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Morphological characteristics of tumours formed by Lewis lung carcinoma-derived cloned cell lines with different metastatic potentials: structural differences in their basement membranes formed in vivo

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Summary. Tumour basement membrane (BM) is an extracellular matrix produced by tumour cells of epithelial origin. We examined the structure and function of the tumour BM of tumour tissues formed by Lewis lung carcinoma-derived cloned cell lines (P29, LM12-3 and LM60-D6 cells) with low, medium and high metastatic potentials, respectively. Immunohistochemical staining of major BM constituents laminin and type IV collagen demonstrated that all the cell lines produced and deposited these materials extracellularly in vivo. However, the continuity of the tumour BM composed of these materials was much greater in the higher metastatic LM12-3 and LM60-D6 tumours than in those with the low metastatic P29 tumour. Electron microscopic examination revealed that in the higher metastatic tumours, especially the LM60-D6 tumour, the tumour BM had a highly organized structure consisting of lamina densa and lamina rara. Parallel bilayers of BM and their fusion were often observed and tumour cells were in direct contact with the BM. In the vicinity of tumour blood vessels, similar interactions between the tumour BM and the vascular BM were observed, and the tumour cells rested on their own BM, the fused BM or the vascular BM. In contrast, in the low metastatic tumour in which the tumour BM was not clearly defined, this close contact between tumour cells and the vascular BM was not observed. In vitro studies showed that the higher metastatic cells adhered more firmly than the LMP cells to a subendothelial matrix. These results suggest that the adhesiveness of tumour cells to the vascular BM in vivo is correlated with their ability to form an integrated BM in vivo, and that this adhesiveness of the tumour cells may be mediated in part by the tumour BM via BM fusion.

Key words: Lewis lung carcinoma – Basement membrane – Cell attachment – Metastasis

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Introduction

The basement membrane (BM) is a complex extracellular matrix that mediates cell attachment, isolates tissue compartments and maintains tissue architecture throughout adult life (Liotta et al. 1983). During embryonic development and adult tissue remodelling, the BM also serves as an inductive interface and mediates morphogenetic interaction between epithelial cells and mesenchymal cells (Banerjee et al. 1977; Grobstein 1967). In malignant neoplasms, the BM shows a variety of structural abnormalities, such as thinning, multi-layering, discontinuity and total loss (Frei 1978; Gould et al. 1975). There are several reports that the tumour BM is lost in invasive carcinomas (Barsky et al. 1983; Havenith et al. 1988) and so may act as a barrier against tumour invasion. However, many invading carcinomas retain their BM in both primary and secondary sites (Carter et al. 1985; Gusterson et al. 1984), suggesting that the tumour BM does not always serve as a barrier. Furthermore, tumour BM has been shown to mediate the neoplastic organization of the epithelial tissue architecture (Ingber et al. 1985). Thus at present, the role of tumour BM in neoplastic development is controversial.

Mouse Lewis lung carcinoma (3LL) and its derived cell lines have been widely used as metastatic models (Brodt et al. 1983; Lamesweerde et al. 1983; Pal et al. 1983). The 3LL tumour, suggested to originate from granular pneumocytes, is equivalent to human alveolar cell carcinoma, whose development is dependent upon the BM (Garcia-Sanz et al. 1989; Grigioni et al. 1987). Therefore, the 3LL tumour should be useful for studying the relationship between tumour cells and the BM in tumour development. One of the present authors has cloned 3LL-derived cell lines with different metastatic potentials, and demonstrated several differences in the in vitro properties of these cell lines in their growth, adhesiveness and proteinase activities (Takenaga 1984). However, little is known about the ability of tumour

cells to form a BM in vivo or the relationship of the BM with metastasis in this tumour system.

In the present immunohistochemical and electron microscopical studies, we compared the structures of the tumour BM in tumour tissues formed by subcutaneous injection of Lewis lung carcinoma-derived cells with different metastatic potentials, and examined the morphogenetic interaction of the tumour cells with the BM in these tumours.

Materials and methods

Cell lines, P29 and LM12-3, with low and medium metastatic potentials, respectively, were established from the 3LL tumour as described previously (Takenaga 1984). The high metastatic LM60-D6 cell line was established by 60 episodes of in vivo selection on the basis of the spontaneous metastasis, followed by double cloning by the limiting dilution method as described for establishment of the LM12-3 line (Takenaga et al. 1988). The cloned cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, New York), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37° C in a humidified atmosphere of 5% carbon dioxide in air. Bovine pulmonary arterial endothelial cells (American Type Culture Collection, Rockville, Md.) were cultured in DMEM containing 20% fetal bovine serum. The subendothelial matrix was prepared by lysis of confluent endothelial monolayer cultures with 0.5% triton X-100 in phosphate-buffered saline (PBS), and then treatment with 0.025 N ammonium hydroxide and four washes with PBS.

In attachment assay, subconfluent cultures of each clone were prelabelled with [^{3}H]thymidine (New England Nuclear, Boston, Mass.; 82.7 Ci/mmol) for 24 h in complete medium. The labelled cells were detached from culture dishes by treatment with 2 mM EDTA in PBS for 10 min at 37° C. They were then suspended at 5×10^5 cells/ml of medium, and plated in culture dishes coated with subendothelial matrix and allowed to adhere. At the times indicated, unattached cells were carefully removed by three washings with 2 ml of warm PBS and the remaining adherent cells were lysed with 0.1 N sodium hydroxide. The lysate was collected and its radioactivity was counted. In detachment assay, cells were inoculated onto the subendothelial matrix, and 20 h later the cell layer was washed twice with PBS and then rotated on an orbital shaker in PBS at 150 rpm, PBS containing 2 mM EDTA at 100 rpm, and PBS containing 0.002% (w/v) trypsin at 100 rpm. After removal of detached cells, adherent cells were detached from the substratum by vigorous pipetting and the numbers of detached cells and adherent cells were counted.

The spontaneous metastatic potentials of the clones were measured by injecting single-cell suspensions of 2.5×10^5 or 4.0×10^5 cells, respectively, in 0.25 ml of Hanks' balanced salt solution intramuscularly into the left thigh or subcutaneously into the right abdominal flank of 5-week-old syngenic C57BL/6 male mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan). The animals were sacrificed 4 weeks later and the number of visible parietal nodules in the lung fixed in Bouin's solution was counted.

Primary subcutaneous tumours in the right abdominal flank and lung metastatic tumours were fixed in ethanol/acetic acid (98/2, v/v) at 4° C for 24 h and embedded in paraffin. For immunohistochemical examination of type IV collagen and laminin, deparaffinized sections were pretreated with 2 μg/ml trypsin (Sigma, St. Louis, Mo.) solution containing 50 mM TRIS-hydrochloride, pH 7.4 and 5 mM calcium chloride for 15 min at room temperature. Immunostaining was performed by the indirect immunoperoxidase method with peroxidase-conjugated swine immunoglobulin to rabbit immunoglobulins (Dakopatts, Copenhagen, Denmark). Colour was developed with 3,3′-diaminobenzidine as substrate, and specimens were counter-stained with methyl green. For negative controls, the primary antiserum was replaced by non-immune serum. The mono-specific antisera used as primary antibodies were rabbit antiserum to bovine kidney type IV collagen (Advance Co., Tokyo, Japan), and rabbit antiserum to laminin from an Engelbreth-Holm-Swarm tumour (Bethesda Research Labs, Bethesda, Md.).

Morphometric analysis was performed by using a computerized image analyser (Vidas, Carl Zeiss). The length of tumour BMs (except for the vascular BM) at five different fields in one specimen stained with antiserum to type IV collagen was measured directly on the video image input from a light microscope using a video camera with 20 magnifying objective lens. The tumour BMs exhibiting various continuities were classified into 15 classes with different lengths, and the counts of the BMs pooled in each class were expressed as histograms. Statistical differences between different metastatic tumours in frequency distribution of the BMs with different lengths were evaluated on the basis of the results obtained by chi-square test. A value of $P < 0.05$ was evaluated to be significant.

For electron microscopy, primary subcutaneous tumour tissues were fixed in 2.5% glutaraldehyde/2% paraformaldehyde/50 mM phosphate buffer, pH 7.3, for 2 h, post-fixed in 25% osmium tetroxide, and then embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate followed by lead citrate and examined in an electron microscope (Nihondenshi, JEM-1200EX).

Results

The spontaneous metastatic potentials of the three clones were tested by injecting the cells intramuscularly or subcutaneously (Table 1). Irrespective of the site of injection, the three clones exhibited significantly different numbers of lung metastatic nodules, the order of their metastatic potentials being LM60-D6 > LM12-3 > P29. Significant differences in their metastatic potentials were also observed using Balb/C nude mice (data not shown), indicating that these differences were not due to differences in sensitivity of the tumour cells to T-cell-mediated immunity.

On plating at an initial density of 2.0×10^4 cells/35-mm dish, cells of the P29, LM12-3 and LM60-D6 clones grew with significantly different doubling times of 10, 19 and 18.5 h, respectively, and reached significantly different saturation densities of about 4.2×10^5, 7.6×10^4 and 6.4×10^4 cells/dish, respectively, at 3 days after inoculation. The growths of tumours formed in vivo from these clones, monitored by caliper measurement, also

Table 1. Spontaneous lung metastatic potentials of cloned Lewis lung carcinoma cells

Cell line	Route of injection	No. of cells injected ($\times 10^5$)	Lung metastasis		
			Incidence	No. of nodules	
				Mean	Range
P29	i.m.	2.5	1/7	0.1	0–1
LM12-3	i.m.	2.5	6/7	5.9	0–17
LM60-D6	i.m.	2.5	7/7	49.6	18–125
P29	s.c.	4.0	0/7	0	0
LM12-3	s.c.	4.0	3/5	2.0	0–7
LM60-D6	s.c.	4.0	7/7	11.6	5–19

i.m., Intramuscular; s.c., subcutaneous

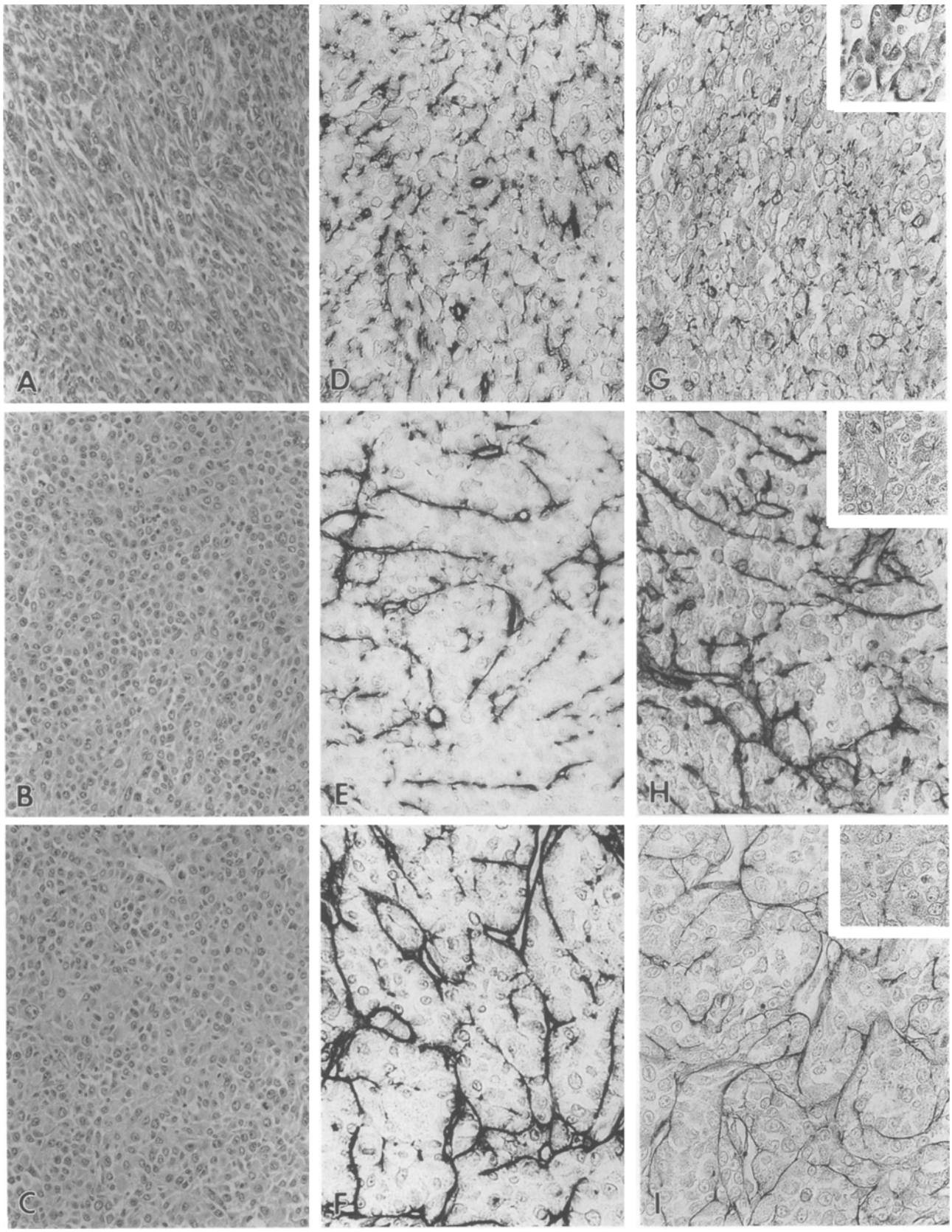

Fig. 1 A–I. Immunohistochemical staining of primary tumour tissues formed by subcutaneous injection of cloned cells with different metastatic potentials. **A–C** Histological appearance of the tumour tissues formed by the low (P29), medium (LM12-3) and high (LM60-D6) metastatic cells, respectively. The low metastatic tumour had a sarcomatous appearance, whereas the higher metastatic two tumours showed a linear or alveolar-like structure. H & E, $\times 170$. **D–F** Immunostaining with anti-type IV collagen of trypsin-treated specimens of P29, LM12-3 and LM60-D6 tumours, respectively. **G–I** Immunostaining with anti-laminin of trypsin-treated and untreated (*insets*) specimens of P29, LM12-3 and LM60-D6 tumours, respectively. The structures reacting with the two antisera were more continuous in the higher metastatic tumours. Indirect immunoperoxidase methods, $\times 340$

differed significantly in this order, as described previously (Takenaga 1984). The sizes of the primary subcutaneous tumours formed by the low and higher metastatic clones reached 3.0 cm in maximal diameters at 25 and 30 days, respectively, after implantation.

Histologically, all the primary subcutaneous tumours had poorly differentiated or undifferentiated carcinomatous appearances, but showed clear differences in histological patterns. The P29 tumour consisted of both large spindle-shaped tumour cells and small fibroblast-like cells arranged in an interlacing pattern (Fig. 1 A). The higher LM12-3 and LM60-D6 tumours were composed of polygonal or columnar tumour cells arranged in a linear or alveolar-like pattern and showed loss of cohesion (Fig. 1 B, C).

To examine the abilities of tumour cells to form a BM in vivo, we stained primary tumour tissues with antisera to type IV collagen and laminin, the major BM constituents. Immunostaining with anti-type IV collagen showed that in addition to the vascular BM, positively stained extracellular structures were observed between tumour cells in all the three different metastatic tumours, but in different patterns. In the P29 tumour, the structures appeared to be punctate or fragmented (Fig. 1 D), whereas in LM12-3 and LM60-D6 tumours, they were continuous, (Fig. 1 E, F), representing a BM-like structure. To clarify the differences in the continuity of these BM-like structures between the three tumours, we performed morphometric analysis using the specimens stained with anti-type IV collagen. The histograms were obtained by counting 1,113-, 881- and 955-immunoreactive structures (tumour BMs) in five random fields of the P29, LM12-3 and LM60-D6 tumours, respectively (Fig. 2). The results showed that frequency of the more continuous BMs was significantly greater in the higher metastatic tumour ($P<0.01$ between the P29 and LM12-3 tumours and $P<0.05$ between LM12-3 and LM60-D6 tumours), and the extent of BM continuity was also greater in the higher metastatic tumour. We concluded that the higher metastatic clone had an ability to form a more continuous BM in vivo.

Similar staining patterns to those with anti-type IV collagen were observed in specimens stained for laminin after enzyme treatment (Fig. 1 G–I). In specimens without enzyme digestion, only the cytoplasm of P29 cells was stained (insets in Fig. 1 G–I). This difference in the staining patterns of the specimens before and after treatment with trypsin suggests that the extracellular antisera-reactive materials are assembled to form BM with other BM components, whereas materials in the cytoplasm are not present as complexes and are sensitive to enzyme treatment. Alignment of tumour cells on the vascular BM as well as on the tumour BM-like structure was observed in the higher metastatic tumours, but not in the low metastatic P29 tumour. In spontaneous lung metastatic tumours, the staining patterns were essentially the same as those in the individual primary tumours, even in the rare P29 metastatic tumours (data not shown), indicating that the ability of the individual clones to form a BM is an inherent property of the cells.

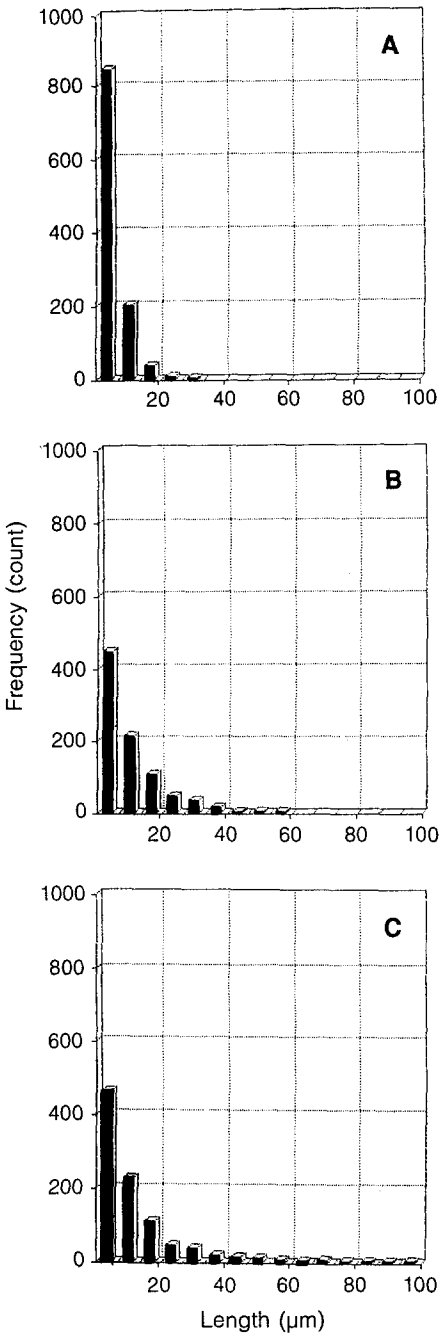

Fig. 2 A–C. Histograms of frequency distribution of the tumour basement membranes (BMs) with different continuities in primary tumour tissues formed by subcutaneous injection of cloned cells with different metastatic potentials. Length of the tumour BMs in the P29 **A**, LM12-3 **B** and LM60-D6 **C** tumours was measured using a computerized image analyser. The tumour BMs with various lengths (see Fig. 1 D–F) were classified into 15 classes with different lengths as indicated. The maximum continuity of the BMs observed was about 100 μm in length. The BMs with length less than 7 μm were the most abundant in all the three tumours. However, in the higher metastatic LM12-3 and LM60-D6 tumours the frequency of such short fragmentous BMs decreased markedly and that of more continuous BMs with length more than 20 μm increased significantly. Furthermore, the extent of the continuity of the tumour BMs was greater in the higher metastatic tumour

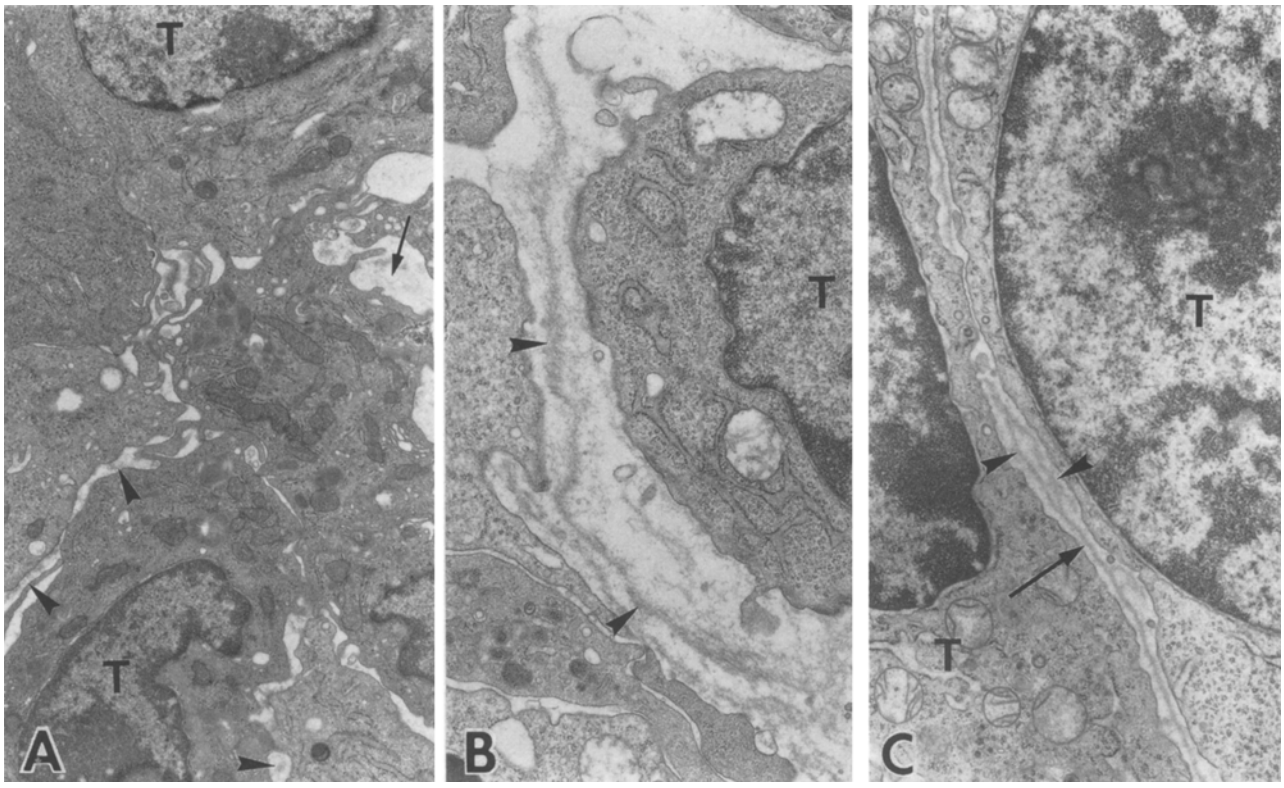

Fig. 3A–C. Electron micrographs of BM-like structures formed by P29, LM12-3 and LM60-D6 cells in primary subcutaneous tumour tissues. **A** Electron-dense extracellular amorphous deposits in a P29 tumour (*arrowheads*). Collagen fibrils were also seen (*arrow*). **B, C** BM-like structures in LM12-3 and LM60-D6 tumours, respectively (*arrowheads*). The organization of the BM-like structure was greater in the LM60-D6 tumour than in the LM12-3 tumour. Fusion of parallel layers of tumour BM is seen in the LM60-D6 (*arrow*). **A** ×6000; **B–C** ×10000. *T*, Tumour cell

The fine structure of the BM-like extracellular matrix formed by tumour cells was examined by electron microscopy. In the P29 tumour, electron-dense amorphous materials were observed between the tumour cells (Fig. 3A). This deposit seemed to correspond with the punctate structures that stained with antisera to type IV collagen and laminin. In addition to this amorphous deposit, collagen fibres or fibrils were seen around the tumour cells. In contrast, in LM12-3 and LM60-D6 tumours, a continuous BM composed of a lamina densa and lamina rara was observed between tumour cells. The BM formed by LM12-3 cells was irregular in thickness, whereas that formed by LM60-D6 cells had a defined structure, the lamina densa having a uniform thickness of 60–70 nm (Fig. 3B, C). Thus, the integrity of the BM formed by LM60-D6 cells was greater than that of the BM formed by LM12-3 cells. In the LM60-D6 tumour parenchyma, the well-defined tumour BMs ran parallel with each other and fused in places to form a single BM, which was shared by tumour cells on both sides of it (Fig. 3C). Similar regions were occasionally seen in the LM12-3 tumour with an ill-defined BM (Fig. 3B), but were not seen in the P29 tumour as there was no integrated BM (Fig. 3A).

At the interface between tumour cells and tumour blood vessels, similar BM-BM interaction was observed. In the LM60-D6 tumour, the well-defined tumour BM ran parallel with the BM of tumour blood vessels and often fused with it. Tumour cells came in direct contact with the fused BM or the vascular BM with extension of their cytoplasm along the BM (Fig. 4C–E). In the LM12-3 tumour, interaction between the tumour BM and vascular BM was also observed, but less frequently and less extensively than in the LM60-D6 tumour (Fig. 4B). In the P29 tumour, collagen fibrils were often present between tumour cells and the vascular BM. Tumour cells extended cytoplasmic villous projections toward the vascular BM and polarized contact of the tumour cells with the vascular BM was never observed (Fig. 4A).

In vitro analysis showed that the initial rate and extent of attachment of LM60-D6 cells to a subendothelial matrix were higher than those of the other two clones. The attachments of LM12-3 cells and P29 cells were not significantly different (Fig. 5). The strengths of cell attachment to the matrix, which were estimated as the extents of resistance to detachment on treatments with PBS, EDTA and trypsin, were well-correlated with the metastatic potentials of the tumour cells (Fig. 6A–C).

Discussion

In this work we found that Lewis lung carcinoma-derived cell lines with different metastatic potentials showed strikingly different abilities to form a BM in vivo, indicating that cloning of the cell lines depending

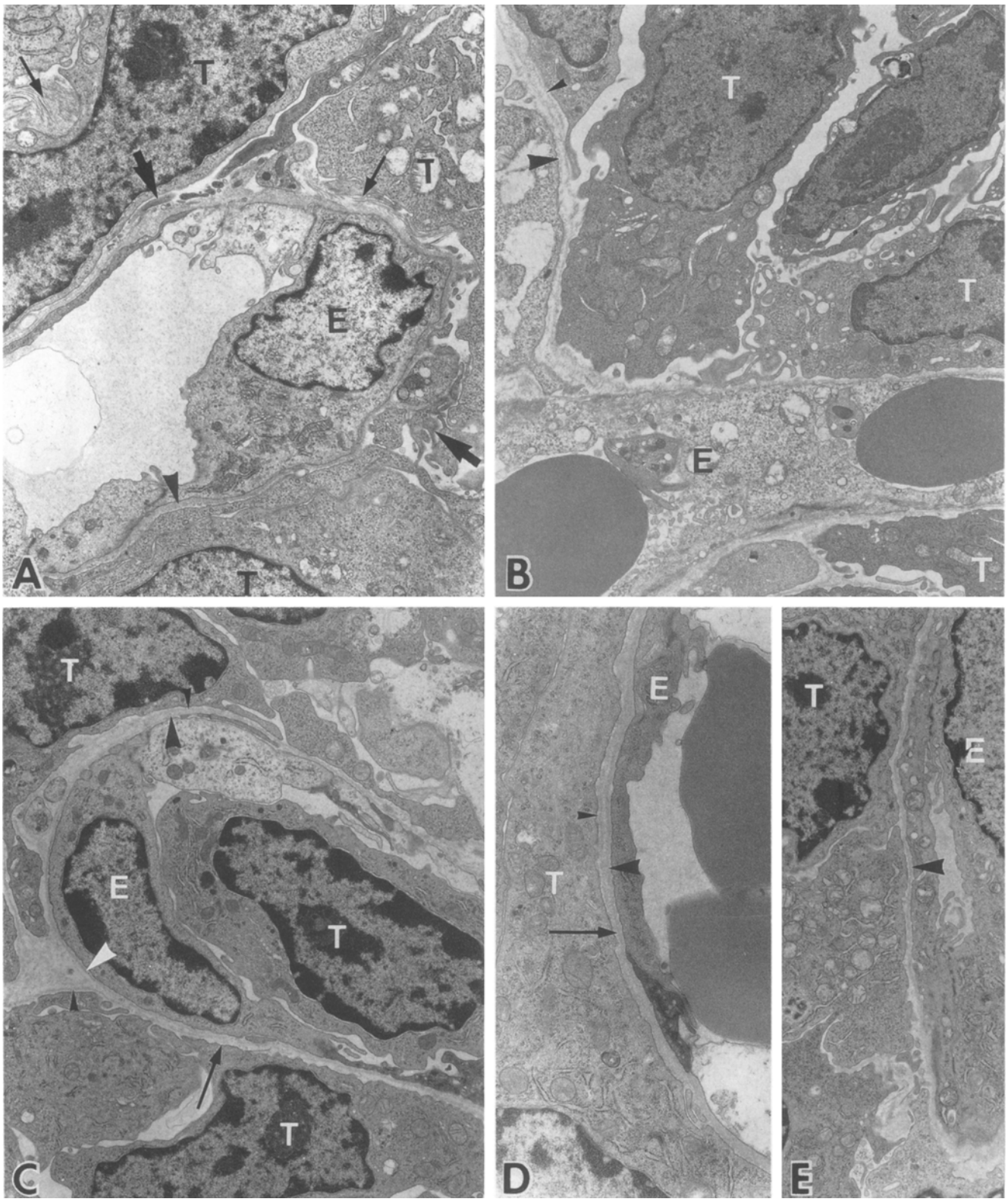

Fig. 4 A–E. Electron micrographs of the interface between tumour cells and the vascular BM in primary subcutaneous P29, LM12-3 and LM60-D6 tumours. **A** P29 tumour. Collagen fibrils (*small arrows*) and cytoplasmic villous projections (*large arrows*) were frequently observed at the interface. No close contact of tumour cells with the vascular BM is seen. **B** LM12-3 tumour **C–E** LM60-D6 tumour. The tumour BM (*small arrowheads*) and vascular BM (*large arrowheads*) run parallel and in place fused to form a single BM (*arrows*). LM60-D6 cells showed closer contact with the vascular BM than LM12-3 cells. **A–C, E** ×4000; **D** ×8000. *T*, Tumour cell; *E*, endothelial cell

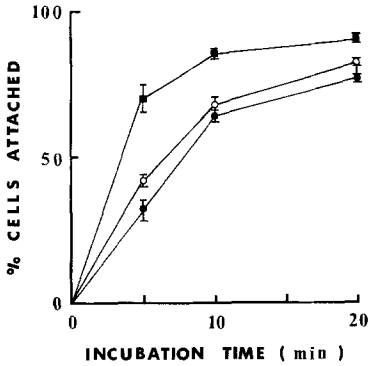

Fig. 5. Adhesions of P29, LM12-3 and LM60-D6 cells to a subendothelial matrix. Cells labelled with [^{3}H]thymidine were inoculated into dishes coated with subendothelial matrix. At the indicated times, unattached cells were carefully removed and adherent cells were lysed and their radioactivities measured. Values are means ± SD. P29 (○), LM12-3 (●) and LM60-D6 (■) cells

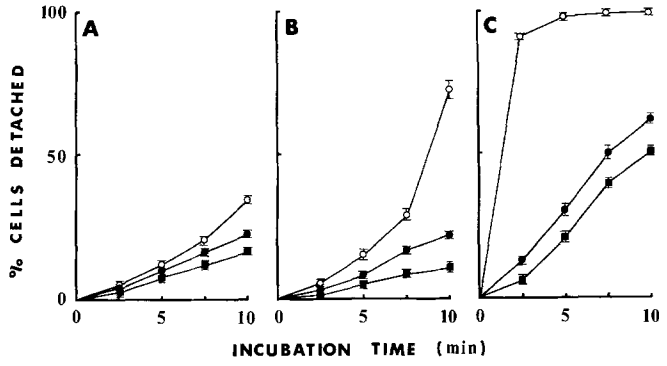

Fig. 6A–C. Detachment of P29, LM12-3 and LM60-D6 cells from a subendothelial matrix by treatments with phosphate-buffered saline (PBS), EDTA and trypsin. Cells were inoculated into dishes coated with the subendothelial matrix, and 20 h later the cell layer was rotated on an orbital shaker for the indicated times in PBS **A** or PBS containing EDTA **B** or trypsin **C**. After removal of detached cells, adherent cells were released from the substratum by vigorous pipetting and the numbers of detached cells and adherent cells were counted. Values are means ± SD. P29 (○), LM12-3 (●) and LM60-D6 (■) cells

on their spontaneous metastatic potentials resulted in selection of their abilities to form a BM. That is, the higher metastatic LM12-3 and LM60-D6 clones formed well-organized BMs, although the continuity and integrity of the BM formed by LM60-D6 cells were greater than those of the BM formed by LM12-3 cells. In contrast, the low metastatic P29 cells failed to form a BM-like structure, but instead formed disorganized aggregates of BM constituents extracellularly. Thus the present tumour system is useful for study of the relationship between the structure and the fuction of tumour BM during tumour development and/or progression.

Formation of a BM is one of the differentiated phenotypic characteristics of epithelial tumour cells (Forster et al. 1984; Stenback et al. 1985) and in this respect, the higher metastatic cells are relatively more differentiated than those with the low metastatic cells. This finding is consistent with a recent observation that the metastatic 3LL tumour is a carcinoma derived from type II pneumocytes and is equivalent to the human alveolar cell carcinoma as judged by its ultrastructure (Garcia-Sanz et al. 1989). These results suggest that the low metastatic P29 cells may be an undifferentiated variant lacking BM forming ability, derived from the higher metastatic cells.

LM60-D6 cells with an integrated BM were in close contact with the vascular BM. Similar but somewhat looser contact was observed between LM12-3 cells with a less integrated BM and the vascular BM. In contrast, P29 cells, which did not form a continuous BM, showed no contact with the vascular BM. These findings suggest that the ability of tumour cells to form a well-defined BM is positively correlated with their adhesiveness to the vascular BM in vivo. This morphological correlation was supported by in vitro functional analyses, demonstrating that the higher metastatic cells adhered to a subendothelial matrix more firmly than the low metastatic cells. In relation to this, it is of note that in the LM60-D6 tumour, the well-defined tumour BMs fuse with each other, and the fused regions are shared by tumour cells on either side. This type of interaction of the BM was also observed between the tumour BM and the vascular BM in the perivascular region and resulted in close contact of tumour cells with the vascular BM, although the fused BM was of dual origin; tumour cells and endothelial cells. It is noteworthy that fibronectin is a component of the vascular BM, but not of the tumour BM (data not shown). Similar fusions of the BM were observed in the LM12-3 tumour, although to lesser extents. Thus, it is conceivable that the attachment of the higher metastatic cells to the vascular BM in vivo may be mediated by the tumour BM via its fusion with the BM of endothelial cell origin, and that this BM fusion may depend upon the integrity of the tumour BM. This kind of BM fusion, back-to-back fusion of BMs of dual origin, has been reported in the formation of the glomerular BM in newborn rat kidney (Abrahamson 1985). Fusion of the alveolar BM with that of the capillary also occurs in adult rat lung (Vaccaro and Brody 1981). Furthermore, fusion of the tumour BM with the pulmonary epithelial BM was observed when murine mammary carcinoma cells invaded the lung parenchyma (Pitelka et al. 1980). Thus, it is likely that fusion of BMs of heterogeneous origins may be important in both normal and neoplastic development. The tumour BM has been regarded as a barrier that separates tumour cells from other tissue compartments. However, the present results strongly suggest that a well-organized tumour BM may be a dynamic structure that mediates morphogenetic interaction between tumour cells and the pre-existing BM of host tissues.

Loss of BM formation in the low metastatic P29 tumour may be due to lack of synthesis of some BM constituents (Burtin et al. 1982), defective assembly of BM components or local proteolytic degradation of these materials (Liotta et al. 1980; Nakajima et al. 1983). In the present study we found that P29 cells could produce and secrete type IV collagen and laminin, and even formed aggregates of these components, although they

did not form a continuous BM. The absence of a BM in this tumour is, therefore, probably attributable to a defect in the integrated assembly of BM components, rather than to their impaired production, although the possibility of increased destruction of the BM by some degradation enzyme(s) could not be excluded. These results suggest structural abnormalities of BM constituents produced by the low metastatic P29 cells and indicate that these cell lines are useful for studying the molecular basis of organization of the BM. We are now investigating this subject.

It is noteworthy that the ability of tumour cells to form a BM is correlated with their metastatic potential. The higher metastatic cells exhibited greater attachment than low metastatic cells to the vascaular BM both in vivo and in vitro. higher metastatic tumour cells have been shown to attach preferentially to vascular BM or BM constituents in vitro (Kramer et al. 1980; Murray et al. 1980). Furthermore, human gastric cancers with liver metastasis were found to show close contact with the vascular BM in the primary tumours, more frequently than those without it (Nakanishi et al. 1991). It is, therefore, conceivable that the metastases of LM12-3 and LM60-D6 cells are closely related to their marked adhesiveness to the vascular BM, which is mediated in part by the interaction of their BM with the vascular BM. At present, we have no evidence that the attachment of tumour cells to the vascular BM results in intravasation of the tumour cells. However, the frequent alignment of tumour cells on the vascular BM may increase the chance of intravasation of tumour cells, permitting their dissemination to distant organs.

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