoal precipitation. Control tubes were incubated in each assay (specific antibody omitted). Protein effects were controlled by the addition of 50 µl per tube of affinity stripped control plasma (17). Hematocrit values for normal adult WAG/Rij and Wistar rats were also estimated.

Histological Procedures. The autoradiographic procedure was completed by the staining of the slides with toluidine blue-eosin (Mann-Dominici procedure) in order to localize more precisely the [125I]sCT-binding sites at the histological level.

RESULTS

CT-Binding Sites in Normal Adult Wistar and WAG/Rij Rats, and in Tumoral WAG/Rij Rats

In Vitro Binding of [125I]sCT to Kidney Sections. Fig. 1 shows the displacement curves of [125I]sCT binding by unlabeled sCT in normal and transplanted WAG/Rij rats. Increasing concentrations of unlabeled sCT resulted in a dose-dependent inhibition of [125I]sCT binding from kidney sections in all rats. Maximum displacement was obtained with 10^-7 M sCT equivalent to 80–85% of the total [125I]sCT binding. Scatchard analysis of the data showed that, under our experimental conditions, [125I] sCT bound to a single class of binding sites with a dissociation constant of 1.5 ± 0.5 x 10^-9 M and a number of sites of N = 263 ± 27 fmol/mg protein for normal Wistar adult rat. The number of sCT-binding sites was significantly reduced (42%, P < 0.02) in normal adult WAG/Rij rat as compared to the Wistar strain (N = 153 ± 4 fmol/mg protein). The reduction is numerically, but not significantly, even greater for the WAG/Rij rat bearing a transplanted tumor (51%, N = 134 ± 6 fmol/mg protein, P < 0.01 versus Wistar/CF). Nevertheless, the dissociation constants were not significantly different (1.5 ± 0.5, 1.5 ± 0.1, and 2.0 ± 0.1 nM, respectively).

Plasma calcitonin levels were not significantly different between the two strains: for the young rats, the values were 0.29 ± 0.03 ng/ml in the Wistar strain and 0.32 ± 0.02 ng/ml in the WAG/Rij strain. In the adult Wistar and WAG/Rij rats, they were, respectively 0.23 ± 0.02 and 0.30 ± 0.04 ng/ml. The CT plasma level was highly increased only in rats with a transplanted tumor (18.6 ± 3.6 ng/ml; P < 0.01). Furthermore, the hematocrit values in Wistar adult rats were significantly lower than in the WAG/Rij rats (respectively 45.6 ± 0.96% and 52.8 ± 0.82%, P < 0.01).

Autoradiographic Studies. Autoradiograms obtained from kidney sections of control adult Wistar rat (Fig. 2a) showed that [125I]sCT-binding sites were highly concentrated in the outer medulla. Fairly intense labeling was also observed in the cortical area but with a patchy distribution seen mostly in the superficial zone. Nonspecific binding obtained with 10^-7 M unlabeled sCT showed an almost complete displacement of the radioactive ligand (Fig. 2b).

Autoradiograms of normal WAG/Rij kidney sections showed clearly an almost completely disappearance of medullary sCT-binding sites (Fig. 2c). Concomitantly, the cortical binding sites of the inner zone were also decreased. Those phenomena were
Table 1 Concentration of [125I]sCT binding sites in kidney sections in young adult Wistar WAG/Rij rats and tumor-transplanted WAG/Rij rats determined by quantitative autoradiography

<table>
<thead>
<tr>
<th>Weights</th>
<th>Cortex</th>
<th>Outer zone</th>
<th>Inner zone</th>
<th>Outer zone</th>
<th>Middle zone</th>
<th>Inner zone</th>
<th>Inner medulla</th>
<th>Papilla</th>
</tr>
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<tr>
<td>(1–17)g</td>
<td>W*</td>
<td>65.50 ± 2.17</td>
<td>23.71 ± 3.15</td>
<td>83.31 ± 3.55</td>
<td>65.14 ± 2.24</td>
<td>83.31 ± 3.55</td>
<td>23.39 ± 3.14</td>
<td>–</td>
</tr>
<tr>
<td>(10–14)W</td>
<td>WR</td>
<td>54.55 ± 3.37</td>
<td>13.50 ± 2.04</td>
<td>32.48 ± 2.98</td>
<td>27.42 ± 1.49</td>
<td>32.51 ± 2.95</td>
<td>18.70 ± 2.14</td>
<td>–</td>
</tr>
<tr>
<td>(21–26)W</td>
<td>WR</td>
<td>47.32 ± 5.53g</td>
<td>17.24 ± 2.42g</td>
<td>79.00 ± 5.59g</td>
<td>31.68 ± 2.74g</td>
<td>55.62 ± 2.88g</td>
<td>8.72 ± 0.87g</td>
<td>–</td>
</tr>
<tr>
<td>(21–26)W</td>
<td>WR</td>
<td>69.15 ± 4.11g</td>
<td>20.88 ± 2.58g</td>
<td>44.04 ± 1.83g</td>
<td>29.89 ± 1.94g</td>
<td>30.99 ± 2.27g</td>
<td>5.59 ± 0.35g</td>
<td>–</td>
</tr>
<tr>
<td>(79–82)W</td>
<td>WR</td>
<td>38.43 ± 5.43f</td>
<td>10.10 ± 0.84f</td>
<td>60.63 ± 3.88f</td>
<td>29.55 ± 2.74f</td>
<td>48.02 ± 4.76f</td>
<td>4.60 ± 0.33f</td>
<td>–</td>
</tr>
<tr>
<td>(40–58)W</td>
<td>WR</td>
<td>57.01 ± 7.19f</td>
<td>4.98 ± 0.53f</td>
<td>12.84 ± 0.87f</td>
<td>6.25 ± 0.56f</td>
<td>12.59 ± 0.91f</td>
<td>4.24 ± 0.42f</td>
<td>–</td>
</tr>
<tr>
<td>(200–250)W</td>
<td>WR</td>
<td>42.90 ± 3.84f</td>
<td>10.58 ± 2.09f</td>
<td>38.85 ± 2.38f</td>
<td>22.31 ± 2.13f</td>
<td>38.05 ± 2.06f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(200–250)W</td>
<td>WR</td>
<td>40.95 ± 3.40f</td>
<td>4.43 ± 0.71f</td>
<td>3.71 ± 0.67f</td>
<td>3.27 ± 0.45f</td>
<td>11.90 ± 1.33f</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range in g.

** P < 0.05 versus 1-week-old rats of the same strain (Student’s t test).

¶ P < 0.01 versus 1-week-old rats of the same strain (Student’s t test).

* P < 0.05 versus 2-week-old rats of the same strain (Student’s t test).

† P < 0.01 versus 2-week-old rats of the same strain (Student’s t test).

‡ P < 0.05 versus Wistar adult control rats (Student’s t test).

§ P < 0.01 versus Wistar adult control rats (Student’s t test).

* P < 0.05 versus normal adult WAG/Rij rats (Student’s t test).

P < 0.01 versus normal adult WAG/Rij rats (Student’s t test).

much more dramatically observed in kidney sections from animals bearing the transplanted tumours (Fig. 2d).

Quantitative measurements by densitometric analysis of the autoradiograms are summarized in Table 1.

sCT Binding to Kidney Sections during Postnatal Development

Kidney sections from developing Wistar and WAG/Rij rats (1, 2, and 4-week-old) were studied by quantitative autoradiography in order to establish the earliest time at which modifications in the localization of the binding sites could be observed. The autoradiograms showed that the sCT-binding sites in the various zones of the outer medulla were not yet differentiated in 1-week-old rats of both strains (Fig. 3, a and b). The differentiation started to appear after 2 weeks of life, and was similar in both strains (Fig. 3, c and d), and similar, for the Wistar rat, to what was observed later (4-week-old and adult Wistar rat: Figs. 3e and 2a). On the contrary, both medullary and inner cortical binding sites, which were present in the 2-week-old WAG/Rij kidney (Fig. 3d), were no longer detectable from the age of 4 weeks in this strain (Fig. 3f). The results of the densitometric analysis are also shown in Table 1.

DISCUSSION

The present data demonstrate that WAG/Rij rats, which are known to develop a high incidence of MTC when aging (6), show a dramatic reduction in sCT-binding sites in both the outer medulla and the inner zone of the kidney cortex. This reduction was not apparent during the first 2 weeks of postnatal development but appeared around 4 weeks after birth.

Scatchard analysis of the data showed that this effect was due to a decrease in the number of CT-binding sites with no change in the apparent affinity for the peptide. A similar phenomenon was reported by Tashjian et al. (18) who found a decrease in CT-binding sites in cultured bone cells in the presence of calcitonin, with no detectable change in the affinity for the hormone. To explain their results, they proposed "an occupancy of the receptors by a tightly bound, poorly dissociable hormone" leading to a loss of available sites. Such an explanation could account for the findings in the transplanted rat with high plasma CT levels, but not for adult normal WAG/Rij.
RENAL CALCITONIN BINDING SITES IN WAG/Rij RATS

Rij rats, as no significant excess in circulating CT level was ever observed.

Whether or not these modifications are related to an eventual development of the MTC remains unanswered. Only 50% of the WAG/Rij population develop the disease, whereas the deficiency in available binding sites was observed in all the kidneys we have examined so far. Our findings, along with the observation that an early C-cell hyperplasia is a common feature in the WAG/Rij rat thyroid, are consistent with the two-mutational-event theory for the initiation of cancer as postulated by Knudson (19) and applied by Jackson et al. (20) to the human familial MTC, i.e., a first-inherited mutation might lead to the susceptibility of protracting the disease, a second further mutation being necessary to initiate the cancer.

From a physiological point of view, the lack of available receptors in the outer medulla of the WAG/Rij kidney might result in modifications in the composition of plasma and/or urine of this strain. The effects of CT on the structures composing the outer medulla, i.e., mostly the ascending limb of the loop of Henle are well known (21, 22): CT causes an increase in the reabsorption of water and electrolytes such as calcium, potassium, and magnesium. A lack of this effect could explain the changes noted in the hematocrit value, significantly higher for the WAG/Rij than the Wistar adult rats.

Further studies will be necessary to establish whether this binding site deficiency results in modifications of plasma and/or urine associated to a probable altered pattern of water and/or electrolyte transfer. Such changes, if they exist, could possibly detect the disease long before the increase in CT circulating levels. In view of the present data, kidney function in MTC patients or in family members at risk of protracting the disease should be reinvestigated.

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