The Extracellular Matrix of Normal Chick Embryo Fibroblasts: Its Effect on Transformed Chick Fibroblasts and Its Proteolytic Degradation by the Transformants

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ABSTRACT Extracellular matrix (ECM), prepared from chick embryo fibroblasts, contains fibronectin as the major structural protein along with collagen and other polypeptides as less abundant protein components. When Rous sarcoma virus-transformed chick embryo fibroblasts are cultured on the ECM in the presence of the tumor promoter tetradecanoyl phorbol acetate, the transformed cells lose their characteristic rounded morphology and align on and within the ECM fibrillar network. This restrictive aspect of ECM is only temporary, however, and with time (24–72 h) the transformed cells progressively degrade the ECM fibers and resume their rounded appearance. The matrix degradation can be monitored by employing biosynthetically radiolabeled ECM. The addition of purified chicken plasminogen to the Rous sarcoma virus-transformed chick embryo fibroblast cultures enhances the rate and extent of ECM degradation, due to the elevated levels in the transformed cultures of plasminogen activator. Plasminogen-dependent and -independent degradation of ECM has been characterized with regard to sensitivity to various natural and synthetic protease inhibitors and to the requirement of cell/ECM contact. Plasminogen-dependent degradation of ECM occurs rapidly when ECM and cells are in contact or separated, whereas plasminogen-independent degradation is greatly reduced when ECM and cells are separated, which suggests that cell surface-associated proteolytic enzymes are involved. A possible role in ECM degradation has been indicated for cysteine proteases, metallo enzymes, and plasminogen activator, the latter as both a zymogen activator and a direct catalytic mediator.

Extracellular matrix (ECM) is a complex network of proteins and carbohydrate polymers that underlies epithelial cells and encompasses the cells of the connective tissue. The components of the matrix, which include fibronectin, collagen, elastin, glycosaminoglycans, and various glycoproteins, are synthesized by cells and after secretion are organized into a stable fibrillar array (for review see references 1 and 2).

Although the ECM is essential for structural integrity of the tissue, it does not appear to be an inert scaffolding but a molecular complex that can regulate proliferation, cellular differentiation, and morphogenetic movement (1, 2). A structural facsimile of ECM can be produced in vitro by various cultured cells including fibroblasts and muscle, epithelial, and endothelial cells (3–10). The ECM produced by these cells has been shown to have a pronounced effect on cellular morphology, adhesion, locomotion, proliferation, and response to exogenous effector molecules such as hormones, growth factors, and enzymes (11–15).

The ECM, which is often adhesive and restrictive in nature as it anchors and enmeshes resident cells, appears to be altered...
in certain disease states. Malignant cells can escape the ECM-imposed restriction by modifying and breaking down the matrix network in vivo. This is often observed in histological sections of tumor tissue as "tongues" of malignant cells clearing away ECM and penetrating surrounding normal tissue (16). As an experimental model for this process a number of investigators have cultured malignant cells on ECM in vitro and observed that the cells actively clear the ECM by solubilizing and degrading the matrix components (17-22). However, a number of laboratories also have demonstrated that when malignant cells are cultured on ECM, the matrix, or its individual components, imposed morphological and locomotory restriction on the cells, causing them to adhere firmly and align on and within the ECM network (9, 23-26). These apparent experimental contradictions may be related to the nature of the malignant cells being examined, the histological origin of the ECM employed as the substratum, and the biological and temporal limitations of the different experimental approaches.

We have been using normal chick embryo fibroblasts (CEFs) and Rous sarcoma virus-transformed chick embryo fibroblasts (RSVCEFs) to examine the role of specific proteolytic enzymes in modulating the behavior of the RSVCEF transformants (27-32). Normal CEFs have been shown to produce an abundant ECM (5), which may have a direct effect on the morphology and behavior of CEF cultures and the transformants that arise after infection of the CEFs with RSV. Therefore we were interested in examining the effect of ECM, isolated from cultures of normal CEFs, on homologous cultures of RSVCEFs, and in determining the effect of the elevated levels of RSVCEF proteolytic enzymes on such matrices. Our results indicate that ECM initially has a pronounced restrictive effect on the behavior of the transformants but that in time the transformed cells overcome that restriction by catalytically degrading the ECM using a number of enzyme systems.

MATERIALS AND METHODS

Cell Cultures: Primary CEFs were prepared from 10-12-d-old embryos (33). The cultures were grown in Eagle's minimal essential medium (MEM) containing high glucose, penicillin, and streptomycin and supplemented with 10% heat-inactivated fetal calf serum (FCS). CEF secondary cultures were infected and transformed with RSV as described previously (27, 28). All experiments were performed on second, third, and fourth passage cultures of CEFs and RSVCEFs.

Preparation of ECM: Trial preparations of ECM were carried out according to a number of different methods (9, 18, 20, 34). The method used for all preparations was a modification of the procedures described by Jones and De Clerk (18). Petri dishes were seeded with CEFs (4 x 10^6 cells in 35-mm dishes) and incubated in MEM supplemented with 10% FCS in the presence or absence of such factors as trypsin inhibitor, wash with PBS, addition of 1 ml collagenase under incubation condition B for 1 h, and termination with EDTA. After each enzyme treatment the radioactivity released from the ECM was determined (see below), and the residual ECM was solubilized and analyzed by PAGE (see below).

ECM Degradation Assay: Radiolabeled ECM preparations were washed with MEM before cells (CEFs or RSVCEFs) were seeded onto them at 1.5 x 10^6 cells/35-mm dish in MEM containing 5% plasminogen-depleted FCS prepared as previously described (35). Approximately 18-24 h after the cultures were established growth factor was added to bring the concentration of such factors to 2 mM, and the cultures were incubated in 2 ml serum-free MEM in the presence or absence of 100 ng/ml of tetradeanol sodium salt (TAP). At selected intervals thereafter triplicate representative dishes were taken for analysis. The supernatant was removed, phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, and a 200-μl aliquot was mixed with 5 ml Aquasol-2 and counted in a beta-liquid scintillation counter. The remaining supernatant was frozen for later analysis or dialyzed and lyophilized in preparation for SDS-PAGE (see below). The remaining ECM and cells were collected from the dishes by scraping in 0.5 ml 1% SDS. A 50-μl aliquot was added to 5 ml Aquasol-2 and counted in a beta-liquid scintillation counter. The total counts per minute in the supernatant and residual matrix were calculated, and the percent radioactivity released was based on counts per minute in supernatant/total counts per minute in supernatant + matrix x 100. The amount of radiolabeled protein in the matrix and released into the supernatant was quantitated by sequential gel electrophoresis and autoradiography. The supernatant was removed, phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, and a 200-μl aliquot was mixed with 5 ml Aquasol-2 and counted in a beta-liquid scintillation counter. The remaining supernatant was frozen for later analysis or dialyzed and lyophilized in preparation for SDS-PAGE (see below). The remaining ECM and cells were collected from the dishes by scraping in 0.5 ml 1% SDS. A 50-μl aliquot was added to 5 ml Aquasol-2 and counted in a beta-liquid scintillation counter.

Preparation of ECM for Separation and Cell Contact Degradation Studies: ECM was prepared on 35-mm dishes as described above. The vertical sides of the dishes were cut away from the base, leaving the ECM intact on a plastic 35-mm disk, which was sterilized in 70% ethanol and air-dried. Under these conditions surface tension allowed the disks to be floated on medium with the ECM facing downward in a 60-mm culture dish 1–2 mm above a monolayer of cells. Alternatively, the plastic dish could be rewetted with medium and submerged on the bottom of a 60-mm culture dish with the ECM facing upward. A cell suspension was added to the dish, allowing the cells to attach directly to the ECM.

SDS PAGE: Samples of supernatant and resuspended matrices were mixed with appropriate volumes of concentrated electrophoresis sample buffer (36) containing 5% beta-mercaptoethanol, boiled, and applied directly to 8.5% polyacrylamide slab gels using the system of Laemmli (36). Alternatively, samples were dialyzed against 500 vol 0.01% SDS, lyophilized, and taken up in electrophoresis sample buffer containing 5% beta-mercaptoethanol, boiled, and analyzed on 8.5% polyacrylamide slab gels. Unless otherwise noted, equal volumes of supernatant and equal volumes of resuspended matrices corresponding to the culture dish or an equal number of cells were loaded onto each gel. After electrophoresis, the gels were stained in 0.25% Coomassie Blue in 50% methanol, 8% acetic acid for 1–2 h at 37°C and destained with several changes of 5% methanol, 12% acetic acid. Autoradiography was performed on gels containing 35S-labeled proteins with Kodak R film, Ready pack. Molecular weight standards were run in parallel lanes and included myosin (200,000), β-galactosidase (135,000), bovine serum albumin (67,000), ovalbumin (43,000), phosphorylase (25,000), and cytochrome c (12,000).

Microscopy: Cells cultured on matrices that had been prepared on strips (6 x 20 mm) of tissue culture plastic cut from Lux petri dishes (Lux Scientific Inc., Newbury Park, CA) were fixed with 1.5% glutaraldehyde in 0.1 M potassium phosphate, pH 7.4, for 1 h and washed overnight at 4°C in the phosphate buffer. Fixed samples were prepared for scanning electron microscopy by critical point drying, coated with evaporated gold, and examined with a JEOL 100C electron microscope in the scanning mode. Samples for phase microscopy were examined with an inverted phase microscope and photographed with a Polaroid camera. ECM-containing dishes were prepared and analyzed for immunofluorescence using an anti-chicken fibronectin antiseraum (37, 38).

Preparation of Plasminogen, Plasminogen-free Serum, and Acid-treated Serum: FCS and chicken serum were selectively freed of plasminogen by two passages over a lissamine–Sepharose affinity column according to established procedures (35). The serum was assayed with purified plasminogen activator (PA) (39) and shown to be 95–100% plasminogen free. Plasmin-
The Journal of Cell Biology

Effect of Chick ECM on the Morphology of Transformed Chick Fibroblasts

It is well established that when CEFs are transformed by RSV their morphology is dramatically altered (42), and this has been ascribed to the diminished production of ECM components by transformed cells (37, 43, 44). It has also been shown that treatment of RSVCEF cultures with the tumor promoter TPA causes pronounced clustering of the transformed cells and a concomitant loss of the residual ECM substratum (28, 29, 45). When transformed CEFs are incubated in serum-free medium on the ECM prepared from ascorbic acid-treated cultures. The ECM preparations were incubated for 1 h at 37°C in buffer alone (lane 1), buffer plus 260 U collagenase (lane 2), buffer plus 0.25 μg trypsin (lane 3), and buffer plus trypsin (0.25 μg) for 1 h before collagenase (260 U) was added for 1 h (lane 4). The ECM was then prepared for SDS PAGE and autoradiography. Marker proteins were run in a parallel lane of the gels, and their positions are indicated. In B overexposure of the autoradiograph caused the 145/155K doublet to resolve only as a single band at 150K.

Results

Preparation and Characterization of ECM from Monolayer Cultures of CEFs

Growing cultures of CEFs were incubated with [35S]methionine to radiolabel the protein components of the ECM. When ECM is prepared from radiolabeled CEF cultures that had been incubated in the absence (lane 1) or presence (lane 2) of 50 μg/ml of ascorbic acid for 48 h before preparation of the ECM. (B) Autoradiograph of radiolabeled ECM polypeptides prepared from ascorbic acid–treated cultures. The ECM preparations were incubated for 1 h at 37°C in buffer alone (lane 1), buffer plus 260 U collagenase (lane 2), buffer plus 0.25 μg trypsin (lane 3), and buffer plus trypsin (0.25 μg) for 1 h before collagenase (260 U) was added for 1 h (lane 4). The ECM was then prepared for SDS PAGE and autoradiography. Marker proteins were run in a parallel lane of the gels, and their positions are indicated. In B overexposure of the autoradiograph caused the 145/155K doublet to resolve only as a single band at 150K.

Materials: Human alpha-2 macroglobulin (α2M) was provided by Dr. Richard Feinman (Downstate Medical Center). Chicken myosin and actin were provided by Dr. Alfred Stracher (Downstate Medical Center). A preparation of chicken procollagen/collagen was provided by Dr. Bjorn Olsen (Rutgers Medical School, New Brunswick, NJ). Na125I (carrier-free) and [35S]methionine (700–1400 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). TPA was purchased from Chemical Carcinogenesis (Eden Prairie, MN). Chick embryonated eggs (Cofal negative) were obtained from Spafas, Inc. (Norwich, CT). FCS was purchased from HyClone Laboratories, Sterile Systems, Inc. (Logan, UT). Collagenase type III was purchased from Advance Biofactors (Lynbrook, NY). Aquasol-2 was purchased from New England Nuclear (Boston, MA). Trypsin, 1,10 phenanthroline, leupeptin, and p-nitro-phenylguanidine benzoate (NPGB) were purchased from Sigma Chemical Co. (St. Louis, MO). E64c was provided by Dr. Joseph Etlinger (Downstate Medical Center).
with or without normal CEFs since only from 5 to 8%, respectively, of the labeled material was released during a 48-h incubation. RSVCEFs, however, induce a progressive breakdown of ECM components, releasing 35% of the protein components after 48 h. Matrix degradation is dramatically increased by TPA-treated RSVCEF cultures, which released 40% of the radioactivity after 24 h and 70% after 48 h. The extensive solubilization of ECM polypeptides in the TPA-treated RSVCEF cultures correlates with the extensive cell clustering observed in Fig. 2h. That the pattern of matrix degradation was similar for ECM prepared in ascorbate-free and ascorbate-containing medium (Fig. 3) indicates that the increased collagen deposition did not affect the overall pattern of ECM solubilization by the transformed cells.

Plasminogen-dependent and -independent Degradation of ECM

The degradation of ECM by transformed cells in serum-free medium observed in Fig. 3 can be enhanced by the addition of purified chicken plasminogen (Fig. 4), and this increased rate of degradation is prevented by trasylol, an inhibitor of plasmin. This indicates that the elevated levels of PA (28) catalytically convert plasminogen to plasmin, which in turn rapidly degrades the ECM. Trasylol, however, had no effect on the cell-mediated ECM degradation, as 70–80% of the radiolabeled ECM was released over 48 h in either the presence or absence of trasylol (Fig. 4). The polypeptides released from the ECM into the culture medium are shown in Fig. 5 and provide evidence that the cell-mediated degradation of ECM is distinct from the plasminogen-mediated degradation. The polypeptide pattern of the culture medium

![Figure 2](image1)

**Figure 2** Morphology of normal and transformed cells incubated on tissue culture plastic (left) or ECM (right). Normal CEF (a and b) and RSVCEFs (c and d) were seeded (1.5 x 10⁶ cells/35-mm dish) in MEM containing 5% plasminogen-depleted FCS onto plastic tissue culture dishes or dishes that contained ECM. After 24 h all cultures were washed in MEM and incubation was continued in serum-free MEM. Some of the RSVCEF cultures were supplemented with TPA (e–h) (100 ng/ml). Phase-contrast photomicrographs were taken 23 (a–f) or 48 h (g and h) after the addition of serum-free MEM.

The PA activity of CEFs, RSVCEFs, and TPA-treated RSVCEFs cultured on ECM was 0.46, 12.9, and 81.2 U/culture, respectively, at 23 h, which demonstrates that the substantial increases in PA activity upon RSV transformation and TPA treatment (28, 29) are not reversed by culturing on ECM.

**Alteration and Degradation of ECM by Transformed and TPA-treated Transformed Cells**

Since the morphological studies indicated that the ECM was absent after a 48-h incubation of transformed cultures in TPA we decided to quantitate the apparent loss of ECM using ³⁵S-radiolabeled matrices. Fig. 3 demonstrates that the ECM was relatively stable when incubated in serum-free medium with or without normal CEFs since only from 5 to 8%, respectively, of the labeled material was released during a 48-h incubation. RSVCEFs, however, induce a progressive breakdown of ECM components, releasing 35% of the protein components after 48 h. Matrix degradation is dramatically increased by TPA-treated RSVCEF cultures, which released 40% of the radioactivity after 24 h and 70% after 48 h. The extensive solubilization of ECM polypeptides in the TPA-treated RSVCEF cultures correlates with the extensive cell clustering observed in Fig. 2h. That the pattern of matrix degradation was similar for ECM prepared in ascorbate-free and ascorbate-containing medium (Fig. 3) indicates that the increased collagen deposition did not affect the overall pattern of ECM solubilization by the transformed cells.

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![Figure 3](image2)

**Figure 3** Degradation of radiolabeled ECM by normal and transformed cells. CEFs and RSVCEFs were seeded (1.5 x 10⁶ cells) onto 35-mm petri dishes containing ³⁵S-labeled ECM (see Materials and Methods). At zero time (18 h after seeding) the cultures were washed with MEM and incubated in serum-free MEM with or without TPA (100 ng/ml). As controls, dishes containing ³⁵S-labeled ECM were incubated in MEM without cells. At the indicated times the medium was removed from the culture dishes and counted for radioactivity. The ECM were prepared from CEF cultures grown in the absence (solid lines) or presence (dashed lines) of ascorbate.

Fairbairn et al. Extracellular Matrix Degradation by Transformed Cells
Effect of Protease Inhibitors on Cell-mediated ECM Degradation

Using selective protease inhibitors we further examined the nature of the cell-mediated, plasminogen-independent degradation (Fig. 6). The protease inhibitors were added to cultures at concentrations that in prior experiments had been shown to have no effect on cell viability, cell plating efficiency, and cellular protein and RNA synthesis (29). DFP, the general inhibitor of serine proteases (46), inhibits ECM degradation by RSVCEF cultures to 32-38% of control. The epoxide derivative E64c, an inhibitor of mammalian cysteine proteases (47), inhibits ECM degradation by 30-43%. The metallo-protease inhibitor, 1,10 phenanthroline, inhibits degradation by 40-53%. The combined use of all three inhibitors reduces ECM degradation by 80% (Fig. 6A). NPGB, a potent inhibitor of arginine-preferring serine proteases (31), inhibits ECM degradation by 35% (Fig. 7B), a percent inhibition similar to that observed for the more general serine protease inhibitor disopropylfluorophosphate (Fig. 6A). Leupeptin, an inhibitor of arginine-preferring serine and thiol proteases (48), also inhibits ECM degradation to 37% of control. The combined use of leupeptin and NPGB did not alter significantly the inhibition pattern exhibited by either compound alone (Fig. 6B). This result is in contrast to the combined use of the more general protease inhibitors (Fig. 6A) and suggests that a specific arginine-preferring serine protease is partially responsible for the degradation of ECM by RSV-transformed cells.

Effect of Serum and Serum Components on ECM Degradation

All of the previously described ECM degradation studies were performed in serum-free medium. Since FCS contains plasminogen (35) is a standard supplement of transformed cell cultures, we examined the effect of FCS supplementation on matrix degradation (Fig. 7). Adding 5% FCS to cultures of TPA-treated RSVCEF incubated on radiolabeled ECM increases the degradation of ECM over that of parallel cultures incubated in serum-free medium. This increase in degradation (5 to 15% after 6 h, 40 to 55% after 24 h) is not
as substantial as the increase observed when purified plasminogen is added to serum-free cultures (Fig. 4). Since this might be due to natural protease inhibitors present in serum, FCS was acid treated and renaturated to inactivate most of the protease inhibitory capacity of FCS (40). Acid-treated FCS enhanced the degradation of ECM by RSVCEFs (25% after 6 h and 70% after 24 h) to levels similar to those observed when purified plasminogen was added to serum-free cultures (Fig. 4). That the increase in ECM degradation in the presence of FCS or acid-treated FCS was dependent on serum plasminogen is illustrated by the reduction in ECM degradation that occurs when plasminogen-depleted FCS or plasminogen-depleted, acid-treated FCS is used as a supplement (Fig. 7). The plasminogen depletion of FCS by lysine Sepharose affinity chromatography used in these experiments selectively removes >98% of the plasminogen in serum (35).

**ECM Degradation by Transformed Cells in Direct Contact with or Separated from ECM: Effect of Plasminogen and the Protease Inhibitor α2M**

Degradation of ECM proteins by transformed cells could be mediated by cell surface proteolytic enzymes or by secreted proteolytic enzymes or both. To examine these possibilities,
transformed cells were incubated either in direct physical contact with radiolabeled ECM or separated from the ECM. We separated cells from ECM by preparing ECM on plastic tissue culture dishes and floating the disks in the medium above a monolayer of 4 x 10^6 RSVCEF cells (separated conditions) or placed in culture dishes (ECM facing downward) with 4 x 10^6 RSVCEF cells seeded on top of the disks (contact conditions). TPA (100 ng/ml) was added to all cultures. The present study demonstrates, however, that this restorative ability of ECM components is only shortlived. The transformed cells, unlike the normal CEFs, can progressively degrade the proteins of ECM and thereby overcome the restrictive properties of the underlying matrix. This is especially pronounced when the transformed cells are treated with the tumor promoter TPA, which induces a highly transformed phenotype (28, 29). Concomitant with matrix degradation, the TPA-treated transformed cells regain their abnormal morphology and migrate or rearrange in culture to form multicellular clusters (Fig. 2h).

The nature of the degradative ability of transformed fibroblasts is complex, but the present study illustrates some aspects of their catalytic activity toward ECM. When a source of plasminogen is present, the degradation of the ECM is rapid and extensive (Fig. 4) due to the conversion of inactive plasminogen to the active protease plasmin by the elevated levels of PA produced by the transformed cells (29, 30). If serum is present, RSVCEF-mediated ECM degradation occurs, but its rate and extent are controlled by the presence of acid-labile protease inhibitors in the serum (Fig. 7). The rapid clearing of matrix in the presence of serum appears to be due mainly to plasmin since selective removal of plasminogen from serum reduces ECM degradation to the level observed in serum-free medium. This plasminogen dependence in the presence of serum was not observed by Kramer et al. (20) in studies on endothelial ECM degradation by B16 melanoma cells. However, studies from other laboratories that demonstrated degradation of endothelial ECM by highly tumorigenic fibrosarcoma cells (18) and lung tissue matrix degradation by metastatic tumor cells (21) indicate that serum plasminogen is involved in transformed cell-mediated matrix degradation.

The present studies also demonstrate that a plasminogen-independent ECM degradation can occur. This degradation is not as rapid as plasmin-dependent degradation, but, it is progressive, and eventually all or most of the ECM present in a culture dish is solubilized (Fig. 4). Although such plasmin-
Malignant cells can bring about a rapid degradation of fibronectin and other matrix proteins through the generation of plasmin. In the absence of plasminogen, the transformed cells can use their locally secreted and surface enzymes, including possibly the direct catalytic action of PA to bring about a progressive degradation of matrix in the immediate vicinity of the cells even in the presence of natural protease inhibitors. Once the matrix under and around the cells has been solubilized, its adhesive and restrictive aspect is thus eliminated, releasing the malignant cells to continue along their migratory, degradative, and invasive pathway.

The authors gratefully acknowledge Dr. Peter B. Armstrong for his help with the immunofluorescence studies and Dr. Richard Feinman for providing purified α2M. We acknowledge also Ms. Kathy Revez and M. Angelo Albano for their highly skilled technical assistance and Ms. Roseann Lingeza and Ms. Rita Gould for their preparation of the manuscript.

This work was supported by grants from the National Institutes of Health (RO1 CA 16740 and RO1 AM 30537) and the American Cancer Society (BC 167).

Received for publication 13 February 1985, and in revised form 1 July 1985.

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FAIRBAIRN ET AL. Extracellular Matrix Degradation by Transformed Cells 1797
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