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Tumorigenicity of Adenovirus-Transformed Cells: Collagen Interaction and Cell Surface Laminin Are Controlled by the Serotype Origin of the E1A and E1B Genes

FERN J. BOBER,[1] DAVID E. BIRK,[1] THOMAS SHENK,[2] AND KAREL RAŠKA, JR.[1]*

Department of Pathology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854,[1] and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544[2]

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A library of cells transformed with recombinant adenoviruses was used to study tumorigenicity and interaction with extracellular matrix. Cells expressing the complete E1 region of highly oncogenic adenovirus type 12 (Ad12) are tumorigenic, adhere preferentially to type IV collagen, and express cell surface laminin. Weakly tumorigenic cells, which express the E1A oncogene of Ad12 and the E1B genes of Ad5, also attach preferentially to type IV collagen but do not contain laminin on their surface. Cells which express the E1A oncogene of Ad5 and the E1B genes of Ad12 are nontumorigenic and do not preferentially attach to type IV versus type I collagen but have laminin on their surface. There is no significant difference in the amounts of laminin secreted into the culture medium among cells expressing the E1B genes of Ad5 or Ad12. In vitro assays show that cells which express the E1B genes of Ad12, irrespective of the origin of the E1A genes, can bind three times more exogenously added laminin than cells expressing the E1B genes of nononcogenic Ad5. The interaction of adenovirus-transformed cells with collagen is controlled by the serotype origin of the E1A oncogene, whereas cell surface laminin is controlled by the serotype origin of the E1B genes.

Human adenoviruses vary in their tumorigenic potential in rodents. Group A serotypes induce tumors with high efficiency, whereas the group C viruses are nononcogenic (8). Tumorigenicity of adenovirus-transformed cells depends on and follows that of the virus used for transformation.

The transforming capacity of adenovirus is associated with the leftward 11.5% of the genome containing the early region E1 (7), which comprises two transcriptional units, E1A and E1B (1). Serotype origin of the E1A genes has profound effects on the properties of the transformed cells. Cells expressing the adenovirus 12 (Ad12) E1A genes have a greatly decreased concentration of class I major histocompatibility complex antigen on their surface (2, 14) and are virtually resistant to cytolysis by natural killer cells (16, 17). The E1A genes also control the function of the adenovirus-specific transplantation immunity, which is induced with comparable efficiency by different serotypes (18). The differences in tumorigenic potential of different serotypes thus cannot be explained in the context of immunogenicity (11). Moreover, the Ad12 E1B region also affects tumorigenicity. Cells expressing only incomplete Ad12 E1B regions or only E1A genes usually induce tumors less efficiently and the induced tumors have a long latency period (5).

Recent studies have established a close correlation between tumorigenicity and the interaction of tumor cells with the extracellular matrix (3, 12). Metastatic potential, invasiveness, and tumorigenicity are usually associated with affinity for type IV collagen versus type I collagen (3, 15, 21) and with high concentrations of the glycoprotein, laminin, on the cell surface (20). Indeed, our earlier studies have shown that highly tumorigenic Ad12-transformed cells attached preferentially to type IV collagen, whereas nontumorigenic Ad2 transformants preferred type I collagen (10). Moreover, tumorigenic cells expressing the entire Ad12 E1 region had higher concentrations of cell surface laminin than weakly tumorigenic cells expressing only the Ad12 E1A genes (3).

Establishment of cell lines which contain and express transforming regions constructed from the E1A and E1B genes of oncogenic and nononcogenic adenovirus serotypes, respectively (17), makes a direct examination of the role of these genes in cell interaction with the extracellular matrix feasible.

In this report, we provide experimental evidence that the serotype origin of the adenovirus E1A oncogene in transformed cells controls the interaction with type IV collagen, whereas the serotype origin of the E1B region controls the level of cell surface laminin.

MATERIALS AND METHODS

Animals. Fischer 344 (F) rats of the RT-1[l] haplotype were purchased from Harlan-Sprague-Dawley Laboratories, Walkersville, Md.

Transformed cell lines. Seven established F-cell lines transformed with viable recombinant adenoviruses used in this study were characterized in detail previously (17). The A1 and A2 lines transformed with the *sub*370-12E1A virus express the E1A genes of Ad12, whereas the expressed E1B region is from Ad5. The B1, B2, and B5 cells transformed with the *sub*370-12E1B virus express the Ad5 E1A oncogene, and the expressed E1B genes are from Ad12. The AB4 and AB5 cell lines transformed with the *sub*370-12E1AB virus contain and express the entire E1 region of Ad12. The EcoC3 cells were transformed with a recombinant plasmid containing *Eco*RI-C (0 to 16.5 map units) of Ad12 DNA (18). All cell lines were grown in monolayer culture in Dulbecco

* Corresponding author.

modified minimum essential medium supplemented with 10% fetal bovine serum.

Tumorigenicity assays. Randomized newborn F rats were injected subcutaneously with 2×10^6 syngeneic transformed cells and were screened for the development of tumors over a period of 1 year.

Collagen attachment assays. Collagen was isolated and attachment assays were performed as previously described (3), with several modifications. Bacteriological dishes (35 mm) were precoated by drying 1 ml of a 10-μg/ml solution of type I or type IV collagen in HCl (pH 2.0) and blocked with 2% bovine serum albumin for 2 h at room temperature. Attachment assays were performed with 5×10^5 viable cells per dish. At the times indicated, the attached cells were released by 0.02% EDTA–0.05% trypsin treatment for 15 min at 37°C followed by a wash with phosphate-buffered saline (PBS). Aliquots of the resulting cell suspension were counted in a ZBi counter (Coulter Electronics, Inc., Hialeah, Fla.) to determine the total number of attached cells.

Cell surface immunofluorescence. Detection of cell surface laminin and fibronectin on paraformaldehyde-fixed cells was accomplished by a procedure slightly modified from that described by Bober et al. (3). Cells were removed from monolayer, either by trypsinization (0.025%, 10 min) or with 0.02% EDTA, seeded on glass cover slips in 35-mm culture dishes, and grown in Dulbecco modified minimum essential medium supplemented with 10% fetal bovine serum that was previously depleted of fibronectin by gelatin-Sepharose affinity chromatography. Monolayers were fixed with 4% paraformaldehyde in PBS for 15 min at 4°C and blocked with 2% bovine serum albumin, and laminin and fibronectin were localized by an indirect immunofluorescence technique with a rhodamine-conjugated secondary antibody.

Cell surface laminin and fibronectin on viable cells in suspension were quantitated by flow cytometry. Cells were incubated in fibronectin-free Dulbecco modified minimum essential medium for 24 h and then detached with 0.04% EDTA. Cell suspensions were incubated with rabbit anti-laminin, rabbit anti-fibronectin, or normal rabbit immunoglobulin G (GIBCO Laboratories, Grand Island, N.Y.) at a final dilution of 1:25 in PBS for 1 h at room temperature, washed, and incubated with a 1:25 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G in PBS. Cell fluorescence was analyzed in a computer-linked System 50 Cytofluorograf (Ortho Diagnostics, Inc., Raritan, N.J.).

Laminin quantitation in the culture medium. Secretion of laminin into the culture medium after a 6-h incubation period was determined by a competitive enzyme-linked immunosorbent assay as described in detail previously (3), except that the medium was not concentrated before analysis.

Binding of ^{125}I-laminin to cells. Laminin, isolated from the Englebreth-Holm-Swarm sarcoma, was iodinated by the lactoperoxidase method (22). Cells were removed from monolayer by trypsinization (0.025%, 10 min), put into suspension culture in Joklik medium supplemented with 10% fetal bovine serum for 2 h to allow for receptor regeneration, and subsequently used for laminin-binding assays in Eppendorf tubes (13). Total binding was assessed at various time intervals by incubating the cell suspensions (1.5×10^5 cells per tube) with a fixed amount of labeled laminin (1.0 μg per tube). Unbound labeled laminin was removed by washing three times with PBS containing 0.1% bovine serum albumin, and radioactivity remaining in the cell pellets was measured with a gamma counter (LKB Instruments, Inc., Rockville, Md.). Labeled laminin specifically bound to cells was calculated by subtracting the amount of labeled laminin bound in the presence of a 100-fold excess of unlabeled laminin from the amount of labeled laminin bound in the absence of excess unlabeled laminin.

RESULTS

Collagen attachment of adenovirus-transformed cell lines. A library of independently established cell lines transformed with the recombinant viruses *sub*370-12E1AB (AB cell lines, containing both E1A and E1B regions from Ad12), *sub*370-12E1A (A cell lines, containing Ad12 E1A and Ad5 E1B regions), and *sub*370-12E1B (B cell lines, containing Ad5 E1A and Ad12 E1B) has been used for analysis of the role of serotype origin of the adenovirus E1A and E1B genes, in controlling the interaction of transformed cells with matrix components. A diagrammatic representation of the recombinant E1 regions of the adenovirus substitution mutants used for cell transformation is shown in Fig. 1. The transformed properties of these cell lines have been described previously (17). The AB4 and AB5 cell lines express the entire E1 region of Ad12 and are highly tumorigenic. With a dose of 2×10^6 cells, tumors are induced in 100% of injected syngeneic newborn animals; the mean latency period is under 85 days. The A1 and A2 cell lines which express only the Ad12 E1A oncogene (the E1B genes are from Ad5) are less tumorigenic. Under the same conditions, they induce tumors in <40% of the animals and the latency period is over 6 months. The cell lines B1, B2, and B5, which express only the Ad12 E1B genes (the E1A oncogene is from Ad5), are nontumorigenic.

Cell attachment to type I and type IV collagens was tested with six of these syngeneic cell lines in the presence of cycloheximide. This is used to inhibit the synthesis of endogenous matrix components which would interfere with the assays. All four tumorigenic cell lines expressing the Ad12 E1A oncogene (both A and AB cell lines) showed a definite preference for type IV collagen, whether the E1B genes were from Ad12 or Ad5. Cell line B2 showed a nearly twofold preference for type I collagen over type IV collagen.

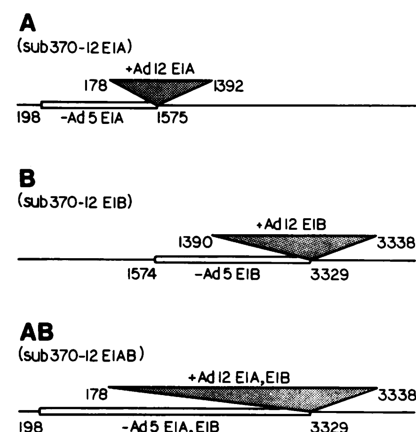

FIG. 1. Diagrammatic representation of recombinant adenovirus E1 regions in viruses used to generate transformed rat cell lines A, B, and AB. The Ad5 chromosome is represented by a line, deletions are shown by empty rectangles, and insertions are shown by shaded triangles. The Ad5 nucleotides present on either side of the Ad5-specific E1 deletions are designated below each chromosome, whereas the Ad12 nucleotides at the extremes of the Ad12-specific E1 insertions are designated above each chromosome.

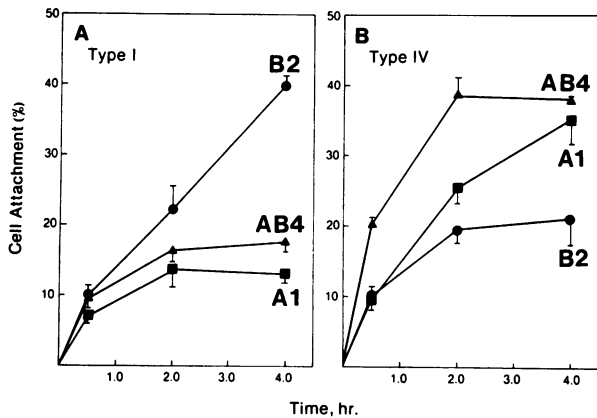

FIG. 2. Attachment of adenovirus-transformed cells to type I (A) or type IV (B) collagen. Cells were pretreated with cycloheximide for 2 h, trypsinized, and added to 35-mm petri dishes (5×10^5 cells) previously coated with 10 μg of type I or type IV collagen. Cell attachment was determined after 0.5-, 2.0-, and 4.0-h intervals by removing adherent cells and counting them with a Coulter ZBi counter. Datum points represent the means plus or minus standard deviation of three independent experiments. ■, A1 cells; ●, B2 cells; ▲, AB4 cells.

The B5 cells showed no preference for either type of collagen. Results of three experiments with A1, AB4, and B2 cells are depicted in Fig. 2.

Cell surface laminin and fibronectin. The presence of laminin and fibronectin on the cell surface was studied by indirect immunofluorescence. Trypsinized cells were plated on glass cover slips and fixed with paraformaldehyde 24 h later. A low-magnification fluorescence micrograph that demonstrates the presence or absence of laminin and fibronectin on the cell surface is shown in Fig. 3. All cell lines, regardless of which adenovirus E1 genes were expressed, exhibited a similar intensity of immunofluorescence for fibronectin. On the other hand, significant staining for cell surface laminin was seen only with cells that expressed the Ad12 E1B region (B and AB cell lines). Detaching the cells without trypsinization with only EDTA, before seeding, or plating at different densities made no difference in the cell surface staining. Cells expressing the Ad5 E1B genes (A cell lines) remained negative for surface laminin.

Quantitative analysis of laminin and fibronectin on the surface of viable cells in suspension was performed by flow cytometry. These analyses show that laminin was present only on the surfaces of cells which express the Ad12 E1B genes (B and AB cell lines), regardless of the origin of the E1A oncogene (Fig. 4). In agreement with earlier observations (6), fibronectin was detected on all of the cell lines, irrespective of which adenovirus E1 genes were expressed.

Laminin secretion into the medium. Laminin secreted into the serum-free culture medium after 6 h of incubation was quantified by an enzyme-linked immunosorbent assay technique. Results presented in Table 1 show that all cell lines studied secreted laminin into the culture medium, and there was no significant difference in the quantities of secreted laminin among the various cell lines studied.

Cell binding of ^{125}I-laminin. To determine whether the presence of cell surface laminin on cells which express the Ad12 E1B region was due to an increased ability of these cells to bind laminin, the binding of radiolabeled laminin to cells in suspension was studied. Laminin binding to all cell lines was concentration dependent. Binding to A1, B2, and AB5 cell lines is shown in Fig. 5. With the selected concentration of radiolabeled laminin (1.0 μg per tube), all cell lines

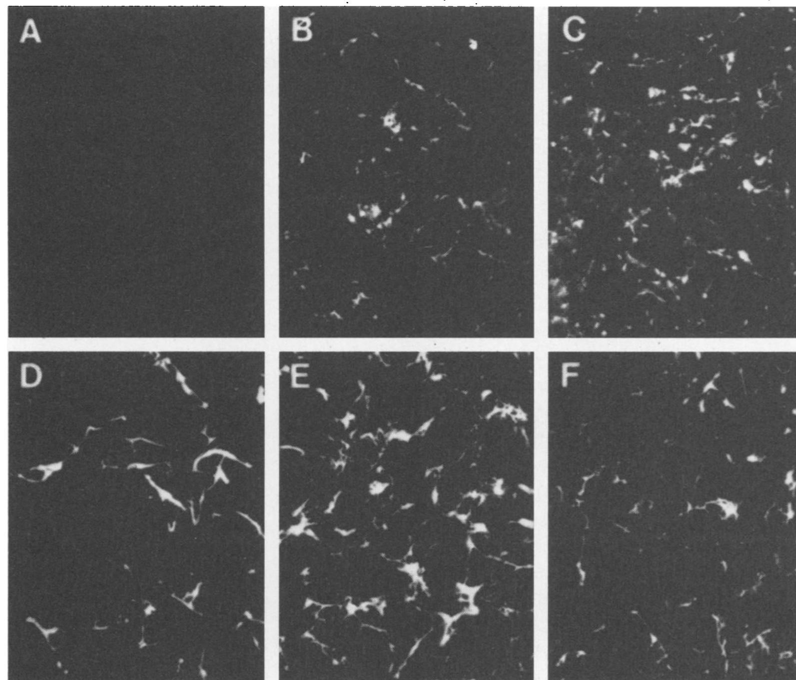

FIG. 3. Low-magnification micrograph showing the immunofluorescent staining for cell surface laminin and fibronectin in a field of adenovirus-transformed cells. After trypsinization and replating, cell monolayers were fixed with paraformaldehyde and stained for cell surface laminin (panels A through C) and fibronectin (panels D through F). A2 cells are shown in panels A and D, B1 cells are shown in panels B and E, and AB5 cells are shown in panels C and F.

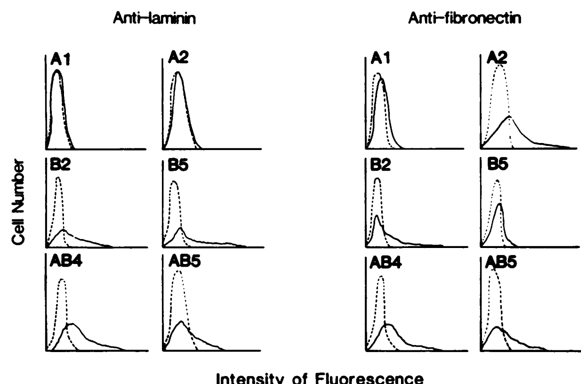

FIG. 4. Flow cytometric quantitation of cell surface laminin and fibronectin in viable cells transformed with recombinant adenoviruses. Normal rabbit immunoglobulin G was used as a control for primary antisera. Immune serum histograms (——) and control serum histograms (- - - -) are shown.

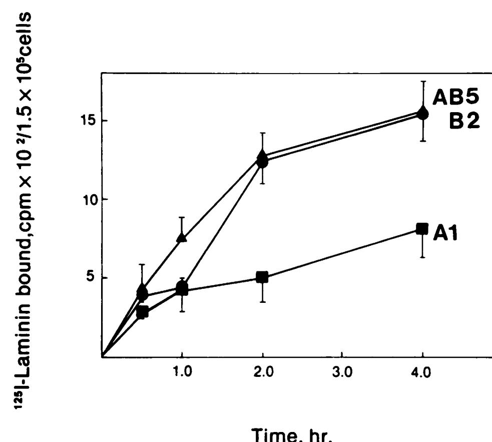

FIG. 5. Time course of ^{125}I-laminin binding to transformed cells. Cell suspensions were incubated with 1 μg of radiolabeled laminin per 0.5 ml at room temperature for the time periods indicated. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled laminin. Each point represents the mean plus or minus standard error of the mean of at least four separate experiments. ■, Cell line A1; ●, cell line B2; ▲, cell line AB5.

approached saturation after 2 h. Therefore, for standard binding assays, an incubation period of 2.5 h was used. Addition of a 100-fold excess of unlabeled laminin was shown to inhibit the binding of the labeled ligand by 75 to 90%.

Table 2 shows the specific binding of ^{125}I-laminin after 2.5 h by rat cells transformed with recombinant adenoviruses. Cell lines expressing E1B genes from Ad12 (B1, B2, and AB5) bound about threefold more radioactive laminin than cell lines expressing the Ad5 E1B genes (A1, A2). The highly tumorigenic EcoC3 cell line, transformed with cloned *Eco*RI-C (E1A plus E1B genes) of Ad12 DNA, was used as a positive control. Collagen-binding preference and tumorigenicity of individual cell lines also are included in Table 2. It is evident that cells which express the Ad12 E1A oncogene show a preference for type IV collagen. Cell lines which express the Ad12 E1B genes show significantly higher binding of laminin to the cell surface than do cells expressing the Ad5 E1B region.

DISCUSSION

Attachment preference for collagen type IV has been shown to be associated with cell metastatic potential in different tumor cell models (12, 21). In the adenovirus system, there is a close correlation between the attachment preference for type IV collagen and tumorigenicity (3, 10). The attachment to type I and type IV collagens in cell lines

TABLE 1. Laminin secretion into the culture medium by cells transformed with recombinant adenoviruses[a]

Cell line	Amt of laminin[b]
A1	15.5 ± 1.4
A2	13.6 ± 4.8
B1	16.3 ± 2.0
B2	12.9 ± 1.3
AB4	14.5 ± 2.1
AB5	16.1 ± 1.8

[a] Laminin secreted into the culture medium was quantitated by using a competitive enzyme-linked immunosorbent assay. Unconcentrated, serum-free medium collected from cell monolayers incubated for 6 h was analyzed.
[b] Laminin is expressed as nanograms secreted per 10^5 cells. Values represent the means plus or minus standard deviation of three determinations.

that express adenovirus E1 genes has now been examined for a total of 26 cell lines: 6 cell lines described here that distinguish Ad5 and Ad12 E1A genes and another 20 cloned adenovirus-transformed cell lines studied previously (3, 10; unpublished results). Only two nontumorigenic rat cell lines transformed with recombinant plasmids containing the Ad12 E1A but not E1B genes (3) failed to fit the generalization and exhibited very low binding affinity for both types of collagen. The products of the Ad12 E1A genes in these two cell lines accumulated to much lower quantities than in any other cell lines used in this study (unpublished results).

The serotype origin of the E1A or E1B regions does not affect the secretion of laminin into the culture medium (Table 1). On the other hand, the concentration of cell surface laminin and cell binding affinity for laminin appear to depend on the serotype origin of the E1B genes. Cells which express the Ad12 E1B genes (B and AB cell lines) carry laminin on their surfaces, whereas cells expressing the corresponding Ad5 E1B genes (A cell lines) are negative for surface laminin in immunofluorescence and flow cytometric assays. The presence of cell surface laminin is not influenced by the serotypic origin of the E1A gene.

It has been shown that the metastatic potential of different tumor cell lines is affected by their ability to bind laminin (13, 19–21). In the adenovirus system, the presence of cell surface laminin alone is not sufficient for tumorigenicity, which appears to depend primarily on the serotypic origin of the E1A oncogene. Cell lines which express only Ad12 E1A genes (A cell lines) are often tumorigenic but induce slow-growing tumors (17). When Ad12 E1B genes are also expressed (AB cell lines), there is a high concentration of surface laminin and the cells are highly invasive and produce fast-growing tumors (17). Although adenovirus-transformed cells are not truly metastatic, cells which express the entire El region of Ad12, when injected intravenously, produce multiple tumor foci, whereas cells expressing only the Ad12 E1A oncogene fail to do so (unpublished results).

To investigate whether higher cell surface concentrations of laminin may reflect a difference in the number of laminin receptors, the binding of laminin was studied on cells first

TABLE 2. Binding of ^{125}I-laminin, collagen attachment preference, and tumor induction by adenovirus-transformed cells

Cell line	Serotype origin of E1 oncogenes		Amt of ^{125}I-laminin bound[a] (ng/10^5 cells)	Collagen-attachment preference[b] (type)	Tumor induction[c]
	E1A	E1B			
A1	12	5	10.9 ± 0.9	IV	+
A2	12	5	12.7 ± 1.8	IV	+
B1	5	12	32.7 ± 3.6	None	−
B2	5	12	31.8 ± 9.1	I	−
AB5	12	12	29.9 ± 10.0	IV	+++
EcoC3	12	12	35.4 ± 3.6	IV	+++

[a] Binding of radiolabeled laminin to transformed cells was measured in suspension for 2.5 h at room temperature. Radioactivity of laminin bound in the presence of a 100-fold excess of unlabeled laminin was subtracted. The data for each cell line represent the means plus or minus standard deviation of six separate experiments.

[b] Collagen-binding preference was determined as described in the legend to Fig. 2.

[c] Tumorigenicity was determined after injection of 2×10^6 cells into newborn syngeneic rats: +, tumors induced in more than 10% but less than 40% of animals; +++, tumors induced in 100% of animals; −, no tumors induced.

trypsinized to remove endogenous surface laminin and subsequently allowed to regenerate laminin-binding proteins. There is a two- to threefold higher binding of laminin by cells which express the Ad12 E1B genes (B and AB cell lines) than those expressing only the Ad12 E1A oncogene (A cell lines). This result suggests that cells expressing the Ad12 E1B genes contain an increased number of laminin receptors.

The Ad12 E1B gene in transformed cells somehow affects the tumorigenic potential of transformed cells. Our results, which show that cell surface laminin is controlled by expression of the Ad12 E1B genes, provide an explanation for the role of the Ad12 E1B region in contributing to tumorigenicity. This result is in excellent agreement with earlier observations that expression of the larger Ad12 E1B polypeptide in transformed cells correlates both with tumorigenicity and the appearance of a new higher-molecular-weight class of glycoprotein-bound carbohydrates on the surfaces of tumorigenic transformed cells (4, 9).

Tumorigenicity of adenovirus-transformed cells, which is affected by their interaction with the extracellular matrix, thus involves at least two separate mechanisms. Differential interactions with collagen types and the level of cell surface laminin are controlled by the serotype origin of the adenovirus E1A and E1B genes, respectively. Experiments are in progress to examine the effects of adenovirus oncogenes on expression of collagen type IV and laminin receptors in transformed cells.

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