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Alterations of basement membrane in di-isopropanolnitrosamine-induced carcinogenesis of the rat thyroid gland: an immunohistochemical study

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Abstract Alterations of basement membrane (BM) in di-isopropanolnitrosamine (DIPN)-induced carcinogenesis of the rat thyroid gland were examined by means of immunohistochemical localization of collagen type IV (CN-IV), laminin (LN), and fibronectin (FN) in pre-nodular and nodular thyroid lesions, correlating with the morphogenesis and proliferative activity of these lesions. Adult male rats of the Wistar strain were injected s.c. in the back with DIPN, and the thyroid glands were removed at the 15th and 30th week of treatment. Each of 133 thyroid lesions was histochemically analyzed. The follicular epithelial BM as revealed by CN-IV and LN was discontinued or completely lost during the progression of thyroid lesions from pre-nodular to nodular lesions and finally overt carcinomas. At the same time, the BM of vascular endothelial cells demonstrated a loss of dense capillary networks of follicles, a sinusoidal dilatation and, predominantly in carcinomas, development of interstitial-type blood vessels. However, FN, which was hardly stained in the normal thyroid tissue, was remarkably deposited in the interstitium of invasive carcinomas. These observations strongly suggested that alterations of BM structure play a key role in the morphogenesis of rat thyroid tumors, and that the expression of FN is an important step in the invasive growth of thyroid tumors.

Key words Di-isopropanolnitrosamine · Carcinogenesis · Rat thyroid gland · Immunohistochemical study

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Introduction

Basement membrane (BM) is a tissue-specific organized component of the extracellular matrix (ECM) [23]. It functions not only to organize tissue-specific structures but also to regulate gene expression [28], morphogenesis in embryos [2], regeneration of tissues [22], cell growth and cell death [2], and the migration, distribution and polarity of cultured cells [12] by transducing signals to integrin and non-integrin receptors on the surface of those cells it is in contact with [26]. Morphologically, the BM of tumor tissues shows a variety of changes, such as frequent discontinuation or complete disappearance, particularly in malignant varieties [25], and an unusual thickening by excess deposition of ECM components [14]. Evidence is accumulating that BM proteins produced by tumor and stromal mesenchymal cells are deeply involved in carcinogenesis [3, 13], differentiation, proliferation, invasion and metastasis of tumor cells [1, 14, 26], and activation of tissue proteinases [27].

In the thyroid glands, ECM molecules are synthesized and secreted both by follicular epithelium and by stromal cells [10, 11]. They stimulate focal proliferation of the thyroid tissue [6] and remodeling of stroma by production of matrix metalloproteinase (MMP) in thyroid tumors [4, 15, 21].

Di-isopropanolnitrosamine (DIPN)-induced rat thyroid tumors provide a useful experimental model with which to investigate the carcinogenesis of human cases, because they clearly demonstrate sequential events from early diffuse hyperplasia of thyroid follicles and the appearance of pre-nodular (type 1) and then sharply demarcated nodular (type 2) lesions consisting of several or more follicles with variable size. Type 2 lesions can be subdivided into hyperplastic nodules (type 2A) and nodules with some atypia (type 2B). Finally, carcinomas (type 3), characterized by higher atypia, proliferative activity, and invasive growth, occupy a large area of thyroid tissue until 30 weeks [18, 21].

We demonstrate herein remarkable alterations of BM as revealed by immunolocalization of collagen type IV

(CN-IV) and laminin (LN) during the carcinogenesis of rat thyroid gland, being accompanied by a phenotypic change of follicular epithelium toward carcinoma, with increased labeling for proliferating-cell nuclear antigen (PCNA) and stimulated expression of fibronectin (FN).

Materials and methods

Animals

The animal experiments were conducted according to the guidelines of Yamanashi Medical University (1993). Male rats (n=48) of the Wistar strain, 130–150 g in initial body weight, were used. They were given a powdered basal diet (Oriental MF, Oriental Yeast Co. Ltd, Tokyo, Japan) and tap water ad libitum. The animals were separated into three groups: group 1 (n=12) injected s.c. in the back with DIPN (Nakarai Tesque Inc., Kyoto, Japan) at a dose of 2,800 g/kg; group 2 (n=24) injected s.c. in the back with DIPN at a weekly dose of 750 mg/kg for 10 weeks; and group 3 (n=12) with no treatment served as the control. The animals of groups 1 and 2 were administered phenobarbital (Kaseikogyo Co. Ltd, Tokyo, Japan) as a promoter for carcinogenesis [18] at a dose of 500 ppm in drinking water from the day of DIPN injection until sacrifice. Half of the animals in each group were sacrificed under anesthesia by ether at the 15th and 30th week of treatment.

Tissues

The thyroid glands were removed immediately after sacrifice. The tissues were immersed in either 20% buffered formalin (pH 7.3) at room temperature, overnight, or 4% paraformaldehyde fixative (pH 7.3) at 4°C, overnight, and embedded in paraffin as usual.

Table 1 Classification of thyroid lesions in di-isopropanolnitrosamine (DIPN)-induced carcinogenesis

Type 1	Pre-nodular lesion of hyperplastic follicles in clusters
Type 2	Nodules consisted of hyperplastic follicles without atypia (2A) or with atypia (2B)*, but no apparent malignancy
Type 3	Carcinoma defined by marked atypia and invasive growth, demonstrating follicular, papillary, and anaplastic patterns

*The type 2B nodules might be classified as “adenoma”, but we avoided using this term, since it is not yet proven that they are true neoplasms and, even if they are, they are definitely benign

Immunoperoxidase

The antisera used in the present study were: anti-CN–IV rabbit antiserum, diluted 1:1000 (LB-1403, LSL); anti-LN rabbit antiserum, diluted 1:100 (L9393, Sigma); anti-FN rabbit antiserum, diluted 1:50 (JBH408, Gibco); and anti-PCNA mouse monoclonal antibody, diluted 1:500 (5A10, MBL). Paraffin sections 2- to 3-µm thick were treated with 0.4% pepsin at 37°C for 30 min. The sections for PCNA staining were pre-treated in an autoclave at 120°C for 5 min in 0.01 M citrate buffer (pH 6.0). In addition, the sections for immunoperoxidase were treated with 3% hydrogen peroxide to neutralize the endogenous activity of peroxidase. All sections were incubated with 1% bovine serum albumin to prevent the non-specific binding of antibodies before the reaction with antibodies. The sections were incubated with each antiserum diluted with phosphate-buffered saline (PBS) at 4°C overnight and, after being washed with PBS (pH 7.3), reacted with peroxidase-labeled anti-rabbit or anti-mouse immunoglobulins (Dako-Japan, Kyoto, Japan) at room temperature for 1 h, stained using 3,3’diaminobenzidine with 0.005% hydrogen peroxide, and counter stained with hematoxylin. For the negative control, the antisera were replaced by the non-immune sera of the corresponding animals.

Classification of the thyroid lesions

Variable histological lesions induced by DIPN were classified according to the criteria proposed in previous papers [18, 21] and shown in Table 1.

Results

Each of 133 thyroid lesions was histochemically analyzed, focusing on BM structure and the expression of FN. The findings are summarized in Table 2.

Thyroid glands of control animals (group 3)

The BM, as visualized by the immunostaining for CN-IV and LN, appeared as a single thin membrane-continuously surrounding the basal surface of follicular epithelium and endothelium of capillary blood vessels which tightly envelop each follicle (Fig. 1a, b). The BM of follicular epithelium at the site of closest contact to capillary blood vessels appeared as a single layer of epithelial and endothelial BM (Fig. 1b). The pattern of immunostaining for CN-IV and LN was essentially the same at the light microscopic level.

Table 2 Summary of representative changes in each lesion

(n=133)	Control	Type 1 (n=12)	Type 2A (n=68)	Type 2B (n=13)	Type 3 (n=40)
Continuity of epithelial basement membrane[a]	++	++	–~+	–~+	–
Networks of blood capillaries[b]	++	+	–~+	–~+	–
Expression of fibronectin[c]	–	–	–	–~+	+~++
(Expression of thyroglobulin[d])	++	+	+	–~+	–~+)

Estimations: the results of histological observation of each lesion (Table 1) were semi-quantitatively estimated according to the following criteria, and representative evaluations accounting for over 50% of each lesion are listed above

[a] Intact continuous ++, discontinuous +, mostly lacking –

[b] Developed ++, reduced +, mostly disappeared –

[c] Negative staining –, weakly positive +, strongly positive ++

[d] Staining of the control ++, reduced staining +, mostly negative –, stronger than the control +++ (cited from the literature [20] with modification)

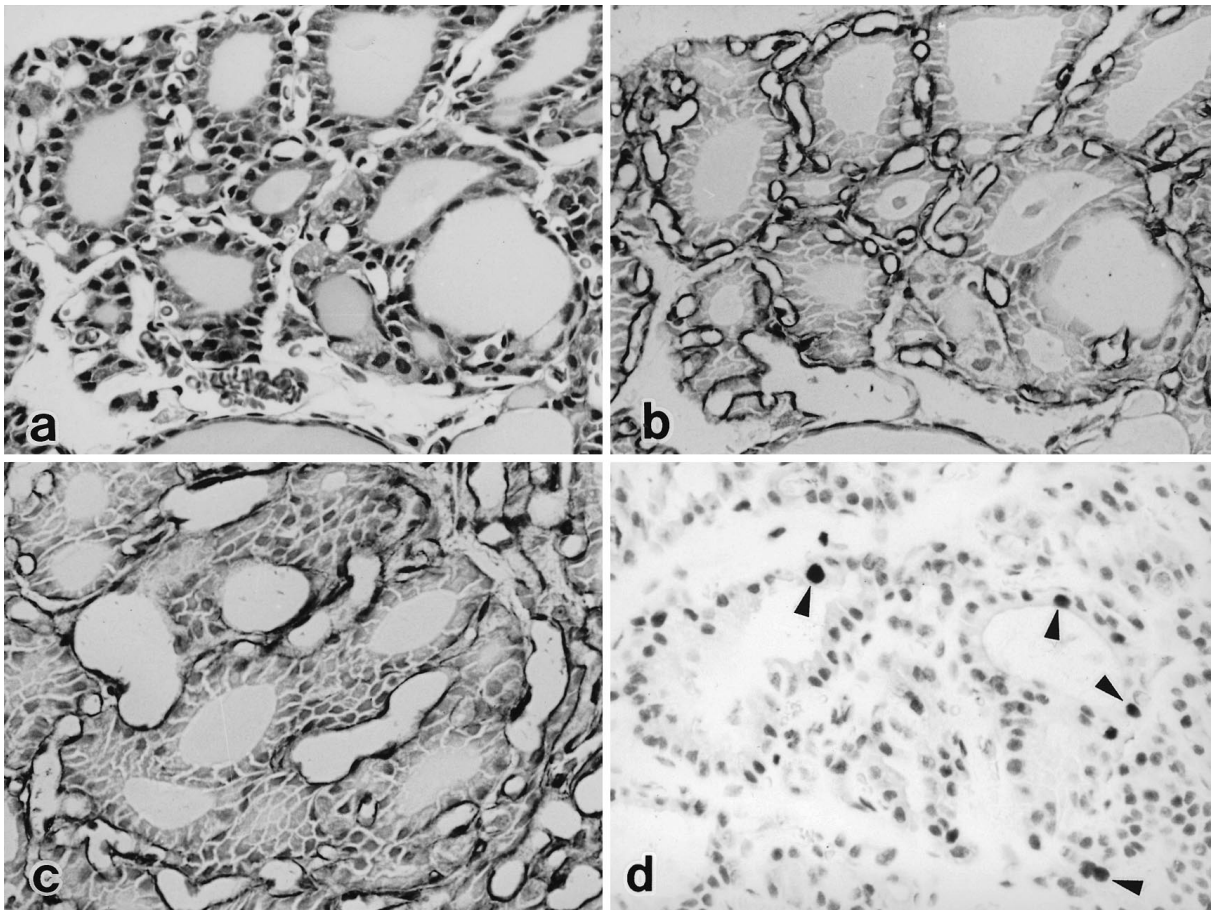

Fig. 1 The thyroid tissue of the control rat (a, b) and a type-1 lesion (c, d), stained by hematoxylin and eosin (a) and for collagen type IV (CN-IV) (b, c) and proliferating-cell nuclear antigen (PCNA) (d). ×340. Note that a fine network of blood capillaries envelops follicles in the control (b) and in the rather coarse and dilated vasculature in type 1 lesion (c). PCNA-positive cells are rare in the control (figure not shown), but sporadically present in type 1 lesions (d, *arrowheads*)

Diffuse hyperplasia

The follicles showed a narrow lumen and irregular shape but no significant change in the staining pattern of BM from the control (figure not shown). The capillary networks were fine and their lumen was dilated.

Type 1 lesions

The BM structure in type 1 lesions (Fig. 1c, d) was almost the same as in diffuse hyperplasia, but with capillary blood vessels more dilated and in a coarse network resulting from increased areas of direct contact between the adjacent follicles (Fig. 1c). A few epithelial cells with PCNA-positive nuclei, which were scarcely detected in the control, existed in these lesions (Fig. 1d).

Type 2 lesions

Type 2 lesions (Fig. 2 and Fig. 3) were characterized by marked dilatation of blood capillaries, particularly in type 2B with a sinusoidal pattern (Fig. 3b). The follicular component of nodules frequently showed cystic dilatation accompanied by remarkable narrowing of blood capillaries, which eventually disappeared as revealed by the immunostaining for BM proteins. At the same time, the follicular epithelium became thinner and disrupted (Fig. 2b). These epithelia showed decreased labeling for PCNA relative to cuboidal cells.

Follicles appeared in type 2 which, despite loss of contact with BM and blood supply, maintained a cuboidal cell shape and proliferative activity (Fig. 2c, d). Interestingly, FN immunostaining, which was negative in the control and type 2A nodules, turned weakly positive in the connective tissue stroma and BM in type 2B lesions.

Type 3 lesions

Most of the type 3 lesions (Fig. 4 and Fig. 5) appeared within type 2 nodules, grew rapidly, and replaced the pre-existing type 2 lesions as type 3 nodules (Fig. 4). These lesions were characterized by a prominent loss of fine networks of capillary blood vessels, which typically develop in endocrine tissues, including the thyroid gland,

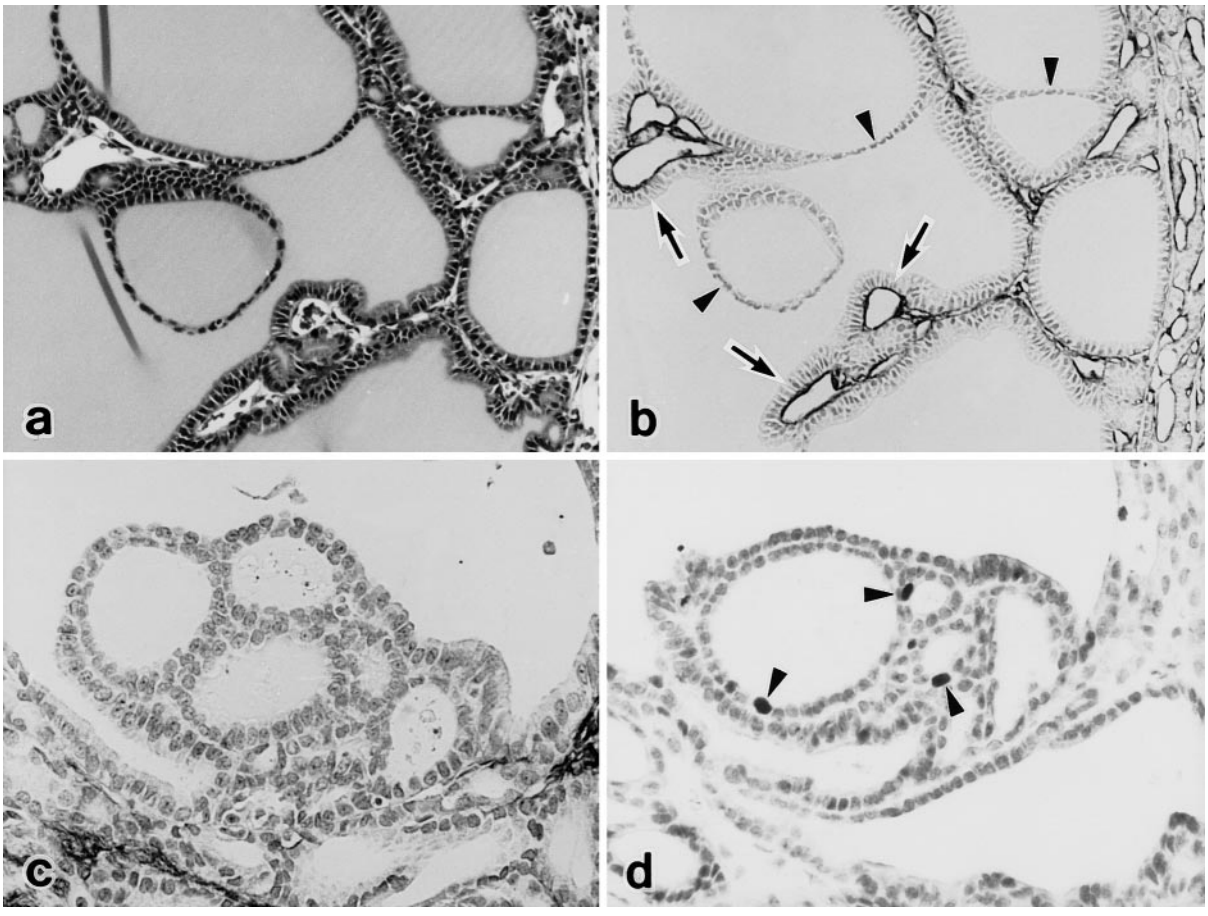

Fig. 2 Type 2A nodules stained by hematoxylin and eosin (a), for collagen type IV (b, c) and proliferating-cell nuclear antigen (d). ×170 (a, b); ×340 (c, d). The nodule is composed of many follicles with variable size, and the distribution of interfollicular blood vessels is irregular and sparse (b, *arrows*). Epithelial cells lacking a basement membrane (BM) lining are extremely flat in shape (b, *arrowheads*) relative to columnar or cylindrical epithelia, which have close contact with the interstitium and blood vasculature. Note follicles in type 2A that, despite loss of the BM lining, preserve cell density, the cuboidal shape of the epithelium (c), and proliferative activity (d, *arrowheads*)

accompanied by involution and disruption of the BM structure of follicles (Fig. 4b and Fig. 5b). Only rarely did lesions of papillary growth demonstrate an incomplete BM structure. The deposition of FN increased markedly in these lesions (Fig. 4c).

In contrast to the decreased density of capillary networks in these malignant tumors, interstitial blood vessels developed markedly and showed expansion of lumen (Fig. 5b). The vascular BM proteins were densely stained at the sites of stromal and capsular invasion of tumors. In the tumor parenchyma, however, BM proteins were incompletely stained except for FN, which was intensively deposited in the stroma (Fig. 5d). The blood vessels in the capsule of thyroid glands were frequently involved in tumor invasion in advanced type 3 lesions, where FN was strongly deposited.

Discussion

The development of DIPN-induced rat thyroid carcinoma assumes a typical pattern of multiple-step carcinogenesis, i.e., from the initial diffuse hyperplasia of follicles to the formation of early pre-nodular lesions (type 1), then either hyperplastic (type 2A) or dysplastic (type 2B) nodules and, finally, overt malignant nodules (type 3). This is accompanied by a gradual increase in proliferative activity, as revealed by the labeling index of BrdU, and a decrease in endocrine function, as revealed by the immunohistochemical detection of thyroglobulin and thyroid hormones [18, 19, 20, 21].

As an extension of our previous studies, we aimed to clarify in the present study alterations of the BM structure of thyroid follicles and blood vasculature during histogenesis of thyroid tumors and to provide further histochemical evidence regarding established roles of BM proteins, particularly CN-IV, LN, and FN in histological alterations, endocrine function [20], growth activity, and invasiveness of tumor tissues at the light microscopic level.

The immunohistochemistry for CN-IV and LN in the control rat thyroid glands demonstrated the presence of a thin layer of BM enveloping each follicle and a fine network of capillary blood vessels, known to be specific to the endocrine organs, as described for the anterior pituitary glands [30]. In nodular lesions of type 2, the mesh

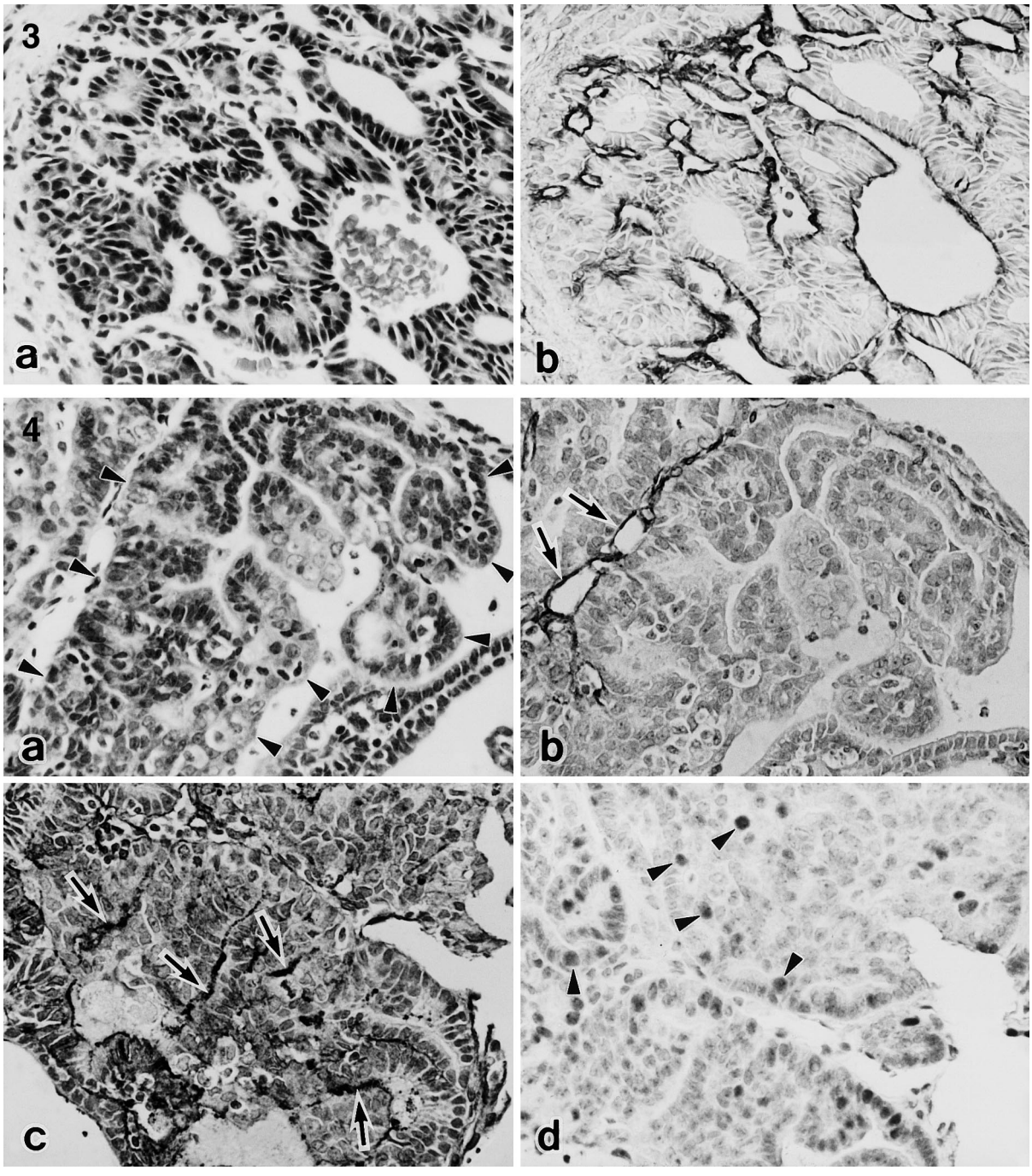

Fig. 3 A type 2B nodule stained by hematoxylin and eosin (a) and for collagen type IV (b). ×340. The interfollicular vasculature is dilated and appeared sinusoidal in shape with increased deposition of basement membrane protein (b)

Fig. 4 Type 3 lesion growing within a type 2B nodule (a, *enclosed by arrows*) stained by hematoxylin and eosin (a) and for collagen type IV (CN-IV) (b), fibronectin (FN) (c) and proliferating-cell nuclear antigen (PCNA) (d). ×340. CN-IV is lost except for basement membrane of preexisting blood vessels (b, *arrows*), but FN is shown to be strongly deposited (c, *arrows*) and PCNA labeling discloses high proliferative activity (d, *arrowheads*)

of capillary blood vessels tended to become more coarse and to take a sinusoidal pattern, particularly in type 2B lesions, and the epithelial BM was frequently shown to be disrupted.

The follicular epithelial cells changed shape, proliferative activity, and expression of endocrine function, depending on the types of nodules. Their shape was flattened in dilated follicles in type 2A nodules and they had decreased growth activity and endocrine function, suggesting dependency on contact with epithelial and/or blood vascular BM for survival. It was reported that the ECM regulates the cell shape of the epithelium and the gene expression related to cell-specific functions, such

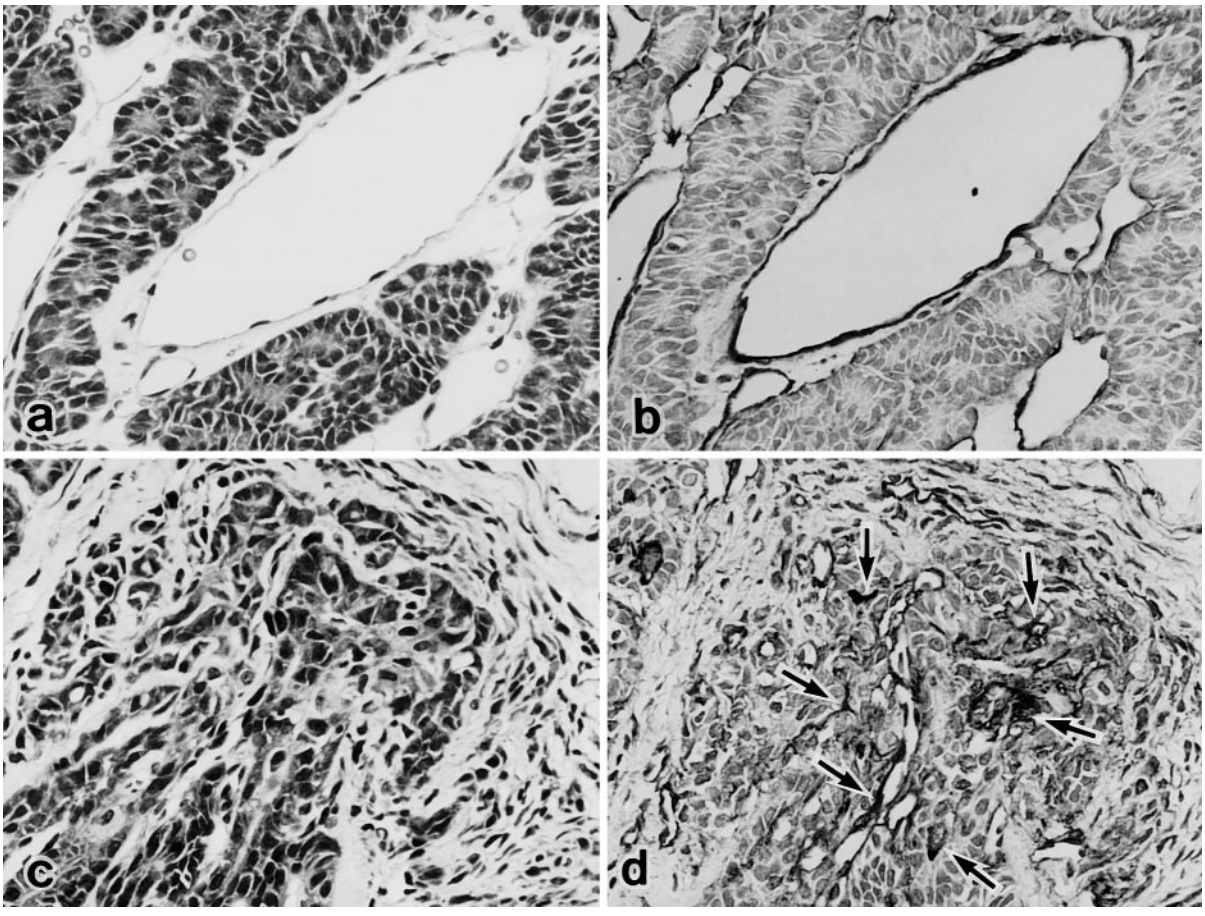

Fig. 5 Type 3 nodules stained by hematoxylin and eosin (a, c) and for collagen type IV (CN-IV) (b) and fibronectin (FN) (d). ×340. The vascular lumen is dilated and basement membrane is thickened by increased deposition of CN-IV (b). The capillary meshes of blood vessels are poorly developed, and tumor parenchyma is separated from endothelium by a layer of loose connective tissue. Note strong deposition of FN in the stroma of tumor tissues at the site of invasion (d, *arrows*)

as the synthesis of the milk protein beta-casein in cultured murine mammary epithelial cells [9].

In contrast to these epithelial cells, cells appeared particularly in type 2B nodules, which maintained a cuboidal shape and proliferative activity without contact with the BM. The observation of these epithelia in type 2B and, more prominently, in type 3 lesions favors the hypothesis of a decrease or loss of dependency of follicular epithelium on BM and further acquisition of a malignant nature to continue proliferation independent of contact with BM proteins. The circumvention of anchorage dependence is thought to play an important role in tumorigenesis 30. Thus, type 2B lesions seemed to demonstrate a transitional form from hyperplastic nodules of type 2A with BM dependency to malignant nodules of type 3 with BM independence.

Changes of BM have been reported in human thyroid tumors, including neoplastic and non-neoplastic lesions [5, 16, 24]. The epithelial BM was frequently interrupted in papillary and anaplastic carcinomas [16], accompanied by higher growth activity in carcinomas than in the other benign lesions of thyroid [17].

The alterations of blood vessels in malignant nodules were characterized by vasculature of a predominantly interstitial type having a thickened BM, as reported in human pituitary adenomas [7] and regarded as characteristic of neovascularization in tumors [8]. In addition, it was revealed that vascular BM was more prominent in follicular carcinomas than in adenomas of human thyroid [16].

Stimulated expression and deposition of FN have been demonstrated in tumor tissues originating from various organs. A restricted expression of oncofetal FN mRNA was shown in human papillary and anaplastic carcinomas of thyroid glands, which are characterized by frequent metastases in lymph nodes and early invasive growth to the surrounding tissues [29]. These observations support the explanation that contact between the tumor cells and the interstitium and vascular endothelium mediated by FN is an essential step in the tumor invasion of interstitium, capsule, and vasculature [13, 14]. Although the present observation that FN began to be expressed in type 2B lesions and is strongly deposited in carcinomas at the peripheral site of invasion affirms the current view on the role of FN in tumors, the exact mechanism by which FN functions, particularly in tumor tissues, remains to be elucidated.

Regarding the mechanism of FN function, it seems worth noting that (1) patients with ovarian cancer who

had a high concentration of FN in ascites showed early metastasis and wide spread of the tumor and (2) FN stimulated cultured ovarian cancer cells to express and activate MMP-9 [27]. This observation supports the possibility that lytic destruction of BM or ECM by activated MMP-9 could promote tumor invasion and metastasis in these patients.

In summary, remarkable alterations of BM structure occurred during the process of experimental carcinogenesis of rat thyroid gland, being accompanied by a phenotypic transformation of follicular epithelium to carcinoma. To clarify the underlying mechanism of the alteration of BM, further studies need to be performed on the subunit structure of BM proteins, alterations in integrin and non-integrin receptors, and the dynamics of expression of MMPs in tumor tissues.

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