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Specific alterations of the basement membrane and stroma antigens in Human pituitary tumours in comparison with the normal anterior pituitary. An immunocytochemical study

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Summary. Our report is the first immunocytochemical study of the principal elements of the basement membrane (BM) and connective tissue in normal and adenomatous human anterior pituitaries. In normal tissues, both the parenchymatous BM limiting the endocrine cell cords and the endothelial BM around the capillaries were continuous and were stained with anti-laminin (LM), anti-type IV collagen (CIV) and anti-fibronectin (FN) antisera. Antiserum to type I collagen (CI) stained the connective tissue only. The same antigens were investigated in 23 human pituitary adenomas, 6 of them having been diagnosed as locally invasive by the radiologist and the neurosurgeon. In all cases a lack of cordal structure was observed and the parenchymatous BM was completely absent (9 cases) or fragmented (14 cases). No correlation could be established between the extent of parenchymatous BM alterations and the invasive behaviour of the tumour. In contrast, a continuous endothelial BM was observed around the blood vessels in all cases and its presence was confirmed in double immunofluorescence experiments using anti-von Willebrand factor and anti-LM or anti-CIV antisera. Anti-FN and CI also stained the wall of the vessels. The tumours showed arterial development, in addition to the capillaries found in normal tissue. The present results favour the hypothesis of a decreased synthesis of parenchymatous BM by human adenomatous pituitary cells in comparison with normal cells and show that these tumours are the site of an active arterial neovascularization.

Key words: Pituitary adenoma – Human anterior pituitary – Basement membrane – Immunocytochemistry – Neovascularization

Introduction

Human pituitary tumours are the most frequent intracranial neoplasms and can be responsible for different endocrine and tumour symptoms. In most instances hormonal hypersecretion is associated with cellular proliferation. In non-secreting pituitary tumours, however, which represent 20–30% of cases, a mass effect is the only symptom that reveals the neoplasm. The majority of pituitary adenomas are considered to be benign; metastatic pituitary carcinomas are exceptional (Kovacs and Horvath 1986). However, pre-operative radiological features and macroscopic findings at surgery show that a large proportion of these tumours display locally aggressive behaviour, invading local structures such as the optic chiasm, the diaphragma sellae and the sphenoidal or cavernous sinus.

Most studies on pituitary tumours have focused on the secretory activity of the adenoma cells. Few concern the connective tissue compartment, the importance of which is widely recognized in the study of the invasiveness and growth of tumours (Liotta et al. 1986). Routine pathological examinations based on reticulin staining or electron microscopy have shown that pituitary adenomas are devoid of a capsule and that they lack the reticulin network or the parenchymatous basement membrane (BM) which surrounds every cell cord in the normal anterior pituitary (Racadot et al. 1975; Velasco et al. 1977). Two recent studies have concerned the vascularization of pituitary adenomas. These have shown that in contrast with the normal gland, the blood supply of which is provided almost exclusively by capillaries fed by the hypothalmo-hypophyseal portal venules, systemic arteries were seen in the large majority of the different types of pituitary adenoma routinely processed for light (Racadot et al. 1986) or electron microscopy (Schechter et al. 1988).

The purpose of the present study is: (1) to deepen our knowledge of the organization of the connective tissue compartment in secreting or non-secreting adenomas of different clinical grades and the normal tissue by the immunocytochemical localization of various antigens of the extracellular matrix, connective tissue and endothelium; (2) to attempt to correlate the immunocytochemical findings with the invasive behaviour of the tumour and the process of neovascularization.

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Table 1. Clinical and pathological data

Case no.	Sex	Age (years)	Grade[a]	Local[b] invasion	Histological appearance
ACTH-secreting adenoma					
1	F	60	I	–	Papillary
TSH-secreting adenoma					
2	F	21	II	–	Trabecular
GH-secreting adenoma					
3	F	42	0	–	Trabecular
4	F	33	I	–	Massive
PRL-secreting adenoma					
5	M	41	III	–	Trabecular
6	M	53	I	st+ss	Massive
7	F	45	0	–	Massive
PRL-GH-secreting adenoma					
8	M	32	III	st+ss+cs	Massive
9	M	42	0	–	Trabecular
10	F	37	0	–	Massive
LH/FSH-secreting adenoma					
11	M	60	II	–	Massive
12	M	41	II	st+ss+cs+d	Massive
Non-secreting adenoma					
13	M	65	III	–	Massive
14	M	75	III	st+ss	Massive
15	M	59	0	–	Massive
16	M	53	II	–	Massive
17	M	30	I	ss	Massive
18	M	51	I	–	Trabecular
19	M	36	I	–	Trabecular
20	M	76	I	–	Trabecular
21	M	54	III	ss	Massive
22	M	41	III	–	Massive
23	F	43	II	–	Trabecular

[a] 0, Microadenoma; I, tumour which reached the optic chiasm; II, suprasellar extension amputating the anterior recess of the third ventricle; III, tumour which reached the foramen of Monro

[b] –, No invasion; st, sellae turcica destroyed; ss, sphenoidal sinus invaded; cs, cavernous sinus invaded; d, diaphragma sellae destroyed

Materials and methods

Twenty-three pituitary tumours were obtained from 16 men (30–76 years old) and 7 women (21–60 years old) operated on at the neurosurgery unit, Hôpital Foch, Suresnes, France. The samples included 3 prolactin (PRL), 2 growth hormone (GH), 3 bihormonal PRL and GH, 1 corticotropin (ACTH), 1 thyroid-stimulating hormone (TSH) and 2 gonadotropin (LH/FSH) secreting adenomas. The other 11 tumours were not associated with clinical or biological evidence of anterior pituitary hormone hypersecretion (for more details, see Table 1). Two autopsy specimens of normal anterior pituitaries were obtained less than 9 h after death. All tissue samples were immediately snap-frozen at −80° C.

Indirect immunoperoxidase or immunofluorescence was performed using the following antisera: rabbit purified polyclonal antisera raised to human type IV collagen (CIV), human laminin (LM), human fibronectin (FN) and to human type I collagen (CI) from Pasteur Diagnostic, Lyon, France; mouse monoclonal antibody against human von Willebrand factor (WF), donated by Dr. D. Meyer (INSERM U. 143, Paris, France); goat anti-rabbit immunoglobulin (Ig) antiserum and rabbit anti-mouse Ig antiserum conjugated to horseradish peroxidase (Biosys, Paris, France); goat anti-rabbit Ig antiserum conjugated to fluorescein isothiocyanate (Biosys); and sheep anti-mouse Ig antiserum conjugated to Texas red (Amersham, Paris, France).

Cryosections, 8 μm in thickness, were dried and fixed in absolute ethanol. The indirect immunoperoxidase technique was used to localize a single antigen on a section according to the following protocol. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in ethanol for 30 min and the sections were washed in phosphate buffer saline (PBS) pH 7.4 (3 × 5 min). The sections were covered with 10% bovine serum albumin (BSA) in PBS for 30 min. The excess of BSA was removed and the sections covered with the primary antibody overnight at 4° C in a moist chamber. Polyclonal primary antibodies to CIV, LM, FN and CI were used at a 1:500 dilution in PBS to which 1% BSA and 0.05% sodium azide were added. After incubation, the sections were washed in PBS (2 × 20 min) and covered with 10% BSA for 30 min. The excess of BSA was removed and the sections covered with the peroxidase labelled anti-rabbit Ig antibody at a 1:200 dilution in PBS 1% BSA for 2 h at room temperature. The sections were washed in PBS (3 × 10 min).

Peroxidase activity was visualized by applying to the sections a freshly prepared mixture of 0.5 mg/ml 3-3′diaminobenzidine (Sigma, Paris, France) and 0.005% hydrogen peroxide in PBS. The sections were washed in distilled water (3 × 5 min). Before mounting, the sections were counterstained with 1% haematoxylin.

On control sections non-immune rabbit serum was substituted for the first antibody.

Double labelling was performed on frozen sections using the indirect immunofluorescence method (Burtin et al. 1983). At first, the sections were treated with the mouse monoclonal anti-WF antibody that was revealed by the sheep anti-mouse Ig serum conjugated to Texas red. A second immunostaining was carried out with

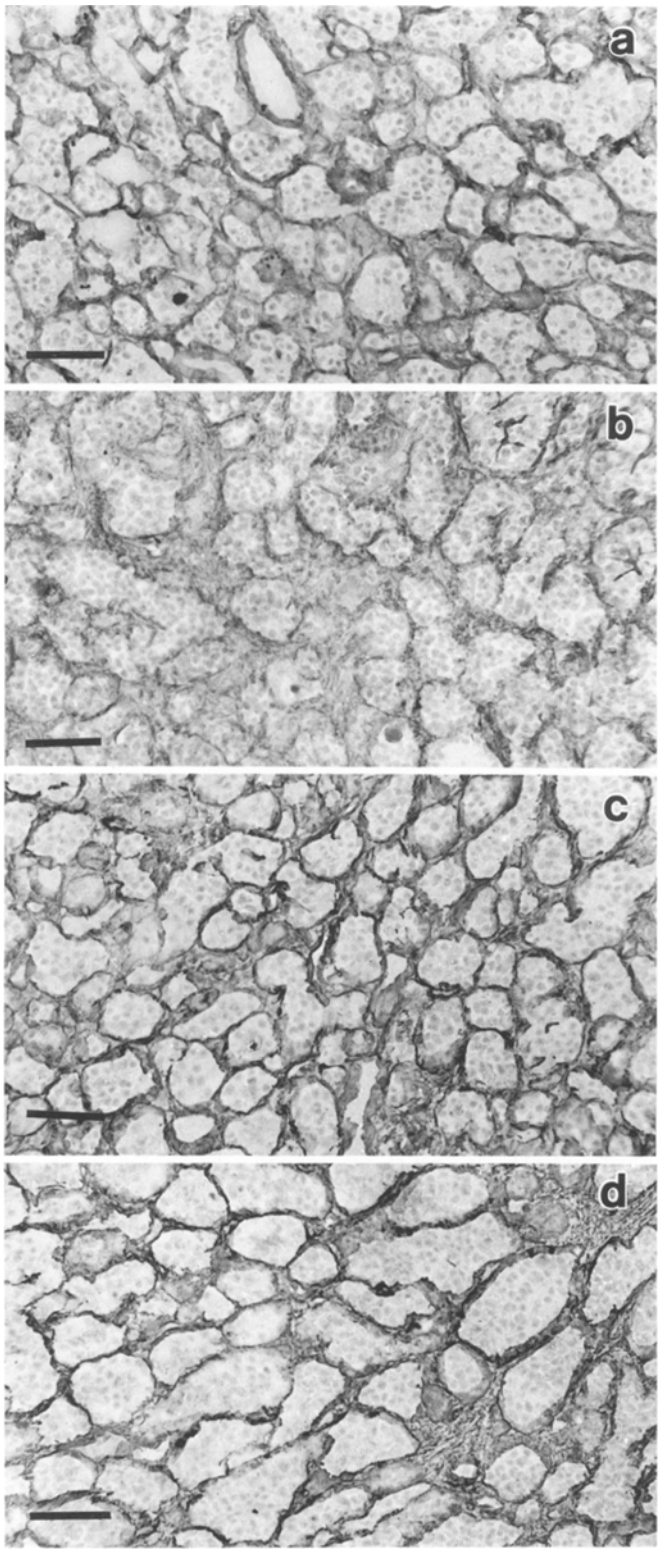

Fig. 1. Human normal anterior pituitary stained by anti-fibronectin (FN) (**a**), anti-type I collagen (CI) (**b**), anti-laminin (LM) (**c**) and anti-type IV collagen (CIV) (**d**) antisera. FN, LM and CIV are present in basement membranes (BM) and connective tissue, but CI is essentially localized in connective tissue. Indirect immunoperoxidase on frozen sections. ×100; *bar* = 100 μm

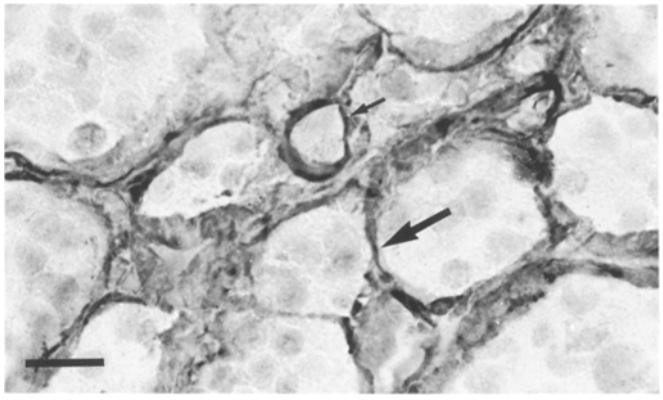

Fig. 2. Human normal anterior pituitary stained by anti-LM antiserum showing the two types of BMs; the parenchymatous BM (*long arrow*) around the endocrine cell cords, and the endothelial BM (*short arrow*) around the capillaries. Indirect immunoperoxidase on frozen sections, ×400; *bar* = 25 μm

one of the rabbit polyclonal antibodies followed by the goat anti-rabbit Ig serum conjugated to fluorescein. Control experiments showed that the various immunoreagents did not cross-react.

The sections were examined with a Zeiss photomicroscope. Photographs were taken on APX25 Agfa film for immunoperoxidase staining and TriX 400 Kodak film for immunofluorescence with an automatic Orthomat camera.

Results

In normal tissue, two different BMs were regularly observed with the anti-LM and anti-CIV antisera. One was the epithelial or parenchymatous BM limiting the cell cords comprising the various endocrine cells of the anterior pituitary and the other was the endothelial BM surrounding the numerous capillaries interspersed between the cell cords. These BMs were continuous and delimited the connective tissue of the anterior lobe, the perivascular and pericordal spaces. Both BMs were strongly stained with antisera against LM or CIV. In addition, the connective tissue presented weak staining for LM but was more intense for CIV. Antiserum against FN, while staining the connective tissue and the vascular BM strongly, stained the epithelial BM weakly. In contrast, the anti-CI antiserum stained a fibrillary material in the connective tissue compartment only (Figs. 1, 2, 5a).

The four antisera raised to the extracellular matrix components gave positive results on frozen sections of all 23 pituitary tumours that were studied (Table 1). Whatever the type, the architecture varied, but in contrast to the normal gland, adenomatous anterior pituitary cells were no longer organized in individualized cell cords (Figs. 3, 4, 5b–d). Most adenomas had a massive parenchyma (14 cases). Alternatively, the adenomatous cells had a trabecular architecture, the cells forming palisades in the neighbourhood of blood vessels (8 cases). Papillary organization of the tumour cells was observed in only 1 case. The comparison of adjacent sections showed that anti-LM and anti-CIV antisera stained the same structures. The common feature of these different

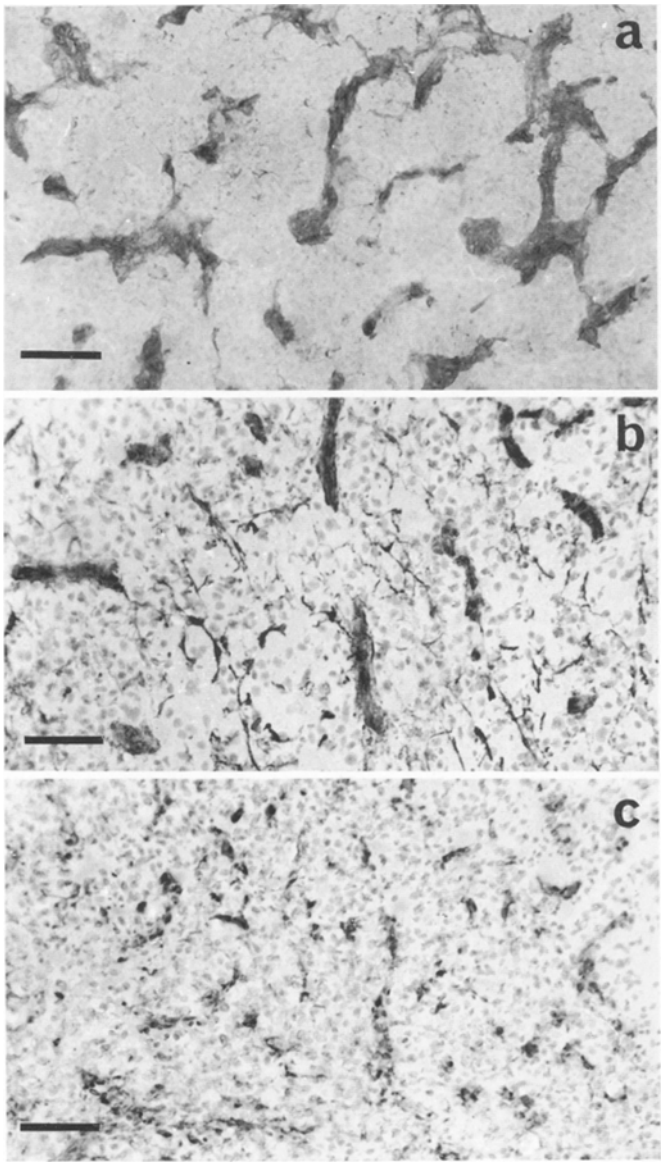

Fig. 3. Pituitary adenomas (**a** case 14, **b** case 2, **c** case 4; nos. refer to Table 1) stained by the anti-LM antiserum. Thick strips of immunoreactive material associated with the vessels are seen in the three cases. In **b** residual thin structures of parenchymatous BM are present between the adenomatous cells. Indirect immunoperoxidase on frozen sections, ×100; *bar* = 100 μm

patterns of tumour organization was the systematic absence of the regular cell cords that were observed in the normal tissue. Along with the absence of cell cords, abnormalities of the parenchymatous BM were always seen. Regular parenchymatous BM was not seen in any of the tumours. The most prominent anomaly was complete absence, noted in 9 cases (Fig. 4). In the other 14 cases (2 prolactinomas, 1 somatotropic adenoma, 1 corticotropic adenoma, 1 gonadotropic adenoma, 1 thyrotropic adenoma and 6 non-secreting adenomas) the immunoreactive material consisted of variably fragmented strips of BM that did not define a consistent cordal structure (Fig. 3). Immunoreactive FN and CI were often seen in association with the abnormal paren-

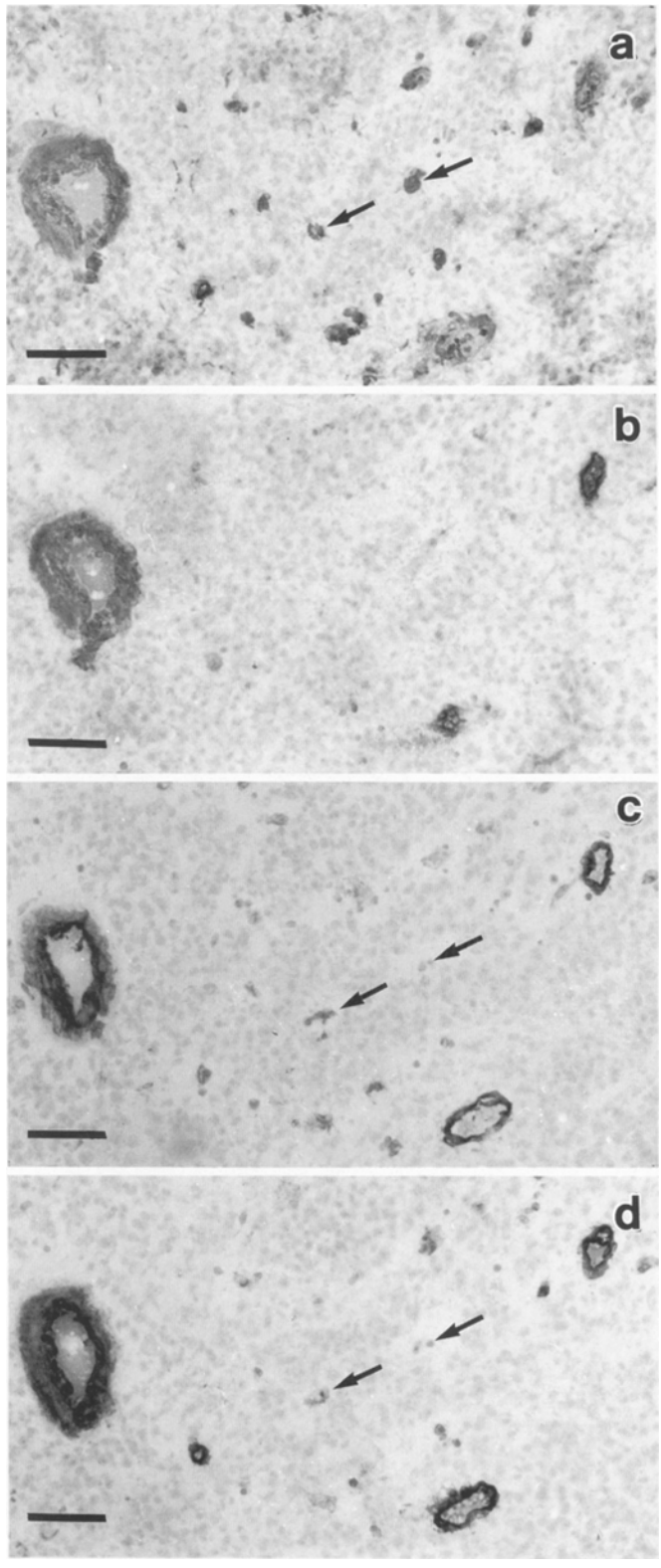

Fig. 4a–d. Pituitary adenoma (case 12 of Table 1). Adjacent sections are stained by anti-FN (**a**), anti-CI (**b**), anti-LM (**c**) and anti-CIV (**d**) antisera. Small vessels are intensely stained by the anti-FN antiserum but are weakly immunoreactive to the anti-LM and anti-CIV antisera (*arrows*). The anti-CI antiserum only stained the arterioles. The latter are also stained by the three other antisera. Indirect immunoperoxidase on frozen sections, ×100; *bar* = 100 μm

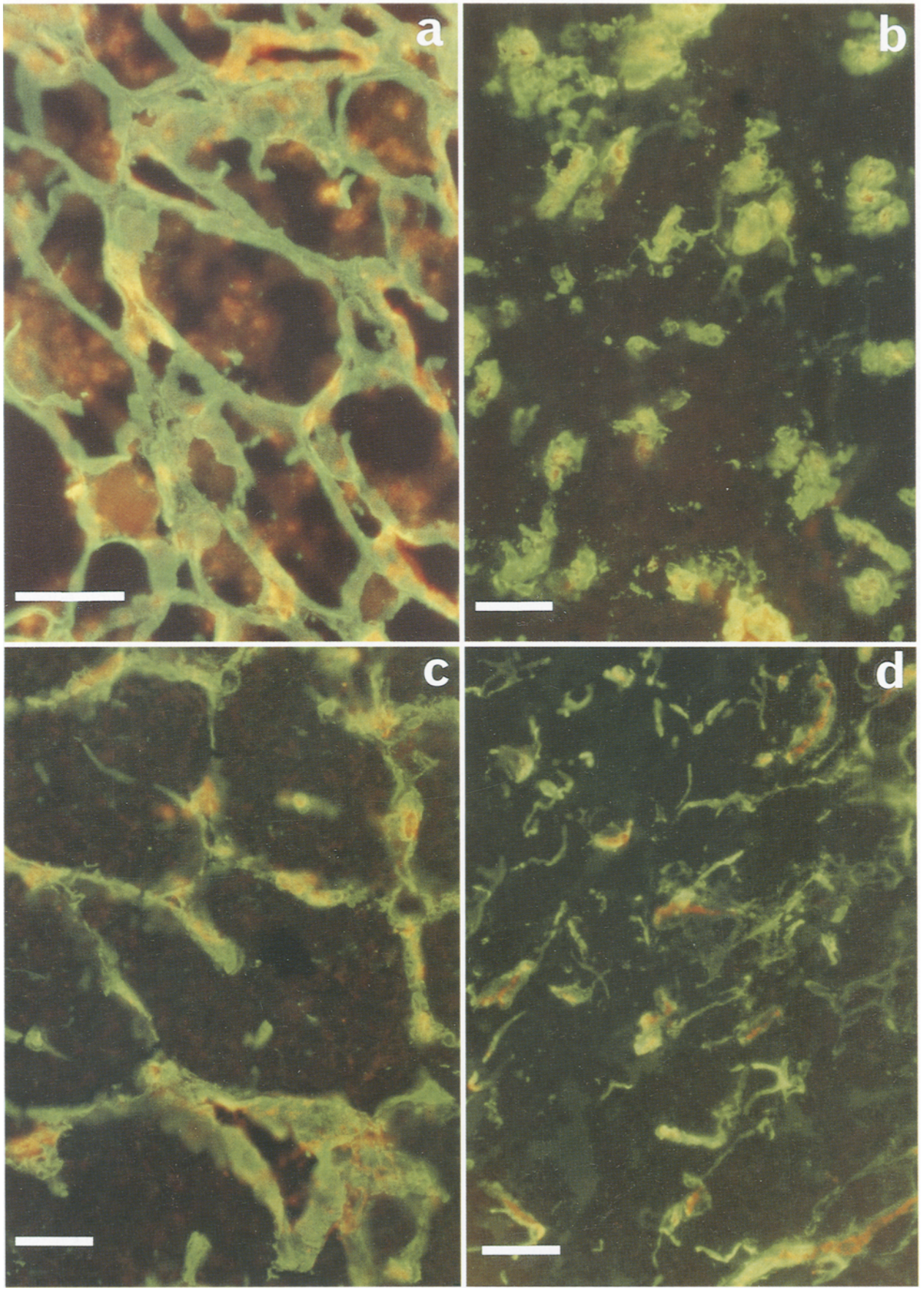

Fig. 5. Human normal anterior pituitary (**a**) and pituitary adenomas (**b** case 12, **c** case 14, **d** case 2; nos. refer to Table 1). The endothelial cells are stained by the anti-von Willebrand factor antibody (Texas red fluorescence) and the BMs by the anti-laminin antiserum (FITC fluorescence). **a** In the normal tissue, the cell cords and the capillaries or venules are delimited by regular BMs. Some endocrine cells display a non-specific red fluorescence. ×400; *bar* = 50 μm. **b–d** A continuous BM is present around the blood vessels. In **c** and **d**, short strips of BM, interspersed among the adenomatous cells and not always associated with blood vessels, are frequently seen. Double immunofluorescence experiments on frozen sections, ×300; *bar* = 50 μm

chymatous BM. Specific alteration of the parenchymatous BM could not be related to the type of adenoma or to the local invasiveness.

An obvious difference from normal tissue was the presence of systemic arteries in all the tumours studied (Fig. 4). In contrast with the parenchymatous BM, a continuous vascular BM was clearly seen around all the blood vessels by the anti-LM and anti-CIV antisera. Different patterns of staining were, however, noted with the anti-FN and anti-CI antisera with regard to the nature of the vessels. While the anti-FN staining was intense around the capillaries, it was generally weak around arterioles. The anti-CI antiserum gave an intense staining only around the arterioles (Fig. 4).

In immunofluorescence experiments, the anti-WF stained the endothelial cells whatever the type of vessels. When anti-LM or anti-CIV staining was performed on the same sections, double immunofluorescent staining confirmed the presence of a continuous BM around the blood vessels. The perivascular BM represented the bulk of the immunoreactive material in the tumours. In addition, double staining experiments revealed that, when they were present, the short strips of BM that were interspersed among the adenomatous cells were not always associated with capillaries and might represent sparse elements of parenchymatous BM (Fig. 5).

Discussion

In normal tissues LM and CIV are considered to be specific components of the BM; FN is localized in the BM and the connective tissue while CI is only present in the connective tissue (Laurie et al. 1983; Leblond and Inoue 1989; Martin and Timpl 1987). In the normal anterior pituitary, our observations on FN and CI localizations fit into the general picture. Our results, however, indicate the presence of LM and CIV in both the BM and the connective tissue. Evidence for LM in the connective tissue compartment of the normal rat anterior pituitary has been reported previously (Tougard et al. 1985).

In pituitary adenomas, the absence of a regular parenchymatous BM (the BM that surrounds every cell cord in the normal gland) was a consistent finding. This confirms previous data obtained by routine reticulin staining or electron microscopy (Racadot et al. 1975; Velasco et al. 1977). The contribution of the present immunocytochemical localization of specific markers of the BM and the extracellular matrix was to demonstrate that the disruption or the absence of the parenchymatous BM is the consequence of the simultaneous disappearance of the antigens sought. This disruption of the parenchymatous BM may have several consequences. At first, due to the fact that they are no longer anchored to the BM, adenomatous cells loose the functional polarity that plays an essential role in their differentiation and secretory activity (Holck et al. 1986). Subsequently, adenomatous cells lack that structural barrier to invasion which the BM represents (Liotta et al. 1986). The loss of parenchymatous BM appears to be a general and cardinal feature of pituitary tumours, occurring in all adenomas regardless of their secretory activity, size or clinical invasiveness. In contrast, our results showed that the vascular BM was continuous both in invasive and non-invasive adenomas. It is noteworthy that the integrity of the vascular BM and of the vessel wall is consistent with the very low metastatic potential of pituitary tumours.

The lack of a well-defined parenchymatous BM may result either from a decreased synthesis of the BM components or from an increase in proteolysis. However, given the similar alterations of the parenchymatous BM occurring in invasive and non-invasive tumours and the integrity of the vascular BM seen in both types, our results favour the hypothesis of a decreased parenchymatous BM synthesis in all pituitary tumours.

Small and large new vessels in the adenomas displayed different immunostaining patterns, most obviously in FN and CI immunoreactivities. Small vessels were intensely stained by the anti-FN antiserum but were devoid of immunoreactive CI. In contrast, large vessels were weakly stained by the anti-FN antiserum but heavily stained by the anti-CI antiserum. These data are in accordance with several models of angiogenesis in which FN has been shown to be the first component to appear around newly formed vessels and is followed by deposits of LM, CIV and, finally, by CI and CIII. During the process, FN concentrations diminish as the vessels become more differentiated (Ansprunk et al. 1991; Dvorak 1986; Nicosia and Madri 1987; Paku and Paweletz 1991). Evidence for the development of an arterial network in all pituitary adenomas is a morphological marker of the functional disconnection of tumour tissue from the hypothalamic neuroendocrine control exerted in the normal gland, via the blood of the hypothalamo-hypophyseal portal venules (Racadot et al. 1986). This arterial neovascularization also indicates autonomy of the control of tumour growth.

This study shows that striking differences of extracellular matrix organization and vasculature exist in pituitary tumours when compared with normal anterior pituitary tissue. Further studies are needed to define criteria for malignancy and to determine the dynamics of tumour progression in human pituitary adenomas.

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