PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 176, 490–502 (1984)

Early Nuclear Structural Changes of Transformed Human Amniotic Cells (WISH) in Presence of Type IV Collagen Detected by the Nuclear Refringency Assay (41903)

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Abstract. An early nuclear activation of transformed human amniotic epithelial (WISH) cells triggered by type IV collagen is visualized by a modification of the nuclear refringency obtained by mercury binding on condensed chromatin. This phenomenon is quantified by the nuclear refringency test. The nuclear activation of WISH cells by basement membrane collagen is also shown by the DNase I sensitivity of chromatin and by the measurement of mRNA synthesis. These nuclear phenomena are concomitant with WISH cell attachment and laminin synthesis. Reversible effects on nuclear refringency, cell attachment, and laminin synthesis are tested by the addition and removal of different metabolic inhibitors.

In the past few years, it has been shown that the malignant behavior of tumor cells is correlated with cell surface alterations and defective interactions with extracellular matrix (1, 2). Normal and transformed cells differ in their ability to adhere to collagenous matrices (see (3) for review) when a choice of collagen substrates is given to them. Epithelial cells attach slowly but preferentially to type IV basement membrane collagen (4–6), whereas transformed cells derived from carcinoma bind rapidly and selectively to this latter in the absence of serum (7–10). Attachment of transformed cells, propagated *in vitro*, to basement membrane collagen is followed by spreading and flattening of the cells (7, 10). In fact, this initial recognition step is inhibited by specific amino-sugars, reminiscent of a lectin-like interaction (7). Such differences in the cell recognition of basement membrane collagenous structures could be related to the normal or transformed character of the cells.

It has also been reported that cell surface stimulation of lymphocytes by lectins induces an early morphological modification of nuclei. Indeed, the condensed chromatin of nonstimulated lymphocyte nuclei converts into a dispersed state when lymphocytes are activated by phytohemagglutinin (11). This cell activation is detected by a decrease of nuclear refringency obtained by mercury binding on condensed chromatin (12, 13). This phenomenon can be quantified by the nuclear refringency assay (14). Therefore, using this method, we have attempted to analyze the priming nuclear response of normal (HA) and transformed human amniotic epithelial (WISH) cells closely related with the membranous effect of type IV collagen. The early nuclear activation of WISH cells triggered by type IV collagen has been followed by the study of further nuclear modifications, such as DNase I sensitivity of chromatin and the synthesis of mRNA. Moreover, nuclear activation of WISH cells treated by type IV collagen is associated with cell attachment and laminin synthesis. This latter is a glycoprotein attachment factor identified in the culture medium of most epithelial and carcinoma cells (4, 9, 15–18).

Materials and Methods. *Cells and medium.* Primary cultures of human epithelial amnion cells (HA) were prepared from full-term placentas. Cells obtained during subsequent trypsinizations were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% newborn calf serum (GIBCO Bio-Cult Ltd., Paisley PA34EP, Scotland).

The WISH cell line, derived from human amnion tissue (ATCC CLL25), was cloned *in vitro* from an altered colony, and maintained in EMEM with 15% newborn calf serum (GIBCO) (19).

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0037-9727/84 $1.50

Collagen preparation. Type I collagen was purified from human amnion and type IV collagen from human placenta villi, according to the method of Burgeson *et al.* (20). Preparation and chemical characterization have been previously described (10).

Nuclear refringency. HA or WISH cells (5×10^5 cells/ml) were incubated in EMEM without serum supplemented with 10 μg/ml of either type I or IV purified collagen. After varying incubation times at 37°C, the cells were harvested by scraping and suspended in the medium before cytocentrifugation in a Shandon Cytospin at 800 rpm for 8 min. The cells were projected on triplicate glass slides at the same density in an 6-mm diameter area. The slides were immediately immersed in 5% acetic acid–Zenker reagent (25 g potassium dichromate, 50 g mercuric chloride, and 10 g sodium sulfate dissolved at 80°C in 1 liter of distilled water) at room temperature for 60 min. Then the temperature was raised to 90°C in the same reagent for 10 min. After washing in tap water for 10 min, air-drying overnight, and clearing in toluene, the slides were mounted using a mounting medium with a refractive index of 1.650 (Technicon Industry) (12, 13). The cells, viewed on slides with a phase-contrast microscope, appeared more or less refringent and three groups could be individualized according to their refringency: low (A), intermediate (B), and high (C) refringent cells.

The morphological patterns of nuclear refringency of WISH cells attached in the presence of type IV collagen (5 μg/2.5×10^5 cells) and laminin (1 μg/2.5×10^5 cells) were directly observed on the glass slides after 1 hr of incubation. The slides were immersed in Zenker reagent and submitted to the same treatment as described above.

Quantitative analysis. The diagram of the measurement system is shown in Fig. 1. In order to quantify the results, the phase-contrast microscope (Leitz G × 400) was connected with a Plumbicon 720 line camera and a Cambridge Quantimet 720 (QTM) image analyzer (21, 22, 23). Data obtained from the image analyzer was further processed with a PDP 11/34 computer, a VT video terminal, and a LA 120 line printer. The image produced by the microscope was swept by the camera and submitted to the QTM as 600,000 image points. Each screening point was assigned an integer from 0 to 63, according to its brightness. Three binary images, taken from the brilliancy thresholds of the same nucleus, were processed in parallel. The first image included the darkest chromatin; the second, the medium refringent chromatin, and the third, the most refringent chromatin. The first image was submitted to dilation to be correlated with

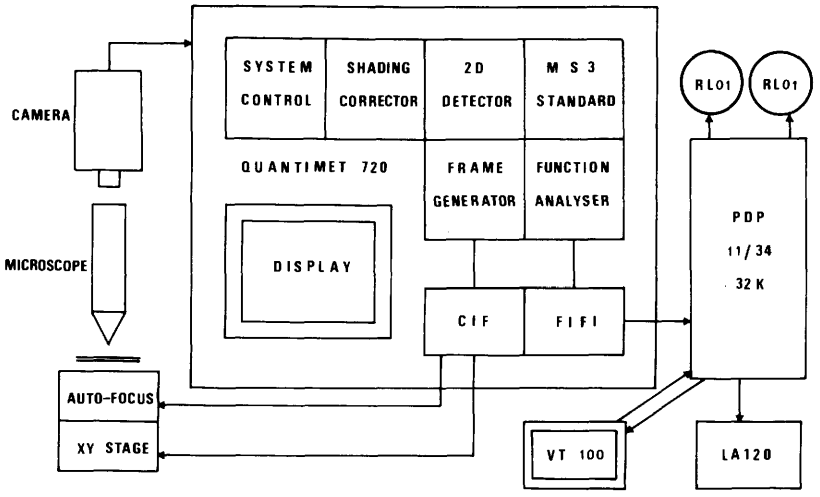


FIG. 1. Schematic representation of optic automation equipment used for the nuclear refringency assay. The measurement involved a Zeiss microscope, a Plumbicon 720 line camera, a Quantimet 720 image analyzer, a PDP 11/34 computer, a VT 100 video terminal, and an LA 120 line printer.

the two others. The analyzer relays to the computer the areas of the three gray levels, allowing the cells to be classified into the three groups. The computer software was written in Fortran IV and used 24K words of 16 bits. Therefore, a threshold of brightness, called r, could be selected from the lowest refringent cells (performed by Dr. Schovaert in the Laboratoire d'Anatomie Pathologique, Hôpital St. Vincent-de-Paul, Professor A. Pompidou).

Nuclear refringency index (NRI). A thousand cells were observed on each slide and a nuclear refringency index (NRI) was defined according to the formula:

$$\text{NRI} = \frac{a(r \times 1) + b(r \times 2) + c(r \times 4)}{a + b + c},$$

a, b, and c were the percentage of cells of each group (a = percentage of A cells, b = percentage of B cells, c = percentage of C cells: consequently, $a + b + c = 100$). The Quantimet analyzer allowed the determination of the brightness of the A, B, and C cell groups: ($r \times 1$) for A cells; ($r \times 2$) for B cells; and ($r \times 4$) for C cells. In our experimental conditions, r equals 16.

The results are expressed as the means of the three NRI values calculated on the triplicate slides for one test.

Nuclear refringency test (NRT). NRT was expressed as a ratio of the NRI of treated cells/NRI of control cells:

$$\text{NRT} = \frac{\text{NRI}_t}{\text{NRI}_c} = \frac{1a_t + 2b_t + 4c_t}{1a_c + 2b_c + 4c_c},$$

t = treated cells, and c = control cells. The standard deviation was established from seven tests. Statistical significance was evaluated by the nonparametric Wilcoxon test.

DNase I digestion. WISH cells were suspended at a concentration of 5×10^5 cells/ml and 100 ml of suspension was incubated for 5 hr with 25 μCi of [*methyl*-^{3}H]thymidine (Commissariat à l'Energie Atomique—CEA, Saclay, France; sp act 26 Ci/mmole). The cells were washed in medium, suspended at the same concentration, and grown in nonradioactive medium for 20 hr. The cells were then harvested by trypsinization and divided into two equal samples separately incubated for 4 hr at 37°C in EMEM supplemented or not with type IV collagen (5 μg/ml) in absence of serum.

Nuclei were isolated by the procedure of Weintraub and Groudine (24) using reticulocyte standard buffer (RSB, 10 m*M* NaCl, 3 m*M* $MgCl_2$, 10 m*M* Tris, pH 7.4) containing 0.5% NP40 (Bethesda Research Laboratories, Rockville, Md.) and 2 m*M* phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, Mo.) to inhibit protease activity.

Kinetics of DNA degradation by DNase I were performed on aliquots of the final nuclear suspension containing 1 mg DNA/ml. After 10 min preincubation time at 37°C, bovine pancreatic DNase I (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 1 U/ml for 0–15 min. The reaction was stopped at the indicated times by adding EDTA, pH 8, to a final concentration of 10 m*M*. Acid insoluble products were precipitated in cold 7% (w/v) perchloric acid. Aliquots of the supernatant were withdrawn for determination of ^{3}H activities in a liquid scintillation counter (Intertechnique SL30).

Poly(A)$^+$ RNA synthesis. The rate of RNA synthesis was determined by the incorporation of [^{3}H]uridine. Cells (5×10^6/25-cm^2 tissue culture flask, Falcon) were labeled with 10 μCi/ml of [^{3}H]uridine for 60 min (CEA; sp act 20 Ci/mmole). Cytoplasmic RNA was obtained by lysis of the cells in 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 20 m*M* Tris, pH 8.3, containing 5 μl/ml of diethyl pyrocarbonate (DEPC, Sigma) and 0.5% Nonidet P40 (NP 40, Bethesda Research Laboratories), extracted by phenol chloroform (v/v) and collected by ethanol precipitation. Polyadenylated cytoplasmic RNA from the extracted cytoplasmic RNA was isolated by the use of oligo-(dT)-cellulose columns (25). Poly(A)$^+$ RNA was then precipitated with 2 vol of ethanol, lyophilized, and the radioactivity was measured by liquid scintillation (Intertechnique SL30).

Cell attachment assay. Attachment assay was adapted from the method of Klebe (26). Multiwell tissue plates (2 cm^2, Falcon, Div. Becton, Dickinson & Co., Oxnard, Calif.) were filled with 0.5 ml of EMEM supplemented with 200 μg/ml of bovine serum albumin (BSA, Sigma) to prevent nonspecific binding. Then 0.5 ml of cell suspension containing 2.5 $\times 10^5$ cells were added and incubated in presence of 10 μg/ml of either type I or IV collagen at 37°C. After selected incubation periods, the unattached cells were discarded; the remaining

cells were dispersed with trypsin (Difco Laboratories, Detroit, Mich.) and treated with trypan blue solution (0.5%, Boehringer-Mannheim, France). The viable cells were unstained and counted in an hemocytometer. Assays were performed in triplicate. The standard deviation did not exceed 10%.

Immunoenzymatic characterization of laminin. Laminin was detected by indirect immunoperoxidase labeling in the culture medium after 3 hr of attachment test. Four microliters of the solution were placed on a 13-mm GCWP filter with 0.22-μm pore diameter (Millipore Corp., Bedford, Mass.) and air-dried. The filters were then immersed in phosphate-buffered saline (PBS) with 2% BSA at 4°C overnight. After washing with PBS, filters were incubated with laminin antisera (Bethesda Research Laboratory) at a dilution of 10^{-3} for 2 hr at 20°C or overnight at 4°C. The antisera were then eliminated and filters washed with PBS before a second incubation of 40 min at 20°C with peroxidase-labeled goat anti-rabbit gamma globulins (Miles Yeda Ltd., Kiryat Weizmann, Rehovolt, Israel). After the elimination of this serum, filters were washed and the bound peroxidase activity was revealed with α-chloronaphtol (3 mg/1 ml methanol/4 ml PBS, Merck Darmstadt, FRG). The quantitation of laminin was performed by this method which allowed the detection of 1 to 40 ng of laminin. No cross-reaction was observed between collagen type I or IV preparations and laminin.

Inhibitors. Nocodazole (Aldrich Europe B.2340, Beerse, Belgium) was used to inhibit the polymerization of microtubules. The biological effects were identical within a large dose range (0.04–100 μg/ml) (27). The antimicrotubular effect was immediately active in cultured cells and also rapidly reversible (27). Cells were incubated in EMEM without serum supplemented with 0.3 μg/ml of Nocodazole for 1 hr at 37°C. For testing reversible effect, Nocodazole was removed and the cells were washed in fresh medium before being seeded into wells containing type I or IV collagen.

Cytochalasin B is known to dissociate microfilament bundles (28). Cells were pretreated for 1 hr with 5 μg/ml of cytochalasin B (Sigma). To remove the inhibitor, the cells were incubated in EMEM for 2 hr.

N-Acetyl neuraminic acid (NANA) (Sigma), which inhibits the biological effect of type IV collagen on the cell membrane, was also tested (7, 10). The pretreatment of the cells with a 5 m*M* NANA solution in EMEM was performed at 20°C for 1 hr.

During the inhibitor treatments, the cells were maintained in suspension by gentle agitation.

In all assays using inhibitors, treated and untreated control cells were seeded in wells without collagen fractions. In all cases, cell viability was higher than 90% as evaluated by trypan blue exclusion.

Results. *Nuclear refringency.* The nuclear refringency patterns of cytocentrifuged cells are illustrated in Fig. 2. Three groups of cell nuclei were observed on each slide: A cells appeared with a completely black nucleus on the gray cytoplasmic area; intermediate B cells appeared with clumps of bright chromatin; C cells exhibited a brilliant chromatin surrounded by a rim of dark cytoplasm. The percentage of each group of cells was counted and allowed the NRI determination as described under Materials and Methods. The assay was performed in both normal (HA) and transformed (WISH) amniotic cells at varying incubation times (20, 30, 60, 120, and 180 min). NRI was determined for untreated cells (NRI control) and for treated cells (NRI treated). The ratio NRT = NRI treated/NRI control was calculated for each cell type and for each incubation time.

In Fig. 3 we show the kinetics of nuclear refringency variation of WISH and HA cells treated or not by type I or IV collagen. A majority of C refringent cells were constantly observed on untreated HA or WISH cell slides. For these control cells, the NRT value was 1. HA cells treated by either type I or IV collagen exhibited only a slight variation of the nuclear refringency at 3 hr and the decrease did not exceed 0.9. WISH cells treated by type I collagen also showed a NRT of 0.9 after 3 hr. In contrast, WISH cells treated by type IV collagen exhibited an early impairment of the nuclear refringency and A cells were prevalent on the slides. A marked decrease in nuclear refringency (NRT = 0.7) was observed after 20 min of incubation with type IV collagen. A maximum variation of 0.5 was reached within 30 min. Two hours later, the NRT was stabilized between 0.65 and 0.7.

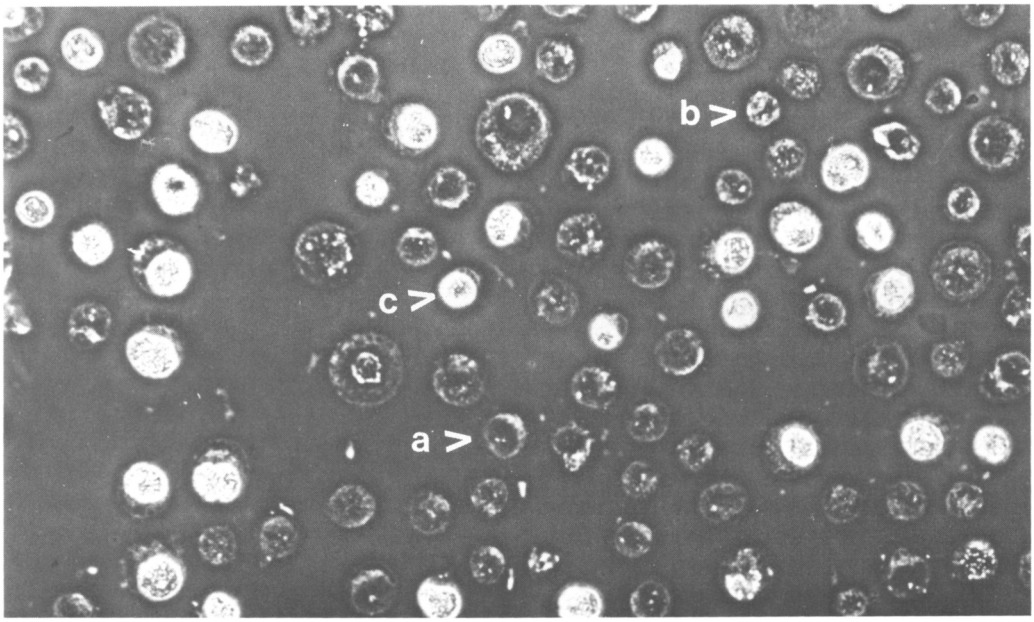


FIG. 2. Pattern of nuclear refringency. Three categories of WISH or HA cells were observed on each slide according to their refringency. Arrows indicate: (a) low refringent A cells; (b) intermediate refringent B cells; (c) high refringent C cells.

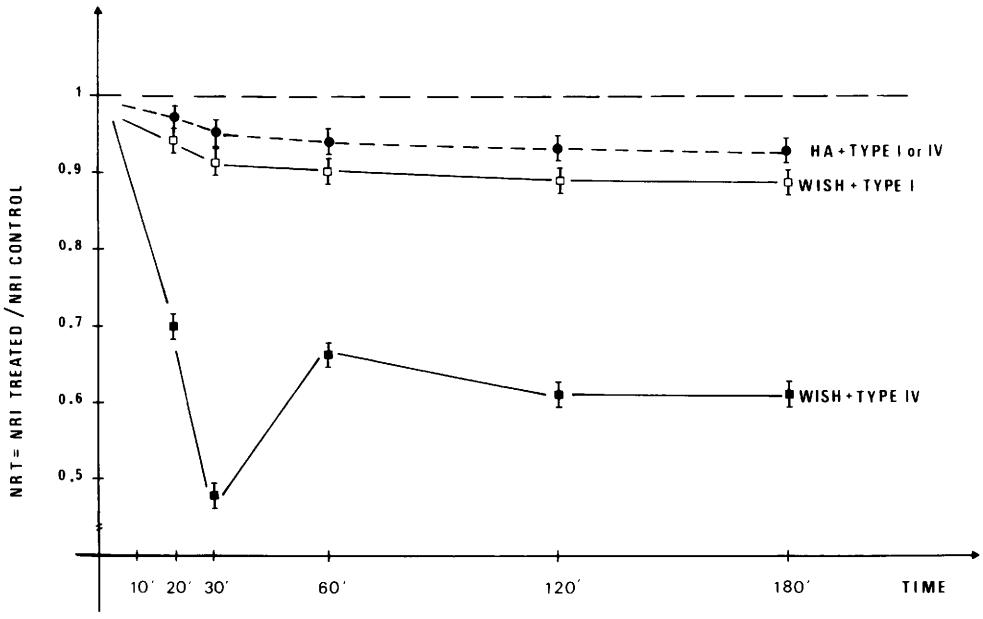


FIG. 3. Time course of the nuclear response of HA and WISH cells treated by type I or IV collagen. After varying incubation times, cells were fixed for NRI determination as described under Materials and Methods. NRT = NRI treated/NRI control cells, was calculated for HA cells in presence of type I or IV collagen fractions (10 μg/ml) (●) and for WISH cells in presence of type I (□) or IV (■) collagen fractions (10 μg/ml). Each time point represents the mean of seven experiments. Bars denote the SD ($P < 0.01$).

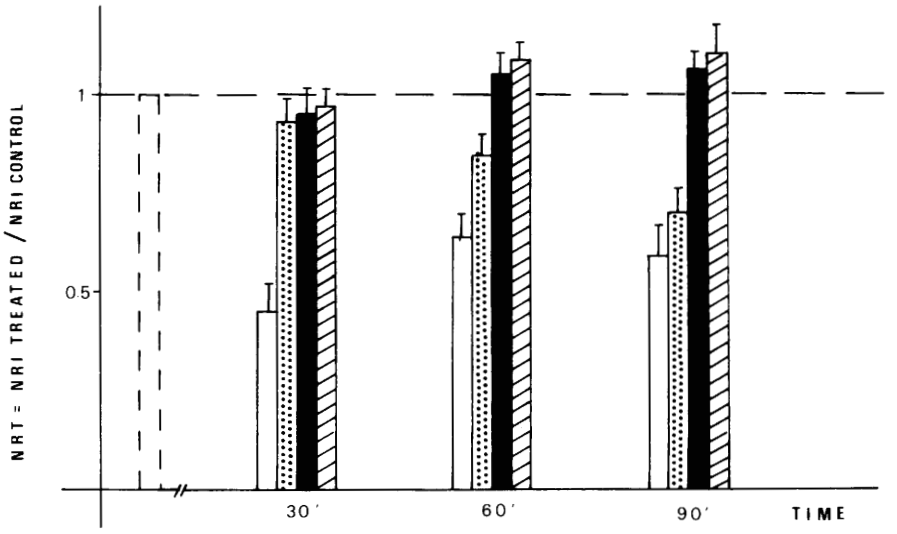


FIG. 4. Effect of inhibitors on nuclear refringency of WISH cells treated with type IV collagen. NRT was evaluated after varying incubation times in presence of type IV collagen (10 μg/ml) for WISH cells preincubated with NANA (▨), Nocodazole (■), cytochalasin B (▨), or without inhibitors (□). NRT = 1 for control untreated WISH cells (— —).

The effects of three different inhibitors on the nuclear refringency of WISH cells treated by type IV collagen is shown in Fig. 4. NANA, known to inhibit the cell surface effect of type IV collagen (7, 10), also inhibited the nuclear activation and NRT value of the cell population, almost reaching that of control cells. The cytoskeleton inhibitors, Nocodazole and cytochalasin B, also suppressed nuclear activation and the NRT values were close to 1.

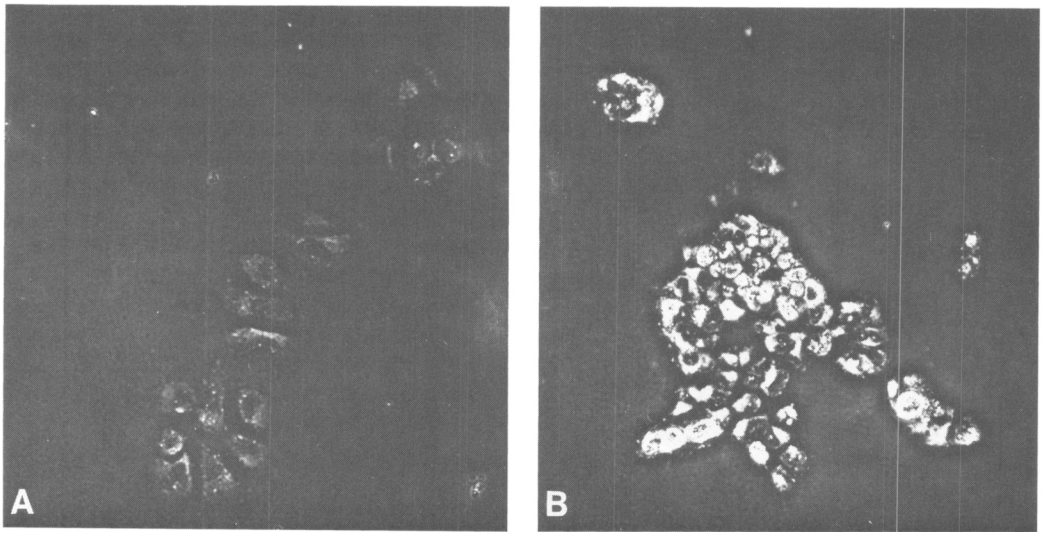


FIG. 5. Morphological appearance of nuclear refringency of WISH cells seeded on glass slides and directly fixed on the slide with Zenker reagent after 1 hr of incubation at 37°C. (A) WISH cells treated by 5 μg/ml of type IV collagen. (B) WISH cells treated by 1 μg/ml of laminin. ×400.

The morphology and nuclear refringency of cells laid on type IV collagen differed from those of cells attached to laminin. WISH cells attached in the presence of type IV collagen were very flat and the nuclei appeared dark on the cytoplasmic area (Fig. 5A). In the presence of laminin, on a glass substratum, WISH cells clustered together, remained round, and the nuclei were brilliant (Fig. 5B). The NRT value of this latter cell population differed little or none from the one of control cells.

Kinetics of limited DNase I digestion. The kinetics of DNase I nuclear degradation of WISH cells treated for 4 hr by collagen I or IV are compared in Fig. 6. The chromatin of cells attached in presence of type IV collagen was particularly sensitive to DNase I digestion. The increase of [^{3}H]thymidine counts per minute in the acid-soluble phase of digest was about 50% higher in WISH cells treated by type IV collagen than in cells treated by type I collagen or untreated control cells for an incubation time of 10 min. Primary HA cells

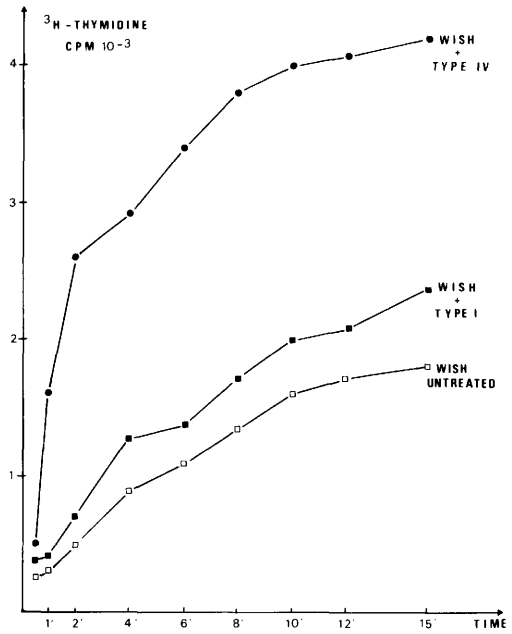


FIG. 6. Comparative kinetics of DNase I nuclei digestion of untreated, type I-, or type IV-treated WISH cells. The nuclei were digested with DNase I for increasing periods of time as described under Materials and Methods. Aliquots of acid soluble digest products were taken for measurement of ^{3}H activity. This result was confirmed in seven separate experiments.

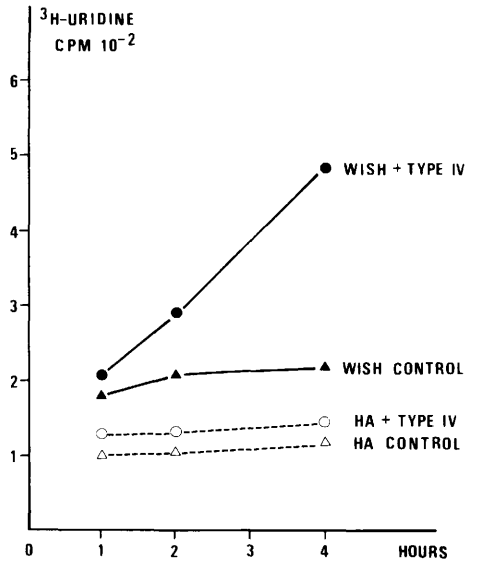


FIG. 7. Effect of type IV collagen on poly(A)$^+$ RNA production in HA and WISH cells. The synthesis of poly(A)$^+$ RNA was determined at different times after contact of the cells with type IV collagen and pulse-labeled for 60 min with [^{3}H]uridine. The poly(A)$^+$ RNA was measured in the cytoplasm of WISH or HA cells treated with type IV collagen; and of untreated WISH or HA cells.

treated or not by type I or IV collagen did not show any notable difference with DNase I sensitivity (data not shown). Similar experiments using staphylococcal nuclease instead of DNase I showed no difference in the degradation rates of nuclei from control and type IV collagen-treated cells (data not shown). These results were confirmed in seven separate experiments.

mRNA synthesis. The effect of type IV collagen on RNA synthesis was also investigated. As shown in Fig. 7, a significant increase in poly(A)$^+$ RNA levels was observed in WISH cells after 4 hr of treatment with type IV collagen. There was an increase of 55% in the poly(A)$^+$ RNA levels in treated cells as compared to the untreated ones. In HA cells, under the same conditions, no significant incorporation of isotopes into poly(A)$^+$ RNA was noted in cells treated or not by type IV collagen.

Cell attachment and laminin synthesis. The attached HA or WISH cells were counted after the same incubation times as those selected for nuclear refringency assay. Attachment ki-

netics in the presence and absence of type I or IV collagen were performed in EMEM without serum. The results are shown in Fig. 8. Few HA cells bound to the substratum in absence of serum at 180 min of incubation. Type I collagen slightly enhanced the cell attachment which did not exceed 8% after 180 min. In presence of type IV collagen, cell attachment reached 12% at the same incubation time.

On the other hand, 20% of WISH cells untreated by collagens adhered to the substratum after 180 min but the cells remained round and dispersed. The attachment increased up to 40% by 180 min when WISH cells were treated by type I collagen. In presence of type IV collagen, this value reached 85% in 3 hr. In addition, as previously mentioned, morphological changes of the cell, such as clustering and spreading out on the substratum, occurred in a few minutes (7, 10). The same discrepancy in the attachment characteristics of normal and transformed cells to type IV collagen was repeatedly observed.

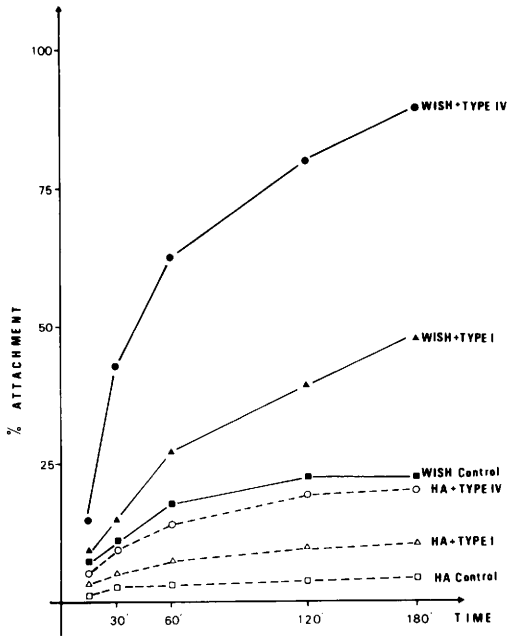


FIG. 8. Kinetics of the attachment of HA and WISH cells on collagen type I or IV substrates. WISH cells (2×10^5/ml) were incubated on type IV collagen (10 μg), type I collagen (10 μg), and uncoated polystyrene wells of tissue culture plates (control) for 20 to 180 min without serum. HA cells (2×10^5/ml) were incubated on type IV collagen (10 μg), type I collagen (10 μg), and uncoated polystyrene wells (control) for 1–24 hr without serum.

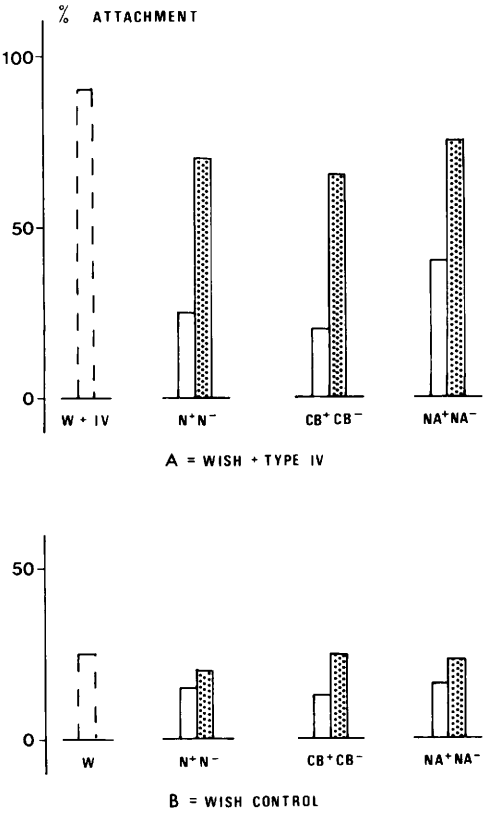


FIG. 9. Effect of inhibitors on the attachment of WISH cells in presence or absence of type IV collagen. WISH cells (2×10^5 cells/ml), preincubated for 60 min with Nocodazole (NA^+, 0.3 μg/ml), cytochalasin B (CB^+, 5 μg/ml), or NANA (NA^+, 5 mM), were plated in triplicate wells in medium supplemented with type IV collagen (5 μg/0.5 ml). WISH cells were allowed to attach for 180 min. After removal of the inhibitors, WISH cells were replaced in new culture wells containing medium with type IV collagen and incubated for 180 min. Controls without type IV collagen were effected. (□) attachment of WISH cells incubated with inhibitors; (▨) attachment of WISH cells after inhibitor removal; (— —) attachment of WISH cells after 180 min incubation.

After 24 hr incubation in the absence of serum, WISH cells were counted and the morphological patterns were assessed (Fig. 10). The viability of attached cells was determined by trypan blue exclusion. During this period, WISH cells attached to the substratum in the presence of type IV collagen, spread out and formed a confluent monolayer (Fig. 10B, a) with a cell attachment of 80% (Fig. 10A, a).

In parallel, WISH cells, not treated with collagen, attached poorly, remained round (Fig. 10B, b), and degenerated, as revealed by the number of surviving viable cells (Fig. 10A, b). WISH cells routinely grown with 15% calf serum proliferated (Fig. 10A, c) with local piling up of cells (Fig. 10B, c).

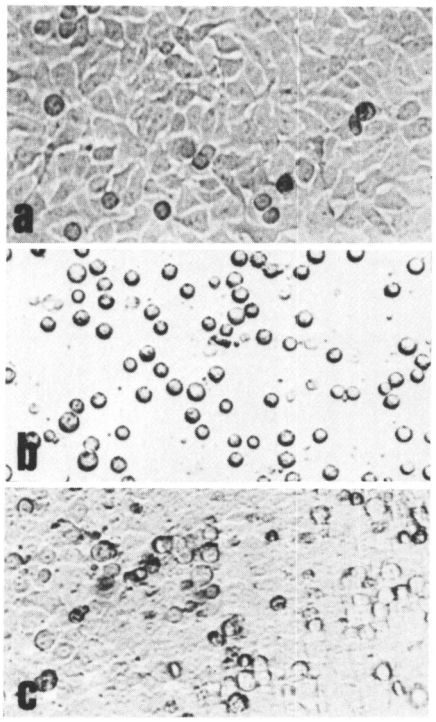
As shown in Fig. 5B, the addition of laminin (1 μg/ml) to the culture medium induced a clustering effect of cells. The cell aggregates attached loosely to the substratum and only a small degree of cell flattening was observed after 24 hr. The percentage of the cells that attached did not exceed 25%.

Attachment of WISH cells in presence of type IV collagen was reduced by 50% after Nocodazole or cytochalasin B and by 30% after NANA pretreatment (Fig. 9A). In absence of type IV collagen, the attachment of WISH cells was not modified by inhibitors (Fig. 9B). After removal of the inhibitor, in presence of type IV collagen, the cells recovered their capacity to attach up to 50% in 180 min (Fig. 9A).

Laminin was identified and determined by immunoenzymatic detection in the incubation medium of attached type IV collagen-treated WISH cells (Table I). Laminin synthesis was

Medium	Cell number
(a) + Type IV without serum	$1.9 \times 10^5 \pm 354$
(b) Without type IV or serum	$4 \times 10^3 \pm 458$
(c) + Serum without type IV	$4 \times 10^5 \pm 856$

A



B

FIG. 10. Effect of type IV collagen or serum on the attachment (A) and morphological patterns (B) of WISH cells. (A) Numeration of WISH cells. On each well of Falcon plates, 2.5×10^5 cells were seeded. After 24 hr of culture, the cells were removed by trypsinization and the number of WISH cells (mean ± SD) was estimated by trypan blue exclusion. (B) Morphological patterns. (a) WISH cells maintained on type IV collagen (10 μg/2.5×10^5 cells) for 24 hr in culture medium without serum form a confluent layer of flattened cells with an epithelial cell-like morphology. Ten to 15% of cells were trypan blue positive. (b) WISH cells incubated without serum or type IV collagen remain round and unattached. Thirty to 45% of the cells were trypan blue positive. (c) In the presence of serum without type IV collagen, WISH cells proliferated and exhibited morphologically transformed foci with the piling up of cells. Magnification: B (a–c), ×200.

TABLE I. IMMUNOENZYMATIC CHARACTERIZATION OF LAMININ

INHIBITOR	% VIABLE CELLS	% ATTACHED CELLS	LAMININ SYNTHESIS
NONE	98.75	81.75 ± 5.58	
NOCODAZOLE +	99	21 ± 1.15	
NOCODAZOLE -	99	52 ± 3.47	
NONE	98.8	84.6 ± 7.06	
CYTOCHALASIN B +	98.2	25 ± 1.49	
CYTOCHALASIN B -	96.8	76.46 ± 3.35	
NANA +	98	40.5 ± 2.31	
NANA -	98.8	79.3 ± 1.35	

Note. Numbers represent the percentage of cells attached in six replicate assays. Laminin synthesis was detected by immunoenzymatic labeling (as described under Materials and Methods) in the 3-hr attachment assay medium of WISH cells treated by type IV collagen: in the absence of inhibitor, after inhibitor treatment (+), and after inhibitor removal (−). Type IV collagen preparation did not cross-react with anti-laminin.

inhibited by Nocodazole and cytochalasin B and only impaired after treatment with NANA. Removal of the inhibitors allowed the recovery of laminin synthesis (Table I). In contact with type IV collagen, comparable amounts of laminin were seen in the 3-hr culture medium of WISH cells without inhibitor treatment or after inhibitor removal. The 3-hr HA cell culture medium contained only traces of laminin under the survival conditions of the test (EMEM without serum).

It was noteworthy that laminin antibodies inhibited the attachment of WISH cells to type IV collagen.

Discussion. Collagen is a major constituent of the extracellular matrix: type I collagen is found predominantly in connective tissues, whereas type IV collagen is present in base-

ment membranes. *In vitro,* differences are observed in the recognition process of type IV collagen by normal and transformed epithelial cells (4–8). Previous studies have reported similarities between the effect of lectins (29) and type IV collagen fractions (7, 10) on transformed cells reacting to these agents. In this study we describe an early modification of the nuclear structure in transformed cells triggered by the membranous effect of type IV collagen. An analogous early nuclear effect has been observed and quantified in lymphocytes stimulated by mitogenic lectins (11, 30).

Nuclear refringency is a property of condensed chromatin which contains nonexpressed genes. Its decrease is due to chromatin dispersion associated to nuclear activation (11). In normal cells, type IV collagen does not modify in a detectable manner either cell metabolism or nuclear refringency. Despite this absence of nuclear modification, a difference in the attachment properties is observed in regard to the two types of collagen.

HA cells, like other epithelial cells (4, 5), bind slowly but specifically to type IV collagen and laminin is synthesized normally. The contact of HA cells with type IV collagen *in vitro* does not stimulate new nuclear functions and the condensed chromatin remains quiescent. This result was expected since epithelial cells are closely associated to basement membrane *in vivo.*

On the contrary, the contact of WISH cells with type IV collagen induces a quenching of nuclear refringency in a few minutes. In the same time, attachment properties are restored and WISH cells, deposited on type IV collagen, exhibit morphological differences compared to their untreated counterparts. The morphological changes of WISH cells stimulated by type IV collagen could be related to the synthesis of laminin, a molecule promoting cellular attachment (15–17) and spreading (18).

The use of NANA, which impairs the first clustering of cells in presence of basement membrane collagen fractions (7, 10), also hinders the stimulation of cell nuclear refringency, as well as the attachment of the cells and laminin production. The recovery of these properties is observed when transformed WISH cells are replaced with type IV collagen after the removal of NANA.

In addition, as shown by the blocking effect of Nocodazole and cytochalasin B, the integrity of the cytoskeleton framework is prerequisite for nuclear activation and attachment processes. The removal of these inhibitors leads to the decondensation of chromatin and to the reinduction of metabolic events which include spreading out and attachment of cells.

It is also of interest that laminin antibodies inhibit the attachment and the nuclear refringency of WISH cells treated by type IV collagen (data not shown). These data suggest that laminin synthesis is indeed the result of the contact of WISH cells with type IV collagen.

The chromatin conformation changes of WISH cell nuclei treated by type IV collagen is confirmed by their accessibility to limited DNase I digestion. Indeed, DNase I is known to digest preferentially the active parts of decondensed chromatin (23, 31–34). This effect on chromatin is accompanied by a stimulation of gene expression as revealed by the increase of mRNA synthesis.

It is of interest that the simple contact of transformed epithelial cells with basement membrane collagen might modulate the expression of various sets of genes, enabling the cell to produce attachment factors usually synthesized in their normal counterparts. Transformation processes involve alterations both in the synthesis of cell surface proteins and in the interactions with matrix proteins (35, 36). The laminin synthesis induced by the matrix signal of type IV collagen restores an extracellular environment for the cells. The inclusion of the cells in a common matrix changes the surface properties of transformed cells and provides narrow connections between the cells which are necessary to modify cell growth properties (as shown by the patterns of WISH cells grown on type IV collagen) (Figs. 5 and 9). This cell-to-cell contact mediated by the newly formed matrix (i.e., laminin bound to type IV collagen) may thereby trigger the metabolic events that lead to the cell attachment through cell membrane, cytoskeleton, and nucleus interactions.

The nuclear refringency test is an easy and rapid method for the detection and estimation of early structural changes in the nucleus of transformed epithelial cells induced by the recognition of type IV collagen. The nuclear response of tumoral cells to basement mem-

brane collagen could express their capacity to restore an extracellular environment able to limit the cell proliferation.

We are grateful to M. Sarragne, J. Lopez, D. Esnous, P. Michel, and F. Coutances for their excellent technical assistance and to C. Girard for her contribution to the preparation of the manuscript and illustrations. This work was supported in part by Grant 8279.114 from the Institut National de la Santé et de la Recherche Médicale (Unité 43) and by Grants 7915182 from the Institut National de la Santé et de la Recherche Médicale and 8070225 from the Délégation Générale à la Recherche Scientifique et Technique (Laboratoire Central d'Anatomie pathologique).

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Received December 6, 1983. P.S.E.B.M. 1984, Vol. 176.
Accepted May 11, 1984.