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Reduced Expression of Laminin α3 and α5 Chains in Non-small Cell Lung Cancers

Takumi Akashi,[1,3] Eisaku Ito,[1] Yoshinobu Eishi,[1] Morio Koike,[1] Kyoichi Nakamura[1] and Robert E. Burgeson[2]

[1]The Department of Pathology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519 and [2]Cutaneous Biology Center, Harvard Medical School, Charleston, Massachusetts, USA

The basement membrane is considered to act as a barrier which hinders cancer cells from invading the surrounding stroma. In order to assess changes in essential components during neoplasia in the lung, we immunohistochemically studied distribution patterns of laminins α3 and α5 in 40 adenocarcinomas and 8 squamous cell carcinomas. The α5 chain was generally preserved at the periphery, frequently disrupted in foci with alveolar collapse and absent in foci of fibroblastic proliferation within adenocarcinomas. Fragmentation and absence of laminin α3 chain were more prominent than for α5 chain. Laminin α3 chain was partially fragmented or absent in peripheral areas of adenocarcinomas, being significantly different from α5 chain. Non-small cell lung cancers with reduced α5 chain showed a tendency for greater lymph node metastasis. In cultured normal air way epithelial cells, both laminin α3 and α5 chains were found to be expressed by northern analysis. Eleven of the twelve cultured lung cancer cell lines did not express α3 chain and expression of α5 chain was reduced in three. Quantitative RT-PCR analysis also demonstrated expression of laminin α3 chain in adenocarcinoma tissues to be significantly lower than in normal lung tissues. These results suggest that expression of laminin α chains is often reduced in lung cancer cells and this might contribute to basement membrane fragmentation and subsequent proliferation of stromal elements, as well as play some role in the process of cancer cell invasion.

Key words: Lung — Cancer — Basement membrane — Laminin

In neoplastic tissue, the basement membrane is considered to act as a barrier which hinders the cancer cells from invading the surrounding stroma.[1)] With adenocarcinomas of the lung, disruption of basement membrane and linkage with lymph node metastasis and a poor prognosis have been reported.[2–5)]

Because the epithelial basement membrane is known to be renewed through turnover over several weeks,[6)] two putative processes leading to fragmentation can be hypothesized. One is increased degradation of basement membrane by proteinases, such as collagenase, secreted by the cancer cells themselves or by the surrounding stromal cells.[3, 7, 8)] The other possibility is that a reduced capacity of cancer cells to synthesize basement membrane components might exert an influence.

The epithelial basement membrane is considered to be formed from materials secreted by both epithelial and stromal cells.[9–11)] Laminins, one of the major components of the basement membrane, are heterotrimeric glycoproteins composed of one large α chain and two smaller β and γ chains.[12)] In the process of heterotrimer assembly in the endoplasmic reticulum, laminin α chains are considered to be limiting factors.[13–15)] There are 5 isoforms of laminin α chains and α2, 3, 4, 5 are considered to be expressed in the mouse and human lung (we could not detect the expression of α2 chain immunohistochemically in this study).[16, 17)] Among them, α3 and α5 chains appear to be derived from epithelial cells.[16)] We recently found that some gastrointestinal cancer cells can not form a basement membrane and this is correlated with reduced expression of laminin α3 and α5 chains.[18)] Thus, we hypothesized that reduced expression of laminin α3 and α5 chains might affect the basement membrane formation also in lung cancers.

In this research, in order to clarify the significance of loss of basement membrane in the process of cancer cell invasion and to clarify the mechanism of loss of basement membrane, we examined expression patterns of laminin α3 and α5 chains in non-small cell lung cancer tissues and cell lines. With progression of adenocarcinomas, active proliferation of fibroblasts occurs in tumors,[19)] which is considered to be related to cancer cell invasion.[20)] We therefore examined the relationship between basement membrane fragmentation and histological patterns of adenocarcinomas. We also examined the relationship with lymph node metastasis.

In order to know the expression and synthesis pattern of laminin α3 and α5 chains in lung cancer cells, cultured normal small airway epithelial cells (SAE cells) and can-

[3] To whom correspondence should be addressed.
E-mail: akashi.path@med.tmd.ac.jp

cer cell lines were examined first by northern analysis and western analysis. Since a marked reduction of α3 chain was found in the cancer cell lines, expression of α3 chain mRNA was next examined in surgically resected lung cancers by RT-PCR analysis.

MATERIALS AND METHODS

Lung cancer cases T1 or T2 cancers (tumor staging by the World Health Organization system) of 40 adenocarcinomas and 8 squamous cell carcinomas, surgically resected in Tokyo Medical and Dental University Hospital were examined.[21] Fresh specimens were embedded in OCT compound (Sakura, Tokyo) and frozen in liquid nitrogen. The histology of the carcinomas was determined according to the World Health Organization system.[21] For the RT-PCR analysis, one large cell carcinoma and one small cell carcinoma were also examined.

Immunohistochemistry Frozen sections of tumor tissues were fixed with acetone and reacted with first antibodies (Table I), biotinylated second antibodies, streptavidin-peroxidase complex and diaminobenzidine in succession. Staining was graded as intact, fragmented when there were several tumor alveoli which showed discontinuity (Fig. 1E), and absent when there were several alveoli which showed complete loss of staining (Fig. 1G).

Most adenocarcinomas were the mixed subtype and showed peripheral replacement growth (bronchioloalveolar pattern) and central scarring with collapsed alveoli or fibroblastic responses.[21] Immunohistochemical findings were assessed for bronchioloalveolar, collapsed and fibroblastic areas separately in such cases.

Basement membrane patterns were correlated with lymph nodes metastasis. Fisher's exact probability test was used to evaluate the difference between cancers with fragmented or absent basement membrane and those with intact basement membrane.

Table I. Antibodies, cDNA Probes and Primers

Antigen	Clone	Source
Laminin α2	5H2	Engvall[22]
Laminin α3	BM2	Burgeson[23]
Laminin α5	4C7	Engvall[22, 24]
Gene		
Laminin A_3[25]	1.6 kb	Domain II/I
Laminin A_5[26]	1.0 kb	Domain G
Gene	**Primer sequence**	
GAPDH	5′-CATCACCATCTTCCAGGAGC-3′	
(913 bp)	5′-ACATGGCAACTGTGAGGAGG-3′	
Laminin A3[25]	5′-TCTTGCTGAACCGGATAAGG-3′	
(549 bp)	5′-TGAGGATGTTCTCGTAGGCG-3′	

Cell cultures A549, PC3, RERF-LC-MS, RERF-LC-OK, VMRC-LCD, ABC1: human lung adenocarcinoma-derived cell lines. LC-1-sq, EBC1: human lung squamous cell carcinoma-derived cell lines. Lu-134-A-H, RERF-LC-MA, SBC1: human small cell carcinoma-derived cell lines. TIG1: human fibroblast cell line originating from fetal lung tissue (Japanese Cancer Research Resources Bank). SAEC: normal epithelial cells, which were obtained from healthy adult volunteers by bronchoalveolar lavage and demonstrated characteristic features of basal cells and differentiated into Clara cells or ciliated epithelial cells (Clonetics, San Diego, CA).[27] All cultured cells were grown in equal-volume mixtures of Dulbecco's modified Eagle's medium with 10% fetal calf serum and SAE basal medium (Clonetics) with growth factors.[27]

Northern blot analysis of α3 and α5 chain expression For northern analysis, Nylon membranes with 1 μg of poly-A mRNAs extracted from cultured cells were hybridized to random priming ^{32}P-labeled probes (Table I), exposed to imaging plates for 48 h (laminin α3 and α5) or 12 h (glyceraldehyde phosphate dehydrogenase, GAPDH) and visualized with an image analyzer (Fuji Bas 2500, Fuji Film, Tokyo). The molecular sizes of the hybridizing signals were estimated with the aid of RNA size markers.

Western blot analysis of α3 chain expression Tris-buffered saline containing 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors (Sigma, Saint Louis, MO) was added to the cultured cell lines. After centrifugation, 25 μg of proteins was electrophoresed on a 7.5% acrylamide gel and transferred to a nitrocellulose membrane. An affinity-purified rabbit antiserum raised against a synthetic peptide (CGRTQNEDFKKALTDADNSVNK) derived from the II/I domain of the human laminin α3 chain was hybridized.[25] Bound antibodies were visualized with the peroxidase-conjugated anti-rabbit Ig and enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

RT-PCR analysis of laminin α3 chain expression Poly-A mRNA was extracted from thin sections of OCT-embedded tissue (10 μm×15 slices) with acid guanidine (Stratagene, La Jolla, CA) and oligo-dT latex (TaKaRa, Kusatsu). cDNA reverse-transcribed from 2 to 6 ng poly-A mRNA with random hexamers was amplified with 0.4 p*M* primers (Table I) in 24-cycle (laminin α3) and 20-cycle (GAPDH) reactions. PCR products were electrophoresed on agarose gels, stained with ethidium bromide and quantified with a CCD (charge-coupled device) image sensor (Gel Doc 1000 apparatus and Molecular Analyst Software; Bio Rad, Richmond, CA). External standard curves for both laminin α3 and GAPDH were processed from diluted lines of control mRNA derived from SAE cells. Amounts of sample mRNAs were adjusted so that the PCR reaction would not reach a plateau level.[28] The

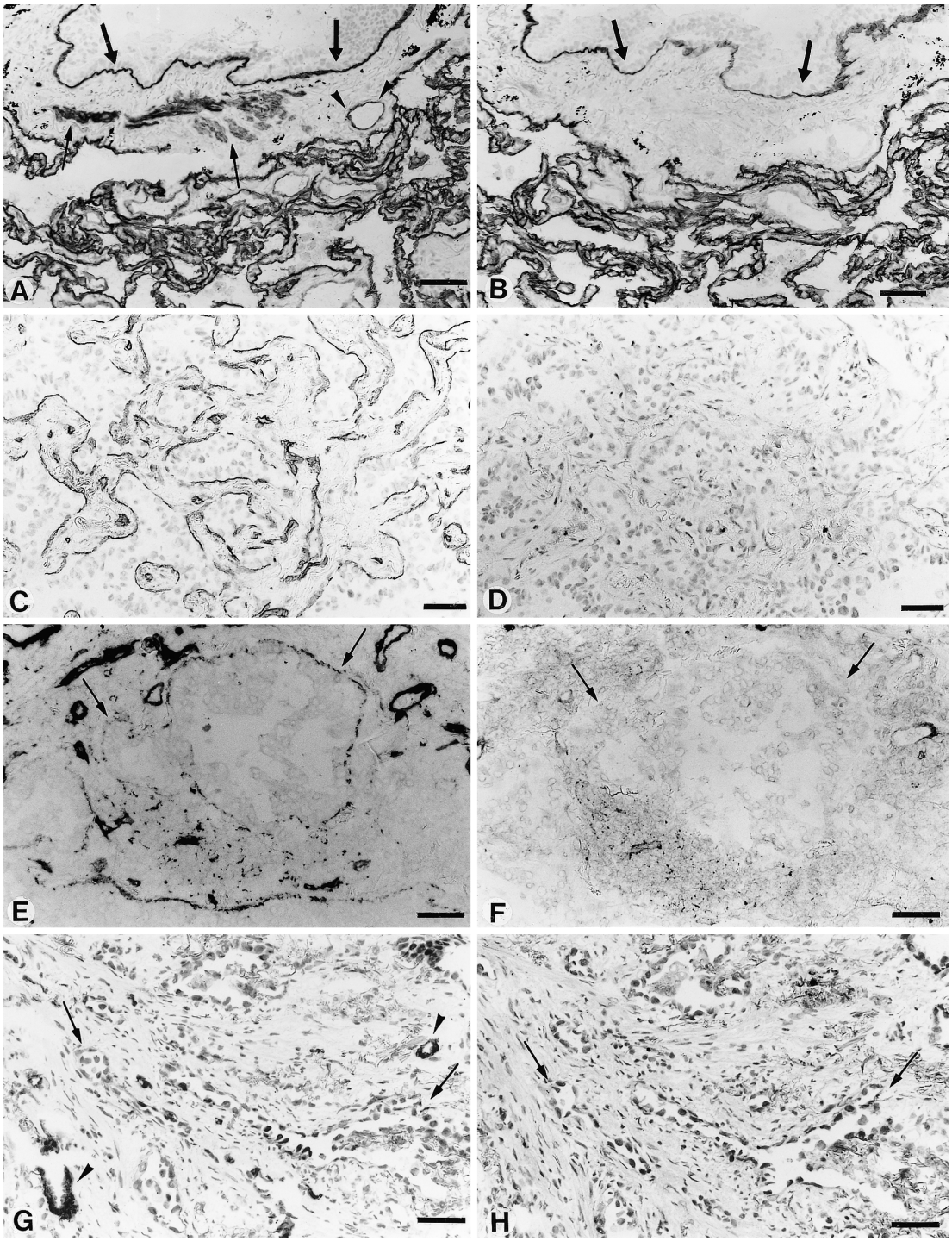

Fig. 1. Laminin α5 (A, C, E, G) and α3 (B, D, F, H) chains in normal lung (A, B) and adenocarcinomas (C–H). Linear staining is evident in both normal bronchiolar epithelium (thick arrows) and alveolar walls. In addition, positive α5 chain staining is shown in a vascular wall (arrowheads) and smooth muscle cells (thin arrows). In bronchioloalveolar areas of adenocarcinomas (C, D), laminin α5 lines the alveolar walls covered by cancer cells (C), whereas α3 chain is absent (D). In a collapsed area (E, F), laminin α5 is discontinuous along the cancer alveolus (E) and laminin α3 chain is absent in the serial sections (F). In an area of fibroblast proliferation (G, H), cancer cell alveoli lack both laminin α5 and α3 chains. Positive staining of α5 chain is apparent in vascular walls (G, arrowheads) (Bar, 25 μm).

amounts of both laminin α3 and GAPDH in each sample were estimated with the aid of the external standard curves and expressed in terms of the amount of corresponding control mRNA. The means of the α3/GAPDH ratios from two independent reactions were used as the values for each sample. Student's *t* test was used to compare data for normal tissues and adenocarcinomas.

Table II. Summary of Immunohistochemical Findings

			Intact (%)	Frag. (%)	Absent (%)
α5	Adeno	BA area	28 (93.3)	2 (6.7)	0 (0.0)
		Col. area	3 (37.5)	3 (37.5)	2 (25.0)
		Fb. area	0 (0.0)	6 (40.0)	9 (60.0)
		Solid	3	0	4
	SCC		4	3	1
α3	Adeno	BA area	20 (66.7)	6 (20.0)	4 (13.3)
		Col. area	1 (11.1)	2 (22.2)	6 (66.7)
		Fb. area	0 (0.0)	4 (26.7)	11 (73.3)
		Solid	1	1	5
	SCC		4	3	1
α5	Adeno	N (−)	13	5	9
		N (+)	2	3	6
	SCC	N (−)	4	0	0
		N (+)	0	3	1
	Sum	N (−)	17	5	9
		N (+)	2	6	7
α3	Adeno	N (−)	7	5	15
		N (+)	1	3	7
	SCC	N (−)	3	1	0
		N (+)	1	2	1
	Sum	N (−)	10	6	15
		N (+)	2	5	8

Adeno: adenocarcinoma, BA area: area with bronchioloalveolar pattern, Col. area: area with collapse, Fb. area: area with fibroblast proliferation, SCC: squamous cell carcinoma, N: lymph node metastasis, Frag: fragmented.

RESULTS

Expression of laminin α3 and α5 chains in lung cancers (Table II) As previously reported,[17] laminin α5 and α3 chains were found to be expressed in both bronchiolar epithelium and alveolar walls of the normal lung (Fig. 1, A and B). The α5 chain was generally preserved in the areas of bronchioloalveolar pattern of the adenocarcinomas (Fig. 1C). It was frequently fragmented (Fig. 1E) or absent in foci of alveolar collapse and often absent in foci with fibroblastic proliferation (Fig. 1G). Fragmentation and absence of laminin α3 chain were more widely observed than was the case for α5 chain, for example in bronchioloalveolar areas of adenocarcinomas, where the α5 chain was intact (Fig. 1, C and D). The α3 chain was also frequently absent in foci with alveolar collapse (Fig. 1F) where fragmented α5 chain persisted (Fig. 1E), as well as in foci with proliferation of fibroblasts (Fig. 1H). Both laminin α5 and α3 chains were well-preserved in the residual bronchiolar epithelium in foci with collapse (data not shown). Solid adenocarcinomas frequently showed absent α3 and α5 chains with proliferation of fibroblasts. Some squamous cell carcinomas also showed fragmented α3 and α5 chains in the invading fronts (Fig. 2, A and B).

Other basement membrane components, laminin β1, γ1 chains, collagen type IV and core protein of heparan sulfate proteoglycan (HSPG), showed distributions similar to that for the laminin α5 chain (data not shown).

Relationship between loss of basement membrane components and lymph node metastasis (Table II) Adenocarcinomas and squamous cell carcinomas with disrupted

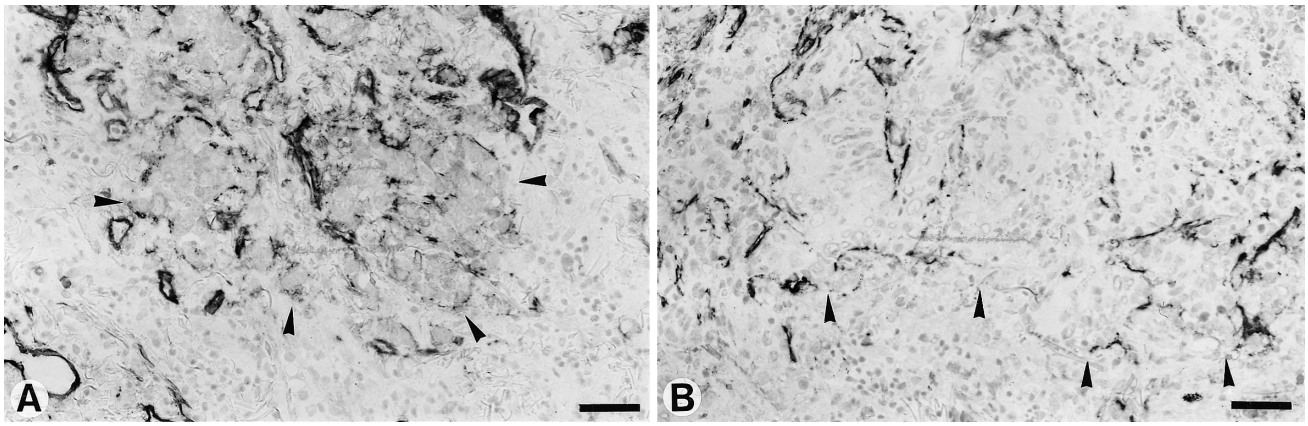

Fig. 2. Laminin α5 and α3 chains in a squamous cell carcinoma (A, B). Both are discontinuous in the invading front (arrowheads) of the squamous cell carcinoma (Bar, 50 μm).

or absent α3 or α5 chain tended to show higher incidence of lymph node metastasis than those with intact basement membrane. The total of adenocarcinomas and squamous cell carcinomas with fragmented or absent α5 chain showed a higher incidence of lymph node metastasis than those with intact α5 chain (P value<0.01). Because laminin α3 chain was disrupted in most of the cancers, including the cases without lymph node metastasis, there was no statistically significant relationship between α3 chain and lymph node metastasis (P value=0.15).

Northern analysis (α3 and α5 chains) and western analysis (α3 chain) in normal and cancer cell lines (Fig. 3) In order to know the expression and synthesis pattern of laminin α3 and α5 chains in lung cancer cells, cultured normal small airway epithelial cells and cancer cell lines were examined first by northern analysis. In normal airway epithelial cells, both laminin α3 and α5 chains mRNA were expressed. In contrast, the fibroblast cell line, TIG1 cells expressed neither. Most of the cancer cell lines did not express α3 chain, and weak expression, about one-eighth that of normal airway epithelial cells, was found in one of three squamous cell carcinoma cell lines. Expression of α5 chain was preserved in nine of the twelve can-

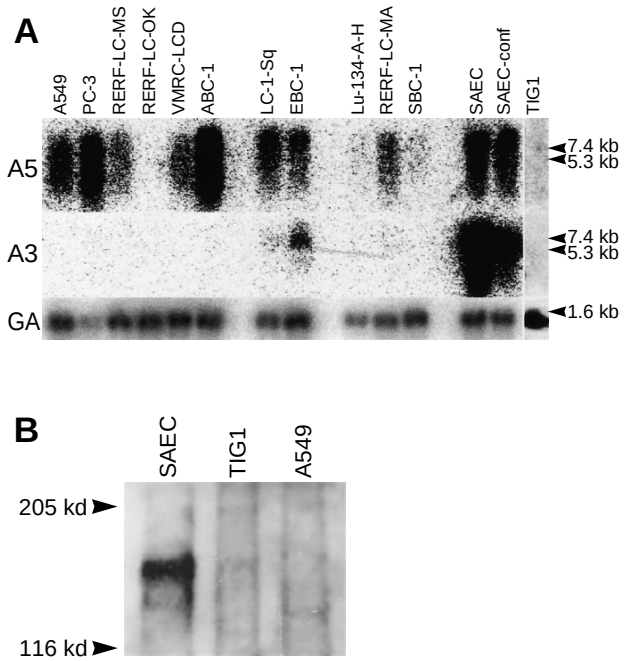

Fig. 3. Northern and western analysis of laminin α chain expression in cultured cell lines. A. Northern blot analysis of transcripts of laminin α5 (A5, 11 kb), α3 (A3, 6 kb), and GAPDH (GA, 1.3 kb) in lung cancer cell lines. Poly-A mRNAs (1 μg) were electrophoresed and hybridized with ^{32}P-labeled probes. A549, PC-3, RERF-LC-MS, RERF-LC-OK, VMRC-LCD, ABC1: adenocarcinomas. LC-1-sq, EBC1: squamous cell carcinomas. Lu-134-A-H, RERF-LC-MA, SBC1: small cell carcinomas. SAEC: normal small air way epithelial cells at 70% confluency. SAEC-conf: at 100% confluency. TIG1: fetal lung fibroblasts. B. Western blot analyis of laminin α3 chain. Proteins (25 μg) were electrophoresed, hybridized with laminin α3 chain antiserum and visualized with the ECL system.

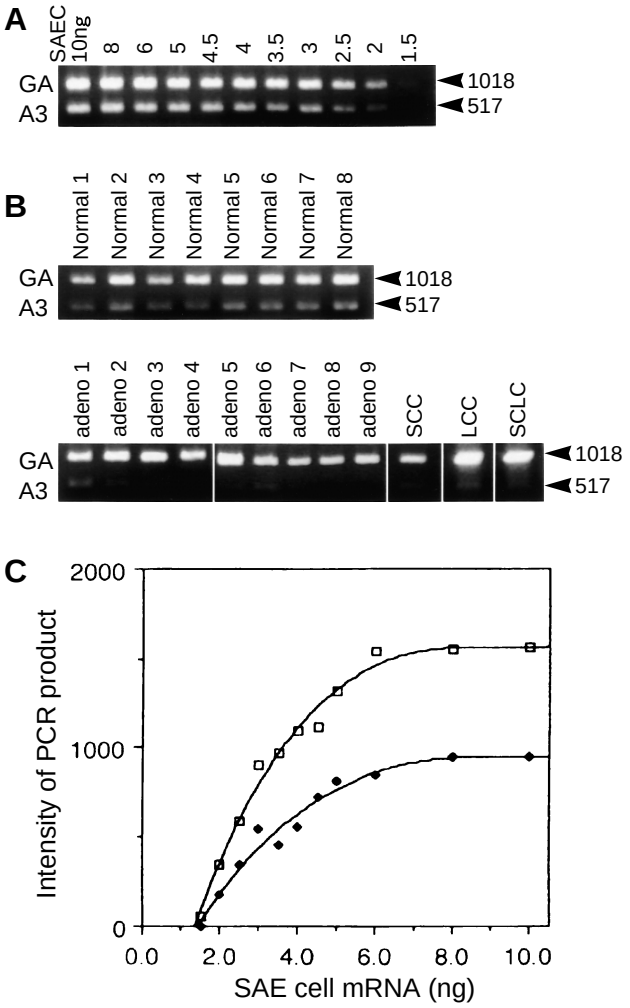

Fig. 4. RT-PCR analysis of laminin α3 chain expression in normal and cancer tissues. A, C: RT-PCR reaction of serially diluted standard mRNA derived from normal small airway epithelial cells (SAEC). A: Gel electrophoresis. GA indicates 913-bp fragment of GAPDH. A_3 refers to 549-bp fragment of laminin α3 chain. B: RT-PCR reaction of normal lung tissues (normal 1–8) and cancer tissues. Adeno 1–4: adenocarcinomas with intact laminin α3 chain immunohistochemically. Adeno 5–9: adenocarcinomas with absent α3 chain. SCC: squamous cell carcinoma. LCC: large cell carcinoma. SCLC: small cell carcinoma. In the adenocarcinoma cases 4, 5, 7, 8, 9 and the small cell carcinoma, PCR products for laminin α3 can not be observed. C: External standard curves based on quantification of PCR products by a CCD image sensor. □ GAPDH, ◆ laminin α3.

cer cell lines, but was reduced or absent in one of the six adenocarcinoma cell lines and two of the three small cell carcinoma cell lines.

In order to confirm that mRNA level really reflected protein synthesis, α3 chain mRNA-expressing cells (SAE cells) and non-expressing cells, lung fibroblast, TIG1 cells and lung adenocarcinoma derived-A549 cells,[29] which showed differentiation to type II pneumocytes *in vitro*, were examined by western analysis. 165 kd α3 chain was detected in SAE cells but not in TIG1 cells or A549 cells.

RT-PCR analysis of laminin α3 chain in cancer tissue (Fig. 4, Table III) Since a marked reduction of α3 chain was seen in the cancer cell lines, α3 chain expression in surgically resected lung cancers was next examined by RT-PCR analysis. PCR products of GAPDH or α3 chain could not be detected when control mRNA was less than 1.5 ng and reached a plateau at 6 ng (Fig. 4, A and C). Thus, amounts of sample mRNAs were adjusted to between 2 and 6 ng. In all the examined 8 cases of normal lung tissue, expression of laminin α3 chain was observed. In 4 of the 5 cases of adenocarcinomas with α3 chain absent immunohistochemically and one small cell carcinoma, PCR products were not observed. As shown in Table III, the laminin α3/GAPDH ratio for adenocarcinomas (0.54±0.13, mean value±standard deviation) was significantly lower than that for normal lung tissue (0.82±0.13) ($P<0.01$, Student's *t* test).

Table III. RT-PCR Results for α3 Expression

Sample	α3/GA	Sample	α3/GA
Normal 1	0.96	Adeno 1	0.71
Normal 2	0.79	Adeno 2	0.41
Normal 3	0.95	Adeno 3	0.45
Normal 4	0.65	Adeno 4	<[a] 0.73
Normal 5	0.63	Adeno 5	<[a] 0.32
Normal 6	0.83	Adeno 6	0.59
Normal 7	0.88	Adeno 7	<[a] 0.55
Normal 8	0.90	Adeno 8	<[a] 0.60
		Adeno 9	<[a] 0.54
Mean	0.82		0.54
SD	0.13		0.13

a) α3 chain, not amplified.

DISCUSSION

With progression of lung adenocarcinomas, active proliferation of fibroblasts occurs within the tumors.[19] This is associated with a higher incidence of lymph node metastasis and a poor prognosis, and thus is considered to be closely related to cancer cell invasion.[20] The present study demonstrated both laminin α5 and α3 chains to be preferentially fragmented or absent in areas of fibroblastic proliferation in adenocarcinomas. This supports the hypothesis that loss of basement membrane elements is closely related to cancer cell invasion,[4, 5] in line with our finding that carcinomas with fragmented or absent α5 chain showed a tendency for a higher incidence of lymph node metastasis.

In cancers other than lung, disruption of basement membrane components has also been reported, such as in gastric,[30] colorectal,[31] breast and head and neck carcinoma.[32, 33] Disruption of laminin was correlated with lymph node metastasis in colorectal, breast and head and neck carcinomas. Thus, disruption of basement membrane is considered to be a common phenomenon in the transformation of epithelial cells and in the process of cancer cell invasion in several organs.

The epithelial basement membrane is considered to be formed from materials secreted by both epithelial and stromal cells. An epithelial origin of laminin α3 and α5 chains[16] was supported by our finding that both genes were expressed in cultured bronchial epithelial cells, but not in fibroblast cell lines. Thus, it can be presumed that mRNA expression in lung cancer cells affects the quantity of α3 and α5 chains.

The northern analysis demonstrated that expression of laminin α3 chain is reduced in lung cancer cells regardless of their histological type. Western analysis of the cultured cell lines confirmed this. Although the reduction in α3 chain mRNA in cancer tissues observed by RT-PCR analysis might have been influenced by increase in stromal components in cancer tissues, it is probable that an absolute decrease occurred in cancer cells. Well-preserved expression of laminin α3 chain was reported previously for intestinal type gastric adenocarcinomas,[34] pancreatic carcinomas[35] and prostatic carcinomas.[36] In contrast, reduced expression of laminin α3 chain in cancer cells was found in breast cancer cell lines,[37] gastrointestinal cancer cell lines,[18] diffuse type gastric adenocarcinomas and papilloma virus transformed foreskin keratinocytes.[25, 34] Thus, it is possible that regulation of laminin α3 chain expression is affected by malignant transformation in certain kinds of epithelial cells.

Because of the reduced gene expression, the metabolism of laminin α3 chain in the cancer basement membrane is presumed to be biased in favor of degradation even if the proteolytic level is equivalent to that in normal alveolar cells. Thus, fragmentation and absence of laminin α3 chain demonstrated immunohistochemically are likely to be a reflection of decreased gene expression in the cancer cells. We consider that laminin α3 chain mostly derived from the normal alveolar cells becomes fragmented in peripheral areas of adenocarcinomas and becomes absent in the central collapsed or fibroblastic areas.

Reduction of α5 chain mRNA expression was also apparent in the cancer cell lines, although to a lesser

extent than for α3 chain. This might also be explained, at least partly, by reduction of mRNA expression in cancer cells, similarly to α3 chain. In addition, we hypothesize that the reduction of laminin α3 chain might result in the subsequent fragmentation of basement membrane. Because reduction of laminin α3 chain was more widely observed than that of α5 chain in immunostainings, the former might precede the latter in the course of basement membrane alteration in the adenocarcinomas. Laminin α3β3γ2 associates with epithelial integrin α6β4 and type VII collagen.[23, 38] In addition, half of the laminin α3β3γ2 is complexed with laminin α3β1γ1/α3β2γ1,[39] and this complex associates with type IV collagen via nidogen.[40] Laminin α3 chain is considered to play important roles in epithelial-stromal attachment, basement membrane assembly and stability[38] and thus its reduction might lead to fragmentation of the collagen and laminin network.

Recently, laminin 5 (α3β3γ2) has been proposed to play roles in hepatocyte growth factor (HGF)-dependent invasion *in vitro*.[35, 41] Thus, the proposal that loss of individual basement membrane components is related to cancer cell invasion may appear controversial. Mutual interactions between cancer cells and fibroblasts through cytokines, such as HGF secreted by fibroblasts, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) secreted by cancer cells, are involved in the lung cancer cell invasion and activation of stromal cells.[42] Negative regulation of these cytokine activities by basement membane components, e.g. by HSPG through HGF[43, 44] and by secreted protein acidic and rich in cysteine (SPARC) through PDGF and bFGF,[45–47] has been reported. It is conceivable that basement membrane loss facilitates effective interactions between cancer and stromal cells through cytokines and results in both increased invasive potential of cancer cells and activation of stromal elements such as fibroblasts.[44]

In summary, both laminin α3 and α5 chains are often fragmented or absent mainly in collapsed and fibroblastic areas of adenocarcinomas of the lung, this being associated with a tendency for greater lymph node metastasis in the α5 chain case. Expression of laminin α chains is often reduced in lung cancer cells and this might contribute to basement membrane fragmentation and subsequent proliferation of stromal elements, as well as play some role in the process of cancer cell invasion.

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