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Expression of laminin-2 by normal and neoplastic rat C cells during the development of medullary thyroid carcinoma

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Abstract Medullary thyroid carcinoma (MTC) originates from C cells, which secrete calcitonin (CT), their specific marker. C cells are located in contact with the basement membrane (BM) of the thyroid follicles, which is partly made up of the laminin-2 isoform synthesized by thyrocytes. During oncogenesis, proliferation of the C cells, invading the centre of the follicles, leads to a break in their normal contact with the BM. As specific interactions of cells with BM components, especially laminins, are important for proliferation and differentiation, we investigated the relationships of normal and neoplastic C cells with laminin in the Wag/Rij rat model of human MTC. Immunocytochemical studies showed a progressive loss of the laminin layer underlying the hyperplastic C cell nodules around the large dedifferentiated tumours. The α2, β1 and γ1 chains of the laminin-2 isoform were synthesized and secreted by rat MTC 6–23 cell cultures and the tumours induced by subcutaneous injection of these cells. In situ hybridization combined with anti-CT immunocytochemistry showed a low expression of α2 mRNA on differentiated C cells and thyrocytes, but an overexpression on immunonegative spontaneous MTC and induced intrathyroid tumours. The high level of *α2* gene expression, together with tumour dedifferentiation, suggests a relationship with malignancy.

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Introduction

Medullary thyroid carcinoma (MTC) is a C cell neoplasm that occurs in humans in sporadic or familial forms associated with mutations of the RET proto-oncogene [22]. C and MTC cells secrete calcitonin (CT), their usual biological marker [20]. C cells, a minor component of the thyroid tissue, are always located at the basal aspect of the follicles in contact with the surrounding basement membrane (BM). The Wag/Rij strain of rat has been validated as the closest model to human MTC: a large proportion of 18- to 24-month-old rats develop spontaneous tumours that are morphologically [4] and functionally very similar to this human disease [15, 17, 19].

In cancer, the importance of extracellular matrix (ECM) molecules has been demonstrated mainly in the metastatic process, via adhesion and/or migration of malignant cells. Moreover, cell–matrix interactions initiate signal transduction processes that regulate cell proliferation and differentiation: basic cellular processes which, when deregulated, are involved in oncogenesis. An influence of altered interaction with ECM in C cell evolution towards MTC can be suggested, since between normal thyroid and large spontaneous MTCs a number of histological steps demonstrate obvious modifications in the relationships between ECM and C cells. All young animals exhibit progressive C cell hyperplasia [12]; that is, their perifollicular C cell foci increase in size, gradually surrounding the follicle beneath the BM completely. These cellular rearrangements in the pre-MTC state raise the problem of the origin of the BM around a C cell nodule in the absence of the follicular thyroid cells that would elaborate its constituents [1]. During evolution towards malignancy, dedifferentiation begins as a late step in cells located in the central part of the nodule, i.e., distant from the BM. Many hyperplastic C cells become progressively negative for anti-CT antibodies [15, 17,

19]. Recently, Lausson showed that this decrease in immunoreactivity is associated with a higher mitotic index, indicating an increasing aggressiveness of the CT-negative cells [13]. In humans, the presence of a large number of such dedifferentiated cells correlates with a bad prognosis [17].

The BM is an architecturally complex structure, composed mainly of laminin and type IV collagen, the other usual components being entactin and heparan sulfate proteoglycans [31–33, 36, 37]. It is thought to play a central part in a variety of physiological and pathological processes, principally via the laminin family, which is implicated in cell attachment, spreading and motility, cell division, differentiation and gene expression [2, 7, 16, 30]. Laminins are large glycoproteins (800 kDa) composed of three polypeptide subunits, one heavy chain, α, and two light chains, β and γ, linked by disulfide bonds (nomenclature in [5]). Eleven different laminin isoforms have been reported. Laminin-1, the first molecule identified in an Engelbreth-Holm-Swarm (EHS) tumour, is characterized by the heavy chain, α1, of 400 kDa linked to the β1 and γ1 light chains (200 kDa each). The laminin-2 isoform has a heavy chain, α2 (380 kDa), instead of α1.

The present work shows that in rat MTC, the basement membrane was maintained around the hyperplastic follicles and C cell nodules but progressively became defective on the large dedifferentiated tumours. The tumour cell line rMTC 6-23 isolated from a rat MTC [38] synthesizes laminin-2 both in cultures and in subcutaneous malignant tumours induced by in vivo injection of these cells. In in situ hybridization, an unexpected overexpression of α2 mRNA was revealed in the dedifferentiated C cells of both spontaneous and induced tumours.

Materials and methods

Rats of the Wag/Rij strain were raised according to the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and French laws on the protection of animals. The breeding stock was obtained from the TNO Institute (Rijswijck, The Netherlands). A spontaneous MTC develops in 50–60% of the 2-year-old rat population.

The rMTC 6-23 cell line, originating from a spontaneous Wag/Rig rat MTC [38], was purchased from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 15% horse serum and 2.5% fetal calf serum, in a humidified chamber at 37°C with 5% CO_2. Tumours were induced in young rats by intrathyroid ($1.5\times10^{5)}$ or subcutaneous injections (2.5×10^{6}) of these tumour cells.

For histological purposes, rats were anaesthetized to death with Nembutal, and their thyroid glands removed by dissection under a microscope. Thyroid and tumour tissue were cut into small pieces. Classic histology was performed on samples fixed by immersion in Bouin's liquid (Hollande's modifications), dehydrated and embedded in paraffin by standard procedures. Sections were cut at 8 µm. For immunocytochemistry, samples were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4, for 1 day at 4°C, washed overnight in PBS containing 15% sucrose, embedded in tissue-Tek, quick-frozen by immersion into isopentane chilled in liquid nitrogen, and finally stored at –80°C until used. The frozen tissues were sectioned (10 µm) in a cryocut (Bright). Morphological control observations were realised by Mann-Dominici staining process (toluidine blue–eosin).

For immunocytochemistry, tissue sections were incubated in: (a) 15% sucrose PBS buffer (30 min), then washed in PBS (3×10 min), (b) 50 mM NH_4Cl in PBS (10 min), then washed in PBS (3×10 min), (c) PBS with 4% BSA to minimize nonspecific labelling (30 min), (d) primary antibody diluted in PBS overnight in a humid chamber at 4°C, (e) appropriate fluorochrome conjugated antibody (2 h), then washed in PBS (3×10 min). For double labelling, both antibodies were incubated one after the other.

Specific CT detection was performed with an antiserum raised in sheep against human CT (generous gift from Dr. A. Jullienne, U 349 INSERM, Hôpital Lariboisière, Paris, France), and an anti-sheep antibody coupled with AMCA (7-amino-4-methyl coumarin-3-acetic acid) purchased from Sigma Immunochemicals (Saint-Quentin Fallavier, France). Anti-laminin serum was produced in a rabbit by immunization with EHS tumour purified laminin (Sigma Immunochemicals). Collagen type IV was revealed with a polyclonal rabbit serum obtained by immunization with an antigen isolated from human placenta (ICN Biochemicals, Orsay, France). The secondary antibody was a fluorescein isothyocyanate (FITC)-conjugated anti-rabbit IgG serum (Interchim, Montluçon, France). The specificity of the reactions was tested by using normal serum or irrelevant antibodies, or specific antibody saturated by an excess of antigen, when possible (CT detection), as a substitute for primary antibodies. All controls were negative.

The in situ hybridization procedure has been described previously [15]. In short, frozen tissue sections were rehydrated for 30 min in PBS containing 15% sucrose, washed in PBS (2×15 min), post-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min, washed with 2xSSC (5 min). Laminin α2 mRNA detection was performed with two different oligonucleotide probes made and purified by Genset (Paris, France). They were complementary to two different parts of the rat laminin α2 sequence [18]. The first sequence, corresponding to nucleotides 301–341, was 5′GCTGGGCACCATACTGGCTTTGGGCCCATCCAAAGCTCTC3′, and the second one was 5′GGAGGTTGTCAGCAACAGCGGTCTTGACATGGACAACG3′, corresponding to nucleotides 76–114. They were end-labelled with ^{35}S-ATP by terminal deoxynucleotidyltransferase (Boehringer, Meylan, France). Prehybridization was carried out with 50 µl of the prehybridization solution (20% formamide, 10% dextran sulfate, 0.2% SDS, 0.3 M NaCl, 2 mM EDTA, 80 mM Tris-HCl pH 7.5, 0.1% Na-pyrophosphate (Merck, Nogent-sur-Marne, France), 0.1 mg/ml heparin 25,000 U (Boehringer, Meylan, France) 10 mM DTT (Sigma, Saint-Quentin Fallavier, France) under a coverslip in a humid chamber, at 42°C for 1 h. Slides were washed rapidly with 4×SSC. Tissue sections were incubated overnight with 30 µl of the hybridization solution (20% formamide, 4×SSC, 5× Denhart's solution, 5% dextran sulfate, 250 µg/ml herring sperm DNA, 250 µg/ml *E. coli* tRNA) (Boehringer, Meylan, France) containing the probe (2.10^5 cpm for each slide), under a coverslip sealed with rubber cement, in a humid chamber at 42°C. Slides were extensively rinsed and shaken, first with 4×SSC, then with solutions of increasing stringency, 1×SSC (1×30 min), 0.1×SSC (2×60 min).

The immunolocalization of CT was performed at this step, as described above, prior to the application of photoemulsion (Amersham LM-1). After exposure for 3–4 weeks at 4°C, slides were developed then mounted in Mowiol. They were observed, and micrographs were prepared, under fluorescent and dark-field microscopy using an Axiophot Zeiss microscope.

To assess the specificity of the signal, hybridization was accomplished with two probes complementary to two different sequences of the *α2* laminin gene: similar labelling was observed for the same tissues. Observations were made on all the tissues in the area. A strong signal was found under the epithelium of the oesophagus. Negative controls were obtained with the hybridization mixture, but without the probes, or through the pretreatment of sections with ribonuclease (20 µg/ml) for 1 h at 37°C.

Metabolic labelling was performed on rMTC 6-23 cells cultured on plastic, on day 6 after plating, and on biopsies of a subcu-

taneous tumour. In the latter case, the tumour was dissected under sterile conditions, cut into small pieces and maintained in culture medium. Then 0.5 mCi/ml of the Tran^{35}S-label metabolic labelling reagent containing L-^{35}S-methionine and L-^{35}S-cysteine (1000 mCi/mmol, ICN Biochemicals, Orsay, France) was added to both culture media for 18 h. At the end of labelling, the spent media were collected and centrifuged to eliminate cellular debris. Cells were washed three times with DMEM, then lysed in buffer 1: 50 mM Tris-HCL, 0.4 M NaCl, 2 mM EDTA, 1% Triton X-100 (w/v), 0.4% SDS (w/v), 1 µg/ml aprotinin, 2 µg/ml leupeptin, 0.1 mg/ml ovomucoide, 1 mM PMSF, pH 8 (Sigma). Samples were kept frozen at –70°C until use. Aliquots of cell extracts (300 µl) and medium (700 µl) were used for laminin immunoprecipitation according to the method previously described (André et al. 1994). All steps were performed at 4°C. Samples were precleared by an overnight incubation and shaking with 50 µl rabbit preimmune serum, then by 2 h with protein A-Sepharose (CL 4B; Sigma) slurry in buffer 1 containing 4% bovine serum albumin (wt/v). Beads were removed by centrifugation (16,000 *g*, 5 min), and the supernatants were incubated for 3 h, with 50 µl of rabbit anti laminin-1 serum obtained by immunization with murine laminin purified from the EHS tumour [1], then with the protein A-Sepharose slurry for 2 h. The antigen–antibody complexes were washed 10 times with buffer 1 and twice with 50 mM Tris-HCl buffer (pH 8.8), then solubilized in buffer for electrophoresis, as previously described [3]. Samples were applied to 3%–12% polyacrylamide gels, and ^{14}C-labelled protein standards (Gibco BRL, Cergy Pontoise, France) were included (1.12 nCi/lane). These were myosin heavy chains, 200 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; lysosyme, 14.3 kDa. Following electrophoresis, proteins were fixed and stained with Coomassie blue R 250 (Fluka). For autoradiography, gels were dried then exposed to Hyperfilm β-Max (Amersham International, Les Ulis, France) at –70°C for 3–4 weeks.

For the polymerase chain reaction, cDNA was synthesized from 1 µg total RNA extracted from 6–23 cell cultures by the guanidium thiocyanate method [6]. The reaction mixture had a final volume of 20 µl, and contained 75 mM KCl, 25 mM Tris-HCl (pH 8.4), 3 mM MgCl2, 10 mM nuclease-free DTT, 20 U of RNAsin, 200 U of reverse transcriptase superscript (BRL), 1 mM of each dNTP and 40 pmol of a 3'oligo-dT primer. Primer extension was performed for 1 h at 37°C. The reaction mixture was then diluted to 100 µl with the same buffer containing 50 pmol of each specific primer and 1 unit of Taq polymerase. PCR primers for the α2 chain were derived from the known rat sequence [18] (antisense 5′GGGAGGAGAATCTGAATGGTGG3′, sense 5′TTGGCCGAGTAGAGTATCCG3′). The amplified sequence was around 157 pb. PCR reactions were performed in a thermocycler with 20 µl of the reverse transcription volume. The cycling conditions were 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, 30 cycles. Amplified fragments were visualized by ethidium bromide after electrophoresis on 2% agarose gel, then transferred to a nylon membrane (GeneScreen NEN). They were hybridized overnight, with a specific oligonucleotide antisense probe ^{32}P-labelled using terminal deoxynucleotidyl transferase, at 42°C, then washed in stringent conditions. For a control, amplification was performed either with no RNA, or after a 1-h treatment with ribonuclease at 37°C.

Results

The histopathological aspect of Wag/Rij rat thyroid and MTC tumours is shown in Fig. 1A, B. C cell diffuse hyperplasia is genetically determined in Wag/Rij rats and can be observed from the age of 3 months onwards [12]. With increasing age, (from 18 months onwards) more than half of the population develops a C cell tumour: during the early steps in oncogenesis, the proliferative C cells progressively invade the periphery of several adjacent follicles, while the thyrocytes are drawn towards the centre (Fig. 1A). This step leads to the formation of small and then larger nodules comprising still differentiated C cells, which are positive to CT antibodies. The tumour increases in size, with more and more nodular follicles located at the periphery of the tumour mass. These newly incorporated follicles are still surrounded by their connective tissue (Fig. 1B). In the meantime, the centre of the tumour becomes disorganized, and the neoplastic cells lose their endocrine phenotype, becoming negative to CT (or CGRP) antibodies [13].

On normal and pathologic thyroid sections, simultaneous immunolocalization of CT and BM constituents was accomplished by double immunolabelling. The immunocytochemical study showed that in normal and hy-

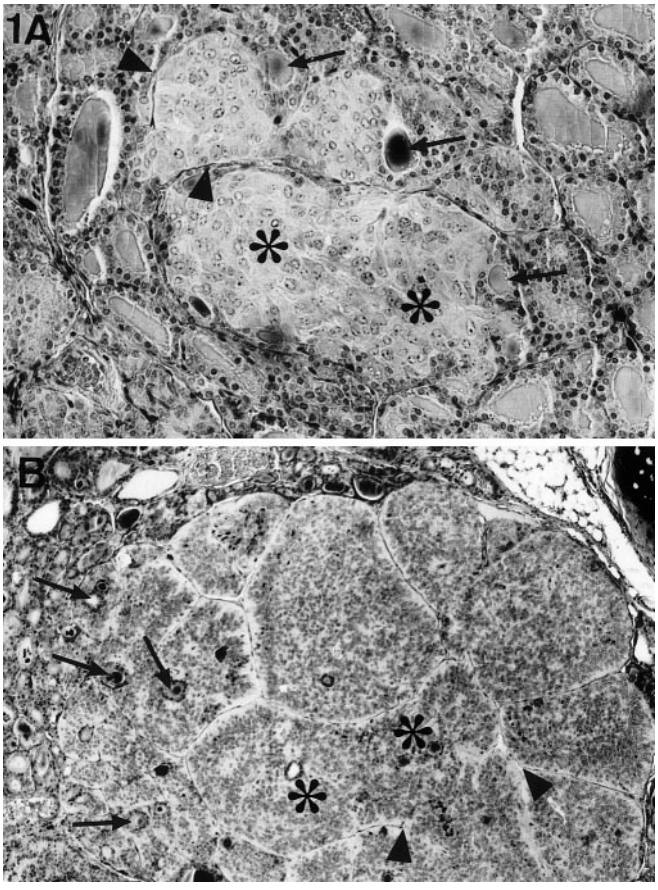

Fig. 1A, B Histological aspects of spontaneous MTC in 18-month-old Wag/Rij rats (routine Mann-Dominici staining). **A** C cell multifocal nodular hyperplasia in a thyroid lobe. This small tumour is composed of five adjacent follicles. In three nodules, the remaining layer of thyrocytes and colloid is observed (*arrows*), and these C cell nodules are clearly delimited by a layer of connective tissue (*arrowheads*). The two others have merged and are enclosed within the same connective envelope (*asterisks*). ×170 **B** Large multilobulated tumour. At the periphery the nodules are clearly distinguishable, separated by connective tissue and showing thyrocytes surrounding colloid in the centre (*arrows*). The central part of the tumour is completely disorganized, showing at least three follicles joined together (*asterisks*) and disrupted connective tissue (*arrowheads*). ×65

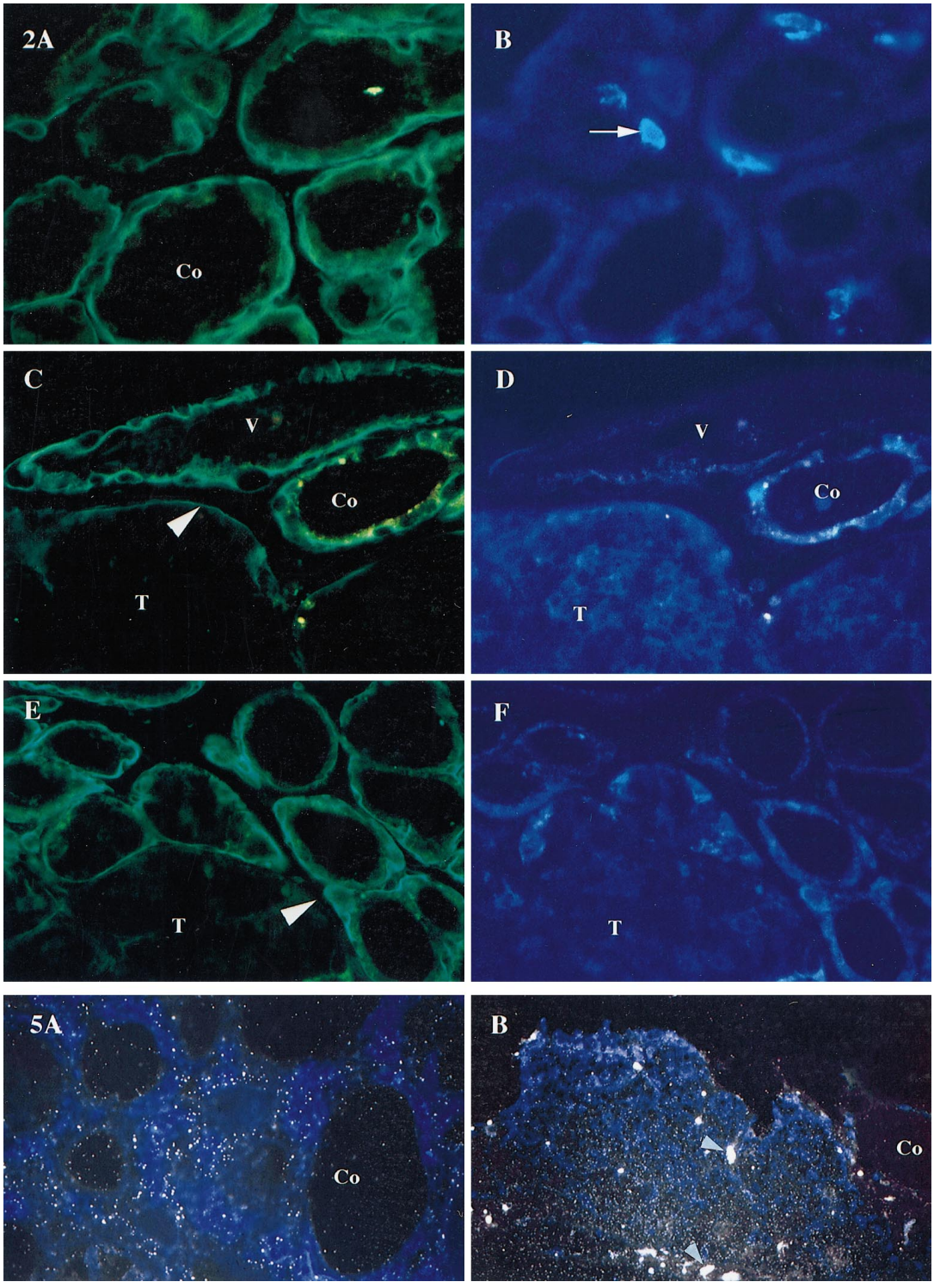

Fig. 2A–F Immunocytochemical localization of laminin in thyroid and MTC. Laminin and CT-positive C cells were detected by double immunostaining, using anti-laminin/FITC (**A**, **C**, **E**) and anti-CT/AMCA (**B**, **D**, **F**). **A**, **B** Thyroid from a 2-year-old rat, showing diffuse C cell hyperplasia; the follicles in which CT-positive C cells are observed (**B** *arrow*) are surrounded by an immunoreactive laminin layer (**A**) follicular lumen containing the colloid (*Co*). ×440 **C**, **D**, **E**, **F** Spontaneous tumours (*T*) in a 2-year-old rat thyroid. An immunoreactive laminin layer is observed, surrounding the thyroid follicles and the tumour (**C** arrow), which appears to be constituted of immunoreactive neoplastic C cells (**D**). ×275 The laminin layer is no longer observed around the large tumour (*arrow*), but is present around the two small nodules developing in the follicles close to the tumour (**E**); the tumour comprises essentially dedifferentiated CT-negative C cells (**F**) (*V* blood vessel). ×275

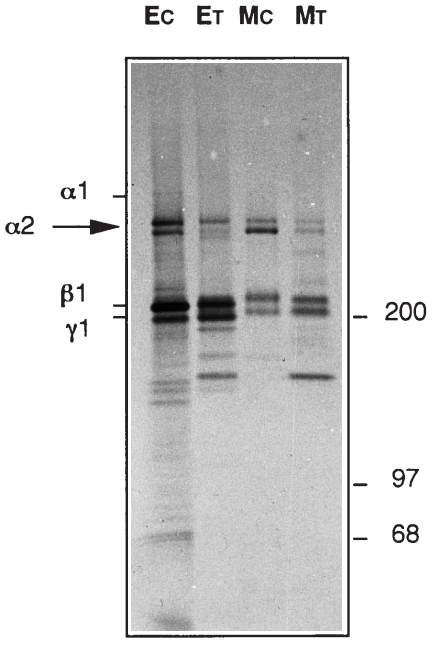

Fig. 3 Demonstration of LN-2 polypeptide neosynthesis by neoplastic C cells. After labelling and immunoprecipitation of extracts of the C cells (*Ec*), tumour tissues (*Et*), and their respective media (*Mc*, *Mt*), autoradiography showed two major bands of about 210 and 220 kDa, the γ and β chains, and a 380–350 kDa doublet corresponding to the α2 heavy chain (*arrow*). The electrophoretic mobility of the α1 chain (400 kDa) was evaluated by the migration of an EHS sample on the gel. The molecular masses of the radiolabelled markers are indicated in the right margin

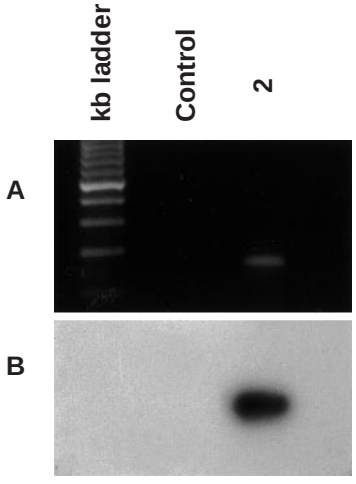

Fig. 4A, B Amplification of *α2* gene of cultured rMTC 6-23 cells by RT-PCR. **A** α2 PCR products (157 bp) were electrophoresed through a 2% agarose gel. **B** Autoradiogram of the Southern blot after hybridization with a ^{32}P-labelled specific probe. The control lane contains no RNA. DNA ladder F5 100 pb (Promega)

perplastic thyroids of young and aged rats, the thyroid follicles were limited by a BM that was immunoreactive both for laminin (Fig. 2A, B) and for collagen IV, which was colocalized (not shown). Immunostaining revealed that a continuous laminin layer was maintained around these nodules and also around tumours that were still differentiated (Fig. 2C, D). However, when the tumour increased in size, the peripheral laminin staining was weakened, fragmented, then became undetectable. The impairment of laminin immunoreactivity around the large tumours occurred gradually with a decrease in CT-positive cells (Fig. 2E, F), characteristic of the neoplastic MTC dedifferentiation process and of the enhancement of malignancy. Meanwhile, these tumours were invaded by blood capillaries, which were lined with an intense immunoreactive laminin layer, totally restricted to the areas of angiogenesis.

In young rats, tumours were induced by injection of rMTC 6-23 cells in an orthotopic position or subcutaneously. As already reported, this cell line is largely dedifferentiated and tumours were always immunonegative to CT antibodies [14, 38]. Fifteen days after grafting, the tumour mass was encapsulated in thick multilayere connective tissue, but no staining with laminin and collagen IV antibodies was detected in the peripheral layer from 2 to 15 days after transplantation.

The persistence of an immunoreactive BM layer around the C cell nodules during the slow process of tumour evolution suggests an implication of these cells in the elaboration of its constituents. Laminins, essential molecules for BM assembly, are frequently synthesized by tumour cells. We therefore investigated the ability of rMTC 6–23 cells to produce laminin molecules in vitro and in vivo, by metabolic labelling performed on tumour cell cultures and on biopsies of tumours induced by a subcutaneous injection of the same cells (two materials that are easily available). In cell lysates and culture media from both samples, laminin molecules were immunoprecipitated with an anti-murine laminin-1 serum, which recognizes the α1, β1 and γ1 laminin-1 chains. Thus, any molecule of the laminin family sharing at least one subunit with laminin-1 was recognized and immunoisolated.

After an 18 h labelling with ^{35}S-methionine, three major polypeptides were detected in cultured rMTC 6/23 cells and biopsy extracts after electrophoresis under reducing conditions and autoradiography. The apparent molecular masses of the light chains (200 kDa) were characteristic for the γ1 and β1 laminin chains. The laminin heavy chain migrated as a protein doublet of 380/350 kDa; identical results were observed with the conditioned media of corresponding samples (Fig. 2). In-

◀ **Fig. 5A, B** In situ hybridization of α2 chain mRNA on a thyroid and a spontaneous tumour in a 2-year-old rat. Epifluorescence of hyperplastic and neoplastic C cells detected by anti-CT/AMCA, combined with dark-field image of the labelled α2 mRNA. **A** Thyroid section: the signal is quite similar in both cell types (immunoreactive C cells and follicular cells), and significantly higher than that of the background on the colloid (*Co*). ×240 **B** Spontaneous tumour: high signal on the central area of the tumour exhibiting a weak CT immunoreactivity (*bottom*) but low signal on the immunoreactive cells located at the periphery (*top*) (*Co* remnants of a thyroid follicle, *arrowheads* groups of red cells seen with the dark field) ×120

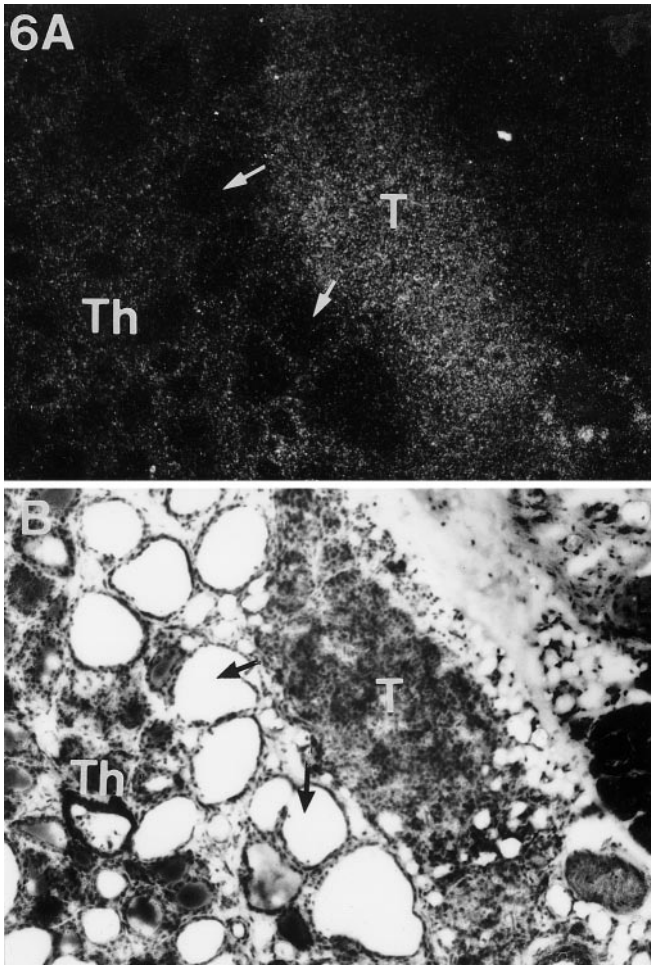

Fig. 6A, B In situ hybridization of α2 chain mRNA on a 7-day rMTC 6-23 orthotopically induced tumour. **A** The dark-field image shows the strong labelling on the tumour (*T*), compared with the weak background signal observed on the colloid (*arrows*) and the low labelling on the thyroid follicular cells (*Th*). This indicates a high expression of the α2 mRNA in neoplastic C cells. **B** Histological aspect of the transplanted tumour mass stained by routine Mann-Dominici method. ×75

terestingly, under the same labelling conditions, a polypeptide doublet with a similar molecular mass has already been observed in thyroid follicular cells [1]: the 380 kDa chain was identified as the α2 heavy chain of laminin-2, which substitutes for the α1 chain of laminin-1, and the 350 kDa polypeptide chain as the product of a proteolytic cleavage of the 380 kDa α2 chain. In cell extracts, β1 and γ1 chains were present in greater amounts than the heavy chain doublet, suggesting the presence of some β1–γ1 complexes devoid of heavy chain. The absence of the α1 heavy chain (400 kDa) was confirmed on a sample of laminin-1, extracted from an EHS tumor, loaded on the gel. Moreover an additional polypeptide chain of 150 kDa was noted in tumour cell extracts as well as in media, suggesting the presence of entactin in these samples (Fig. 3).

The expression of the α2 mRNA heavy chain by rMTC cells was confirmed by hybridization with a rat α2 laminin-specific probe of a cDNA fragment, amplified by RT-PCR (Fig. 4).

In rMTC cell cultures, in vitro immunocytochemical investigations failed to detect laminin-specific deposits around or inside these cells.

The in situ expression of the α2 chain mRNA was investigated in normal thyroid follicles, during the successive steps towards MTC and in the induced orthotopic tumours. To identify the normal C cells in follicles and to determine the differentiated vs dedifferentiated status of the tumour cells, along with the *α2* gene expression, we used a two-step procedure: in situ hybridization was followed by anti-CT serum immunostaining on the same sections.

The results showed that the labelling with the laminin chain α2 probes was similar on thyrocytes and normal immunoreactive C cells and remained low even after a long exposure (Fig. 5A). A similarly low signal was observed on hyperplastic foci and on nodules. Conversely, stronger labelling was observed on the large spontaneous tumours, particularly in the central part (Fig. 5B). As already shown [13, 15], cells located in the centre were largely immunonegative, thus indicating that they were dedifferentiated, while tumour cells located at the periphery and thus close to the remnants of the BM were still immunopositive to anti-CT antibodies.

In the same way, a high level of α2 mRNA transcripts was detected on the induced tumours considering the weak signal observed on the thyroid tissue in the same section (Fig. 6A, B). As already mentioned, these tumours are dedifferentiated and negative to CT antibodies.

Discussion

We have examined those relationships between C cells and the underlying follicular BM that are modified during hyperplasia and MTC evolution. It is well known that oncogenesis is greatly influenced by extracellular signals transmitted via ECM and its receptors [21, 34]. Immunocytochemical localization of laminin showed that the continuous BM lining normal and hyperplastic follicles was maintained around C cell nodules. The slow rate of development of the nodular structures (several months) and their increase in size raise the question of the production and renewal of the BM constituents. In the thyroid, only the follicular cells are known to be implicated in the elaboration of the follicular BM constituents. Immunostaining has revealed that both laminin isoforms, laminin-1 and laminin-2, colocalize in the follicular BM of porcine thyroid [1]. It is generally considered, however, that epithelial and stroma cells participate in the controlled production of ECM molecules [27, 28]. A contribution of C cells to the elaboration of BM components has not been demonstrated before.

We examined the expression of laminin isoforms in the tumour rMTC cell line 6-23 issued from a Wag/Rij tumour, as C cell lines depicting the normal characteris-

tics of the in situ cells are not available. We show here that these cells were able to synthesize and secrete into the medium the α2, β1 and γ1 chains corresponding to the laminin-2 isoform, in cultures or in tumours induced by their injection. The α2 heavy chain was visualized as a doublet of 380–350 kDa. The presence of the same doublet has been reported in primary cultures of thyrocytes: the 380-kDa peptide corresponds to the uncleaved form of the α2 chain, which is often detected as two polypeptides (300 and 80 kDa), while the 350-kDa polypeptide could be the result of a proteolytic cleavage of the 380-kDa molecule, as suggested for the follicular cells [1]. The expression of α2 mRNA in these neoplastic C cells was confirmed by RT-PCR.

Owing to the heterogeneity of the normal thyroid tissue and the paucity of C cells, biochemical investigations on laminin synthesis could not be conducted on normal C cells. We therefore examined the in vivo expression of α2 mRNA on thyroid and MTC tissues by in situ hybridization combined with a CT immunolocalization. This revealed low expression in both thyrocytes and C cells, as well as in small tumours with CT-positive C cells. During hyperplasia, C cells could participate in a coordinated elaboration process of the nodular BM. Conversely, we found an overexpression of laminin α2 gene in the dedifferentiated cells of both induced and spontaneous intrathyroid tumours. This result suggests that during the MTC tumour evolution in vivo cell dedifferentiation is marked by deregulation of the expression of laminin genes, as in tumour cells in vitro. However, we observed that the basal layer was gradually impaired around the large, partly dedifferentiated tumours. Previous ultrastructural studies on human and rat MTC tumours have also described progressive BM fragmentation [8, 9]. In tumour tissues, the loss of BM frequently observed is considered to result from protease destruction, but it might also be due to deregulated assembly of the constituents synthesized by different cell types.

ln vitro, immunocytochemical investigations also revealed no intra- or extracellular laminin deposits in rMTC cells, which synthesize and secrete the three laminin-2 chains into the culture medium. A lot of data has been generated on the ability of tumour cells or cells in primary culture, to express and synthesize different ECM molecules, in particular the laminin isoforms. Considerable heterogeneity concerning the nature of the molecules and their integration into a BM structure has been reported among the different cell types in vitro or in carcinoma [10, 23–26, 35] (Feracci et al., unpublished results). The significance of the deregulation in the expression and synthesis of ECM molecules in tumour cells and the putative relationships with the neoplastic process are difficult to determine. It has been suggested recently that a soluble form of laminin-2 may modulate the oncogenicity and the metastatic capacity of tumour cells [11]. However, studies on the mammary gland in vitro demonstrated that the nature of the substratum influences the expression of different ECM constituents [29, 30]. In MTC, the laminin α2 gene overexpression could result from the progressive loss of ECM, and it may correlate with the C cell dedifferentiation process. The implication of this overexpression in the cancer process requires further investigation.

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References

1. André F, Filippi P, Feracci H (1994) Merosin is synthesized by thyroid cells in primary culture irrespective of cellular organization. J Cell Sci 107:183–193
2. Aumailley M, Krieg T (1996) Laminins: a family of diverse multifunctional molecules of basement membranes. J Invest Dermatol 106:209–214
3. Bartles JR, Feracci HM, Stieger B, Hubbard AH (1987) Biogenesis of the plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. J Cell Biol 105:1241–1251
4. Boorman GA, Van Noord MJ, Hollander CF (1972) Naturally occuring medullary thyroid carcinoma in the rat. Arch Pathol 94:35–41
5. Burgeson RE, Chiquet M, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, Timpl R, Tryggvasson K, Yamada Y, Yurchenko PD (1994) New nomenclature for the laminins. Matrix Biol 14:209–211
6. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thyocianate-phenol-chloroform extraction. Anal Biochem 162:156–159
7. De Arcangelis A, Neuvil A, Boukamel R, Lefebvre O, Kedinger M, Simon-Assmann P (1996) Inhibition of laminin α1-chain expression leads to alteration of basement membrane assembly and cell differentiation. J Cell Biol 133:417–430
8. DeLellis RA, Nunnemacher G, Wolfe HJ (1977) C-cell hyperplasia, an ultrastructural analysis. Lab Invest 36:237–248
9. DeLellis RA, Nunnemacher G, Bitman WR, Gagel RF, Tashjian AHJ, Bloun M, Wolfe HJ (1979) C-cell hyperplasia and medullary thyroid carcinoma in the rat. An immunohistochemical and ultrastructural analysis. Lab Invest 40:140–154
10. Engvall E (1993) Laminin variants: why, where and when? Kidney Int 43:2–6
11. Jenq W, Wu SJ, Kefalides NA (1994) Expression of the α2-subunit of laminin correlates with increased cell adhesion and metastatic propensity. Differentiation 58:29–36
12. Khattab M, Pidoux E, Volle GE, Bouizar Z, Calmetttes C, Milhaud G, Moukhtar MS, Treilhou-Lahille F (1989) Early calcitonin hypersecretion and C cell hyperplasia in rats with high incidence on C cell tumor. Bone Miner 6:249–260
13. Lausson S, Volle GE, Bourges M, Pidoux E, Borrel C, Milhaud G, Moukhtar MS, Jullienne A, Treilhou-Lahille F (1995) Calcitonin secretion, C cell differentiation and proliferation, during the spontaneous development of murine medullary carcinoma (MTC). Virchows Arch 426:611–617
14. Lausson S, Fournes B, Borrel C, Milhaud G, Treilhou-Lahille F (1996) Immune response against medullary thyroid carcinoma induced by parental and/or IL-2 secreting MTC cells in rat model of human familial medullary thyroid carcinoma. Cancer Immunol Immunother 43:116–123
15. Le Guellec P, Dumas S, Volle GE, Pidoux E, Moukhtar MS, Treilhou-Lahille F (1992) An efficient method to detect calcitonin mRNA in normal and neoplastic rat C cells (medullary thyroid carcinoma) by in situ hybridization using a digoxigenin labeled synthetic oligodeoxynucleotide probe. J Histochem Cytochem 41:389–396

16. Liotta LA, Rao CN, Wewer UM (1986) Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 55:1037–1057
17. Lippman SM, Mendelsohn G, Trump DL, Wells SA, Baylin SB (1982) The prognostic and biological significance of cellular heterogeneity in medullary thyroid carcinoma: a study of calcitonin, L-DOPA decarboxylase and histaminase. J Clin Endocrinol Metab 54:233–240
18. Maher JJ, Tzagarakis C (1994) Partial cloning of the M subunit of laminin from adult rat lipocytes: Expression of the M subunit by cells isolated from normal and injured liver. Hepatology 19:764–770
19. Melvin KEW, Miller HH, Tashjian AHJ (1971) Early diagnosis of medullary carcinoma of the thyroid gland by means of calcitonin assay. N Engl J Med 285:1115–1120
20. Milhaud G, Tubiana M, Parmentier C, Coutris G (1968) Epithélioma de la thyroïde sécrétant de la thyrocalcitonine. CR Acad Sci [D] Paris 266:608–610
21. Miyamoto S, Teramoto H, Coso OA, Gutkind S, Burbelo PD, Akiyama SK, Yamada KM (1995) Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol 131:791–805
22. Mulligan LM, Eng C, Healey CS, Clayton D, Kwok JBJ, Gardner E, Ponder MA, Frilling A, Jackson CE, Lehnert H, Neumann HPH, Thibodeau SN, Ponder BAJ (1994) Specific mutations of the RET proto-oncogene are related to disease phenotype in Men 2A and FMTC. Nat Genet 6:70–74
23. Olsen D, Nagayoshi T, Fazio M, Peltonen J, Jaakola S, Sanborn D, Sasaki T, Kuivaniemi H, Chu M, Deutzmann R, Timpl R, Uitto J (1989) Human laminin: cloning and sequence analysis of cDNAs encoding A,B1 and B2 chains, and expression of the corresponding genes in human skin and cultured cells. Lab Invest 60:772–782
24. Remy L, Lissitsky JC, Daemi N, Jacquier MF, Bailly M, Martin PM, Bignon C, Doré JF (1992) Laminin expression by two clones isolated from the colon carcinoma cell line LoVo that differ in metastatic potential and basement membrane organization. Int J Cancer 51:204–212
25. Schuler F, Sorokin LM (1995) Expression of laminin isoforms in mouse myogenic cells in vitro and in vivo. J Cell Sci 108:3795–3805
26. Seebacher T, Medina JL, Bade EG (1997) Laminin α5, a major transcript of normal and malignant rat liver epithelial cells, is differentially expressed in developing and adult liver. Exp Cell Res 237:70–76
27. Simo P, Bouziges F, Lissitzky JC, Sorokin L, Kedinger M, Simon-Assmann P (1992) Dual and asynchronous deposition of laminin chains at the epithelial mesenchymal interface in the gut. Gastroenterology 102:1835–1845
28. Simon-Assmann P, Duclos B, Orian-Rousseau V, Arnold C, Mathelin C, Engvall E, Kedinger M (1994) Differential expression of laminin isoforms and α6-β4 integrin subunits in the developing human and mouse intestine. Dev Dynamics 201:71–85
29. Streuli CH, Bissell MJ (1990) Expression of extracellular matrix components is regulated by substratum. J Cell Biol 110:1405–1415
30. Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz AP, Roskelley C, Bissel MJ (1995) Laminin-mediated tissue-specific gene expression in mammary epithelia. J Cell Biol 129:591–603
31. Timpl R (1989) Structure and biological activity of basement membrane proteins. Eur J Biochem 180:487–502
32. Timpl R, Brown J (1994) The laminins. Matrix Biol 14:275–281
33. Timpl R, Dziadek M (1986) Structure, development and molecular pathology of basement membranes. Int Rev Exp Pathol 29:1–112
34. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ (1997) Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 137:231–245
35. Wewer UM, Wayner EA, Hoffstrom BG, Lan F, Meyer-Nielsen B, Engval E, Albrechtsen R (1994) Selective assembly of laminin variants by human carcinoma cells. Lab Invest 71:719–728
36. Yurchenko PD, O'Rear JJ (1994) Basal lamina assembly. Curr Opin Cell Biol 6:674–681
37. Yurchenko PD, Cheng YC, Colognato H (1992) Laminin forms an independent network in basement membranes. J Cell Biol 117:1119–1133
38. Zeytinoglu FS, DeLellis RE, Gagel RF, Wolfe HJ, Tashjian AH (1980) Establishment of a calcitonin-producing rat medullary thyroid carcinoma cell line. I. Morphological studies of the tumor and cells in culture. Endocrinology 107:509–515