Original Article

DNA Measurement, Proliferation Markers, and Other Factors in Pituitary Adenomas

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Abstract

To assess the proliferative activity of pituitary adenomas, 36 surgically removed adenomas were studied by light microscopical parameters; mitotic count; expression of PCNA, Ki-67, cathepsin D, and EGF; and image cytometry. Three adenomas (9%) showed high, 11 (34%) medium, 17 (53%) moderate, and 1 (3%) low structural differentiation. In 10 adenomas (31%), no mitosis was observed. The average was 2.4 mitoses/100 HPF; the highest count was 7.1 mitoses/100 HPF. Eleven adenomas (33.3%) were PCNA-negative; in 20 adenomas (60.6%), between 0.05 and 3.9, and in 2 adenomas (6.0%), between 10.5 and 16.4 PCNA-positive nuclei were observed. Only a recurrent null-cell adenoma (9%) was Ki-67–negative. Three adenomas (9.1%) were EGF-negative, 28 (84.8%) showed up to 10% positive cells, and 2 (6.1%) showed between 10 and 30% positive cells; 19 adenomas (68%) were cathepsin D–negative, including all endocrine-inactive adenomas. Half the adenomas had an euploid DNA stem line. Endocrine-inactive adenomas displayed a higher rate of euploid DNA stem lines than endocrine-active adenomas. The S-phase fraction varied between 2.97 and 28%, with a mean value of 14.4%. Half the adenomas showed an S-phase fraction of 11.65% or lower. **Endocr Pathol 5:198–211, 1994.**

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Obtaining structural information on the growth rate and the cell proliferation of tumors, especially of endocrine organs, is a very difficult challenge. Counting mitoses and other morphological parameters, such as cell size, cell and nuclear pleomorphy, and the cell structures (e.g., chromatin, endoplasmic reticulum, mitochondria), are broadly used in tumor pathology. Cell cycle–related antigens, such as the proliferating cell nuclear antigen (PCNA) and Ki-67, especially for the S-phase, are of prognostic value [56]. PCNA, first detected in 1978 by Miyachi [44] and described [7,8, 42,59] as the auxiliary protein of the DNA polymerase delta, reaches a maximum in the late G1, and especially in the S-phase [9,14,39]. Ki-67, which is absent in G0 and early G1 [24,25] but present in the other phases of the cell cycle, with a maximum in G2 and M-phase [29], is associated with components of the nuclear matrix [61]. Ki-67 antigen is known to be very sensitive to fixation, and for a long time it was only applicable on frozen tissue. Currently, PCNA and Ki-67 (as the antibody MIB-1) are widely used on paraffin sections [13,26, 28,62].

Flow and image cytometry provide information about the fraction of cells in the G0/G1-phase, the S-phase, and the G2/- and M-phase of the cell cycle. In addition, the DNA index (mode), the 2c-deviation index, and the ratio of 5c-exceeding events can be measured, thus providing information on the stem line, the distribution, and the single cells with a DNA content greater than 2c.

Epidermal growth factor (EGF), primarily described by Cohen in 1962 [15], and

its human equivalent (hEGF) have a mitogenic effect on human foreskin fibroblasts (HF cells) [11]. Kasselberg and associates [38] first detected EGF in the anterior pituitary gland. There are contrary results in different cell lines with regard to mitogenic and other effects of EGF [12,31,45,55,58]. Recently, Renner and colleagues [49] demonstrated that nonfunctioning pituitary adenomas secrete mitogenic substances (EGF and others) in vitro, which stimulate proliferation of pituitary cells, fibroblasts, and endothelial cells.

There is evidence that cathepsin D, an ubiquitous estrogen-regulated lysosomal endopeptidase [48], is associated with proliferation of ductal mammary cells independent of their estrogen receptor status [23]. This fact is in accordance with the observed mitogenic in vitro activity of cathepsin D. The clinical significance remains problematic [17,32,60]. Furthermore, it may support or facilitate tumor invasion and metastasis [46] by digesting the extracellular matrix, either by proteases, secreted in the conditioned medium, or by degradation of the extracellular matrix after endocytosis.

In pituitary adenomas, neither light microscopic nor electron microscopic studies allow reliable conclusions to be drawn regarding their proliferative activity. Therefore, additional methods (i.e., immunohistological expression of PCNA, Ki-67, and image cytometry) were used in a collection of surgically removed pituitary adenomas between 1976 and 1979. This old series of adenomas was selected to provide a long follow-up period. In addition, EGF and cathepsin D were included in this study to evaluate their influence on, or possible correlation with, the proliferation markers or the DNA measurement. The findings were correlated to one another, to the adenoma type [53], and to the clinical course, with the intention of improving evaluation of the proliferative activity of pituitary adenomas.

Materials and Methods

Thirty-six pituitary adenomas mainly derived from trans-sphenoidal hypophysectomy at the Department of Neurosurgery of the University of Hamburg, Germany, were fixed in buffered formalin and embedded in paraffin. The adenomas were classified into 4 grades of differentiation; high (grade 1), medium (grade 2), moderate (grade 3), and low (grade 4).

We defined the mitotic count [29] in our study as the total number of mitoses in 100 microscopic high-power fields (HPF) in a magnification of ×500 (microscopic eyepiece, 12.5; lens, 40). The total number of observed mitoses was divided by the total number of assessed microscopic HPFs in each section, and the result was multiplied by 100. The mean measured HPFs were 118.72 (range, 2–324 HPF).

For immunocytochemistry, we applied the avidin-biotin complex (ABC) technique [35] using for PCNA/Ki-67 normal horse serum (Vector Laboratories; Burlingame, CA), antiproliferating cell nuclear antigen (100 μL/section; dilution, 1:20; monoclonal antibody PC10; Oncogene Science, Manhasset, NY; overnight at 4°C)/anti-Ki-67 antigen (100 μL/section; dilution, 1:10; monoclonal antibody Mib 1; Dianova, Hamburg, Germany; 1 hour at room temperature, microwave-processed sections), biotinylated horse anti-mouse immunoglobulin G (IgG) (Vector Laboratories) avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC standard kit; Vector Laboratories); for EGF/cathepsin D, normal swine serum (Dako, Hamburg Germany), antiepidermal growth factor (100 μL/section; dilution, 1:10; polyclonal antibody, Oncogene Science, Manhasset, NY; overnight at 4°C)/anticathepsin D antigen (100 μL/section; dilution, 1:300; polyclonal; Dako Corporation, Carpinteria, CA; 1 hour at room temperature), biotinylated swine antirabbit IgG (Dako, Hamburg, Germany), ABC complex (Dako, Hamburg, Germany), and diaminobenzidine (DAB) as chromogene.

Each immunohistochemistry was accompanied by 2 positive control sections, consisting of normal human submandibulary gland tissue for EGF, normal human tonsil tissue for PCNA, and normal human and human breast cancer tissue for cathepsin D and Ki-67. As negative control, we used the same tissue as for the positive control, but we substituted the primary antibody, the biotinylated antibody, or the ABC complex by PBS.

The slides of the immunohistochemistry with PCNA and EGF were very weakly counterstained in Mayers haemalaun. We counted every positive stained nucleus or cell, disregarding the grade of staining, in a magnification of ×500 (microscopic eyepiece, 12.5; lens, 40). To compare the mitotic count with the results obtained from immunohistochemistry, we referred the PCNA/Ki-67 index to the total number of PCNA/Ki-67–positive nuclei in each section, divided by the total number of microscopic HPFs, assessed in each section. The number of microscopic HPFs assessed varied in the measurement of PCNA between 3 and 76 (mean value, 35 HPFs) and in the measurement of Ki-67 between 2 and 25 (mean value, 9 HPFs).

To assess the immunohistochemistry of EGF and cathepsin D, the sections were estimated as positive or negative. In addition, the positive sections of EGF were classified by the observer into 2 groups: containing less than 10% positive cells or between 10 and 30% positive cells. For DNA staining, 7-μm sections were Feulgen-stained [19]. For hydrolysis, the slices were incubated for 15 minutes in 1 N HCl at 60°C.

DNA measurement was performed on an Wild Leitz Wetzlar cytophotometry microscope and analysed by a Miamed-DNA image analyzing system. At least 100 adenoma cells and between 6 and 31 reference cells (endothelial cells; mean value, 15.4) were measured. The DNA status (aneuploid or euploid DNA stem line), the DNA index (mode), the 2c-deviation index, and the ratio of 5c-exceeding events were calculated. The DNA stem line was indicated in the histogram by the observer. Aneuploidy of the DNA stem line was assumed if the mode value of diagnosis cells was beyond the limit of 2c +/− times the coefficient of variation of the reference cells [30,33]. Samples with a euploid DNA stem line were also considered to be aneuploid if more than 3 cell nuclei with a DNA content greater than 5c were observed. Adenomas with cell nuclei between 1 and 3 with a DNA content greater than 5c were judged as giving cause for suspicion.

We estimated the S-phase fraction [54] by determining the limits of the S-phase indicated by the observer in the histograms obtained from DNA measurement. The numbers and the percentage of cells were then measured within these limits. We excluded 7 samples because we were not able to determine clearly the limits of the S-phase in the related histogram.

We observed the clinical course of 18 adenomas for at least 5 years; the longest period was 14 years. For statistical analysis, we calculated the coefficient of correlation, cross tabulations, and chi-squared tests [51].

Results

Microscopical Parameters

The light microscopic parameters that are generally connected with an increased growth are increased chromatin content, increased pleomorphy, increased number of mitoses, increased rate of double-nucleated cells, and increased incidence of necrotic cells. From these parameters, we classified 4 grades of differentiation (Table 1): 3 adenomas (9.4%) showed high (grade 1) differentiation, 11 (34.4%) had medium (grade 2) differentiation, 17 (53.1%) had moderate (grade 3) differentiation, and 1 (3.1%) had low (grade 4) differentiation. The average differentiation of the adenomas was between medium and moderate. Endocrine-active adenomas (n = 20) were more highly differentiated than the endocrine-inactive ones (n = 12).

The mitotic count is generally defined as the number of mitoses per 10 HPFs, but due to the low number of mitoses in pituitary adenomas, our number of mitoses is based on 100 HPF. The results are shown in Table 2. In 10 adenomas (31%), no mitosis was observed. The mean value was 2.4 mitoses/100 HPF and the highest count was 7.1 mitoses/100 HPF (Fig. 1). The endocrine-active adenomas had a higher mean value (2.61 mitoses/100 HPF) than the endocrine-inactive adenomas (2.02 mitoses/100 HPF). We found no positive correlation between the grade of differentiation and the number of mitoses. However, the less differentiated adenomas showed a significantly lower number of mitoses ($P < 0.05$) than the more highly differentiated adenomas.

Table 1 Adenoma type and grading

Adenoma type	Grade of differentiation 1	2	3	4
GH-cell adenoma				
Densely granulated (n = 4)[a]	1	6	3	—
Sparsely granulated (n = 6)[a]				
Mixed GH/PRL–cell adenoma (n = 4)[a]	1	1	2	—
GH/PRL/glycoprotein adenoma (n = 3)[a]	1	1	1	—
Other (n = 1)b	—	—	1	—
PRL-cell adenoma, sparsely granulated (n = 5)	—	—	5(2[a]/3[b])	—
ACTH-cell adenoma, sparsely granulated (n = 1)[a]	—	—	—	1
Null-cell adenoma (n = 5)[a]	—	2	3	—
Oncocytic adenoma (n = 3)[b]	—	1	2	—
Total (n = 32)	3 (9%) (3)[a]	11(34%)(8[a]/3[b])	17(53%)(8[a]/9[b])	1 (3%) (1[a])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

Proliferation Markers

The results of PCNA immunostaining are shown in Table 3. We did not find any PCNA-positive nucleus in 11 adenomas (33.3%); in 20 (60.6%), there were between 0.05/HPF and 3.9/HPF, and in 2 (6.0%), there were between 10.5/HPF and 16.4/HPF PCNA-positive nuclei (Fig. 2). The mean value was 1.67 PCNA-positive nuclei/HPF. The endocrine-inactive adenomas showed a higher mean number of PCNA-positive nuclei (3.28/HPF) than the endocrine-active adenomas (0.62/HPF).

The results of the Ki-67 immunohistochemistry (Table 4; Fig. 3) revealed

Table 2 Adenoma type and number of mitoses

Adenoma type	Number of mitoses: Negative	Positive	Mitoses/100 HPF
GH-cell adenoma, densely (n = 4) and sparsely granulated (n = 6)	1	9	0–7.1
Mixed GH/PRL–cell adenoma (n = 3)	1	2	0–4.3
GH/PRL/glycoprotein adenoma (n = 3)	1	2	0–4.9
Other (n = 1)	1	—	0
PRL-cell adenoma, sparsely granulated (n = 6)	5 (3[a]/2[b])	1 (1[b])	0–1.2 (0[a]/0–1.2[b])
ACTH-cell adenoma, sparsely granulated (n = 1)	—	1	5.2
Null-cell adenoma (n = 5)	—	5	1.7–7.1
Oncocytic adenoma (n = 3)	1	2	0–1.7
Total (n = 32)	10(31%)(6[a]/4[b])	22(69%)(14[a]/8[b])	0–7.1 (0–7.1[a]/0–7.1[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

Table 3 Adenoma type and PCNA-positive nuclei

Adenoma type	PCNA-content		PCNA-positive nuclei/HPF
	Negative	Positive	
GH-cell adenoma, densely (n = 4) and sparsely (n = 6) granulated	6	4	0–1.6
Mixed GH/PRL–cell adenoma (n = 4)	2	2	0–0.35
GH/PRL/glycoprotein adenoma (n = 2)	—	2	0.05–2.0
Other (n = 1)	—	1	2.3
PRL-cell adenoma, sparsely granulated (n = 6)	2 ($1^a/1^b$)	4 ($2^a/2^b$)	0–16.4 (0–3.3[a]/0–16.4[b])
ACTH-cell adenoma, sparsely granulated (n = 1)	—	1	1.7
Null-cell adenoma (n = 6)	1	5	0–10.5
Oncocytic adenoma (n = 3)	—	3	0.45–1.3
Total (n = 33)	11 (33%) ($9^a/2^b$)	22 (67%) ($11^a/11^b$)	0–16.4 (0-3.3[a]/0-16.4[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

only 1 negative adenoma (9%) (a recurrent null-cell adenoma). The 10 Ki-67–positive adenomas showed between 0.12 and 10 Ki-67–positive nuclei/HPF. In 2 endocrine-active adenomas, we observed 1 HPF showing only Ki-67–positive nuclei. Excluding these 2 tumors, the mean number of Ki-67–positive nuclei was 2.69/HPF. The endocrine-inactive adenomas had a mean value of 0.70 Ki-67–positive nuclei/HPF, and the endocrine-active adenomas had a mean value of 4.28 Ki-67–positive nuclei/HPF.

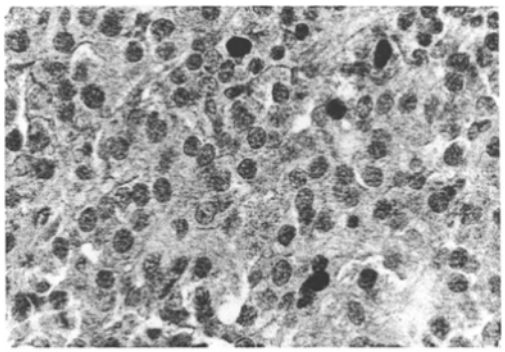

Figure 2. Null-cell adenoma with 4 PCNA-positive nuclei (anti-PCNA-haemalaun, ×400).

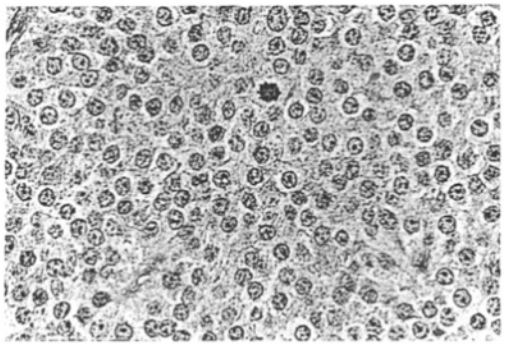

Figure 1. Null-cell adenoma with a mitosis (Hematoxylin-eosin, ×180).

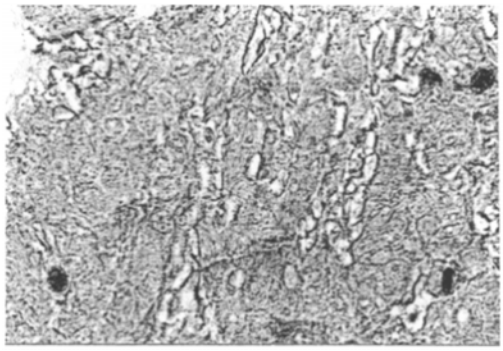

Figure 3. Sparsely granulated PRL-cell adenoma with 4 Ki-67–positive nuclei (anti-Mib1-hematoxylin & eosin, ×400).

Table 4 Adenoma type and Ki-67–positive nuclei

Adenoma type	Ki-67 (Mib-1) Negative	Ki-67 (Mib-1) Positive	Ki-67 (Mib-1)–positive nuclei/HPF
GH-cell adenoma, densely (n = 2) and sparsely (n = 1) granulated	—	3	1–all (in 1 HPF)
Mixed GH/PRL-cell adenoma (n = 2)	—	2	5–10
GH/PRL/glycoprotein adenoma (n = 1)	—	1	4.4
PRL-cell adenoma, sparsely granulated (n = 2)	—	2 ([a]/1[b])	2.3–all (in 1 HPF) (all[a]/2.3[b])
Null-cell adenoma (n = 2)	1	1	0–0.4
Oncocytic adenoma (n = 1)	—	1	0.12
Total (n = 11)	1 (9%) (1[b])	10(91%)(7[a]/3[b])	0–all (in 1 HPF) (1–all[a]/0–2.3[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

DNA Measurement

DNA measurement was performed on 36 adenomas (Table 5); 18 adenomas (50%) had an euploid DNA stem line (Fig. 4), and 18 (50%) had an aneuploid DNA stem line (Fig. 5). The endocrine-inactive adenomas displayed a higher rate of euploid DNA stem lines (61.5 vs 38.5%) than the endocrine-active adenomas (43.5 vs 56.5%). All oncocytic adenomas were euploid, whereas the 4 prolactinomas and the Nelson tumor had an exclusively aneuploid DNA stem line.

Table 5 Adenoma type and DNA status

Adenoma type	DNA stem line Aneuploid	DNA stem line Euploid	mean DNA index (mode)
GH-cell adenoma	5	5	
Densely granulated (n = 4)			1.38
Sparsely granulated (n = 6)			1.15
Mixed GH/PRL–cell adenoma (n = 4)	1	3	1.06
GH/PRL/glycoprotein adenoma (n = 3)	2	1	1.38
Other (n = 1)	—	1	1.00
PRL-cell adenoma, sparsely granulated (n = 7)	5 ([a]/1[b])	2 ([b])	1.34 (1.41[a]/1.25[b])
ACTH-cell adenoma	1	1	
Sparsely granulated (n = 1)			1.25
Densely granulated (n = 1)			1.00
Null-cell adenoma (n = 6)	4	2	1.02
Oncocytic adenoma (n = 3)	—	3	0.96
Total (n = 36)	18(50%)(13[a]/5[b])	18(50%)(10[a]/8[b])	1.18 (1.24[a]/1.06[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

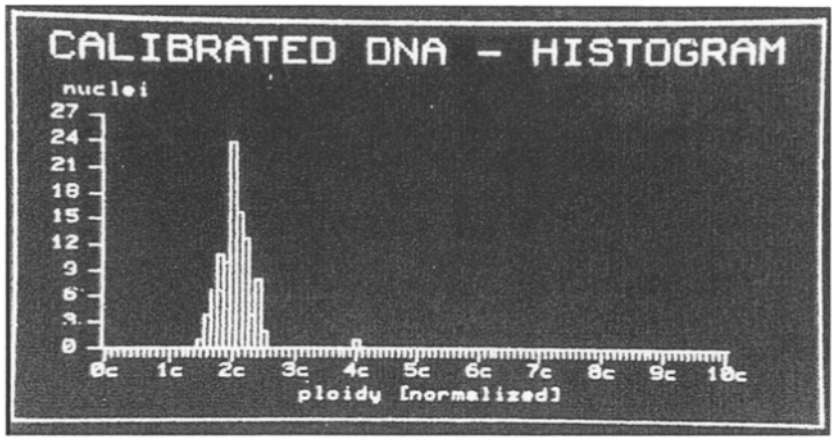

Figure 4. DNA histogram of an euploid oncocytic adenoma.

The DNA index (mode) varied between 0.88 and 2.13, with a mean value of 1.18; 24 adenomas (67.2%) had a DNA index between 0.88 and 1.13, and 14 adenomas (38.9%) revealed a DNA index of 1.00. Only endocrine-inactive adenomas, 2 null-cell adenomas, and 1 oncocytic adenoma had a DNA index lower than 1.00 (0.88).

The 2c-deviation index varied between 0.09 and 5.31, with a mean value of 0.94. The endocrine-active adenomas had a higher mean 2c-deviation index (1.12) than the endocrine inactive adenomas (0.62). The euploid adenomas had a mean 2c-deviation index of 0.59 versus 1.29 in aneuploid adenomas.

In 14 tumors (38.9%), we found cells containing a nucleus with a DNA content between 5c and 9c, and 2 adenomas (5.6%) had a nucleus greater than 9c. One adenoma with a euploid DNA stem line contained 6 nuclei with a DNA content greater than 5c. Eight tumors with an euploid DNA stem line gave cause for suspicion, because they contained between 1 and 3 nuclei with a DNA content greater than 5c. Five adenomas with an aneuploid DNA stem line also showed more than 3 nuclei with a DNA content greater than 5c. The endocrine-inactive adenomas had a lower mean value of nuclei with a DNA content greater than 5c (0.53) than the endocrine-active adenomas (1.95). The null-cell adenomas and the adenoma immunoreactive for other hormones showed no nucleus with a DNA content greater than 5c.

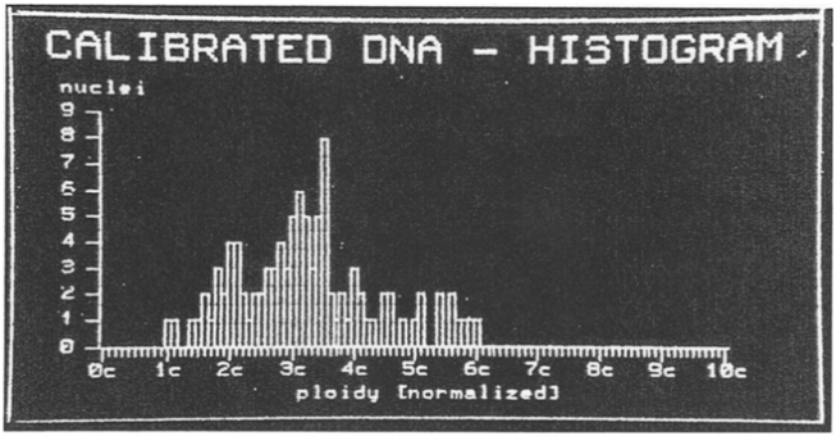

Figure 5. DNA histogram of an aneuploid GH/PRL/glycoprotein adenoma.

The S-phase fraction (Table 6) varied between 2.97 and 28%, with a mean value of 14.4%. Half the adenomas showed an S-phase fraction of 11.65% or lower; 73.3% aneuploid adenomas versus 42.9% euploid adenomas showed more than 10% of cells in the S-phase. Only a slight difference was observed between endocrine-active and endocrine-inactive adenomas. The mean percentage of cells in the S-phase was 14.82% in endocrine-active adenomas and 13.49% in endocrine-inactive adenomas. We found no statistically significant correlation between the S-phase fraction of the adenomas and their immunohistochemical expression of Ki-67 and PCNA.

Other Factors

With regard to EGF expression, we graded the adenomas in 3 groups (Table 7). The first group did not show any EGF-positive cytoplasm, the second group showed up to 10% EGF-positive cells, and the third group showed between 10 and 30% EGF-positive cells (Fig. 6). Adenomas with more than 30% EGF-positive cells were not found. Only 3 tumors (9.1%) were negative; 28 (84.8%) showed up to 10% EGF-positive cells, and 2 (6.1%) showed between 10 and 30% EGF-positive cells. The mean value for the endocrine-active (0.95) and endocrine-inactive (1.00) adenomas were nearly equal.

The cathepsin D content was studied in 28 adenomas (Table 8). Nineteen tumors (68%) presented no cathepsin D–positive cells. In 9 tumors (32%), a cytoplasmic

Table 6 Adenoma type and S-phase fraction

Adenoma type	S-phase fraction	
	Mean value	Range
GH-cell adenoma, densely (n = 2) and sparsely (n = 6) granulated	13.10	2.97–23.00
Mixed GH/PRL–cell adenoma (n = 3)	16.35	11.00–19.23
GH/PRL/glycoprotein adenoma (n = 3)	9.51	4.00–15.53
Other (n = 1)	21.00	
PRL-cell adenoma, sparsely granulated (n = 5)	9.82 (23.39[a]/14.46[b])	8.91–21.00 (15.0–28.0[a]/8.91–20.0[b])
ACTH-cell adenoma, sparsely granulated (n = 1) and densely granulated (n = 1)	14.47	6.93–22.00
Null-cell adenoma (n = 5)	13.81	6.93–26.00
Oncocytic adenoma (n = 2)	7.95	6.00–9.90
Total (n = 29)	14.36 (14.82[a]/13.49[b])	2.97–28.00 (2.97–28.0[a]/6.0–26.0[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

staining was observed (Fig. 7). All endocrine-inactive adenomas were cathepsin D–negative.

Clinical Course

We observed the clinical course of 18 adenomas; 11 (61.1%) did not recur. At the time of study, 3 tumors were the recurrences of a former adenoma, of which 2 recurred again. Four tumors, which were the first occurrence of an adenoma, showed a recurrence after a period of between 3 months and 8 years. Adenomas without any cathepsin D–positive cells had a significantly higher recurrence rate ($p < 0.05$) than the others.

Five of 6 null-cell adenomas recurred.

Table 7 Adenoma type and EGF-positive cells

Adenoma type	EGF-content		
	Negative	Positive	EGF-positive cells/HPF
GH-cell adenoma densely (n = 4) and sparsely (n = 6) granulated	1	9	0–30%
Mixed GH/PRL–cell adenoma (n = 4)	—	4	<30%
GH/PRL/glycoprotein adenoma (n = 2)	—	2	<10%
Other (n = 1)	—	1	<10%
PRL-cell adenoma, sparsely granulated (n = 6)	2 (2[a])	4 (1[a]/3[b])	0–10% (0–10%[a]/<10%[b])
ACTH-cell adenoma, sparsely granulated (n = 1)	—	1	<10%
Null-cell adenoma (n = 6)	—	6	<10
Oncocytic adenoma (n = 3)	—	3	<10%
Total (n = 33)	3 (9%) (3[a])	30(91%)(17[a]/13[b])	0–30% (0–30%[a]/<10%[b])

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.
[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

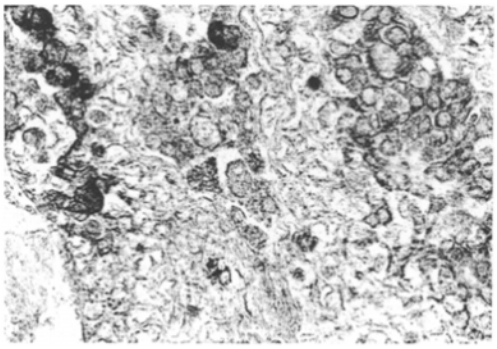

Figure 6. Mixed GH/PRL–cell adenoma with cytoplasmic immunostaining for EGFN (anti-EGF-haemalaun ×400).

Thus, a significantly negative correlation ($p < 0.001$) between endocrine activity and rate of recurrence is evident. Half the observed aneuploid adenomas (n = 8) recurred, whereas only 3 of the euploid adenomas (n = 10) recurred.

Discussion

Counting mitoses is a widely used method for tumor reports, but there are many reservations about it, because delay in fixation allows cells to complete and exit the M-phase, as well as because of non-homogeneous distribution of the proliferative cells, cell size variations within a specimen, and interobserver variation [29]. In addition, the number of mitoses in pituitary adenomas is known to be very low [2,52]. Our highest count was 7.1 mitoses/100 HPF. Despite the fact that one parameter of the grade of differentiation was an increased count of mitotic events, there was no positive correlation between the count of mitoses and the grade of differentiation. However, we found a significantly lower number of mitoses ($p < 0.05$) in less differentiated adenomas than in more highly differentiated ones. Therefore, we conclude that the number of mitoses had a high variance, and we cannot draw conclusions about the grade of differentiation in pituitary adenomas. Furthermore, we did not observe any significant correlations between the number of mitoses and the expression of PCNA and Ki-67.

PCNA has a half-life of approximately 20 hours [9,29]. In quiescent cells 48 hours after entering the G0-phase of the cell cycle, approximately 40% of the PCNA content of proliferating cells could be detected [9]. Therefore, PCNA immunoreactivity can be detected in cells that have recently left the cell cycle. Although Ki-67 had a maximum in G2 and M-phases, it is present in the other phases of the cell cycle. In addition, the M-phase is only a part of the cell cycle. Therefore, it may be that the number of PCNA-positive or Ki-67–positive nuclei is

Table 8 Adenoma type and cathepsin D-positive cells

	Cathepsin D	
Adenoma type	**Negative**	**Positive**
GH-cell adenoma, densely (n = 4) and sparsely (n = 6) granulated	4	6
Mixed GH/PRL–cell adenoma (n = 4)	3	1
GH/PRL/glycoprotein adenoma (n = 2)	—	2
Other (n = 1)	1	—
PRL-cell adenoma, sparsely granulated (n = 4)	4 ($2^a/2^b$)	—
Null-cell adenoma (n = 5)	5	—
Oncocytic adenoma (n = 2)	2	—
Total (n = 28)	19 (68%) ($9^a/10^b$)	9 (32%) (9^a)

[a]All GH-cell adenomas, mixed GH/PRL–cell adenomas, GH/PRL/glycoproteins adenomas, and ACTH-cell adenomas are endocrine-active.

[b]All null-cell adenomas, oncocytic adenomas, and others (FSH/TSH/LH) are endocrine-inactive; 3 PRL-cell adenomas, sparsely granulated, are endocrine-inactive ([b]) and 4 are prolactinomas ([a]).

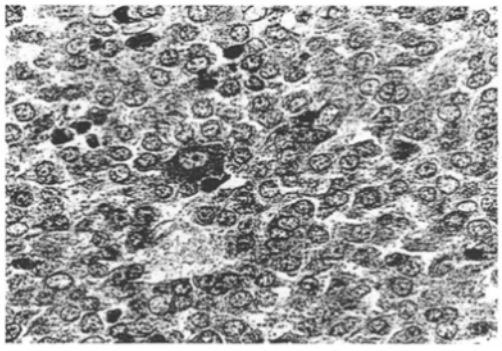

Figure 7. Sparsely granulated GH-cell adenoma with cytoplasmic immunostaining for cathepsin D (anti-cathepsin-D-hematoxylin-eosin, ×400).

greater than the number of actually observed mitoses, which may explain why we did not find a positive correlation between Ki-67, PCNA, and the number of mitoses.

Although a significant correlation existed between PCNA content and Ki-67 in some tumors, no correlation could be obtained in others [10,16,28,50]. Significant correlations between PCNA content and Ki-67 were not demonstrable, which may be influenced by fixation procedures in the immunomethods for PCNA and Ki-67. Ki-67 antigen is known to be very sensitive to fixation [29]. For PCNA, immunohistochemical detection is greatly reduced after formalin fixation longer than 48 hours or after heat exposure [29], whereas the time between tissue removal and formalin fixation does not seem to have a negative influence on the immunoreactivity of PCNA [57]. In our material, these factors may have influenced the immunoreactivity of PCNA, and they may have prevented a significant correlation between PCNA and Ki-67.

Recently Hsu and co-workers [34] reported a significantly higher mean percentage of PCNA-positive cells in recurrent adenomas than in nonrecurrent adenomas. In addition, the recurrent adenomas had a higher mean percentage of PCNA-positive cells than the initial ones. Our investigations included 3 recurrent adenomas and 4 adenomas that recurred later. In nonrecurrent adenomas, the mean value of PCNA-positive nuclei was 0.85/HPF, whereas the 3 recurrent adenomas had a mean value of 1.6 PCNA-positive nuclei/HPF. The 4 initial adenomas that recurred later showed a higher mean value (3.1/HPF) of PCNA-positive nuclei, but significant correlations between the number of PCNA-positive cells and recurrence rate were not found.

Although flow cytophotometry (FCM) and image cytophotometry (ICM) are widely used in assessing the proliferative activity of malignant tumors [6,21,22,43], the results in endocrine tumors vary greatly [37]. In the literature using FCM for measurement, aneuploid DNA patterns were described in 9% (n = 32 [1]). 20% (n = 59 [41]). 37.5% (n = 24 [2]), 41% (n = 29 [3]), and 49% [4]). Zbieranowski and Murray [63] reported between 9 and 55% (average index, 37%) aneuploid DNA patterns. Our results suggest that aneuploidy occurs more often in endocrine-active adenomas, especially in prolactinomas and plurihormonal GH/PRL/glycoprotein adenomas, which corresponds with the findings of other authors [3,4,41]. Within the different adenoma types, Lüdecke and colleagues [41] found the highest rate of aneuploidy (36%) in prolactinomas and the lowest in endocrine-inactive adenomas. Anniko and coworkers [3,4] found the highest rate (57%) [3] and 80% [4], respectively, in GH/PRL-cell adenomas; 70% of the prolactinomas were aneuploid, whereas the endocrine-inactive adenomas showed aneuploid DNA patterns in 20%. In contrast to Anniko and colleagues, who observed hypodiploid DNA patterns only in GH/PRL-secreting adenomas (23%; n = 13 [4]), we demonstrated hypodiploid DNA patterns in 3 endocrine-inactive adenomas.

Using flow cytometric studies to measure the S-phase, Hulting and associates [36] reported an S-phase fraction between 3.3 and 19.5% (n = 33; GH adenomas); Anniko and Wersäll [5], between 3 and 21% (n = 84); and Fitzgibbons and colleagues [20], between 9.1 and 24.7% (n = 13; only euploid adenomas). An S-phase fraction of more than 10% was measured in 36% [36] and in 19 of 84 (23%) adenomas [5]; 40% aneuploid adenomas versus 8% euploid adenomas showed more than 10% cells in the S-phase [5]. Anniko and co-workers [4] described that all tumors with more than 10% cells in the S-phase were aneuploid, disregarding the fact that none of the authors [4,5,36] could establish a correlation between S-phase fraction and DNA status.

In our study, the S-phase fraction varied between 2.97 and 28%, and 73.3% aneuploid adenomas versus 42.9% euploid adenomas showed more than 10% cells in the S-phase. Our higher percentage and range of cells in the S-phase may be based on the lower resolution in histograms obtained by image cytometry compared with flow cytometry, in which more than 10,000 can be measured. Anniko and coworkers [4] and Zbieranowski and Murray [63] emphasize that the S-phase could only be calculated roughly in histograms obtained by image cytometry.

The high percentage found by all au-

thors of the S-phase fraction measured by flow and image cytometry is still in contrast to the clinical behavior of slow-growing pituitary adenomas [40] and to the findings obtained from a study by Nagashima and associates [47], who measured the S-phase fraction in 21 pituitary adenomas by incorporation of 5-bromodeoxyuridine (BUdR). The S-phase fraction was less than 0.1% in 9 tumors, between 0.1 and 0.5% in 7, and greater than 0.5% in 5. Although they did not find a correlation to type of adenoma, tumor size, duration of symptoms, or adenoma recurrence, they described the lowest S-phase fraction in prolactin-cell adenomas (<0.1–0.66%) and the highest in two Nelson tumors (1.26% and 1.46%).

In our study, significant correlations between DNA status, S-phase fraction, and recurrence rate were not found. Even the endocrine-inactive adenomas revealed more often a euploid DNA pattern; the endocrine-inactive null-cell adenomas with the highest rate of recurrence showed 4 (66.7%) aneuploid and only 2 euploid (33.3%) patterns. Both euploid adenomas recurred, whereas 1 aneuploid adenoma did not.

In a study by Fitzgibbons and colleagues [20], euploid nonrecurrent adenomas (n = 7) showed a higher median percentage (19.4%) and range (9.5–24.7%) of cells in the S-phase than euploid, recurrent adenomas (n = 3; median, 13.0%; range, 9.1–16.7%). In our material, there was only a slight difference in the 2 groups: euploid, nonrecurrent adenomas (n = 5) had a median percentage of 15.0% (range, 6.0–22.12%) versus 16.5% in euploid, recurrent adenomas (n = 2; range, 6.93–16.46%). We concluded that the rate of recurrence did not depend on DNA status or S-phase fraction, but on other factors, especially residues of adenoma tissue.

Almost all adenomas showed EGF-positive cells, and no significant correlation was detected between EGF content and rate of recurrence. Seven of the 15 observed EGF-positive adenomas recurred, whereas 8 EGF-positive adenomas did not. A statistically significant difference ($p < 0.01$) in EGF content was found only in prolactinomas, which contained a lower number of EGF-positive cells. Interestingly, all prolactinomas were sparsely granulated, showed only moderate differentiation, had an aneuploid DNA content, and did not show any mitoses. These findings may be explained by the lower mitogenic effects of EGF because of its lower content in these adenomas, but they remain in conflict with the fact that EGF stimulates prolactin mRNA and prolactin secretion in vitro in several cell lines (e.g., GH3 and GH4C1 cells) [12,18,27,64].

The content of the lysosomal endopeptidase cathepsin D in the adenomas seems to correlate with the endocrine activity. We found a significantly higher cathepsin D content ($p < 0.05$) in endocrine-active adenomas than in endocrine-inactive ones. This finding agrees with ultrastructural findings of endocrine-active adenomas containing more lysosomes. Because 6 of 7 recurrent adenomas were endocrine-inactive and contained a lower catephsin D content, a statistically significant correlation ($p < 0.05$) between lower cathepsin D content and higher rate of recurrence was measured. However, we cannot conclude that a lower cathepsin D content is associated with a higher rate of recurrence. This higher rate of recurrence in endocrine-inactive adenomas may be based on the fact that it is difficult to assess whether removal of endocrine-inactive adenomas is total and therefore the propensity to recur is greater.

In conclusion, using DNA measurement, morphological structures, growth factors, and proliferation markers to assess the proliferative activity of adenomas, we were not able to find any discriminating methods.

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