Thyroid nodules in recurrent multinodular goiters are predominantly polyclonal[1]

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ABSTRACT. Not only thyroid adenomas and carcinomas, but also the majority of single and well delimited goiter nodules, even if morphologically heterogeneous, are of clonal origin. However, it is still unknown whether the nodules of rapidly growing, recurrent goiters are clonal or polyclonal. We investigated by PCR-based analysis of exon 1 of the human androgen receptor gene clonality of nodules grown in recurrent multinodular goiters (MNG) of 14 female patients. The total goiter volume varied widely between 15 ml and 170 ml. The mean age of patients undergoing surgery for recurrent goiter at the time of their first operation was significantly lower with 34.6±10.9 yr in comparison to 50 consecutive patients who were operated for MNG for the first time (53.7±13.5 yr). The interval between first and recurrent operation was 18±8.5 yr. The mean volume of well circumscribed nodules selected for the present investigation was 3.8±1.4 ml. Assessment of clonality in at least 2 samples of each lesion revealed a polyclonal pattern in 10 out of 14 nodules, whereas only 3 nodules were clonal and in one case the result remained unclear. The unexpected finding that most nodules within MNG, that had re-grown after a first subtotal thyroidectomy, were of polyclonal rather than clonal composition, suggests that these lesions are generated by *de novo* - proliferation of cohorts of differing thyrocytes sharing the common trait of an exceedingly high intrinsic growth rate or alternatively, by unknown growth stimulating molecular events acting focally on clusters of cells derived from different ancestors. In addition, the relatively young age of patients with recurrent MNG at the time of their first surgery and the comparatively short interval between first and second operation point to a genetic element in the occurrence of growth-prone thyrocytes.

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INTRODUCTION

It is now generally accepted that the large majority of solitary benign and malignant thyroid tumors are of clonal origin (1-3) and the same conclusion applies to multiple benign nodules as long as they are well delimited and of morphologically homogeneous structure (4). These nodules are considered to be adenomas. Still, a minority even of these particular thyroid nodules are clearly polyclonal (4-6).

A different and much less well-explored situation presents with the most common type of multinodular goiters (MNG), where the enlarged gland is studded with numerous macroscopically and microscopically heterogeneous, ill-delimited nodules (7). In this type of thyroid disease, the majority of thyroid nodules have no clear-cut borders making sampling for investigations on clonality more difficult and those nodules that are well-defined can be of polyclonal as well as of clonal origin (6).

As to pathogenesis, only in a minority of all clonal thyroid tumors growth-promoting mutations or molecular aberrations have been defined up to now (8, 9). For the latter type of tumors and for polyclonal tumors no generally accepted pathogenetic concept has been developed up to this time (10). Nodules within goitrous thyroid glands offer a particularly welcome opportunity to search for new pathogenetic insight into the origin of polyclonal tumors. Indeed, many such nodules retain at least one fundamental characteristic of tumoral proliferation, which is autonomous, i.e. TSH independent

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Table 1 - *Clinical data of females with a recurrent multinodular goiter (rec MNG).*

Patient no.	Total volume of rec MNG (ml)	Nodular volume (ml)	Scintigraphy of nodule#	Treatment
1	19	4*	↓	$L\text{-}T_4$
2	170	n.d.	↓	-
3	49	6	↓	-
4	26	4	↓	-
5	26	5	↓	$L\text{-}T_4$
6	46	5*	↓	$L\text{-}T_4$
7	107	4	↓	$L\text{-}T_4$
8	16	2	↑	-
9	39	3*	↑	-
10	15	3	↓	$L\text{-}T_4$+ I
11	46	n.d.	↓	$L\text{-}T_4$
12	20	5	↔	-
13	31	3	↔	-
14	15	1	↓	-

$L\text{-}T_4$, levothyroxine; I, potassium iodide.
*clonal nodules.
#for scintigraphy, treatment with $L\text{-}T_4$ and or iodide was discontinued.
^{99m}Tc uptake: ↓ decreased; ↔ regular; ↑ increased

and often even aggressive growth (11). In particular, this is the case in recurrent nodular goiter.
Several studies have demonstrated a high incidence of recurrent goiters in patients operated for non-toxic nodular goiter, irrespective of treatment with levothyroxine (12-15). In a retrospective study with 143 patients, who underwent subtotal thyroidectomy for MNG some 30 yr ago, reexamination revealed a recurrence rate of about 40 % in both levothyroxine ($L\text{-}T_4$) treated and untreated patients (14). A more favorable outcome, albeit after a mean follow-up of only 6.4 yr, was observed in 104 patients from an iodine deficient area with a recurrent goiter in 28 % of untreated and 8.9 % of treated patients (13).
Although there are - to the best of our knowledge - no systematic studies as to the molecular events at the origin of recurrent MNG nodules, the prevailing view stipulates that this thyroid disease is the late result - unaffected by any medical treatment - of clonal expansion of growth-prone follicular cells that have been missed during initial surgery. In contrast to this assumption, we here present evidence that the majority of all nodules arising in recurrent MNG are of polyclonal rather than of clonal origin.

SUBJECTS AND METHODS

Well circumscribed nodules embedded within the multinodular goiter tissue from 15 patients (1 man, 14 women) undergoing surgery for recurrent goiter were obtained at the time of operation and immediately frozen in liquid nitrogen. All patients had undergone subtotal thyroidectomy at the time of first operation some years or decades before (Table 1). Indications for second operation included the size of recurrent goiter, rapidly growing solitary nodules or cosmetic reasons. All MNG were characterized by ultrasonography and technetium 99m (^{99m} Tc) scanning. Serum free T_3, free T_4 and TSH were measured by RIA-methods to exclude patients who were not euthyroid. Anti-TSH receptor antibodies, antithyroglobulin and antithyroperoxidase antibodies (all Brahms diagnostics, Berlin, Germany) were determined to exclude patients with autoimmune thyroid diseases. Patients with positive antibody titres were excluded from the study. This study had been approved by the ethical committee of Ruhr-Universität Bochum. A written informed consent was obtained from all patients.

Assessment of clonality

DNA was isolated from 2-3 different regions of the same nodule, using standard methods (16). Clonality of nodules was assessed by an X-chromosome inactivation method (17). These methods take advantage of the fact that in females either the maternal or paternal X-chromosome is inactivated by methylation. Since only limited amounts of DNA were available, we used the PCR-based technique described by Allen et al. (17) to analyze a highly polymorphic trinucleotide repeat in exon 1 of the human androgen receptor gene. This technique consists of two steps, the

enzymatic digestion of unmethylated DNA by the methylation-sensitive restriction enzyme Hpa II, and the subsequent amplification of the highly polymorphic region of exon 1 of the androgen receptor gene by PCR. Since unmethylated DNA is cut by Hpa II, only DNA of inactivated X-chromosomes is amplified by PCR reaction.

Briefly, 1 µg DNA was digested with 15 U Hpa II (Boehringer Mannheim, Germany) for 16 hours at 37 C. The reaction was terminated by incubation at 95 C for 10 min. One fifth of the reaction and in parallel 200 ng of undigested DNA was amplified in a 50 µl PCR reaction containing 500 nmol/l of each primer [AR1: 5`-GCTGTGAAGG-TTGCTGTTCCTCAT; AR2: 5`-TCCAGAATCTGTTCCAGAGCGTGC (17)], 200 µmol/l dNTPs (Pharmacia, Uppsala, Sweden), 2 U Taq-DNA-polymerase (Life Technologies, Eggenstein, Germany), 2.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3 and 0.001% (w/v) gelatin. Samples were amplified for 25 cycles (95 C for 1 min, 68C for 1 min, 72 C for 1 min). Three µl of the PCR reaction was loaded on an 8% acrylamide/bisacrylamide gel containing 8 mol/l urea and 0.8 x TBE. After electrophoresis, DNA bands were visualized by silver staining (16).

Statistical analysis

Results are given as median values and range or mean values±SD. Differences between groups were calculated by Student's *t* test. *P*- values <0.05 were considered significant.

RESULTS

Only one out of 15 patients with a recurrent goiter was male. The clinical data of female patients are given in Table 1. The total goiter volume of these patients varied widely between 15 ml and 170 ml (median 31 ml). The mean volume of the well circumscribed nodules selected for the present investigation was 3.8±1.4 mL (median 4 mL; range 1-6 ml). Thyroid scanning revealed a decreased ^{99m}Tc uptake in 10 out of 14 nodules (71 %). Six out of 14 patients were treated with L-T_4, one patient received L-T_4 plus iodide.

The mean age of patients who underwent a second subtotal thyroidectomy for recurrent goiter was 34.6±10.9 yr at the time of their first operation. In contrast, the mean age of a randomly selected series of 50 consecutive patients who were operated for multinodular goiters for the first time was significantly higher with 53.7±13.5 yr (Fig. 1). One of the patients with a recurrent multinodular goiter underwent primary surgery already at the age of 14 yr. The mean interval between first and a recurrent operation was 18±8.5 yr.

Since we were aware of the problem (10) that a false positive result "polyclonal" may result from

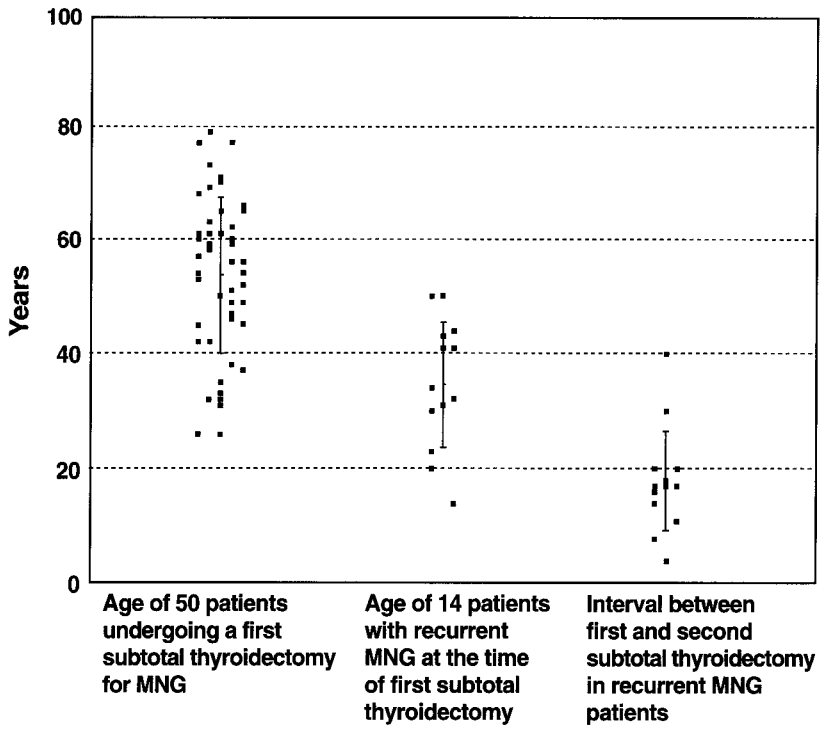

Fig. 1 - *Mean age of patients who underwent surgery for multinodular goiter or for recurrent goiter and mean interval between first and second operation of patients with recurrent goiters. The bars represent the mean value±SD. As revealed by Student's t test there was a significant difference between the mean age of patients operated for multinodular goiters and patients operated for recurrent goiters (p<0.01).*

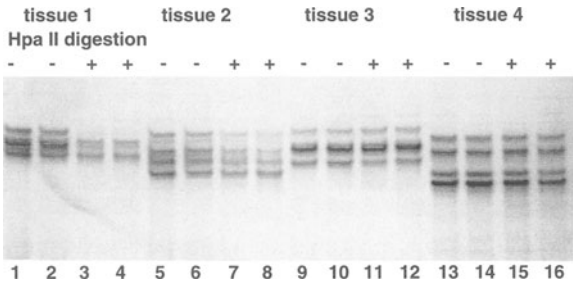

Fig. 2 - *Assessment of clonality of recurrent goiter nodules by PCR-based method. Clonality was determined by digestion of DNA with the methylation-sensitive restriction enzyme Hpa II and subsequent amplification of the highly polymorphic region of exon 1 of the androgen receptor gene (17). At least two samples derived from different areas of the same nodule were analyzed. As control, undigested DNA from each sample was amplified in parallel (lanes 1, 2, 5, 6, 9, 10, 13, 14). The two bands visible for each allele correspond to the two DNA strands since each single-stranded DNA migrates slightly different in the gel (17). In clonal samples (tissue 1), after Hpa II digestion only the two bands corresponding to one allele are visible, while in polyclonal samples (tissues 2, 3, 4) all four bands could be detected after restriction.*

contamination of nodular tissue with surrounding tissue, at least two to three different samples of the same nodule were investigated and the pattern of samples derived from the same nodule had to be identical (Fig. 2). A nodule was considered to be clonal, when after Hpa II digestion only two bands corresponding either to the maternal or the paternal allele were visible, and vice versa to be polyclonal, when all four bands were detectable after Hpa II digestion (Fig. 2). Ten out of 14 nodules clearly showed polyclonal pattern 3 nodules were clonal and in one case the result remained unclear (Table 1). There were no differences in clinical presentation or biochemical parameters in patients with clonal or polyclonal nodules.

DISCUSSION

The unexpected finding that the majority of nodules within MNG that had re-grown after a first subtotal thyroidectomy for MNG were of polyclonal composition is not compatible with the prevailing view that recurrent nodular goiters result from the failure of the surgeon to remove all cells capable of clonal expansion - be it because of growth-promoting mutations or any other, still unknown cause. Rather, the *de novo*-proliferation of polyclonal nodules suggests different pathogenic mechanisms: Either a whole number of follicular cells, derived from different mother cells, must obey in a common and coordinated way to unknown, superordinate molecular events that accelerate their proliferation rate, or, alternatively, polyclonal nodules may arise from clusters of differing cells that share the inborn or acquired trait of a higher than average growth potential (for review see 10). There is presently no solid hint at the true nature of the first of these two mechanisms, while for the second, experimentally founded concepts are about to emerge. Indeed, available evidence suggests that a small but variable number of thyrocytes of the adult gland may have retained the aggressive and autonomous growth behavior of fetal cells and that - in addition - rapidly proliferating cells may *de novo* arise at each mitosis (10, 11). Similar mechanisms have been considered for autonomously proliferating smooth muscle cells in vascular lesions (18).

On the molecular level, constitutive and secondarily acquired resistance of an originally small but variable fraction of thyrocytes toward the growth-inhibiting action of TGF β may be one of many conceivable mechanisms allowing overgrowth of a polyclonal cell population sharing this particular trait (19). TGF β resistant cells are indeed highly prevalent in primary cultures of human MNG samples (19). A recent report (20) describes a more intense expression of TGF β in recurrent as compared to primary MNG tissue, thus supporting the concept of resistance toward the counter-regulatory overexpression of TGF β in aggressively proliferating goiter cells. Another molecular event detected in benign thyroid tumors is the autonomous production of the growth factor IGF-I (21). It is to be expected that other mechanisms beside TGF β-resistance and IGF-I overproduction await to be unraveled in the pathogenesis of MNG.

It is undisputed that clonal adenomas in otherwise normal thyroid glands may arise from a single cell fortuitously hit by a growth-promoting mutation or a sequence of molecular events that confer a growth advantage to the cell. However, the coexistence of polyclonal nodules with clonal lesions of different origin within the same MNG (6) can be taken to indicate that the primary event in many goiters may be a polyclonal proliferation and, thus, that many, if not the large majority of clonal nodules in MNG evolve from polyclonal ones through secondary genetic alterations in cells with a high rate of mitosis. The present findings are certainly in line with such a view. The powerful drive that induced a multitude of genetically differing cell clusters - particularly, but by no means exclusively those forming well-delimited nodules - to proliferate in our patients presenting with recurrent and often voluminous goiters, is impressively illustrated by the comparably young age of these patients at the time of

first surgery and by the interval of only 18 years between first and repeated surgery for regrowth of large MNG (Fig. 1).

Any well-circumscribed, morphologically and functionally homogeneous thyroid nodule containing a clonally expanding cell population is unanimously called adenoma (22). There is no doubt that this type of thyroid nodules is a true tumor. However, there is much uncertainty and confusion in the classification of the incomparably more common ill-delimited, heterogeneous and often polyclonal thyroid nodules (4) that characterize most MNG. Poorly defined terms such as adenomatous nodules are commonly used for these lesions. The predominantly polyclonal nature of nodules in recurrent MNG, described in the present work, adds new evidence to the view that many, if not all, heterogeneous thyroid nodules of MNG, whether clonal or polyclonal, must also be classified within a broad spectrum of autonomously growing benign thyroid tumors rather than to be labeled with the ill-defined term of "hyperplasia" suggesting exogenous stimulation.

We have previously pointed out that long-standing stimulation of the thyroid gland, such as in untreated Graves' disease or in iodine deficiency, may well explain a diffuse enlargement of the thyroid gland but by no means the inevitable nodular transformation of the gland with time (10). Nodules can only develop if some cohorts of cells proliferate at a higher rate than their neighbours. While the differences in the inherent growth potential may be so subtle between certain cell cohorts as to require external stimuli such as iodine deficiency to become manifest in a lifetime, the inherent growth advantage of other cell groups may be large enough to clinically appear as autonomously proliferating nodules. The rather aggressively growing polyclonal nodules described here, just as nodular goiters evolving in children (23) may be examples of thyroid tumors located at one end of the spectrum.

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