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INCREASE IN CALCITONIN mRNA LEVELS IN RATS AT HIGH RISK OF C CELL TUMOURS IS GENETICALLY DETERMINED

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SUMMARY: C-cells tumours are frequent (50%) in old WAG/Rij rats. In comparison to the original Wistar strain, three month old WAG/Rij rats are characterized by higher calcitonin synthesis and secretion, in addition to a genetically transmitted loss of calcitonin binding sites in the outer renal medulla. In order to determine if the increase of calcitonin gene expression is also of genetic origin, we quantified calcitonin and its specific messenger in the thyroid glands of second generation (Wistar x WAG/Rij) hybrids.These parameters ranged from low Wistar like to higher WAG/Rij like values. The amount of calcitonin messenger in the thyroid was highly correlated to the release of the hormone in plasma elicited by a calcium challenge and inversely correlated with the number of its binding sites in the kidney. Our results suggest that an enhanced expression of the calcitonin gene is genetically transmitted, probably as a consequence of the mutation involved in the loss of renal calcitonin binding sites. It may represent the first event leading to malignancy.

Family members at risk of developing the hereditary form of medullary thyroid carcinoma (MTC) (1) are identifed by a hypersecretion of calcitonin (CT) (2), the hypocalcemic hypophosphatemic hormone (3,4), in response to a pentagastrin injection (5) or calcium stimulation (6). Total thyroidectomy, which should be rapidly performed, confirms the existence of C-cell hyperplasia or tumour. WAG/Rij (W/R) rats, a Wistar (W) derived strain, develop C-cell tumours on ageing with a high frequency (50%) (7). Thus young W/R rats have a 1:2 risk of malignancy and represent a potential model for individuals at risk of developing MTC. If compared to the original W strain, these rats respond by a higher secretion of CT to a calcium challenge (8). They have a C-cell hyperplasia (9) and a deficit in CT receptors in the outer zone of the renal medulla (8). We showed recently (10) that the distribution of CT binding sites in the renal medulla of second generation (WxW/R) hybrids (F2) is compatible with a classical mendelian inheritance pattern: about one quater of the animals has kidneys with low CT binding sites (typical W/R), one quarter has the same density of sites as the W strain and the remaining animals have intermediate values. Thus the absence of renal binding sites in the W/R strain is a genetic defect (10). Second generation hybrids responded to a calcium challenge with an increase in circulating CT ranging from low W like to higher or more than W/R like values (10). This increase involves different processes occurring in the

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thyroid gland, such as the biosynthetic activity of the cell, the cellular stores of the hormone and the sensitivity of the cell to calcium challenge. Plasma degradation of the hormone and its fixation on its specific receptors are also important factors.

Our recent studies revealed an increased biosynthetic activity in W/R rats i.e. the thyroidal contents of both CT and its messenger were higher in these rats than in W strain (11). The aim of the present study was to establish if, as in the case of the renal defect which also characterizes the W/R rat, the increase of CT gene expression was transmitted genetically and was linked with the renal defect. We have therefore quantified CT and its messenger in the thyroids of F2 rats and their parental strains.

We report here that the increase in thyroidal stores of both CT and its messenger is genetically transmitted. Furthermore, the negative correlation which exists between the number of CT receptors in kidney and the content of CT messengers in the thyroid suggest a link between the two processes.

MATERIAL AND METHODS

Experimental procedure

Three month old rats were used: 7 W (3 males, 4 females), 7 W/R (3 males, 4 females) and 23 F2 (15 males and 8 females). The distribution of renal binding sites and the response of these animals to a calcium challenge have already been reported (10).

RNA extraction

RNA was extracted from the right lobe of each thyroid by the guanidinium thiocyanate method (12) and purified by a LiCl precipitation. Briefly, each hemithyroid was homogenized with 700 µl of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). A 100 µl aliquot was taken for CT radioimmunoassay and the remaining 600µl homogenate was transferred to a 1.5 ml Eppendorf tube. 60 µl of 2M sodium acetate, pH 4, 600 µl of phenol (water saturated) and 120 µl of chloroform-isoamyl alcohol mixture (49:1) were added with thorough mixing by inversion after the addition of each reagent. After centrifugation, RNA was purified by two successive precipitations of the aqueous phase by 1 volume of isopropanol for one hour. The pellet was washed with 75% ethanol, vacuum dried and solubilized in water treated with diethyl pyrocarbonate (DEPC). To avoid DNA contamination, the RNA was precipitated by addition of 0.25 vol of 10 M LiCl for two hours at 4°C. The resulting pellet was washed three times with 75% ethanol, vacuum dried and dissolved in DEPC treated water. RNA concentration was quantified by absorbance at 260 nm.

Other methods

Radioimmunoassay of plasma and thyroidal CT, northern blot analysis, dot blot preparation and hybridization with CT and actin cDNA [^{32}P]-labeled probes have already been described (10, 11).

Statistical analysis

Quantitative analysis of the autoradiograms was performed by densitometry. Results were expressed in arbitrary units as means ± SEM. The data were submitted to variance analysis and Student's t-test. Multiple intergroup comparison was performed using the nonparametric Kruskal-Wallis test. The results were expressed as mean ± SEM and $p<0.05$ was considered to be significant. F2 rats were classified in four classes using the thyroidal CT mRNA content of W and W/R rats.

Correlation were used to establish the degree of association between the variables.

RESULTS

Circulating and thyroidal CT

As shown in figure 1, the increase in plasma CT levels in response to a calcium challenge was greater in the W/R and F2 than in the W rats. In addition, W/R and F2 have larger thyroidal CT stores than W rats.

Hybridization studies

The quantity of total RNA extracted from each right thyroid lobe did not vary significantly between W (16.6 ± 3.5 µg per lobe), W/R (14.2 ± 1.4 µg) and F2 (15.1 ± 1.6 µg) strains.

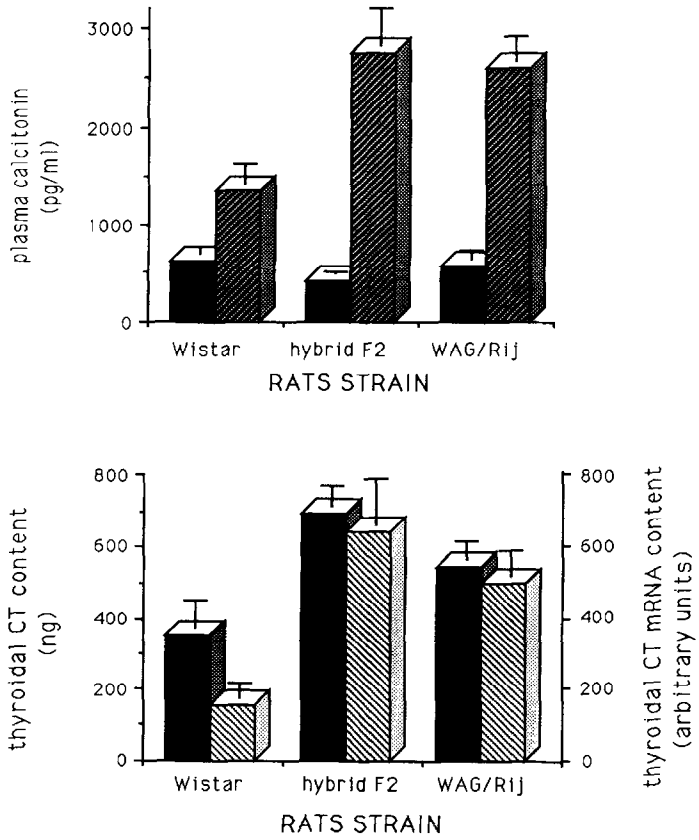

Figure 1: plasma CT levels, before (black column, top) and after (hatched column, top) calcium challenge, and thyroidal CT content (black column, bottom) were measured by radioimmunoassay (9). Thyroidal CT mRNA content (hatched column, bottom) was estimated as follows: for each rat, three aliquots of total RNA (1µg, 2µg, 3µg) extracted from right lobe of each thyroid were spotted by the dot-blot technique. The membrane was hybridized with CT probe, stripped and rehybridized with actin probe in order to detect extraction and loading errors. Autoradiograms were scanned for quantification of CT mRNA and actin mRNA. As the relationship between the amount of RNA spotted and the hybrid image absorbancy was linear (data not shown), the means of the three dilutions were calculated and the amounts of CT mRNA normalized for that of hybridizing actin mRNA. Results were expressed as mean ± SEM of relative CT mRNA quantity per thyroid (arbitrary units).

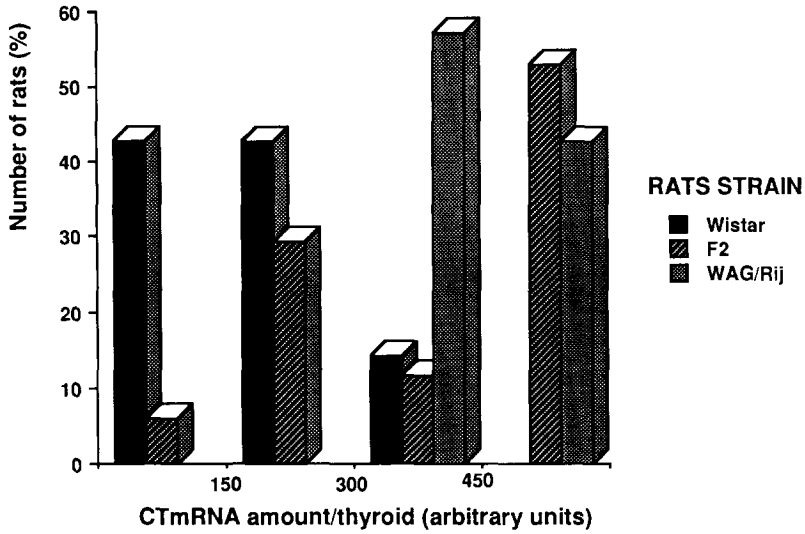

Figure 2: frequency distribution of thyroidal CT mRNA content (arbitrary units) in each rat strain. CTmRNA content were quantified as described in figure 1 and classified into four classes (<150, 150-300, 300-450, >450).

A single hybridization band of the expected lenght (1.1 kb for CT mRNA and 2.1 kb for actin mRNA) was detected by northern blot analysis in all strains (data not shown). In comparison to the W rat (figure 1), CT mRNA amounts were higher in W/R and F2 rats ($p<0.05$, anova and non parametric Kruskal-Wallis tests). This increase was specific for CT mRNA as actin mRNA quantities were similar in all rats (data not shown).

Distribution of thyroidal CT mRNA contents in the parental strains and in F2 rats is shown in figure 2. Results for F2 rats ranged from low W-like to high W/R-like values.

Correlation studies

The correlation coefficients between CT mRNA content, secretion of CT after calcium challenge, thyroidal CT content and the number of binding CT sites in outer renal medulla are given in table 1. The amount of CT mRNA in the thyroid was correlated with

Table 1: Correlation between the thyroidal CT mRNA content and the increase in plasma CT levels after the calcium challenge, the thyroidal CT stores and the number of renal medullary binding sites

rats strain	delta CT	thyroidal CT	renal binding sites
F2 hybrids	0.581 (14)*	0.694 (16)***	- 0.531 (15)*
all rats	0.659 (27)***	0.704 (30)***	- 0.530 (25)**

number in parentheses = number of animals
* $p<0.05$, ** $p<0.01$, ***$p<0.005$

both the increase in plasma CT levels after the calcium challenge and the CT content of the thyroid. No correlation was found between the CT content of the thyroid and the increase in plasma CT after the calcium challenge (10). The number of CT binding sites in renal outer medulla, which has already been reported (10), was inversely correlated with the amount of CT mRNA in the thyroid.

DISCUSSION

The present study shows that the thyroidal stores of both CT and its messenger are higher in second generation (WxW/R) hybrids rats and in parental W/R rat than in parental W rat. Second generation hybrids levels of thyroidal CT mRNA varied from low W-like to higher or more W/R-like values. This suggests that the increased synthesis of CT in the W/R rat, which we described previously (11), is genetically transmitted.

This increase probably reflects an enhanced rate of CT gene transcription. However we cannot exclude the fact that the higher quantities of CT mRNA in W/R thyroid could be due to the stabilization of mRNA or a combination of stabilization with a higher rate of the transcription.

The amount of CT mRNA in the thyroid and the number of binding sites in the outer zone of the renal medulla are negatively correlated. Thus, the genes involved in enhanced CT biosynthesis and those responsible for the stricking decrease in outer renal medullary CT binding sites seem to be linked. The loss of renal CT receptors, which appears in one month old W/R rats, precedes the higher secretion of CT (8). This defect could result from a first mutation affecting all rats of the W/R strain and may trigger an increased transcription rate of the CT gene in a majority of animals. However, though basal plasma levels of the hormone are identical in the two strains, the CT secretion per gram of body weight is almost double in W/R rats than in W rats. Thus we cannot exclude the alternate hypothesis i,e, an increased secretion of CT leading to a down regulation of the receptors (13).

The second generation hybrids, characterized by a larger range of values, are a good model for studying the correlations between CT thyroidal content, its messenger and the amount of hormone secreted in response to a calcium challenge. The existence of a positive correlation between thyroidal stores of CT and its messenger was expected, as protein synthesis is linked directly to the amount of coding messenger. More interesting is the fact that the increase in plasma CT levels after a calcium challenge is correlated to the amount of CT mRNA and not to the amount of CT in the thyroid. This finding suggests that the secretion of CT in response to a calcium challenge is more a function of the biosynthetic activity of the C-cell more than of the CT stores. This fact implies the existence of regulatory networks coupling transcriptional and post-transcriptional activity with secretion capacity (14).

In conclusion, the results we report here suggest that both the renal defect and the enhanced expression of the CT gene can be transmitted genetically. In humans, C-cell hyperplasia is considered as a first stage in the development of MTC (15) and could be

the consequence of a first mutation. A second mutation leads to the malignant development of the tumour, as proposed by Jackson (16). More recently, it was suggested that several mutations could be involved in the development of MTC (17). In the W/R model, the renal defect and resulting increase in CT mRNA could thus be the first event triggering C-cell hyperplasia. Other events, which remain to be identified, would lead to appearance of a C-cell cancer.

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REFERENCES

1- Melvin K.E.W., Miller H.H. and Tashjian A.H. Jr. (1971) *New Engl. J. Med.*, **285**, 1115-1120.
2- Milhaud G., Tubiana M., Parmentier C. and Coutris G. (1968) *C. R. Acad. Sci. (D) Paris*, **266**, 608-610.
3- Copp D.H., Cameron E.C., Cheney B.A., Davidson A.G.F. and Henze K.G. (1962) *Endocrinology*, **70**, 638-649.
4- Hirsh P.F., Gauthier G.F. & Munson P.L. (1963) *Endocrinology*, **73**, 244-252.
5- Hennessy J.F., Wells S.A., Ontjes D.A. and Cooper C.W. (1973) *J. Clinic. Endocrinol. Metab.*, **39**, 487-495.
6- Tashjian A.H. Jr., Howland B.G., Melvin K.E.W. and Hill C.S. Jr. (1970) *New. Engl. J. Med.*, **283**, 890-895.
7- Boorman G A., Van Noord M.J. and Hollander C.F. (1972) *Arch. Pathol.*, **94**, 35-41.
8- Bouizar Z., Rostène W., Treilhou-Lahille F., Pidoux E., Milhaud G. and Moukhtar M.S. (1987) *Cancer Res.*, **47**, 3595-3598.
9- Khattab M., Pidoux E., Volle G.E., Bouizar Z., Calmettes C., Milhaud G., Moukhtar M.S. and Treilhou-Lahille F. (1989) *Bone and Mineral.*, **6**, 249-260.
10- Bouizar Z., Minvielle S., Treilhou-Lahille F., Rostène W., Pidoux E., Feingold N., Milhaud G. and Moukhtar M.S. (1989) *Cancer Res.*, **49**, 5199-5202.
11- Delehaye M.C., Cohen R., Segond N., Pidoux E., Treilhou-Lahille F., Milhaud G. and Moukhtar M.S. (1989) *Biochem. Biophys. Res. Commun.*, **159**, 2, 528-535.
12- Chomczynski P. and Sacchi N. (1987) *Analytical Biochemistry*, **162**, 156-159.
13- Bouizar Z., Rostène W. and Milhaud G. (1987) *Proc. Natl. Acad. Sci USA*, **84**, 5125-5128.
14- Segond N., Delehaye M.C., Jullienne A., Taboulet J., Minvielle S., Milhaud G. and Moukhtar M.S. (1989) *Horm. Metab. Res.*, **21**, 489-493.
15- Wolfe H.J., Melvin K.E.W., Cervi-Skinner S.J., Al Saadi A.A., Juliar J.F., Jackson C.E. and Tashjian A.H. (1973) *New Engl. J. Med.*, **289**, 437-441.
16- Jackson C.E., Block M.A., Greenawald K.A. and Tahsjian A.H. (1979) *Am. J. Hum. Genet.*, **31**, 704-710.
17- Nelkin B.D., de Bustros A.C., Mabry M. and Baylin S.B. (1989) *JAMA*, **261**, 3130-3135.