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Correlation between the Presence of Microvilli and the Growth or Metastatic Potential of Tumor Cells

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We used an electron microscope to examine microvilli which appear on the surfaces of various tumor cells with high or low growth potential and/or metastatic ability. The results show that a greater number of microvilli appeared on the surfaces of tumor cells (QRpP and ERpP) which possess high growth potential than on tumor cells (QR and ER) with low growth potential. We also observed that microvilli were more abundant on the surface of highly metastatic clone cells, i.e. c-SST-2 (cl-2), mouse B16 melanoma (F-10) and human colon carcinoma (KM12SM) than on weakly metastatic clone cells, c-SST-2 (cl-4-2), B16 (F-1) and (KM12C). At the same time, more microvilli were observed on the surface of B16 BL6 cells, which were obtained from the metastatic site of the B16 F10 cells, than on the surface of the parent B16 F10 cells. Immunoelectron microscopy revealed that the c-*neu* oncogene product, which is closely related to an epidermal growth factor receptor, was positively stained in the microvilli of tumor cells (ERpP) with high growth potential and high metastatic ability, whereas the tumor cells (ER) with low growth potential and weak metastatic ability were not stained. These findings suggest that the increased presence of microvilli correlates closely with the growth potential and metastatic ability of tumor cells.

Key words: Microvilli — Growth potential — Metastasis — Scanning electron microscope — Immunoelectron microscopy

Microvilli commonly appear on the surface structures of normal small intestine as well as of malignant cells, and are believed to affect in particular the absorbing function of the small intestine cells.[1] Examinations of various cell surface features have revealed that there are obvious differences between normal embryo cells and embryo cells transformed by a virus,[2] X-irradiation[3] or chemicals.[4] An SEM study has revealed that normal embryo fibroblast cells show smooth surfaces, whereas dimethylbenz[*a*]anthracene-transformation of the same cells generates short and long microvilli which are associated with oncogenic potential.[4] The presence of numerous microvilli on the surfaces of tumor cells along with their pre-malignant lesions has been taken to indicate that microvilli may be characteristic of active cells in general.[5] Furthermore, the disappearance of microvilli from the surface of transformed fibroblasts has been found to correlate with the presence of dibutyryl cAMP[6] or a lipid-depleted medium.[7] Recent SEM investigations have demonstrated two kinds of receptors, transferrin and transcobalamin II, on the microvilli of leukemia cells.[8] However, the studies focusing on the different numbers of microvilli appearing on normal and transformed cells have not substantially explained the relation between the microvilli and the growth or metastatic potential of tumor cells. In the present study, we have investigated the microvilli of tumor cells with high or low growth potential and/or metastatic ability, and have found a possible correlation between abundant numbers of microvilli and the growth or metastatic potential of tumor cells.

The abbreviations used are: QR, weakly tumorigenic cells obtained from BMT-11 cells treated with quercetin; ER, weakly tumorigenic cells obtained from c-SST-2 cells treated with EMS; QRpP, a highly tumorigenic clone derived from QR by implantation of QR cells attached to plastic plates; ERpP, a highly tumorigenic clone derived from ER by implantation of ER cells attached to plastic plates; SHR, spontaneously hypertensive rats; c-SST-2, spontaneously developed rat mammary adenocarcinoma; EMS, ethyl methanesulfonate; FV-KMT-17, KMT-17 cells infected with Friend leukemia virus; SEM, scanning electron microscope.

MATERIALS AND METHODS

Tumor cell lines Mouse tumor clones: Table I lists the tumors whose microvilli were examined by electron-microscopic observation. Tumor cell clones which possess high or low growth potential have been established in our laboratory according to their *in vivo* tumorigenicity and metastatic ability (Table II). The tumor cell (QR) clone with low growth potential is a subclone derived from a quercetin-treated BMT-11 fibrosarcoma cell line which originated in a C57BL/6 mouse. By transplanting into mice the QR cells attached to plastic plates, we have obtained three malignant tumor cell (QRpP) clones with

Table I. Tumors with Microvilli Observed through an Electronmicroscope

Tumor	Growth potential *in vivo*	
	Weakly tum.[a]	Highly tum.[a]
BMT-11 mouse fibrosarcoma	QR	QRpP
c-SST-2 rat mammary carcinoma	ER	ERpP
	Weakly meta.[b]	Highly meta.[b]
B-16 mouse melanoma	F-1	F-10
c-SST-2 rat mammary carcinoma	clone 4-2	clone 2
KM12 human colon carcinoma	C	SM
A bladder metastatic cell clone of B16 F10		B16 BL6
KMT-17 rat fibrosarcoma	FV-KMT-17	KMT-17

a) Weakly or highly tumorigenic.
b) Weakly or highly metastatic.

Table II. Tumorigenicity and Metastatic Ability of Tumor Cell Clones with Low and High Growth Potential

Tumor cell clones	Lethal tumor take[a] (sc)	Lung meta. incidence (No. of colonies)
QR	0/5	1/5 (iv)[b, c] (0, 0, 0, 0, 2)
QRpP	8/8	8/8 (iv)[b, c] (all TNTC)
ER	0/5	0/5 (sc) (0, 0, 0, 0, 0)
ERpP	5/5	5/5 (sc) (3, 7, 26, 34, 35)

a) QR and QRpP tumor cells (2×10^5) were sc inoculated into 7- to 10-week-old C57BL/6 mice. ER and ERpP tumor cells (1×10^4) were sc inoculated into 7- to 10-week-old SHR rats, and the rats were observed for 3 months.
b) QR or QRpP tumor cells (1×10^6) were iv injected into C57BL/6 mice, which were killed and examined 19 days later.
c) Reference 9.

Table III. Metastatic Ability of c-SST-2 Clones in SHR Rats

c-SST-2 clones	Site of inoculation	Pulmonary metastases[b]		
		Incidence	Median (No. of colonies)	Range
Clone 2	sc[a]	5/5	64.0	32–107
Clone 4-2	sc	2/6	0.0	0–10

a) Rats were sc inoculated with 1×10^6 c-SST-2 tumor cells. Pulmonary metastases were examined macroscopically 35 days after the inoculation.
b) Reference 22.

high growth potential.[9, 10] We have examined weakly and highly metastatic cells of F-1 and F-10, which have been derived from the B-16 melanoma,[11] as well as cell clone B16 BL6, a bladder metastatic tumor of the B16 F10 melanoma that possesses high metastatic ability.[12]

Rat tumor clones: The tumor cell line ER, which has low growth potential and weak metastatic ability, was derived from a c-SST-2 cell line; this cell line is from a spontaneously developed mammary adenocarcinoma in an SHR rat, and was produced by treatment of SST-2 cells with ethyl methanesulfonate (EMS). We used the plastic plate method[9, 10] to obtain three malignant tumor cell (ERpP) clones with high growth potential and high metastatic ability. We also studied a weakly metastatic (cl-4-2) clone and a highly metastatic (cl-2) clone, both of which were derived from the c-SST-2 cell line.[13] Table III summarizes the *in vivo* metastatic ability of these clones. KMT-17 is a transplantable fibrosarcoma cell line which we have induced with 3-methylcholanthrene in a WKA rat and maintained in ascites form. The FV-KMT-17 clone cells were obtained from the KMT-17 cell line that had been artificially infected with Friend leukemia virus.[14]

Human carcinoma cell clones: A weakly metastatic clone (KM12C) and a highly metastatic clone (KM12SM) were obtained from a primary human colon carcinoma implanted into nude mice.[15]

Scanning electron microscopic (SEM) studies The tumor cells were cultured on coverslips (10×10 mm) for one or two days before they became attached to the slips. The cells on slips were fixed in 1.25% glutaraldehyde buffered with cacodylate at pH 7.4 at 37°C for 10 min,

and transferred to 2.5% glutaraldehyde in the same buffer for 1 h. After having been fixed with 1% osmic acid for 15 min, they were fixed with 1% tannic acid for 30 min, to which a further 1% osmic acid was then added. Following dehydration in a graded series of alcohol and 3-methylbutyl acetate, the specimens of tumor cells were dried in a critical-point dryer with liquid CO_2 (Hitachi, Tokyo), mounted on aluminum stubs painted with conducting silver, and finally coated with gold-palladium. All the specimens were examined with an S-450 scanning electron microscope at an acceleration voltage of 15 kV (Hitachi Ltd., Tokyo).[16] At 5,000-fold magnification, the quantity of microvilli of 100 randomly chosen cells from each clone was evaluated and calculated per cm^2 of the cell surface area. The significance of differences was analyzed by means of Student's t test.

Immunoelectron microscopic studies Fresh tissue specimens obtained from the tumor cells (ERpP and ER) with high and low growth potential, which had been inoculated sc into SHR rats, were fixed overnight with 4% PFA and an additional 8% succhorose, then washed in 10%, 15% and 20% succhorose for 4 h each. After the specimens had been embedded in an OCT compound (Miles Inc., Elkhart, IN), frozen sections (6 μm thick)

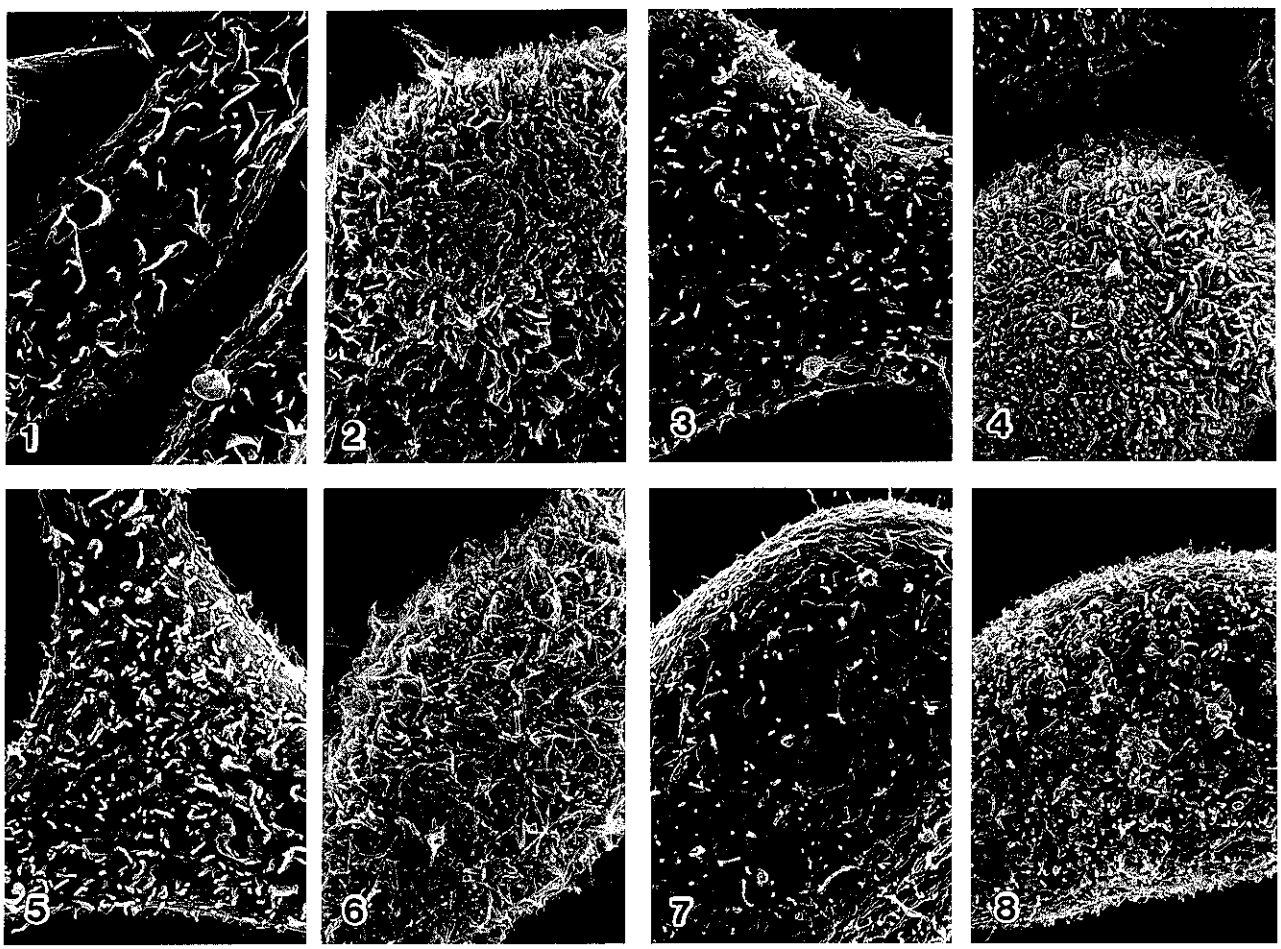

Fig. 1. Surface of a tumor cell (QR), which possesses low growth potential, with few short microvilli. ×7,100.
Fig. 2. Numerous microvilli on the surface of a tumor cell (QRpP), which has high growth potential. ×7,100.
Fig. 3. Surface of a tumor cell (ER), which possesses low growth potential, with few short microvilli. ×7,100.
Fig. 4. A large number of microvilli on the surface of a tumor cell (ERpP), which has high growth potential. ×7,100.
Fig. 5. Surface of a weakly metastatic clone cell (c-SST-2 cl-4-2), with few microvilli. ×7,100.
Fig. 6. Surface of a highly metastatic clone cell (c-SST-2 cl-2), with numerous microvilli. ×7,100.
Fig. 7. Surface of a weakly metastatic F-1 cell, which was derived from B-16 melanoma, with few microvilli. ×7,100.
Fig. 8. Surface of a highly metastatic F-10 cell, which was derived from B-16 melanoma, with numerous microvilli. ×7,100.

were cut in a cryostat and placed on a glass slide. We followed the ABC method of incubation.[17] The sections were incubated in turn for 1 h in each case; the first antibody was a rabbit anti-c-*neu* (1:100, Oncogene Science, Inc., NY), the second antibody consisted biotinylated anti-rabbit immunoglobulins and the third was an avidin-biotin-peroxidase complex solution (Vector Laboratories). Finally, the sections were developed in 3,3′-diaminobenzidine with 0.03% H_2O_2. After immunostaining, the sections were fixed in a 2.5% glutaraldehyde solution (buffered pH 7.4) and post-fixed in 2% osmic acid. Following dehydration, the sections were embedded in Epon 812, detached from the slides after solidification in a 60°C oven for 72 h and cut into ultrathin sections. They were then examined through a Hitachi H-800 electron microscope.

As a negative control, the first antibody was replaced with phosphate-buffered saline.

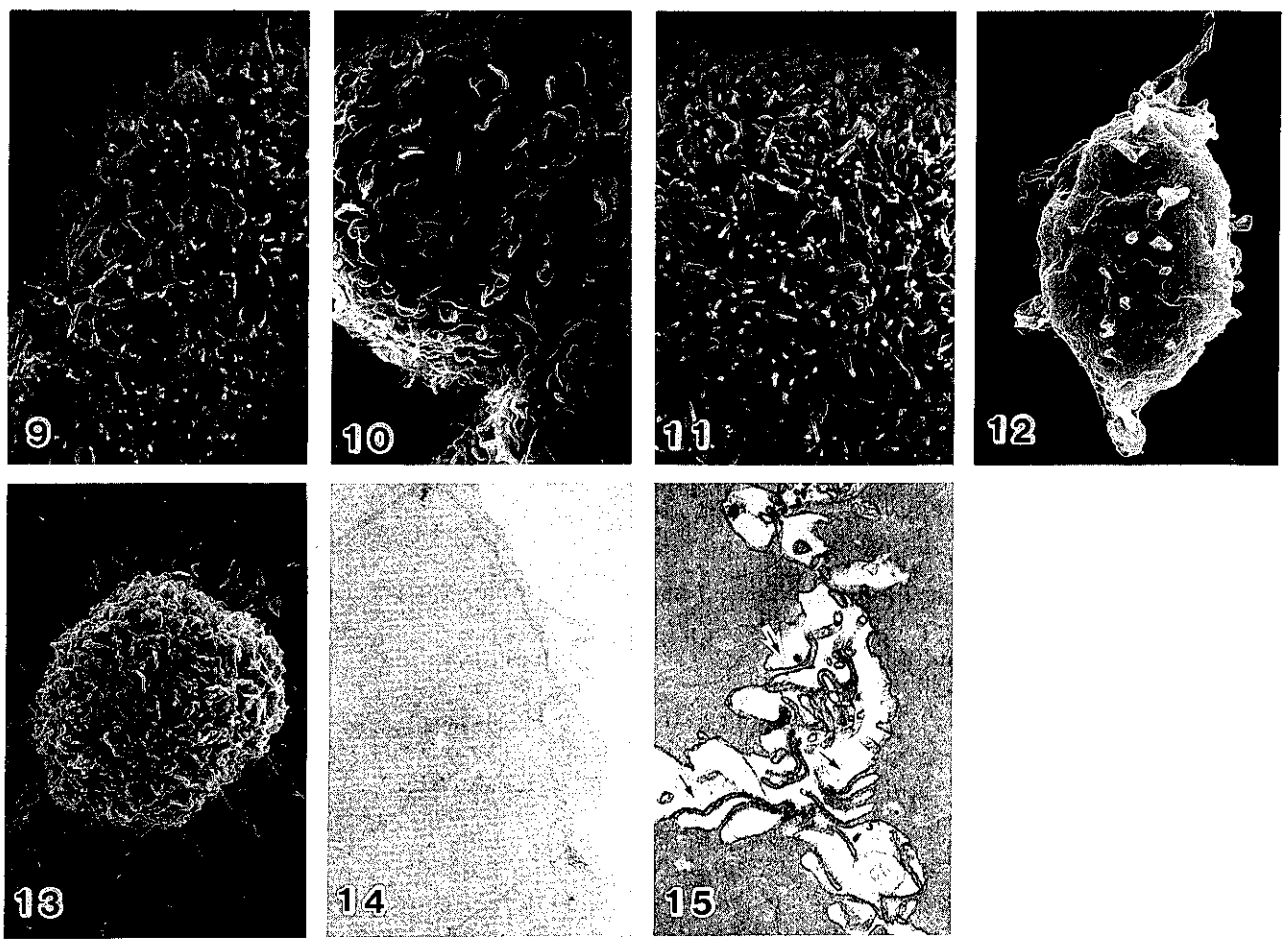

Fig. 9. An increased number of microvilli on the surface of a highly metastatic B16 BL6 cell, which was isolated from bladder metastatic tumor of B16 F10. ×7,100.
Fig. 10. Surface of a weakly metastatic clone cell of a human colon carcinoma implanted in nude mice (KM12C), with few microvilli. ×7,100.
Fig. 11. Surface of a highly metastatic clone cell of a human colon carcinoma implanted in nude mice (KM12SM), with abundant microvilli. ×7,100.
Fig. 12. The nearly naked cell surface of FV-KMT-17 transfected with Friend leukemia virus. ×7,000.
Fig. 13. Untransfected KMT-17 clone cell with a number of microvilli. ×7,000.
Fig. 14. No positive reaction in the membrane of tumor cell (ER). ×5,500.
Fig. 15. Positive staining of a c-*neu* oncogene product in the membrane of a tumor cell (ERpP), especially in the membrane of microvilli (arrows). ×4,650.

RESULTS

Scanning electron microscopic (SEM) findings

1. Microvilli on the surfaces of tumor cells with high or low growth potentiality: The tumor cells (QR) with low growth potential had a smooth surface with a few short microvilli (Fig. 1), whereas the tumor cells (QRpP) with high growth potential displayed a dense covering of microvilli or accumulations of both short and long microvilli (Fig. 2).

These manifestations of microvilli were observed not only in fibrosarcoma of C57BL/6 mice, but also in spontaneous adenocarcinoma of SHR rats. Few microvilli were found on the cell surfaces of tumor cells (ER) with low growth potential and weak metastatic ability (Fig. 3), whereas abundant microvilli were observed on the cell surfaces of tumor cells (ERpP) with high growth potential and high metastatic ability (Fig. 4).

2. Microvilli on the surfaces of tumor cells with high or weak metastatic ability: Distinct differences were also observed in the number of microvilli that appeared on either highly or weakly metastatic tumor cells derived from the c-SST-2 cell line. The surface of the weakly metastatic cell clone (cl-4-2) had few microvilli, whereas the cell surface of a highly metastatic cell clone (cl-2) bore a large number of microvilli (Figs. 5 and 6). Similar results were obtained for nonmetastatic F-1 and metastatic F-10 cells of B-16 melanoma (Figs. 7 and 8). In addition, the B16 BL6 metastatic cells carried more microvilli (Fig. 9) than their parent B16 F10 melanoma cells (Fig. 8).

The cell surface of the weakly metastatic cell clone (KM12C), derived from a primary human colon carcinoma and implanted in nude mice, also carried fewer microvilli than the surface of a highly metastatic cell clone (KM12SM) (Figs. 10 and 11).

3. Microvilli on tumor cells infected or uninfected with Friend leukemia virus: When various types of rat tumor cell were artificially infected with Friend virus complex (FV), they regressed spontaneously.[14] The surfaces of the FV-KMT-17 cells (rat fibrosarcoma cells infected with Friend leukemia virus) bore few microvilli, whereas the uninfected KMT-17 clone cells carried a large number of microvilli (Figs. 12 and 13).

The summary in Table IV shows that the numbers of microvilli in each highly tumorigenic and highly metastatic cell clone were significantly higher than in their weakly tumorigenic and weakly metastatic counterparts.

Detection of the c-*neu* oncogene product by immunoelectron microscopy We examined the expression of the c-*neu* oncogene product on the surfaces of tumor cells (ERpP) with high growth potential and high metastatic ability by immunoelectron microscopy. Positive staining of c-*neu* was observed exclusively in mem-

Table IV. Ultrastructual Differences in the Number of Microvilli between Tumor Cells with Low and High Growth Potential

Weakly tumorigenic clones	Mean ± SD[a]	Highly tumorigenic clones	Mean ± SD[a]
QR	2.29 ± 0.83	QRpP-1	9.52 ± 2.54[b]
		QRpP-2	8.46 ± 1.76[b]
		QRpP-3	9.11 ± 2.16[b]
ER	2.91 ± 0.58	ERpP-1	12.97 ± 1.76[b]
		ERpP-2	12.23 ± 1.65[b]
		ERpP-3	12.50 ± 2.07[b]
Weakly metastatic clones:		Highly metastatic clones:	
clone-4-2(c-SST-2)	3.47 ± 1.56	clone-2(c-SST-2)	11.40 ± 1.77[b]
F-1(B-16)	2.06 ± 0.77	F-10 (B-16)	6.54 ± 1.88[b]
KM12 C (human colon carcinoma)	1.80 ± 0.76	KM12 SM (human colon carcinoma)	11.65 ± 1.22[b]
A bladder metastatic cell clone of B16 F10		B16 BL6	8.22 ± 1.51
FV-KMT-17	0.80 ± 0.42	KMT-17	7.42 ± 1.02[b]

a) Microvilli on 100 individual cells of each clone were observed. Data are expressed as the mean number of microvilli per cm^2 of cell surface area at 5,000-fold magnification.

b) All P values are < 0.01, as calculated by Student's t test, between highly and weakly tumorigenic clones, and between highly and weakly metastatic cell clones.

branes, especially in the membrane of the microvilli of tumor cells (ERpP) with high growth potential and high metastatic ability (Fig. 15) whereas the tumor cells (ER) with low growth potential and weak metastatic ability were not stained (Fig. 14). We did not observe any positive immunoreaction to the c-*neu* product at the ultrastructural level either in the cytoplasmic sites, or in the nuclear membrane and small organelles.

DISCUSSION

Our present electronmicroscopic investigation has revealed significant differences in the numbers of microvilli between tumor cells with high growth potential and low growth potential, and between those with high metastatic ability and weak metastatic ability. Greater numbers of microvilli were found on the surfaces of tumor cells with high growth potential than on those with low growth potential, and on the surfaces of highly metastatic cells than on those of weakly metastatic cells. We also observed more microvilli on the surfaces of B16 BL6 cells, which were isolated from the metastatic site of B16 F10 and possess high metastatic ability, than on the parent B16 F10 cells. The growth and metastatic potential of the tumor cells we examined correlate closely, although we describe them separately in this study. We therefore suspect a relationship between the microvilli and the tumor growth potential and/or tumor metastasis.

It is well known that microvilli which appear on the cell surface of the mammalian small intestine show high metabolic ability. A positive correlation was found between the oncogenic potential of transformed mouse embryo fibroblasts and the concentration of long microvilli.[3,4,18] Cohen *et al.*[19] found that pleomorphic microvilli appeared on the cell surface of bladder tumors and the hyperplastic bladder epithelium of rats after they had been fed with the carcinogen FANFT for six weeks plus saccharin for six weeks; and they suggested that the long microvilli are associated with the malignancy of the bladder tumor. In other SEM investigations, Jacobs *et al.* interpreted the presence of pleomorphic microvilli as an indicator of abnormal or neoplastic proliferative changes in the bladder epithelium of experimental animals[20] and humans.[21] We ourselves have observed a larger number of microvilli on highly metastatic tumor cells than on weakly metastatic tumor cells.[22] Our observations imply that microvilli may be necessary for the active growth and metastasis of tumor cells.

The microvilli are protrusions on membranous structure, and they perform a variety of functions. Many membranous enzymes, cytoskeletal proteins and receptors have been found in a variety of microvilli.[8,23-25] Glenney *et al.*[24] studied microvilli biochemically in chicken intestinal epithelial cells and found that the 110K cytoskeletal protein in the microvilli was an integral membrane protein. The cytoskeletal protein in the microvilli implies that the presence of microvilli is associated with cell growth. Recent studies have focused on the correlation between the microvilli and the growth factor receptors for tumor cells, although few reports have so far been published. Kishimoto *et al.*[8] used transferrin- and transcobalamin II-coated latex particles in their SEM experiments on the microvilli of leukemia cells and found receptors for transferrin and transcobalamin II. An ultrastructural study has revealed a gene product (c-*erb*B-2), which is closely related to the epidermal growth factor (EGF)-receptor, in the microvilli of an adenocarcinoma in the human stomach.[26] In our own ultrastructural studies, we have observed a positive immunoreaction of a c-*neu* oncogene product, which is closely associated with the EGF-receptor, in the microvilli on the surfaces of those tumor cells (ERpP) which possess high growth potential and high metastatic ability, whereas the tumor cells ER showed no such reaction. These findings seem to support our speculation that the microvilli may play an important role in tumor growth and metastasis, and that the increased presence of microvilli on the surface of tumor cells is closely associated with the increased ability of the tumor cells to progress and to metastasize. Further studies of the relation between the microvilli and growth factor receptors or oncogenes are in progress to validate our hypothesis.

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