The Mesothelial Keratins: A New Family of Cytoskeletal Proteins Identified in Cultured Mesothelial Cells and Nonkeratinizing Epithelia

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Summary

The cytoskeletal proteins of cultured normal human mesothelial cells were found to consist of six major components, including actin, vimentin, the 40 kd keratin and the 44, 52 and 55 kd proteins, plus a minor 46 kd protein. Two-dimensional gel electrophoresis, peptide mapping and immunoprecipitation tests showed that the 40–55 kd mesothelial proteins are a family of keratins distinct in size, charge or peptide map from the “epidermal keratins” synthesized by cultured keratinocytes. Unique combinations of keratins from the epidermal and mesothelial keratin families were found to be synthesized by cultured bladder, esophageal, conjunctival, mammary, exocervical and ovarian surface epithelial cells. Mesothelial cells were the only epithelial cell type that synthesized vimentin at more than trace levels. We have also found that many carcinoma cell lines express keratins different from those of their cell type of origin.

Introduction

The concept of a “cytoskeleton” as a basic cellular structure developed from the discovery of detergent-resistant cytoplasmic filament networks (for example, see Brown et al., 1976; Cooke, 1976; Lenk et al., 1977; Osborn and Weber, 1977; Small and Sobieszek, 1977) and from the detection of a three-dimensional filamentous matrix in cultured cells by stereoscopic electron microscopy (Wolosewicz and Porter, 1976; Heuser and Kirschnern, 1980). The cytoskeleton is defined biochemically as those structures that remain insoluble after extraction with a neutral detergent, such as Triton X-100 or Nonidet-P-40, in a neutral buffer. The protein composition of the cytoskeleton varies with the salt concentration, temperature and presence of divalent cations in the extraction buffer (Small and Sobieszek, 1977; Lenk et al., 1977; Isenberg and Small, 1978; Steinert et al., 1982). The most insoluble components are the 100 Å diameter intermediate filaments (IFs). IF proteins of some type are present in all differentiated somatic cells examined (reviewed by Lazarides, 1980) but have not been detected in totipotent, embryonic inner cell mass cells (Paulin et al., 1980; Jackson et al., 1980, 1981). IFs are therefore probably not essential for cell division, but are likely to be necessary for differentiated function.

The abundant, insoluble proteins of the epidermis, known classically as keratin, were found to be the subunits of one class of IFs (Steinert et al., 1976; Sun and Green, 1978a; Franke et al., 1978a). Keratins are present in virtually all epithelia, identifiable by immunofluorescence with antisera raised against epidermal keratins (Sun et al., 1979; Franke et al., 1979a; Schmid et al., 1979). The human epidermal keratins are proteins of 46, 50, 52, 56, 58, 63, 65.5 and 67 kd, most of which have been shown to be translated from different mRNAs (Sun and Green, 1978b; Fuchs and Green, 1978, 1979, 1980). A 40 kd keratin has been found in some epidermal and oral squamous carcinoma cells and in normal conjunctival and corneal epithelial cells (Wu and Rheinwald, 1981; Fuchs and Green, 1981; see also Sun and Green, 1977). Several nonepidermal keratins have been identified in the liver, intestine and early embryo (Jackson et al., 1980; Bruet al., 1980; Franke et al., 1981a, 1981b, 1981c; Oshima, 1982). Characterization of the IF proteins expressed by different epithelia is a prerequisite to understanding how particular filament proteins affect differentiated function, and will serve as a basis for the study of mechanisms by which gene expression is controlled by the state of cell differentiation. We began our attack on these problems by studying an epithelial cell type of very simple histology—the mesothelial cell.

The mesothelium is a single layer of extremely flat cells lining the pleural, pericardial and peritoneal cavities and covering the outer surfaces of the lungs, heart and viscera (reviewed by Cunningham, 1926). It is thought to function as a slippery, nonadhesive surface for the internal organs to slide upon. No specific differentiated product or structure has been identified as a marker distinguishing this cell type, although it has been noted to possess abundant, and sometimes very long, microvilli (Cunningham, 1926; Baradi and Hope, 1964; Andrews and Porter, 1973). It has long been known that mesothelial cells slough off into the peritoneal cavity and are therefore present in ascites fluid (for example, Cunningham, 1922; Domagala and Koss, 1979). Limited division in primary culture has been reported for human mesothelial cells obtained from ascites fluid, and their morphology has been described in detail (Castor and Naylor, 1969; Monif and Daly, 1975; Gotz, 1979; Singh et al., 1978). While attempting to culture ascitic human ovarian carcinomas, we found that our culture conditions

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selectively promoted the growth of normal mesothelial cells from most ascites fluid samples. Aware of the reports by Schmid et al. (1979) and Schlegel et al. (1980) that histological sections of mesothelium and mesothelioma react with antisera raised against epidermal keratins, we examined the cytoskeletal proteins of our cultured mesothelial cells. We report that mesothelial cells synthesize a family of keratin proteins different from, but related to, those expressed by epidermal keratinocytes. These mesothelial-type keratins are expressed together with certain epidermal keratins by many nonkeratinizing epithelia.

Results

Morphology and Growth of Mesothelial Cells in Culture

Cultures were initiated with cells concentrated from ascites fluid of patients with ovarian cancer. Only mesothelial cells grew from most fluid samples, and yielded a confluent culture in 1–2 weeks. Most samples contained few proliferative ovarian carcinoma cells, which usually degenerated in the primary culture. The mesothelial cells grew with a distinctive morphology (Figure 1a), generally appearing circular or oval when viewed from above. Most cells were flat and covered a large area of substratum, with the periphery of each cell virtually devoid of phase-dense or stainable cytoplasm. These characteristics have been described by others for mesothelial cells in short-term culture (Castor and Naylor, 1969; Gotzos, 1977; Singh et al., 1978). Neighboring cells never overlapped, even in regions of high cell density and in cultures refed for several weeks after reaching confluence.

The requirement of mesothelial cells for fibroblast support was not stringent, as it is for normal keratinocytes (Rheinwald and Green, 1975), and was helpful only for enhancing mesothelial cell growth from very low plating densities in DMEM + FCS medium. Population doubling time was 2–3 days in this medium. The cells could be serially subcultured for at least six passages and 15 population doublings, and they remained diploid until they senesced. They did

Figure 1. Morphology and Antikeratin Staining of Cultured Human Mesothelial Cells

(a) Phase contrast view of a confluent, third-passage culture of strain LP-3; (b) the same field as in (a), stained with anti-s. corneum keratins; (c) rare formation of delicate, desmosome-like intercellular contacts revealed by anti-s. corneum keratins staining; (d) mesothelial cell filaments stained with anti-40 kd keratin.
not form tumors in nude mice. Cells with the same growth properties and cytoskeletal composition as described below could also be cultured from peritoneal saline lavages of surgical patients without cancer, but far fewer proliferative mesothelial cells were present in these washes. As described elsewhere, improvement of the culture medium substantially enhances mesothelial cell growth rate and replicative potential (N. D. Connell and J. G. Rheinwald, submitted manuscript).

Detection of Keratin Filaments and the 40 kd Keratin in Mesothelial Cells
Confluent cultures were probed with keratin antibodies via indirect immunofluorescence. An antisera raised against total keratins of human epidermal stratum corneum stained a dense filamentous network in the cells (Figure 1b). The keratin filaments were concentrated in the perinuclear region of cells that were not greatly flattened but were more easily resolved in very flat cells. Filament bundles often formed concentric rings around the nucleus. Fine desmosome-like contacts between a minority of the cells were detected by the staining of associated keratin filaments (Figure 1c). An antisera which was raised against the 40 kd keratin of SSC-15, and which does not cross-react with any of the epidermal keratins (Wu and Rheinwald, 1981), stained in mesothelial cells a filamentous network indistinguishable from that stained by anti-s corneum keratins (Figure 1d). Anti-40 dk keratin also stained the desmosome-like junctions (not shown). Mesothelium in vivo also contained high levels of 40 kd keratin, disclosed by immunoperoxidase staining of paraffin sections of lung and peritoneum (G. Pinkus, J. Corson, O. Alberti and J. Rheinwald, unpublished observations).

Electrophoretic Characterization of the Mesothelial Cytoskeleton
Cultures of mesothelial cells, dermal fibroblasts, umbilical vein endothelial cells, epidermal keratinocytes, and SCC-15 were labeled with 3H-methionine, and their Triton-insoluble proteins were separated electrophoretically on SDS-polyacrylamide gels (Figure 2). Mesothelial cells (lane d) synthesized a 40 kd protein; actin; a 44 kd protein incompletely resolved from actin; variable amounts of material migrating diffusely around 46 kd; and three proteins of molecular weights 56, 55 and 56 kd. In contrast, as reported by Brown et al. (1976) and Franke et al. (1979a), the Triton-insoluble fraction of fibroblasts (lane c) and endothelial cells (lane b) contained only vimentin (56 kd) and actin (43 kd). As described previously (Wu and Rheinwald, 1981), SCC-15 (lane a) synthesized the 46, 50, 52, 56 and 58 kd "epidermal" keratins (which are also made by normal epidermal keratinocytes in culture; Sun and Green, 1977, 1978b; Fuchs and Green, 1978, 1981) plus the 40 kd keratin.

Two-dimensional gel electrophoresis revealed more clearly the differences in cytoskeletal composition of mesothelial cells, fibroblasts and keratinocytes (Figures 3 and 4). Mesothelial cells possessed none of the major epidermal keratins of molecular size 46, 50 and 52 kd (which are isoelectric within the pH 4.8 to 7.4 range of the gel; Figure 3) or 56 and 58 kd (which are more basic and can be retained quantitatively within nonequilibrium pH gradient gels; Figure 4). The 40 kd keratin was a major mesothelial protein. The 44 kd mesothelial protein was nearly isoelectric with actin. The 44 kd protein was also present at a moderate level in SCC-15 (Figure 3e), although we had not noticed it in the less radioactive cell extracts of our earlier study (Wu and Rheinwald, 1981). Neither the 40 kd keratin nor the 44 kd protein was seen in comparable gels of normal epidermal keratinocyte extracts (Figure 3c). Both, however, were present at the very limit of detection in overloaded and overexposed gels (Figure 3d). In other experiments we have also detected very faint spots probably representing the 52 kd and 55 kd mesothelial proteins in overloaded epidermal keratinocyte gels.

Vimentin was a major cytoskeletal protein of cultured mesothelial cells. Vimentin was not detectable in normally loaded gels of either SCC-15 or normal epidermal keratinocyte cytoskeletal proteins, but could be seen in overloaded and overexposed gels (Figure 3d). However, since indirect immunofluorescence detected only trace amounts of vimentin in cultured keratinocytes (N. D. Connell and J. G. Rheinwald, submitted manuscript), much of the vimentin seen in Figure 3d is likely to be from the small number of 3T3 fibroblast feeder cells remaining in the epithe-
vimentin was solubilized. The insolubility of the 40–55 kd mesothelial proteins in low salt is inconsistent with belonging to the vimentin or desmin class of IF proteins (Steinert et al., 1982).

**Immunoalogical Relatedness of Mesothelial and Epidermal Keratins**

Anti-40 kd keratin precipitated only the 40 kd keratin from mesothelial extracts (Figure 2, lane 3). Both the 40 kd keratin and the 44 kd protein were precipitated very efficiently by anti-46 kd epidermal keratin (lane f) and by anti-total s. corneum keratins (lanes g and h). Anti-total s. corneum keratins cross-reacted somewhat less strongly with the 55 kd protein and weakly, yet specifically, with the 52 kd protein. The 46 kd mesothelial protein, when present in sufficient amounts, was also detectably precipitated. Anti-vimentin specifically precipitated vimentin (lane j), it did not cross-react detectably with any other mesothelial cytoskeletal protein. The 44, 46, 52 and 55 kd me-
soothelial proteins should therefore be considered keratins. (For convenience, we will henceforth refer to these proteins as the ‘mesothelial keratins,’ abbreviated as 40Kυ, 44Kυ, 46Kυ, 52Kυ and 55Kυ, to distinguish them from the ‘epidermal keratins’ synthesized by cultured epidermal keratinocytes, which will be abbreviated as 46Kε, 50Kε, 52Kε, 56Kε and 58Kε.)

Peptide Mapping of Mesothelial Keratins
The 46Kυ, 52Kυ and 55Kυ of mesothelial strain LP-9; the 46Kε, 52Kε and 56Kε of SCC-66; and vimentin from fibroblast strain IMR-90 were compared with respect to the peptides produced by the Cleveland proteolysis method (see Experimental Procedures). 44Kυ was not analyzed because it was too difficult to excise cleanly from actin in the preparative gels. Each band cut from the one-dimensional preparative gels consisted of a single protein except for the 52 kd band of SCC-66. This was used as the best available source of sufficient amounts of 52Kε, even though about one quarter of the protein was 52Kυ (see Figure 6h). As shown below, this contamination did not confound interpretation of the result.

The map of 40Kυ was identical with that of the 40 kd keratin isolated from SCC cells (data not shown). The map of 46Kυ was very similar to that of 46Kε (Figure 5). It differed only in the absence of the largest digestion intermediate of 46Kε. Peptide maps of 52Kυ and the 52 kd protein of SCC-66 (mostly 52Kε) also differed only by a single peptide, which was much more prominent (more resistant to the protease) in 52Kε. Thus 46Kυ and 52Kυ each differed from its epidermal counterpart, at this level of analysis, by only a single V8 protease cleavage site. The map of 55Kυ was significantly different from that of 56Kε and was very different from that of vimentin. The pattern of 55Kυ was similar to the patterns of all the the epidermal keratins, having two prominent clusters of lower and higher molecular weight protease-resistant peptides (Fuchs and Green, 1978).

Synthesis of Mesothelial Keratins by Simple, Glandular and Stratified Nonkeratinizing Epithelia
To determine whether histologically more complex, nonkeratinizing epithelia might also express keratins of the mesothelial family, we cultured the epithelial cells from various normal human tissues, labeled them with 35S-methionine and separated the Triton-insoluble proteins on two-dimensional gels.

The ovarian surface epithelium is derived from embryonic mesothelium (Pinkerton et al., 1961) and is therefore the closest relative of the mesothelial cell. We were unable to culture surface epithelial cells from several surgical specimens of normal adult ovary, so we instead characterized the keratins of cells cultured from two clinically low-grade, histologically well differentiated epithelial tumors of the ovary. These tumors are neoplastic variants of ovarian surface epithelium (Woodruff and Julian, 1970). It was necessary to purify selectively clumps of ovarian cells from the single mesothelial cells on a Nitex filter before plating so as to avoid mesothelial cell overgrowth. The ovarian cells were not stimulated by epidermal growth factor, hydrocortisone or the 3T3 feeder layer, and were inhibited by cholera toxin; they were therefore cultured without them. As shown in Figure 6a and Table 1, the pattern of keratins expressed by ovarian epithelial cells was very similar to that of normal mesothelial cells. They expressed 40Kυ, 44Kυ, 52Kυ and 55Kυ at high levels. However, vimentin was absent, and a small amount of 46Kε was present.

Figure 5. Proteolytic Digestion Maps of the Mesothelial and Epidermal Keratins and of Vimentin
35S-methionine-labeled Triton-insoluble proteins of cultured mesothelial strains LP-2 and LP-9, lung fibroblast strain IMR-90 and SCC-66 were purified by preparative SDS-polyacrylamide slab gel electrophoresis. SCC-66 was used as a source of 48 kd, 52 kd and 56 kd epidermal keratins, and IMR-90 as a source of vimentin. In separate experiments (B, C and D), pairs of mesothelial and epidermal keratins of the same or similar molecular size were incubated with S. aureus V8 protease, and the digestion products were separated side-by-side on an SDS-polyacrylamide slab gel. These experiments plus digestion maps of 40 kd keratin (A) and vimentin (E) are displayed together for comparison. In experiments (A), (C) and (D), 0, 50 and 100 ng V8 protease were added. In experiments (B) and (E), 0, 5 and 10 ng V8 protease were added. Note the presence of only single peptide differences between the 46Kυ and 46Kε patterns and between the 52Kυ and 52Kε patterns (arrows). The peptide maps of neither pair could be made identical by adjustment of enzyme concentration.
As reported previously by others, conjunctival (Sun and Green, 1977), esophageal (Doran et al., 1980) and exocervical (Stanley and Parkinson, 1979) epithelial cells grew well in the 3T3 feeder layer system and formed stratified colonies visually indistinguishable from those of epidermal keratinocytes. We found that urinary bladder epithelial cells also formed colonies from low-density platings and could be serially cultured for at least five passages and 30 cell generations in this system with our improved medium (T. O’Connell and J. Rheinwald, unpublished observations). The bladder epithelial cells formed unstratified colonies of closely packed, slightly elongated cells.

Normal bladder and mammary epithelial cells synthesized unique combinations of epidermal and mesothelial keratins (Figures 6b and 6g and Table 1). Bladder cells contained moderate to high levels of all the epidermal and mesothelial keratins except for 50K\text{E}, which was completely absent. Mammary cells also contained an acidic 50 kd protein which was indistinguishable by Cleveland digest analysis from 50K\text{E} (data not shown). Conjunctival, esophageal and exocervical epithelial cells expressed very similar keratin patterns, differing from epidermal keratinocytes by increased synthesis of 52K\text{E} and the mesothelial keratins. They were barely distinguishable from one another by small differences in the relative amounts of 55K\text{E} and 50 kd acidic protein they synthesized. Esophageal cells also synthesized a 47 kd protein of the same pl as 46K\text{E}. Careful examination of Figures
Table 1. Pattern of Keratin and Vimentin Expression by Normal Human Epithelial Cell Types during Growth in Culture

<table>
<thead>
<tr>
<th>Normal Epithelial Cell Type</th>
<th>'Mesothelial' Keratins</th>
<th>'Epidermal' Keratins</th>
<th>Other</th>
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<tr>
<td></td>
<td>40 kd</td>
<td>44 kd</td>
<td>46 kd</td>
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<tr>
<td>Mesothelial</td>
<td>++</td>
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<tr>
<td>Epidermal</td>
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<tr>
<td>Mammary</td>
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<tr>
<td>Conjunctival</td>
<td>+</td>
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<td>Esophageal</td>
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<tr>
<td>Exocervical</td>
<td>±</td>
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<tr>
<td>Bladder</td>
<td>+±</td>
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<tr>
<td>Ovarian (low-grade tumor)</td>
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Data summarized from Figures 3 and 6, and from other one-dimensional, two-dimensional and nonequilibrium pH gradient gels. Results are from three normal mesothelial, two epidermal keratinocyte, two mammary, one conjunctival, two esophageal, one exocervical and three urinary bladder epithelial strains (all normal diploid) and from two clinically low-grade ovarian epithelial tumor cell strains. 50 kd* is the acidic 50 kd protein. (++) high level; (+) moderate level, ½ as much as (++); (±) low level, ¼ as much as (+); (±) trace, ⅛ as much as (+); (blank) undetectable in normally loaded gels. Levels were reproducible plus or minus one graded level (as defined above) between experiments with the same cell strain and among different strains of the same cell type.

3 (c, e) and 6 (c, f, g) reveals that the 46 kd epidermal keratin of most cell types actually consists of a major 46 kd component and a minor, incompletely resolved, 47 kd component. Every cell type studied except for mesothelial and ovarian cells also synthesized high levels of 56K* and 58K* detected on one-dimensional and nonequilibrium pH gradient gels (not shown).

Abnormal Levels and Types of Keratins Synthesized by Some Malignant Epithelial Cell Lines

Our earlier study of Triton-insoluble proteins synthesized in culture by SCCs of the epidermis and oral epithelium revealed that several lines abnormally synthesized 40K* (Wu and Rheinwald, 1981). Closer scrutiny of SCC-15 Triton-insoluble proteins separated on two-dimensional gels revealed moderate amounts of 44K* and 52K* as well (Figure 3c). We therefore analyzed the keratins of eight other oral and epidermal SCC lines. All expressed low to moderate levels of one or more mesothelial keratins, but most continued to express the normal keratinocytic pattern of high levels of 46K*, 50K*, 56K* and 58K* (data not shown). Vimentin was expressed by the SCC lines at very low levels, no higher than the level of normal keratinocytic types. Two of the nine SCC lines synthesized greatly reduced levels of an epidermal keratin. SCC-4 had greatly reduced levels of 46K*, SCC-66 (Figure 6h) lacked 50K* and synthesized 52K*, 44K* and 52K* at much higher levels than normal epidermal cells, which is the normal bladder epithelial pattern except for the absence of 55K*. One line, SCC-40, synthesized the acidic 50 kd protein at a level similar to that expressed by mammary epithelial cells (data not shown).

We also examined the Triton-insoluble proteins of three established bladder carcinoma cell lines: EJ, J82 and RT112 (see Marshall et al., 1977, and Hastings and Franks, 1981, for properties of these lines and their derivations). RT112 synthesized the normal bladder pattern of keratins and very little vimentin (Figure 6d). EJ and J82, in contrast, synthesized high levels of vimentin, and very low amounts of 44K* as the only recognizable keratin (data not shown).

Discussion

A Novel Keratin Family Expressed by Mesothelial Cells

The set of keratin proteins made by cultured mesothelial cells is remarkable in that it lacks the 46, 50, 52, 56 and 58 kd "epidermal" keratins, which are the major cytoskeletal components of cultured epidermal keratinocytes. Mesothelial cells contain, instead, vimentin and five other Triton-insoluble proteins (40, 44, 46, 52 and 55 kd) that we have shown to be keratins. For convenience, we refer to these proteins as the "mesothelial keratins," because all are major proteins of cultured mesothelial cells and none is normally expressed by epidermal keratinocytes.

The 40 kd mesothelial keratin is the same as that synthesized by some SCCs (Wu and Rheinwald, 1981). 40K* also is normally expressed by conjunctival cells and is inducible in cultured epidermal keratinocytes by high concentrations of vitamin A (Fuchs and Green, 1981). It has been found to be a major keratin of intestinal epithelium (Franke et al., 1981b) and of many other nonkeratinizing epithelia (Tseng et al., 1982), of embryonic epidermis (Banks-Schlegel, 1982) and of Novikoff hepatoma cells (Schmidt et al., 1982).

The other Triton-insoluble mesothelial keratins can be placed in the general "keratin" class of IF proteins by their immunological cross-reactivity and peptide maps. 40K* and 44K* cross-reacted strongly with anti-46K*. The specific, albeit weaker, cross-reaction
of anti-total s. corneum keratins with the 52 and 55
kd proteins suggests that they are keratins, but are not as similar as 40Kw and 44Kw to the epidermal s.
corneum keratins (primarily processing products of
56Kw, 58Kw, 65.5Kw and 67Kw; Fuchs and Green,
1980) that were the immunogen for this serum. 52Kw
is clearly very similar to 52Kw, since they differed only
by the greater resistance of one proteolytic intermediate to further digestion. Enzymatic modification of the same translation product could yield these two proteins, although not even a trace amount of 52 kd keratin of the other charge type is detectable in normal mesothelial or epidermal cells. Thus the two 52
kd keratins may be the products of closely related genes.
The same conclusions can be made about the relation between 46Kw and 46Kw. The peptide map of 55Kw,
similar to its close relative 52Kw, was not observed in any one of the epidermal keratins. Further evidence that
the 55 kd protein is a keratin is its coordinate expression and repression with 40Kw, 44Kw and 52Kw in
mesothelial cells during different stages of growth (N. D. Connell and J. G. Rheinwald, submitted manuscript). Hybridization of mesothelial keratin mRNAs to epidermal keratin cDNA sequences has also confirmed the relatedness of these two keratin families
(K. H. Kim, J. G. Rheinwald and E. V. Fuchs, submitted manuscript).

Specific Patterns of Mesothelial and Epidermal Keratins Expressed by Different Cultured Epithelial Cell Types

The wide range of tissues that express keratin proteins (apparently all of the epithelium except lens) was discovered only recently (Franke et al., 1979a, 1979b; Sun et al., 1979; Schmid et al., 1979), following the identification of keratins as the subunits of epithelial tonofilaments (Sun and Green, 1978; Franke et al.,
1978b). In several tissues and cell types keratins have been identified that are not expressed by normal epidermis (Wu and Rheinwald, 1981; Fuchs and Green, 1981; Franke et al., 1981a, 1981b, 1981c; Milstone and McGuire, 1981; Oshima, 1982; Schmidt et al., 1982; Tseng et al., 1982). It is clear that the in vivo and in vitro keratin patterns expressed by epithelial cells are not necessarily identical (Sun and Green, 1977; Fuchs and Green, 1978, 1980; Wu and Rheinwald, 1981). The vitamin A present in serum represses the synthesis of 63–67 kd keratins by epidermal cells during growth in culture (Fuchs and Green, 1981). Nevertheless, the keratin pattern expressed by cells even during growth in unnatural environments is significant, as demonstrated by Doran et al. (1980).

These authors confirmed the intrinsic divergence of differentiated state among even closely related epithelia by showing that cultured epidermal, esophageal and corneal epithelial cells regained their tissue-specific histology and in vivo pattern of keratin synthesis when implanted subcutaneously in nude mice. Although Doran et al. reinterpreted their one-dimensional gels as indicating that these three cell types adopt virtually identical patterns of keratin synthesis in culture, our two-dimensional gel analysis shows that unique patterns of mesothelial and epidermal keratins are expressed in culture by the eight epithelial cell types we examined. It remains to be determined what patterns of synthesis most of these epithelia, including the mesothelium, adopt in vivo. Besides being extremely useful for identification of cells in culture, our results have important implications for the study of gene regulation. Our culture conditions are sufficiently permissive to generate from small biopsies quantities of at least a gram of pure, actively dividing epithelial cell types for studies of the molecular basis of these differences in gene expression.

These results also clearly show that most of the keratin genes can be turned on or off independently.
Although 46Kw, 50Kw, 56Kw and 58Kw are coordinately expressed at high levels in epidermal, mammary, esophageal, exocervical and conjunctival epithelial cells, 50Kw is not expressed in bladder cells. 52Kw is never expressed at high levels unless 40Kw is also expressed (bladder, conjunctiva, exocervix and esophagus), but 40Kw is expressed without 52Kw in the simple epithelia (mesothelial and ovarian). The four major mesothelial keratins are coordinately expressed at high levels in mesothelial and ovarian carcinoma cells, but are expressed in very different relative amounts in other cell types. The absence of certain epidermal keratins in SCC-66 and SCC-4 demonstrates that keratin genes that are normally expressed constitutively as a feature of cell differentiation can become repressed independent of the other keratin genes.

Diversity and the Role of Keratins in Determining Cell Differentiation

The total number of different keratin genes is now at least 12, including the 40, 44 and 55 kd "mesothelial" keratins; the 46, 50, 52, 56, 58, 63, 65, 67 and 67 kd "epidermal" keratins; and the 47 kd keratin present in several cell types, but most prominently in esophageal cells. The number is 15 if 46Kw, 52Kw and the acidic 50 kd mammary keratin are independent gene products. The different keratin pattern of each epithelial cell type suggests that the precise subunit content of the keratin filaments is important for performance of differentiated function. One may speculate that different keratins confer different physical and chemical properties to the filaments, such as flexibility, bundling with neighboring filaments or association with other macromolecules and organelles in the cytoplasm and with desmosomes at the cell surface. These may in turn affect cell shape and histological organization. The importance of correct cell shape for
synthesis of differentiation-related proteins has been demonstrated convincingly for mammary epithelial cells (Shannon and Pitelka, 1981).

Experimental Procedures

Derivation and Culture of Mesothelial Cells

Cells were concentrated by low-speed centrifugation from ascites fluid of patients with ovarian carcinoma. Cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM, Flow Laboratories, Inc.) plus 20% fetal calf serum (Sterile Systems, Inc.) plus 0.4 μg/ml hydrocortisone and were plated at a density of 10^9 to 10^10 non-erythrocyte cells per 100 mm diameter plastic culture dish, with a feeder layer of about 10^9 mitomycin-treated 3T3 mouse fibroblasts. Alternatively, cells were plated without feeders in a 1:1 (v/v) mixture of fresh medium that had been conditioned for 8–24 hr by confluent 3T3 cultures. Under these conditions, only mesothelial cells grew progressively from most ascites samples. 3T3 fibroblast support was only helpful for mesothelial growth at low density. For these experiments, mesothelial strains LP-2, LP-3 and LP-9 (derived from different patients) were grown at high density in fresh medium without feeders and were used between the second and fifth passages. Cells were disaggregated for subculture by a 10 min incubation with 0.05% trypsin and 0.01% EDTA in pH 7.3 phosphate-buffered isotonic saline (PBS) at 37°C.

Other Cell Strains and Lines

The derivation of human squamous cell carcinomas (SCC) lines SCC-4 and SCC-15 was reported previously (Rheinwald and Beckett, 1981). SCC-40 and SCC-66 were derived from SCC of the soft palate and floor of the mouth, respectively. SCC cells were cultured in DMEM plus 5% fetal calf serum plus 0.4 μg/ml hydrocortisone with a 3T3 feeder layer. Ovarian epithelial tumor cell strains BIN-14 and BIN-36 were cultured from ascites fluid of patients with clinically and histopathologically low-grade, well-differentiated ovarian epithelial tumors. These cells were grown in DMEM/F12 (3:1 v/v) plus 20% FCS. 3H-methionine-labeled cell suspensions of the normal mammary epithelial cell strains H97 and H214 were obtained from M. Stumpfer, Lawrence Berkeley Laboratories, who had cultured the cells by her published method (Stumpfer, 1982). Bladder carcinoma lines EJ, J82 clone 9 and RT112 (see Marshall et al., 1977, for derivation) were obtained from L. B. Chen and were cultured either in complete keratinocyte medium with a 3T3 feeder layer or in DMEM plus 10% FCS medium.

Normal adult human bladder epithelial strains HBI-4 and HBI-5 were cultured from urinary bladder removed at autopsy, and HBI-7 from a spontaneously aborted 22 week old fetus. Esophageal epithelial strain HE-1 was cultured from a surgical specimen of adult esophagus and HE-2 from the esophagus of the same fetus as HBI-7. Ancova epithelial cell strain HC-8, cultured from a biopsy of normal adult cervix, was provided by R. Rice and P. Gline, Harvard School of Public Health. Fetal conjunctival epithelial cell strain AX was provided by H. Green, Harvard Medical School. Nearly confluent, exponentially growing cultures in the second to fourth passage were used in these experiments.

Normal human newborn foreskin epidermal keratinocyte strains N and A–Ep and bladder, conjunctival, esophageal and esophageal epithelial cells were cultured with a 3T3 feeder layer, 0.4 μg/ml hydrocortisone, 20 ng/ml EGF, 10^-10 M cholera toxin, 5 μg/ml transferrin, 5 μg/ml insulin and 2 x 10^-11 M triiodothyronine, as described previously (Rheinwald and Green, 1975, 1976; Green, 1978; Rheinwald, 1980; Watt and Green, 1981), with the following modifications that greatly improved growth and extended lifespan (T. O’Connell, M. A. Beckett, B. L. Allen-Hoffmann and J. G. Rheinwald, unpublished observations)—a 1:1 mixture of DMEM and F12 was used as the defined medium component, fetal calf serum was reduced to 5%, and 1.8 x 10^-11 M adenosine was added. (This adenosine concentration was used by Peetel and Ham, 1980, in their defined closed growth medium permissive for a single passage of human keratinocytes.)

Normal human fetal lung fibroblast strain IMR-90, obtained from the Institute for Medical Research, Camden, New Jersey, and newborn foreskin fibroblast strain A–F were cultured in F10 + 15% fetal calf serum. Primary cultures of human umbilical vein endothelial cells were provided by J. Pober and M. Gimbine, Brigham and Women’s Hospital, Boston, who isolated and grew them as described previously (Gimbine et al., 1974).

Cell Labeling, Extraction and Electrophoresis

Cultures were incubated for 4–6 hr in methionine-free medium to which 100 μCi/ml [35S]-methionine [35S] was added. When present, 3T3 feeder cells were then selectively removed by a brief incubation with EDTA (Rheinwald and Green, 1975). Cytoskeletal fractions were isolated by a modification of previously described methods (Brown et al., 1978; Starger and Goldman, 1977; Osborn and Weber, 1977; Sun and Green, 1978a; Franke et al., 1978). The human cell layer was rinsed and scraped with a rubber policeman into a Dounce homogenizer containing 10 ml of 20 mM Tris-HCl (pH 7.4), 0.6 M KCl and 1% Triton X-100. The cells were broken by homogenization and sonication at 4°C, and the insoluble fraction was pelleted by centrifugation at 10,000 x g for 20 min. The pellet was resuspended in the same buffer and extracted and centrifuged again. For one-dimensional gel electrophoresis, the pellet (cytoskeletal fraction) was dissolved in 0.3 ml Tris buffer, 2% SDS and 10 mM diithiothreitol by boiling for 5 min. The proteins were then separated electrophoretically on an SDS–polyacrylamide (10%) slab gel by the Laemmli technique, as described in Wu and Rheinwald (1981). For two-dimensional gel electrophoresis (O’Farrell, 1975), the pellet was dissolved in urea and Triton isoelectric focusing sample buffer. The proteins were then separated in the first dimension by isoelectric focusing and in the second dimension by SDS–polyacrylamide (10%) gel electrophoresis. To detect basic proteins, samples were instead run in the first dimension for 3.5 hr by the nonequilibrium pH gradient electrophoresis method (O’Farrell et al., 1977). Labeled proteins were identified by autoradiography amplified by infiltration of the gels with a fluor (EnHance, New England Nuclear) before they were dried and applied to Kodak SB-5 film. Pulse-chase experiments and comparison between patterns obtained with 4 and 24 hr labeling periods showed that the relative labeling levels of the keratins and vimentin remained constant, and were therefore proportional to content.

Peptide Mapping by Partial Proteolysis

[35S]-methionine-labeled Triton-insoluble proteins of SCC-66, mesothelial cell strain LP-9 and human lung fibroblast strain IMR-90 were separated electrophoretically on preparative 8.5% polyacrylamide–SDS slab gels. (The Triton-insoluble fraction of mesothelial strain LP-9 was extracted several times in low salt buffer—that is, lacking 0.6 M KCl—in order to solubilize and remove vimentin and permit the isolation of pure 55 kd mesothelial keratin.) The prominent bands were identified by staining for 15 min in a water solution of Coomassie blue, and were cut out individually with a razor blade. Eight millimeter segments of the bands were placed at the bottom of adjacent sample chambers of a 12.5% polyacrylamide–SDS slab gel. Staphylococcal V8 protease (Worthington Biochemicals; 0–100 ng) was added to the chambers, and the proteins and their cleavage peptides were then separated electrophoretically by the method of Cleveland et al. (1977).

Immunocchemical Techniques

Antiserum against total human plantar stratum corneum keratins and against 40 kd keratin of SCC-15 were raised in rabbits. Indirect immunofluorescence employed a rhodamine-conjugated, goat anti-rabbit IgG secondary antibody. Immunoprecipitation was achieved with a protein A adsorbant. Methods are described in Wu and Rheinwald (1981). Antiserum against pure 48 kd epidermal keratin was provided by E. Fuchs, University of Chicago. Antiserum against vimentin was provided by R. Hynes, MIT.
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