Presence of Proteolytically Processed Keratins in the Culture Medium of MCF-7 Cells

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ABSTRACT

MCF-7 human mammary epithelial cells are known to express three distinct cytoskeletal intermediate filament proteins designated numbers 8 (M, 52,000), 18 (M, 45,000), and 19 (M, 40,000) keratin. Using a panel of monoclonal antibodies and peptide mapping, we have determined that MCF-7 cells release proteolytic derivatives of keratins 18 and 19 into the culture medium. These extracellular keratins consist of multiple isoforms (M, 38,000–45,000, isoelectric point 5.0–5.2) on two-dimensional gels, many of which are smaller and more acidic than the predominant intracellular forms and appear to be associated together as a soluble, large molecular weight complex. These results may potentially be correlated with observations of keratin-like antigens in body fluids of cancer patients and the presence of antikeratin antibodies in individuals with certain autoimmune diseases and acute viral infections.

INTRODUCTION

MCF-7 cells represent the most extensively studied human breast cancer cell line. At the genetic level, this cell line has been utilized in studies of oncogene activity (1–3), hormone receptors (4), and hormone action (5). In tumor immunology, MCF-7 cells have found frequent use among investigators seeking to develop monoclonal antibodies to potential antigenic markers for breast cancer (6–13). In cell biology, MCF-7 has been widely studied for its proliferative responses to various exogenous hormones and growth factors (14–16).

Proteins released into the culture medium by MCF-7 cells have also been of major interest to investigators as potential products of hormonally regulated secretion (17–19) or as growth regulatory agents (20, 21). At present, however, most of these extracellular proteins are not very well defined in terms of their identity and their structural and functional relationships to other components in the cell.

We report here the identification and characterization of a major group of polypeptides released into the culture medium by MCF-7 cells. These proteins apparently are breakdown products of intracellular keratin proteins. Keratins represent a family of relatively water-insoluble proteins that form elaborate networks of cytoskeletal intermediate-sized filaments in epithelial cells (22). Nineteen human keratins have been cataloged based upon their distinct electrophoretic mobilities in two-dimensional gels (23). Within a given tissue or epithelial cell type, characteristic patterns of keratin expression are observed (23–25). MCF-7 cells express three keratin proteins, designated keratins 8 (M, 52,000), 18 (M, 45,000), and 19 (M, 40,000) (23).

The finding of extracellular keratin may have potential relevance to several clinical observations. Patients with various viral infections (26), alcoholic liver disease (27), and autoimmune syndromes (28) have been reported to have moderate titers of circulating antikeratin antibodies. Also, the tumor-associated marker designated tissue polypeptide antigen, which was detected in earlier studies of serum and urine of cancer patients (29), now actually appears to be a keratin or a keratin-related protein (30, 31).

MATERIALS AND METHODS

Monoclonal Antibodies. UCD/AB 6.01 and UCD/AB 6.11 were generated from BALB/c mice immunized with MCF-7 cells as described previously (10). AE1 (42) and 35/H11 (32) were gifts from T.-T. Sun (New York University) and A. M. Gown (University of Washington), respectively.

UCD/PR 10.11 was generated from mice immunized with UCD/AB 6.11 immunoaffinity-purified MCF-7 culture medium antigen. Briefly, splenic lymphocytes from BALB/c mice immunized with 10 μg antigen were fused with P3X63Ag8.653 myeloma cells using polyethylene glycol as described previously (10), and hybrid clones were selected for reactivity to the immunizing antigen by solid-phase radioimmunoassay. Cells from positive clones were injected back into mice for ascites production. UCD/PR 10.11 was isotyped as an IgG1, using specific antisera (Miles Laboratories, Inc., Elkhart, IN) in a double-immunodiffusion assay.

Cell Culture. MCF-7 cells (obtained from G. M. Cooper, Sidney Farber Cancer Institute, Boston, MA) and HeLa cells (obtained from D. Etchison, University of California, Davis, CA) were routinely grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 5% calf serum and gentamicin (25 μg/ml). For serum-free culture, 1:1 Dulbecco's modified Eagle's medium:Hams F-12 nutrient mixture (GIBCO) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (5 ng/ml) (Collaborative Research, Waltham, MA) was used (33). Cell toxicity experiments were performed by adding specified amounts of sodium azide to the culture medium.

Gel Electrophoresis and Immunoblotting. One-dimensional polyacrylamide gel electrophoresis was performed as described by Laemmli (34). Two-dimensional gels were run using a modified version (35) of the O'Farrell technique (36). Proteins were electroblotted to nitrocellulose (37), incubated with the various antibodies, and visualized by a 125I-labeled rabbit anti-mouse immunoglobulin secondary antibody and autoradiography.

Keratin Purification. Intracellular keratins 8, 18, and 19 were purified by electrodialysis (38) of specific bands in polyacrylamide gels of detergent-extracted (0.1% (v/v) Triton X-100, 5 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.1, 2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N'',N'''-tetraacetic acid, 2 mM MgCl2) pellets of MCF-7 cells. Keratin-enriched urea extracts were prepared as described by Steinert et al. (39).

Keratin from serum-free MCF-7 culture fluid was prepared from clarified (750 g × 10 min) media, free of cells and gross debris, which was dialyzed against distilled H2O and concentrated by lyophilization. For amino acid composition analysis, this material was run on polyacrylamide gels, and the entire M, 38,000–45,000 region was excised and electroeluted.

Keratin from serum-containing MCF-7 culture fluid was purified by affinity chromatography over a UCD/AB 6.11 column. Briefly, clarified culture medium was passed through a 2.5 × 10-cm column of UCD/AB 6.11 coupled to Affi-gel 10 (Bio-Rad, Richmond, CA) beads according to the manufacturer’s protocol. The column was then washed extensively with 1 M NaCl, 0.1 M acetic acid, followed by equilibration buffer (20 mM sodium phosphate, pH 7.6, 0.15 M NaCl, 0.05% Tween 20, 0.02% NaN3). Bound proteins were eluted with 2 M guanidine HCl, 0.1 M Tris, pH 9.0, 0.5% Tween 20. Immunoreactivity of the purified proteins was confirmed by immunoblotting of all preparations.
Immunofluorescence. Intracellular keratin filaments were visualized by indirect immunofluorescence using fluorescein-labeled goat anti-mouse IgG (Antibodies Inc., Davis, CA) on cells grown on glass coverslips, fixed, and permeabilized by 3.7% parafomaldehyde, 0.2% Nonidet P-40 in phosphate buffer (133 mM NaCl, 7 mM NaHPO₄, 7H₂O, 3 mM Na₂HPO₄·H₂O, pH 7.2). Coverslips were mounted for viewing under ultraviolet light in 70% glycerol, 2% n-propylgallate in phosphate buffer and photographed.

IRMA. Two IRMAs were developed for the routine assay of keratin levels in experimental samples. The first utilized UCD/AB 6.11 as the catch antibody bound to plastic microtiter plates, and rabbit polyclonal antikeratin followed by [¹²⁵I]Protein A as the detection system. The second, more sensitive and more recent assay, utilized UCD/PR 10.11 as the catch antibody and [¹²⁵I]UCD/AB 6.11 as the detection system. All IRMAs were performed at room temperature.

Microtiter wells were coated overnight with 0.5 μg of the first antibody in 50 μl, washed three times in 0.9% NaCl, and blocked with IRMA buffer (0.5% bovine serum albumin, 0.01% NaN₃, 0.15 M NaCl, 0.05 M sodium phosphate, pH 7.5). In the first assay, sample diluted in IRMA buffer was added, incubated for 2 h, and washed in 0.9% NaCl. Rabbit antikeratin antibody was then added, incubated for 1 h and washed, followed by [¹²⁵I]Protein A (50,000 cpm/well) for 1 h and a final wash. In the UCD/PR 10.11-UCD/AB 6.11 assay, the sample and the [¹²⁵I]UCD/AB 6.11 detection system (100,000 cpm/well) were coincubated for up to 18 h and then washed. Results were analyzed on a LKB 1260 gamma counter (LKB Instruments, Gaithersburg, MD), and standard curves were routinely generated with known aliquots of UCD/AB 6.11 affinity-purified MCF-7 culture fluid antigen.

The UCD/AB 6.11-rabbit antikeratin IRMA is sensitive to 500 ng/ml, whereas the UCD/PR 10.11-UCD/AB 6.11 IRMA is routinely sensitive to 5 ng/ml.

Gel Exclusion Chromatography and Sedimentation Studies. A 2.0 ml aliquot of concentrated MCF-7 culture fluid were applied to a 90-ml bed volume of Sephacryl S-300 (Pharmacia, Piscataway, NJ) equilibrated in 0.25 M NaCl, 0.1 M sodium phosphate, pH 7.5. Fractions (2.9 ml) were eluted from the column and assayed for keratin immunoreactivity by the UCD/AB 6.11-rabbit antikeratin IRMA. Blue dextran, bovine serum albumin, and cytochrome c were used as calibration standards.

The buoyant density of the culture medium antigens was determined by dissolving 0.8 g of CsCl in 3.2 ml of MCF-7 culture medium to yield a sample containing 20% (w/w) CsCl. The sample was layered on top of a 40% CsCl cushion, overlayed with 5% CsCl in buffer (10 mm Tris, pH 7.5, 10 mm NaCl, 10 mm EDTA) and centrifuged for 21 h at 120,000 x g at 20°C. Fractions were collected by bottom puncture, weighed, and assayed by the UCD/AB 6.11-rabbit antikeratin IRMA. Blue dextran, bovine serum albumin, and cytochrome c were used as calibration standards.

The sedimentation coefficient was determined by layering a 0.7-ml aliquot of MCF-7 culture medium over a 10–40% linear sucrose gradient in 10 mm Tris, pH 7.5, 10 mm NaCl, and 10 mm EDTA. Samples were centrifuged, collected, and assayed as above.

Amino Acid Composition Analysis. Purified keratins were lyophilized and submitted to the Protein Structure Laboratory, University of California, Davis. The proteins were acid hydrolyzed and analyzed on a Durrum D-500 (Dionex, Sunnyvale, CA) or Beckman 6300 amino acid analyzer (Beckman Instruments, Palo Alto, CA) as described by Moore and Stain (40).

Peptide Mapping. Staphylococcal V8 peptides were generated from aliquots (10³ cpm) of proteins purified by electrodialution, coupled to a N-[¹⁵S]hydroxysuccinimide labeling reagent (Amersham, Arlington Heights, IL), and incubated with 1 μg of enzyme for 30 min at 37°C. Peptide fragments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% acrylamide gels and visualized by fluorography.

Cyanogen bromide peptides were generated by first reducing the 50–100 μg of protein in 20 μl of 0.2 M dithiothreitol, 50 mM Tris, pH 8.2, for 30 min at 35°C, and alkylating in 20 μl of 0.5 M iodoacetic acid in 50 mM Tris, pH 8.2, for 20 min at 22°C. The sample was then dialyzed in 1 mM dithiothreitol, 1% formic acid, adjusted to 70% formic acid, and cleaved by adding a 100-fold molar excess of CNBr:protein

The abbreviation used is: IRMA, immunoradiometric assay.

RESULTS

Characterization of Monoclonal Antikeratin Antibodies. Three monoclonal antibodies generated in our laboratory, UCD/AB 6.01, UCD/AB 6.11, and UCD/PR 10.11, and two monoclonal antibodies obtained from other laboratories, AEI (42), and 35/HH1 (32) were screened for their immunoreactivity to keratin-enriched detergent-urea extracts of MCF-7 cells. The results (Figs. 1A and 2A) indicate that the various antibodies show different degrees of selectivity for the three keratins found in MCF-7 cells: keratins 8, 18, and 19. UCD/AB 6.01 binds to keratin 8, UCD/AB 6.11 recognizes primarily keratin 18, UCD/PR 10.11 recognizes both keratins 8 and 18, 35/HH1 recognizes all three keratins, and AEI mainly identifies keratin 19. Multiple isoforms of each keratin type can be resolved by two-dimensional gel electrophoresis (Fig. 2A). These patterns were 30 times by lyophilization.

A. 1 2 3 4 5
66- 43- 31-

B. 1 2 3 4 5
66- 43- 31-

Fig. 1. Reactivity of various monoclonal antikeratin antibodies to intracellular and extracellular MCF-7 keratin antigens. A. Immunoblots of keratin-enriched urea extracts of MCF-7 cells (3 μg total protein per lane) using UCD/AB 6.01, UCD/AB 6.11, UCD/PR 10.11, 35/HH1, and AEI (lanes 1–5, respectively). The positions of keratins 8, 18, and 19 are indicated (arrows). B. Immunoblot of concentrated serum-free MCF-7 culture supernatant using the same antibodies as above. Medium was from a 48-h collection, clarified, dialyzed, and concentrated 20 times by lyophilization.
MCF-7 CULTURE MEDIUM KERATINS

Fig. 3. Visualization of the MCF-7 keratin cytoskeleton by UCD/PR 10.11 indirect-immunofluorescence. MCF-7 cells grown on coverslips were fixed, extracted with 0.2% Nonidet P-40, and incubated with UCD/PR 10.11 and fluorescein-labeled goat anti-mouse antibody. A characteristic, branched, wavy filament network typical of keratin is demonstrated.

Fig. 2. Two-dimensional immunoblots of intracellular and extracellular MCF-7 keratin antigens using 35βH11. In A, 100 µg of total MCF-7 cell proteins were separated by isoelectric focusing (horizontal dimension) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of the keratin 8 (M, 52,000, isoelectric point 6.1–6.0), keratin 18 (M, 45,000, isoelectric point 5.8–5.3), and keratin 19 (M, 40,000, isoelectric point 5.2–5.0) isoforms are indicated. B, 16 µg of total protein from concentrated 48-h MCF-7 serum-free culture medium. C, mixture of 50 µg (A) and 16 µg (B) showing the overlap of the extracellular antigens with the number 18 and 19 keratin region.

of immunoreactivity are consistent with the concept that the different keratin types are unique proteins but share major regions of homology (22).

Indirect immunofluorescence of detergent-extracted MCF-7 cells confirms that these monoclonal antibodies identify keratin proteins. The antibodies bind to a rich cytoskeletal network with the characteristic appearance of cytokeratins as illustrated in Fig. 3 for UCD/PR 10.11.

Monoclonal Antikeratin Antibodies Identify Antigens in MCF-7 Tissue Culture Medium. A previous report demonstrated that UCD/AB 6.11 identifies an antigen in cell-free culture supernatants of MCF-7 cells (10). We now report that this antigen is among a group of polypeptides spread over a molecular weight range of 38,000–45,000. Antigens in this molecular weight range are identified by UCD/AB 6.11, UCD/PR 10.11, 35βH11, and AE1, whereas UCD/AB 6.01 shows no reactivity (Fig. 1B). Based on the immunoreactivity patterns of these antibodies on the three intracellular keratins in Fig. 1A, the predominant antigenicity identified in the culture medium resembles keratins 18 and 19.

The culture medium antigens can be selectively purified using a UCD/AB 6.11 affinity column. This affinity-purified material contains keratin 19 epitopes detectable by AE1 (data not shown), even though UCD/AB 6.11 itself does not recognize this keratin (Fig. 1A). On two-dimensional gels, the extracellular antigens have an apparent isoelectric point of 5.0–5.2 (Fig. 2B), which overlaps with the keratin 19 region, making them slightly more acidic than most of the intracellular keratin 18 isoforms (Fig. 2C).

The affinity-purified antigens were used as immunogens to develop monoclonal antibodies against the extracellular antigens. The resulting antibodies reacted against intracellular keratins. Most of those antibodies that were characterized identified keratins 8 and/or 18. UCD/PR 10.11, used in this paper, is one such antibody (Fig. 1).

Biophysical Properties of MCF-7 Culture Medium Antigens. When concentrated MCF-7 culture medium is fractionated by Sephacryl S-300 gel filtration and assayed by IRMA, the antigenic peak is observed very near the void volume (Fig. 4), suggesting a molecular weight well above 100,000. Sedimentation analysis of the culture fluid antigen yields an S20,w value of approximately 3.8S which is more consistent with a smaller molecular weight. This discrepancy could be due to the disaggregation of a multimeric complex under certain conditions, or indicate that the antigens behave as nonglobular proteins. On CsCl density gradients, the culture fluid antigen displays a buoyant density of 1.25 g/ml. Treatment with nonionic detergents does not alter these characteristics, suggesting that the antigens are not lipid associated.

Comparisons of the MCF-7 Culture Fluid Antigens with Intracellular Keratins. The amino acid composition of the extracellular antigens was compared with intracellular keratins 8, 18, and 19 purified individually from MCF-7 cells by electroelution (Table 1). Considering an average standard deviation of 0.5% for each residue, the amino acid compositions are remarkably similar. The estimates for methionine are probably low due to oxidative damage from preparative gel purification (38).

The extracellular antigens also share peptide fragments with...
Fig. 4. Gel exclusion chromatography profile of extracellular antigens. Serum-free culture medium (7.5 ml) from a 72-h incubation with MCF-7 cells was concentrated 4-fold by placing the sample in a dialysis bag and dehydrating in dry Ficoll prior to loading on a 80 x 2-cm column of Sephacryl S-300. Fractions were eluted and assayed as described in "Materials and Methods." The relative elution positions of the void volume ($V_0$, indicated by blue dextran, $M_r$ 2,000 x $10^6$), bovine serum albumin (BSA) ($M_r$ 66,000), and cytochrome c (Cyto. C) ($M_r$ 12,500) are indicated.

Table 1 Comparisons of amino acid compositions of purified keratins 8, 18, and 19 and extracellular MCF-7 antigens

<table>
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<tr>
<th>Amino acid</th>
<th>Keratins No. 8</th>
<th>Keratins No. 18</th>
<th>Keratins No. 19</th>
<th>Extracellular antigens</th>
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<tr>
<td>Asp + Asn</td>
<td>9.8 mol%</td>
<td>10.3</td>
<td>9.7</td>
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<tr>
<td>Thr</td>
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<tr>
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<td>6.6</td>
<td>6.9</td>
<td>7.5</td>
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<tr>
<td>Glu + Gin</td>
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<td>15.2</td>
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<td>14.9</td>
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<tr>
<td>Pro</td>
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<td>4.1</td>
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<tr>
<td>Val</td>
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<td>6.0</td>
<td>5.9</td>
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<tr>
<td>Met</td>
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<td>Trp</td>
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* Values are reported in mol/100 mol free amino acid in sample.
* ND, not determined.

the intracellular keratins by one-dimensional electrophoretic gel analysis (Fig. 5). For example, three peptide fragments are shared in staphylococcal V8 digests of purified keratin 19 and the extracellular antigens (Fig. 5B, lanes 1 and 2). In a like manner, cyanogen bromide digests of purified keratins 8 and 19 and the extracellular antigens also contain keratin 8 peptides (Fig. 5B, lanes 4 and 5). The complexity of the peptide patterns, however, makes it difficult to exclude the possible presence of keratin 8-related proteins in the culture medium of MCF-7 cells.

Processing and Kinetics of Extracellular Antigen Release. The soluble extracellular antigens could, in theory, be products of genes distinct from those encoding the larger intracellular keratins, or be the result of alternative mRNA splicing. We consider this to be unlikely because of evidence that only full-sized keratin proteins appear to be made by in vitro translations of MCF-7 mRNA (43). Instead, it appears that rather specific and controlled proteolytic cleavages are involved. When MCF-7 cultures are poisoned with sodium azide, large amounts of soluble keratin immunoreactivity are detected. Interestingly, when such poisoned cultures are fractionated by a simple low-speed centrifugation, the pellet contains mostly the larger molecular weight keratins characteristic of the full-sized intracellular keratins of nonpoisoned cells (Fig. 6, lane 1). In contrast, the supernatant contains mostly the smaller molecular weight forms identical in size to those found in the culture fluid of nonpoisoned cells (Fig. 6, lane 2). The addition of EDTA to extracts of MCF-7 cells appears to inhibit this conversion (Fig. 6, lanes 4 and 5).

In order to understand the phenomenon of antigen release, we examined the kinetics of keratin release in MCF-7 and...
another keratin-containing cell line, HeLa. The results, Fig. 7, shows that the most rapid rate of keratin release in both cell lines occurs during the first few hours following a culture medium change, either in the presence or absence of sodium azide. In terms of overall antigen release, the azide-treated MCF-7 cells in Fig. 7 released 25 μg/10^6 cells compared with 2 μg/10^6 cells in the nonpoisoned culture over 24 h. HeLa cells behaved similarly, except that they released only one-fourth to one-eighth as much antigen as MCF-7 cells.

As shown by the HeLa cells in Fig. 7, the release of keratin antigens into the culture medium is not limited to MCF-7 cells. We have also detected keratins in culture supernatants from other human mammary cell lines BT-20, T47-D, and SK-BR3, as well as in primary cultures of normal and malignant human mammary cells.

**DISCUSSION**

These experiments describe the accumulation of extracellular soluble protein antigens in the media of MCF-7 breast carcinoma cells and several other epithelial cell lines. By immunological, biochemical, and peptide mapping criteria, these antigens appear to be the degradation products of intracellular cytokeratins. MCF-7 cells express three distinct keratin polypeptides: keratins 8, 18, and 19. The evidence presented here clearly indicates that keratin 18 and 19-derived polypeptides are released into the culture fluid of MCF-7 cells. Since we are unable to detect keratin 8-derived polypeptides in the culture medium using UCD/AB 6.01, the epitope recognized by this antibody could potentially be degraded during release. Therefore, the presence of proteolytically processed forms of keratin 8 in the extracellular medium of MCF-7 cells cannot be excluded.

The release of these antigens apparently involves a conversion of the polypeptides into smaller, more acidic forms. Our data suggest that the conversion is accomplished by proteolytic cleavage. An endogenous protease capable of generating slightly smaller, more acidic variants of bovine and rodent keratin A by removal of positively charged terminal sequences has been described (44). Proteolytic modifications of keratins during terminal differentiation of the epidermis has also been noted (45). Such enzymes could be responsible for the types of molecular alterations documented here in MCF-7 cells. A calcium-dependent intermediate filament protease has also been reported (46). The inhibition of MCF-7 keratin degradation by the addition of EDTA to extraction mixes supports the notion that proteolytic cleavage may be involved in producing the extracellular isoforms we observe. Whatever the mechanism, the degradation results in a very stable, relatively soluble keratin complex. It can be stored in spent culture media for weeks without evidence of further breakdown.

While we cannot demonstrate a specific mechanism of release, extracellular keratin-derived proteins can clearly be released by damaged and dying cells, as illustrated in the azide-poisoning experiments. The basis of their release from cultures of apparently viable cells is less clear. Data have been presented that suggest that keratin-like antigens are released metabolically (10) or during cell division (29). Ultrastructural studies indicate an association of keratin proteins with secretory granules (47) and mucin droplets (48). Our measurements of keratin release from cultures of MCF-7 and HeLa cells demonstrate a dimin-
ishing rate of release. This pattern does not correspond to a constant steady-state release or release during cell division. Rather, our data suggested a release of keratin-derived proteins due to cell damage inherent in the changing of tissue culture media or an inducible release due to the introduction of nutrients or factors in fresh culture medium. On a quantitative basis, we observe more keratin in the medium of viable cultures than we can account for by the complete lysis of the few dead cells we detect by trypan blue staining.

The keratin 18- and 19-related polypeptides in the culture media appear to be associated in a multimeric complex. This is supported by the apparent large molecular weight of the antigens in gel exclusion chromatography, and by the observation that the extracellular antigens eluted from an immunoaffinity column made with the keratin 18-specific UCD/AB 6.11 also contain proteins that react with the antibody AE1, which is keratin 19-specific in the MCF-7 system. Proteolytic processing and the formation of oligomeric complexes may allow these normally water-insoluble proteins to exist in an apparently soluble form. Quinlan et al. (49) have demonstrated that relatively soluble tetrameric complexes of keratins A and D (comparable to keratins 8 and 18 in the human catalog) can be isolated from rat hepatoma and other cell types. Not only is the formation of spontaneous aggregates characteristic of keratins, but such aggregates can be formed using heterologous keratins (50, 51).

The possibility that keratins are also released by cells in vivo has important clinical implications. For example, extracellular “amyloid” deposits found in certain human skin diseases are composed of epidermal keratins (52). Autoantibodies against keratins are found in patients with a variety of diseases (26–28), and low levels of antikeratin antibodies may be present in all individuals (53). These observations suggest that extracellular keratin does occur in vivo and may be increased under certain circumstances, possibly in conjunction with epithelial tissue damage.

Several lines of evidence indicate that the in vivo release of keratin may be associated with some types of malignancy. First, keratins have been detected by immunooassays of sera and bronchial lavages of patients with bronchogenic carcinoma (54, 55). Second, recent reports suggest that the tumor-associated marker tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). Third, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). Fourth, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). Fifth, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). Sixth, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). Sehond, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31).Thond, rats immunized with serum related to tissue polypeptide antigen is a keratin or a keratin-related peptide (30, 31). These observations suggest that extracellular keratin does occur in vivo and may be increased under certain circumstances, possibly in conjunction with epithelial tissue damage.

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