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Rapid Communication

Increase of β1-6–Branched Oligosaccharides in Human Esophageal Carcinomas Invasive Against Surrounding Tissue *In Vivo* and *In Vitro*

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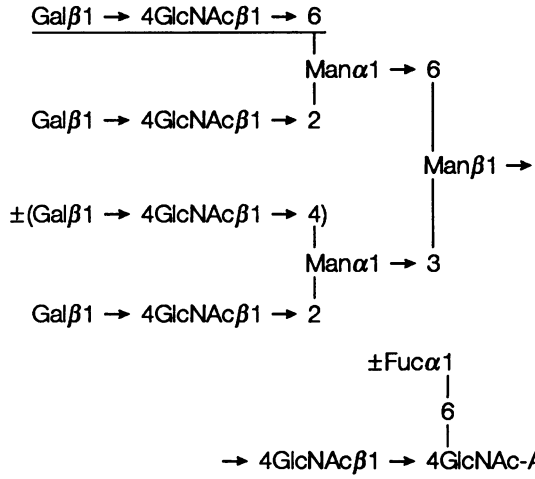
The −GlcNAc β1-6Man- (β1-6) branched N-glycosidic oligosaccharides expressed on tumor cells have been found to contribute to malignant and metastatic potential in experimental tumor models. Phaseolus vulgaris ***leukoagglutinin (L-PHA) requires the β1-6–linked lactosamine antenna for high-affinity binding and was used histochemically to characterize the distribution of these sugar structures in human esophageal squamous cell carcinomas from 42 patients. Leukoagglutinin-reactive carcinoma cells in the invasive tumors were distributed predominantly on the outer surface of the tumor adjacent to the surrounding tissue. Furthermore, when TE 1 cells, a human esophageal squamous cell carcinoma line, were cultured in a collagen gel matrix to obtain colonies in a three-dimensional form, these colonies exhibited high affinity for L-PHA binding only in the outer cell layer facing the collagen matrix, unrelated to the cell growth cycle. These findings suggest that the increase in β1-6–branched oligosaccharides in esophageal carcinomas is an important trait of the tumor in the invasion into the surrounding tissue. (Am J Pathol 1990, 137:1007–1011)***

N-glycosidic oligosaccharides on tumor cells are important structures for recognition by T cells,[1] NK cells,[2,3] and macrophages,[4] for their adherence to endothelial cells[5] and for tumor organ recognition,[6] all of which are vital steps in tumor metastasis. Most importantly, the increase in -GlcNAc β1-6Man- (β1-6) branching of complex type N-glycosidic oligosaccharide structures (as represented below) on the tumor cell surface is thought to contribute to malignant and/or metastatic potential in experimental tumors[7,8] and human breast tumors.[9]



Recently, we suggested that substitution of cell-surface oligosaccharides on human esophageal carcinomas by β1-6–branched oligosaccharides contributes to the ability of these cells to escape recognition by macrophages.[10] Thus it is likely that the increase in β1-6–branched oligosaccharides in carcinomas contributes significantly to the invasion of these tumors into the surrounding tissue, including the bypassing of host surveillance.

In the present paper, we continued studies on the histo- and cello- pathologic features of β1-6–branched oligosaccharides in human esophageal carcinomas *in vivo* and in human esophageal carcinoma cell colonies in collagen

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gel matrix cultures *in vitro,* using *Phaseolus vulgaris* leukoagglutinin (L-PHA), which is specific for β1-6-branched carbohydrate residues.[11] We present evidence that L-PHA-reactive carcinoma cells in tumors *in vivo* and *in vitro* are distributed predominantly on the external surface of the tumor adjacent to the surrounding tissue.

Materials and Methods

Tissue Specimens Used

Tissue specimens of esophageal carcinoma from the 42 surgical cases were fixed in 3.7% formaldehyde/50 mmol/l (millimolar) phosphate-buffered saline (PBS), pH 7.2, and paraffin embedded. All esophageal carcinomas obtained were histopathologically defined as squamous cell carcinoma and were subdivided into three groups; well differentiated (13 cases), moderately differentiated (17 cases), and poorly differentiated (12 cases).[12]

Histochemistry with L-PHA

Based on the procedures of Hsu et al,[13] the sections were immersed in 0.3% hydrogen peroxide-methanol solution for 15 minutes to inactivate endogenous peroxidase, rinsed in PBS, and then treated with biotinylated L-PHA (Vector Labs Inc., Burlingame, CA)/PBS solutions (20 μg/ml) supplemented with 1% bovine serum albumin, Fraction V (Sigma Chemical Co., St. Louis, MO) at room temperature for 60 minutes. After thorough washing with PBS, the treated sections were incubated with horseradish peroxidase (HRP)-labeled avidin-biotin complex (ABC kit PK 400, Vector) at room temperature for 60 minutes washed with PBS, and then immersed in 0.05% TRIS-HCl buffer (pH 7.2) containing 2 mmol/l hydrogen peroxide, 0.05 mmol/1 3.3′-diaminobenzidine, and 3 mmol/l sodium azide for 5 to 15 minutes. The stained sections were counterstained with hematoxylin. Controls were performed using the same procedure but without biotinylated L-PHA. These were negative in all experiments.

The ratios of L-PHA–positive cells to at least 200 carcinoma cells counted were determined under light microscopy. Statistical analysis was done using Student's *t*-test and $P > 0.05$ was determined to be not significant.

Esophageal Carcinoma Cell Culture in Collagen Gel

TE-1 cells, a human esophageal squamous cell carcinoma line,[14] were cultured in a collagen gel matrix according to the method described,[15] to achieve three-dimensional cell growth. The gel was prepared from a mixture of 18 ml of Type I collagen (Cellmatrix 1-A, Nitta Gelatin Co., Ohsaka, Japan) and 2 ml of tenfold concentrated DM 160 medium (Kyokuto Pharmaceutical Co., Tokyo, Japan), pH 7.5. TE-1 Cells dispersed in the collagen mixture (2×10^5/ml), were maintained for 30 minutes at 37°C for gelatinization. Then equal amounts of the complete DM 160 medium supplemented with 5% fetal bovine serum (Flow Labs, Woodcock Hill, UK), 100 μl/ml piperacillin sodium, and 100 μg/ml streptomycin, were added and cultured for 7 days. The collagen gel containing TE-1 cell colonies was fixed in 3.7% formaldehyde/PBS, pH 7.2 for 12 hours, and paraffin embedded. The sections were stained with L-PHA according to the histochemical procedure described above. Any sections treated without L-PHA were negative for diaminobenzidine reaction.

To detect TE-1 cells in the S phase in these colonies, TE-1 cells cultured in the collagen gel were pulse labeled with 20 μM mmol/l (mic molar) 5-bromo-2′-deoxyuridine (BrdU) (Sigma) for 2 hours. BrdU-labeled carcinoma cells were immunocytochemically detected according to the method described,[16, 17] using anti-BrdU mouse IgG antibodies (Becton-Dickinson, Mountain View, CA) followed by treatment with biotinylated horse anti-mouse IgG antibodies (Vector). Control studies performed using the same procedure but without anti-BrdU antibodies were negative.

Results

Distribution of L-PHA–Reactive Carcinoma Cells in a Tumor Mass

Leukoagglutinin-reactive squamous cells were confined to the carcinoma cells (Figure 1A) and to the dysplastic cells adjacent to the normal squamous epithelium (Figure 1B). Normal squamous cells were negative (Figure 1A, left upper and B, left). Most prominent in L-PHA staining were the peripheral margins of infiltrating carcinomas in contact with the stroma (Figure 1C and D). Small nests in the stroma composed of a few carcinoma cells also reacted strongly with L-PHA (Figure 1E).

Carcinoma cells in a tumor mass seem to be strongly stained by L-PHA in the deeply invasive region, D, and the lateral region adjacent to the normal esophageal epithelium, L, compared to the central region, M (Figure 1F), and the ratio of L-PHA–reactive cells to at least 200 carcinoma cells per region was calculated.

The ratios in the D and the L regions were higher than that in the M region (Table 1, Total). When the ratio of L-PHA–reactive carcinoma cells in each region was evaluated according to the histopathologic grouping of squamous cell carcinoma, well differentiated, moderately differentiated, and poorly differentiated, significant difference was found between the D or L region and the M region in all histopathologic groups (Table 1).

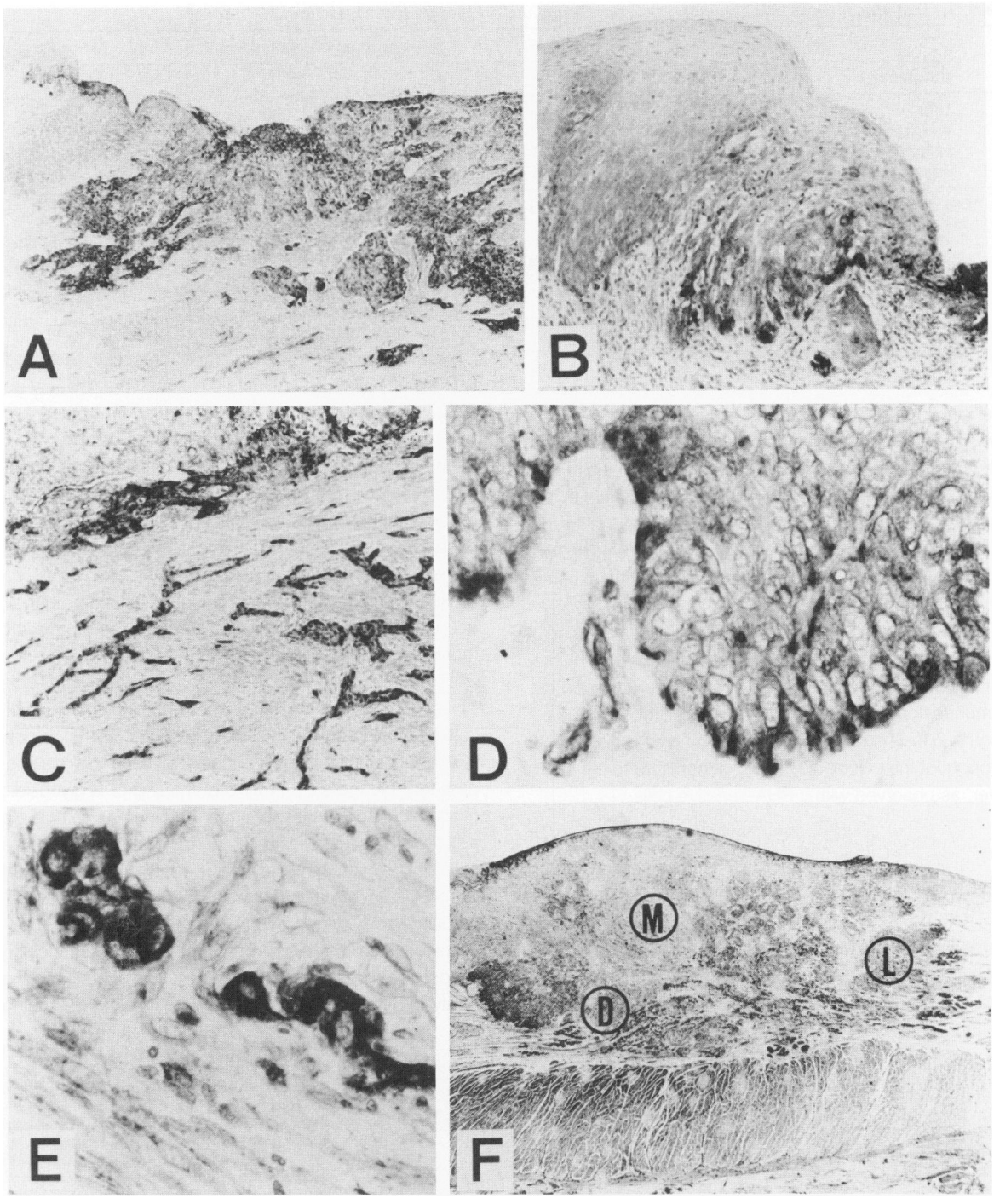

Figure 1. *L-PHA staining of esophageal squamous cell carcinomas (ABC method).* **A:** *L-PHA-reactive squamous cells are confined to carcinoma cells and are more prominent in the infiltrating area. No reactive cells among the normal squamous cells are observed (left upper) (x22).* **B:** *A high-power view of the left upper region of* **(A).** *Dysplastic squamous cells are positive for L-PHA and are clearly differentiated from adjacent normal squamous epithelium (x110).* **C:** *L-PHA-reactive carcinoma cells are evident in the deeper region of the tumor mass in contact with the stroma (upper) and in the small nests scattered throughout in the stroma (x44).* **D:** *Carcinoma cells are positive for L-PHA predominantly in the front of an invasive carcinoma mass (x440).* **E:** *An entire cytoplasm of carcinoma cells reacts strongly with L-PHA when carcinoma cells invade the stroma in a small cell nest or a single-cell profile.* **F:** *A low-power view of esophageal carcinoma shows that L-PHA-positive carcinoma cells are predominant in the deeper D and the lateral L regions, but not in the central region M. Muscularis propria are significantly positive for L-PHA (x440)*

Table 1. *Distribution of L-PHA–Reactive Carcinoma Cells in Esophageal Carcinoma Tumors* *

Squamous cell carcinomas	Cases	Region in an esophageal tumor†		
		L	D	M
Total	42	57.1 ± 5.6‡	62.0 ± 5.6‡	37.9 ± 5.4
Well diff.‖	13	59.6 ± 10.0‡	63.3 ± 10.8‡	39.0 ± 10.0
Moderately diff.‖	17	62.5 ± 8.1‡	67.7 ± 7.4‡	38.0 ± 9.2
Poorly diff.‖	12	49.5 ± 10.4‡	55.4 ± 10.7‡	33.7 ± 7.3

* Each value represents the percentage ratio of L-PHA–positive cells to at least 200 carcinoma cells counted in each region.
† Indicated in Figure 1F.
‡ Significance *versus* M value: $P < 0.01$.
‖ Histopathologic subgroups of squamous cell carcinomas (Materials and Methods).

Distribution of L-PHA–Reactive Carcinoma Cells Cultured in Collagen Gel

We then cultured TE-1 cells in collagen gel to produce three-dimensional colonies, and then examined the distribution of L-PHA–reactive TE-1 cells in one of these colonies. Leukoagglutinin-reactive cells were confined to the outer layer of the colony. The cell membrane facing the collagen matrix was strongly stained, while the inner surface of the cell membrane in contact with other carcinoma cells was not (Figure 2A).

To examine whether the reactivity of TE-1 cells with L-PHA is related to their cell cycle, TE-1 cells in the S phase were assayed by pulse labeling with BrdU followed by immunocytochemical staining with anti-BrdU antibodies (Figure 2B). The stained cells were randomly distributed throughout the colony and were not confined to the outer layer, as observed with L-PHA–reactive cells.

Discussion

Carbohydrate alteration with an increase in -GlcNAc β1-6Man- (β1-6) linked antennae at the trimannosyl core of N-glycosidic oligosaccharides has been observed in viral and oncogene-transformed rodent fibroblasts *in vitro*.[18–20] Glycosylation mutants of the highly metastatic tumor cell line, MDAY-D2, deficient in β1-6GlcNAc transferase V activity, show loss of metastatic potential.[7] In human systems as well, L-PHA–reactive oligosaccharides have been shown to be elevated in primary human breast carcinomas.[9] Our findings that L-PHA–reactive squamous cells in the esophagus are confined to carcinoma cells and dysplastic cells (Figure 1) can be evaluated along the same line as in the previous reports.

Through further studies, we showed here that 1) esophageal carcinoma cells adjacent to the stroma are more prominent in L-PHA staining (Figure 1, Table 1), and this is unrelated to their histopathologic features (Table 1), and 2) L-PHA–reactive carcinoma cells cultured in collagen gel are confined to the outer layer of the colony, unrelated to their cell cycle (Figure 2). These findings suggest that the increase in β1-6 branching of N-glycosidic oligosaccharides in esophageal carcinomas may be a functional trait necessary for invasion of the carcinoma cells into surrounding tissue. The cell growth front of esophageal carcinomas may be resistant to macrophage-mediated surveillance because one mutant clone of TE-1 cells, which exhibited an increase in β1-6–branching oligosaccharides, also reduce macrophage-triggering ability.[10] Structural characteristics of the L-PHA-binding

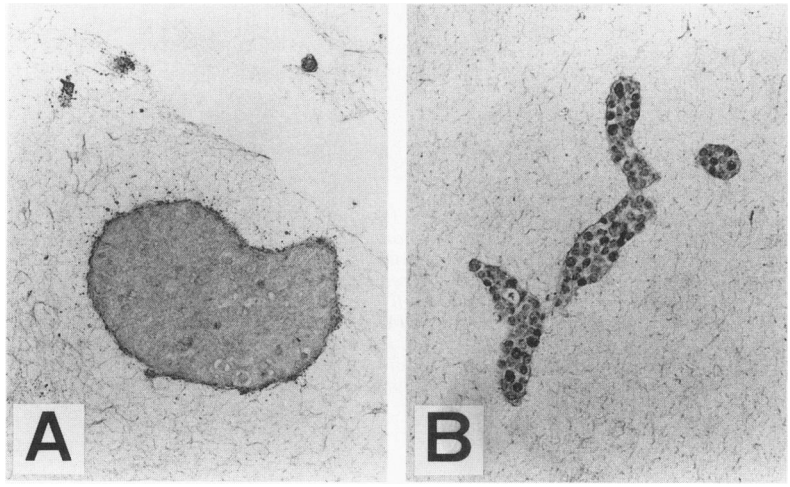

Figure 2. *Thin sections of TE-1 cell colonies in the collagen gel matrix culture (x200).* **A:** *L-PHA–reactive cells are distributed in the outer layer of the colony. All cells in the small clusters, which are facing the collagen matrix, are positive for L-PHA (upper). Micro vesicles localized around the colonies and the small cell clusters also are significantly stained, which seem to be cross-sections of the cellular microprojections. (L-PHA staining, ABC method.)* **B:** *TE-1 cells pulse labeled with BrdU were detected by anti-BrdU antibodies. Positive cells are randomly distributed throughout the colonies (ABC method).*

glycoproteins in esophageal carcinomas should be further studied, compared with those of the previously identified L-PHA–binding glycoproteins, such as leukosialin[21, 22] and lysosomal-associated membrane glycoprotein 1.[21, 23, 24]

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