

Regulation of Human Mesothelial Cell Differentiation: Opposing Roles of Retinoids and Epidermal Growth Factor in the Expression of Intermediate Filament Proteins

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Abstract. We report here the discovery that retinoids are potent regulators of epithelial morphology and keratin expression in cultured human mesothelial cells. When LP-9 cells are cultured in medium supplemented with vitamin A-depleted serum, they grow with an extreme spindle-shaped morphology and synthesize abundant levels of vimentin, but very little keratin. When retinoic acid is added to the medium at 1×10^{-8} to 1×10^{-6} M, keratin synthesis is increased, vimentin synthesis is decreased, and the cells assume an epithelioid morphology. Keratin synthesis, but not epithelioid morphology, seems to be dependent on cell

density as well: even when vitamin A is present, sparse cultures cannot fully maintain keratin synthesis. In contrast, epidermal growth factor (EGF) acts in an antagonistic fashion to suppress both keratin synthesis and epithelial morphology. The effects of vitamin A, EGF, and cell shape on intermediate filament (IF) expression seem to occur in a growth-independent manner, and they appear to be at the level of transcription or mRNA stability. Even so, their effects on IF expression do not appear to be rapid ones, and hence it is unlikely that these agents interact directly at the gene level to cause changes in IF gene expression.

RETINOIDS are potent agents for control of differentiation and proliferation in epithelial tissues. Morphologic studies indicate that vitamin A deficiency promotes the keratinization process characteristic of epidermis, whereas vitamin A excess promotes the differentiative processes typical of mucus-secreting or simple epithelia (Wolbach, 1954; Lotan, 1980; Wolf, 1980; Elias and Williams, 1981).

Among the major biochemical markers of epithelial differentiation are the keratin proteins. There are more than 20 different keratins (40–70 kD) which have been assigned numbers according to their size and isoelectric mobilities (Moll et al., 1982a). Different keratins are expressed in different epithelial tissues and at various stages of development and differentiation (Fuchs and Green, 1980; Moll et al., 1982a, b; Wu et al., 1982; Nelson and Sun, 1983; Kim et al., 1983; Sun et al., 1984). These proteins all have the capacity to assemble into 8-nm cytoskeletal filaments, called intermediate filaments (IFs),¹ and the filament networks composed of different keratins are thought to have different structural and functional properties (for reviews, see Lazarides, 1982; Fuchs and Hanukoglu, 1983; Steinert et al., 1985). As such, the pattern of keratins expressed by a particular epithelial cell can serve as a valuable biochemical indicator of the state of differentiation of a cell.

The availability of methods to culture mammalian epidermal cells in vitro has led to extensive biochemical investigations aimed at exploring the molecular changes involved in the vitamin A-mediated regulation of terminal differentiation. When vitamin A is depleted from the epidermal culture medium by delipidization of the serum, the terminal differentiation-specific keratins K1 (67 kD), K10 (56.5 kD), and K11 (56 kD) are induced (Fuchs and Green, 1981; Eckert and Green, 1984; Kim et al., 1984; Eichner et al., 1984). Both in vivo and in vitro, the expression of these large keratins is confined to the suprabasal epidermal layers, and they are among the major biochemical markers of the keratinization process (Fuchs and Green, 1980; Viac et al., 1980; Asselineau et al., 1985; Kopan et al., 1987). In addition to expressing terminal differentiation-specific keratins, vitamin A-deficient cultures also show an increase in their intercellular adhesiveness, a decrease in cellular movement and an increase in the population of cornified envelope-producing cells (Yuspa and Harris, 1974; Fuchs and Green, 1981; Green and Watt, 1982; Gilfix and Green, 1984).

When epidermal cells are cultured in the presence of retinoids at concentrations which are ~ 10 times higher than physiological, most biochemical features of keratinization are suppressed, and the cells express two new keratins, K13 (52 kD) and K19 (40 kD), which are not normally synthesized by epidermal cells (Fuchs and Green, 1981; Eckert and Green, 1984; Kim et al., 1984). One of these keratins, K13, is expressed in a fashion that correlates with a vitamin A-me-

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; IF, intermediate filament.

diated weakening of intercellular contacts (Kopan et al., 1987). The other keratin, K19, seems to be expressed in only a subset of the suprabasal cells of the population. This protein is unusual in that it is the smallest keratin thus far identified. It is frequently found in simple epithelial tissues (Moll et al., 1982a; Wu et al., 1982).

The biochemical effects of retinoids on simple epithelial cells have not yet been explored. Human mesothelial cells provide a promising model system, because normal primary cells can be readily cultivated from the ascites fluid of patients with ovarian carcinoma (Wu et al., 1982). The mesothelial strain LP-9 has been shown to express four keratins: K7 (55 kD), K8 (53 kD), K18 (44 kD), and K19 (40 kD) (Wu et al., 1982). In addition, these cells express a related intermediate filament protein, vimentin (57 kD), which is typical of fibroblast-like cells, but is rarely associated with cells of epithelial origin. The ability of mesothelial cells to express vimentin as well as keratin may stem from their embryonic origin: whereas most epithelial cells arise from the ectoderm or the endoderm, mesothelial cells are of mesodermal origin (Connell and Rheinwald, 1983).

Several factors have previously been suggested to influence the growth and differentiation of cultured simple epithelial cells. One such factor is epidermal growth factor (EGF), which increases the growth of mesothelial cells, prompts them to adopt a fibroblast-like morphology, and enhances their expression of vimentin (Connell and Rheinwald, 1983). In the absence of EGF, cells appear to grow more slowly, assume an epithelioid morphology, and synthesize keratin (Connell and Rheinwald, 1983). Two additional regulatory factors that have been studied are cell shape and cell-cell interactions: In kidney epithelial (Madin-Darby bovine kidney [MDBK]) cells, which are similar to mesothelial cells in embryonic origin and in keratin and vimentin patterns, cell shape appears to have a significant influence on vimentin synthesis, whereas cell-cell interactions may be more important for keratin expression (Ben-Ze'ev, 1984).

In this article, we investigate the role of vitamin A on growth, differentiation, and keratin/vimentin synthesis in human mesothelial cells. Our results demonstrate clearly that retinoids and EGF have both profound and opposing effects on the biochemistry and morphology of mesothelial cells. Moreover, the molecular mechanisms underlying the effects of cell shape and cell-cell interactions on mesothelial cells may be governed by the action of vitamin A.

Materials and Methods

Growth and Epithelial Cell Cultures in Medium of Defined Vitamin A Concentration

Human mesothelial cell strain LP-9 was derived from the ascites fluid of a patient with an ovarian carcinoma (Wu et al., 1982). Cells were grown in a 3:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium (Gibco, Grand Island, NY). Retinoids are not constituents of either of these media. The medium was supplemented with 0.4 μ g/ml hydrocortisone (Calbiochem-Behring Corp., San Diego, CA) and 20 ng/ml epidermal growth factor prepared by the method of Savage and Cohen (1972). The medium was also supplemented with 20% fetal calf serum (Sterile Systems, Inc., Salt Lake City, UT). Where delipidized serum was required, serum was treated with an acetone/alcohol mixture according to the procedure of Rothblat et al. (1976). Vitamin A, which is soluble in polar organic solvents, was removed quantitatively, together with most lipids (Fuchs and Green, 1981).

In experiments where retinoids were added back to the nutrient medium,

trans-retinoic acid (Sigma Chemical Co., St. Louis, MO) was used at concentrations varying from 1×10^{-8} to 1×10^{-6} M (Gilfix and Green, 1984).

In experiments where cell-cell contact was not desirable, cells were grown suspended in 1.35% methyl cellulose as described (Rheinwald and Green, 1974).

Radiolabeling and Extraction of Proteins

Whenever radiolabeled proteins were desired, cultures were labeled with 100 μ Ci/ml [35 S]methionine (sp act 1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). Extraction of intermediate filament proteins was performed using the method previously described by Wu et al. (1982). Cells were removed by scraping with a rubber policeman, and transferred to a centrifuge tube containing 10 mM Tris-HCl (pH 7.3), 0.6 M KCl, 1% Triton X-100, and 1.7 mM phenylmethylsulfonyl fluoride, 4°C. Cells were lysed with the aid of sonication, and the insoluble fraction was pelleted by centrifugation at 15,000 g for 20 min. The pellet was resuspended in the same buffer, and extracted and centrifuged repeatedly. Intermediate filament proteins were solubilized by dissolving the washed pellet in 8 M urea, containing 10% β -mercaptoethanol. Total protein extracts were obtained by lysing cells directly in 8 M urea, 10% β -mercaptoethanol.

Proteins were resolved by electrophoresis through SDS polyacrylamide (8.5%) gels (Laemmli, 1970). Radiolabeled proteins were identified by fluorography followed by autoradiography.

Immunoblot Analysis

A polyclonal antisera against human K19 was obtained by injecting rabbits with gel-purified 40-kD keratin from cultured human conjunctival cells (Kim et al., 1984). Polyclonal antisera against hamster vimentin was obtained from Dr. Richard Hynes (Hynes and Destree, 1978). A polyclonal antibody against chicken actin was obtained from Biomedical Technologies, Inc., Cambridge, MA. A polyclonal antibody against tyrosinylated α -tubulin was obtained from Dr. Chloe Bulinski (Gundersen et al., 1984). A monoclonal antibody (DM1B) against β -tubulin was obtained from Dr. Don Cleveland (Blose et al., 1984).

Immunoblot analysis was conducted according to the procedure of Towbin et al. (1979). 125 I-labeled *Staphylococcus aureus* protein A (Amersham Corp.) was used to detect specifically bound IgG molecules.

Isolation and Translation of Poly(A) + mRNA

Typically, mRNAs from LP-9 cells were isolated from two 100-mm dishes. For cloning, mRNAs from MCF-7 or LP-9 cells were isolated from 20 \times 100-mm dishes essentially according to the procedure of Berk and Sharp (1978). Cells were lysed in the presence of 4 M guanidinium isothiocyanate, 14% β -mercaptoethanol, 5 mM EDTA, 0.1 M Tris-HCl, pH 7.5. After centrifugation at 7,000 g for 10 min to remove cellular debris, lysates were layered onto a 3-ml cushion of CsCl (final density 1.7), 0.1 M EDTA, 0.25 M NaOAc, pH 5.0, and spun at 36,000 rpm in a model SW40 rotor (Beckman Instruments, Inc., Fullerton, CA) for 22 h at 20°C. The pellet containing total RNA was resuspended in guanidinium isothiocyanate solution as above and precipitated with one volume ethanol (-20° C). The final RNA precipitate was dissolved in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.6, and subjected to chromatography on oligo dT-cellulose.

0.5 μ g of purified mRNA was used for each translation (15 μ l). Rabbit reticulocyte lysates were obtained from Bethesda Research Laboratories, Gaithersburg, MD. [35 S]methionine was added (2 μ Ci/ μ l) and lysates were incubated for 1 h at 30°C.

Preparation of cDNA Libraries

MCF-7 Library. Poly(A)+ RNAs were isolated from the human breast carcinoma line MCF-7. Double-stranded cDNAs were constructed according to the procedure of Gubler and Hoffman (1983). Eco RI linkers were added to the ends of these cDNAs, and the inserts were ligated to the Eco RI ends of the arms of bacteriophage λ strain gt10. The hybrid λ DNAs were then packaged into empty phage heads (Stratagene, Inc., La Jolla, CA), and *Escherichia coli* strain C600 hFl⁻ was infected with the packaged hybrid phage DNAs. About 1.2×10^5 plaques with an average insert size of 1,000 bp were isolated. Radiolabeled probes to the human KB-2 (K14) cDNA (Hanukoglu and Fuchs, 1982) and the mouse endoglycosidase B (endo B) keratin (Singer et al., 1986) were used to screen the library for K18 sequences (Benton and Davis, 1977). Inserts from positive clones were subcloned into pGEM4-blue (Promega Biotec, Madison, WI) for further analysis.

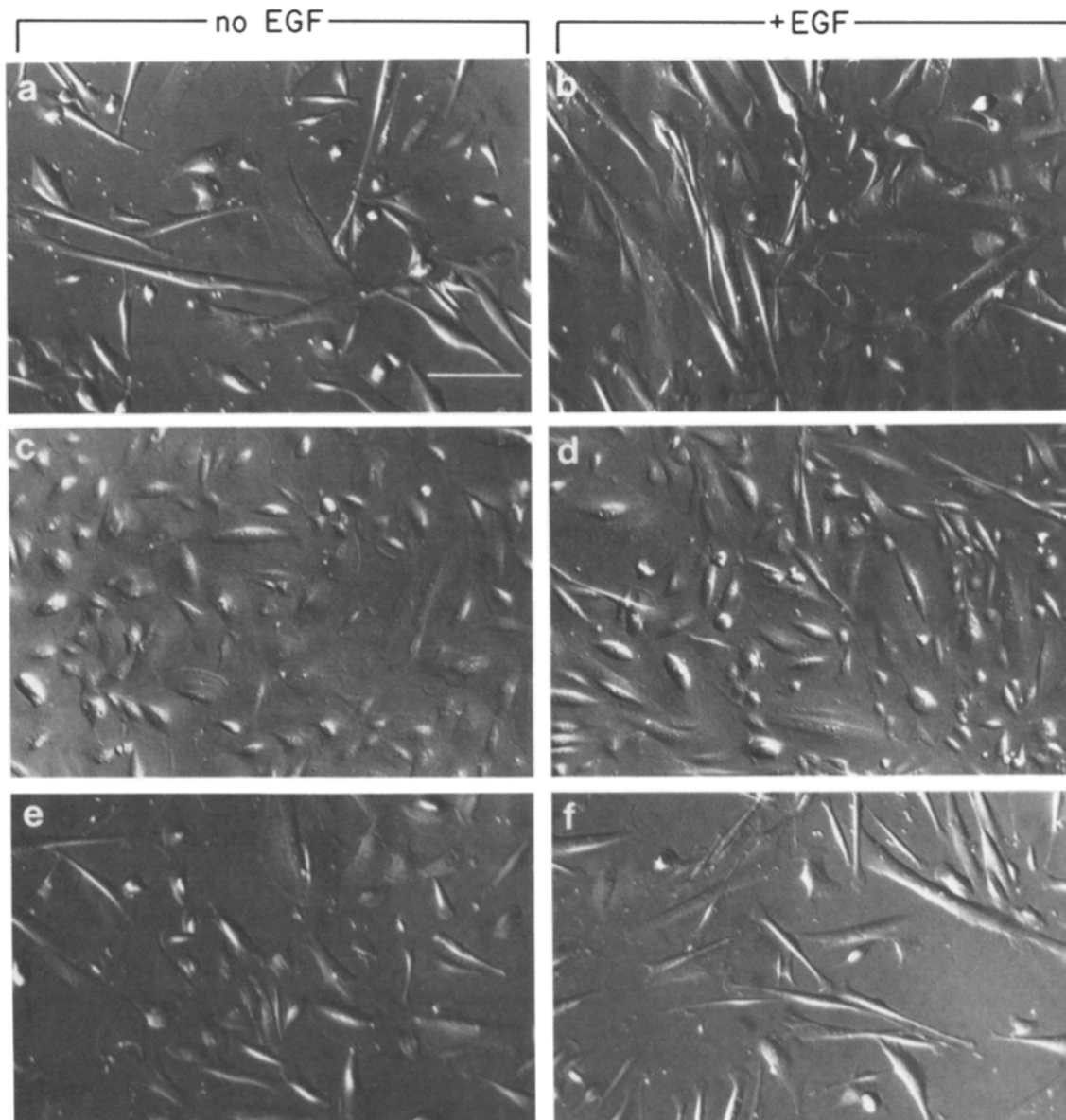


Figure 1. Vitamin A- and EGF-induced changes in the morphology of cultured human mesothelial cells. Cultures of LP-9 cells were grown to one-half confluence in medium containing delipidized serum (*a* and *b*), normal serum (*c* and *d*), or delipidized serum supplemented with 1×10^{-6} M retinoic acid (*e* and *f*). EGF was either included (*b*, *d*, and *f*) or omitted (*a*, *c*, and *e*). Bar, 100 μ m.

Mesothelial Library. Poly(A)⁺ RNAs were isolated from the human mesothelial cell strain LP-9. Double-stranded cDNAs were constructed according to the procedure of Gubler and Hoffman (1983). cDNAs were exposed to terminal transferase (PL Biochemicals, Piscataway, NJ) and dCTP to add a poly dC stretch at the 3' ends of the inserts. Bacterial plasmid pUC8 was linearized with Pst I, and exposed to terminal transferase and dGTP to add a poly dG stretch at the 3' ends of the plasmids. After annealing, the hybrid plasmids were transformed into *E. coli* strain TB1 (Maniatis et al., 1982). About 1,000 ampicillin-resistant clones with an average insert size of 500 bp were isolated. Clones were screened with radiolabeled probe to pVim-2 (Quax-Jeuken et al., 1983) as described above.

Results

In the Absence of Vitamin A, Mesothelial Cells Assume a Spindle-like Morphology

When human mesothelial cells from strain LP-9 were cul-

tured in medium containing serum that had been delipidized by extraction with polar solvents (Rothblat et al., 1976), many of the cells appeared highly elongated and spindle-shaped whether or not EGF was present (Fig. 1, *a* and *b*). In contrast, as shown previously by Connell and Rheinwald (1983), LP-9 cells cultured in the presence of normal serum were more epithelioid (Fig. 1 *c*) and only assumed an elongated and fibroblast-like appearance when EGF was added to the culture medium (Fig. 1 *d*).

Irrespective of the medium used to culture the LP-9 cells, the cell density at which cells were seeded did not seem to have an appreciable effect on their morphology. Hence, in normal medium without EGF, cells were epithelioid whether they were seeded at 50 cells/cm² or 5,000 cells/cm². Similarly, cells cultured in medium containing delipidized serum and EGF maintained their spindle-shaped morphology at all

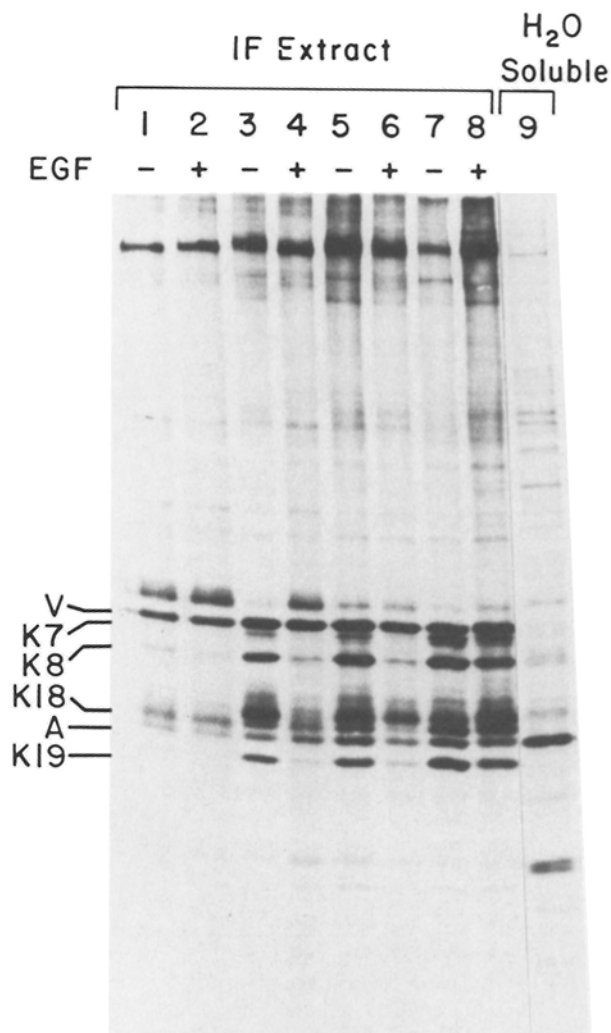


Figure 2. Effects of vitamin A and EGF on IF protein synthesis in LP-9 cells. LP-9 cells were cultured in medium containing either delipidized serum, normal serum, delipidized serum supplemented with 1×10^{-8} M retinoic acid, or delipidized serum supplemented with 1×10^{-6} M retinoic acid. EGF was either added or omitted. At three-quarters confluence, [35 S]methionine (1,000 Ci/mmol) was added at 100 μ Ci/ml to one dish of cells cultured under each condition. After 8 h of incubation IF proteins were extracted, and the samples were resolved by SDS polyacrylamide (8.5%) gel electrophoresis. The gel was subjected to fluorography and autoradiography and exposed to x-ray film. IF preparations were from cells cultured in medium containing: lane 1, delipidized serum; lane 2, delipidized serum + EGF; lane 3, delipidized serum + 1×10^{-8} M retinoic acid (RA); lane 4, delipidized serum + 1×10^{-8} M RA + EGF; lane 5, delipidized serum + 1×10^{-6} M RA; lane 6, delipidized serum + 1×10^{-6} M RA + EGF; lane 7, normal serum; lane 8, normal serum + EGF. Lane 9 shows a radiolabeled water-soluble extract from the cells grown under the same condition as lane 8. Whereas the amount of IF extract loaded represented 2% of the total IF extract from the dish, the amount of water-soluble extract loaded represented 0.02% of the total protein from the dish. It can be seen that the actin in IF extracts was clearly a contaminant, and represented roughly 1% of the actin present in the water-soluble extracts. Keratins are indicated at the left according to the numbering system of Moll et al. (1982a). V, vimentin. A, actin. Note: the presence of the band appearing just above K18 (see, for example, lanes 3 and 5–8) has been described before (Wu et al., 1982) and is not an IF protein. The identities of the additional bands at \sim 60 kD (above vimentin) and at >100 kD were not determined.

densities. Even confluent cultures showed cell morphologies similar to those of sparse cultures.

To determine whether the spindlelike LP-9 cell morphology might be attributed specifically to the removal of vitamin A during the delipidization process, we cultured the cells in the presence of delipidized medium supplemented with 1×10^{-8} to 1×10^{-6} M retinoic acid. The presence of retinoic acid did seem to promote a more epithelioid morphology in the absence of EGF (Fig. 1 e) and a fibroblastic morphology in the presence of EGF (Fig. 1 f). However, retinoic acid alone did not completely restore the epithelioid phenotype observed for cells cultivated in normal medium (compare Fig. 1 c and d with e and f). Either retinoic acid is not as proficient as the serum form of vitamin A (i.e., retinol complexed with serum retinol binding protein; Goodman, 1981) in eliciting these structural changes, or alternatively, other hydrophobic factors in addition to retinoids are involved in orchestrating the morphology of the LP-9 cell.

The delipidization of serum did not seem to affect the viability of mesothelial cells during the time frame in which the experiments were conducted. Nevertheless, it did seem to increase the division time of LP-9 cells from 48 to 60 h. In contrast, EGF decreased the doubling time of LP-9 cells to 36 h. Thus, although both delipidization of serum and EGF promoted a more elongated, spindle-shaped morphology, one agent retarded the growth rate of the cells, while the other agent increased the growth rate. These data indicate that the fibroblast-like morphology of LP-9 cells cannot be determined solely by relative growth rate.

Retinoic Acid and EGF Markedly Alter Keratin and Vimentin Synthesis in Cultured Human Mesothelial Cells

Previously, Connell and Rheinwald (1983) had demonstrated that keratin synthesis is suppressed and vimentin synthesis is increased when LP-9 cells become elongated as a consequence of EGF treatment. If a fibroblast morphology necessitates low levels of keratin and high levels of vimentin, then our morphologic studies would suggest that keratin expression might be suppressed whether or not EGF is administered to LP-9 cells grown in delipidized medium. To test this hypothesis, cells were cultured for 7 d in medium containing delipidized serum, with and without EGF. After an 8-h radiolabeling with [35 S]methionine, IF proteins were extracted and subjected to SDS polyacrylamide gel electrophoresis, fluorography, and autoradiography (Fig. 2). Because two-dimensional gel electrophoresis of IF proteins did not reveal the presence of any other proteins with molecular weights identical to vimentin or to any of the keratins (not shown), one-dimensional gels sufficed for these studies. In radiolabeled IF extracts from cells grown in the absence of vitamin A, a band corresponding to vimentin (57 kD) could readily be seen, whereas bands for the keratins K7 (55 kD), K8 (53 kD), K18 (44 kD), and K19 (40 kD) were barely visible (lane 1). Addition of EGF to the culture medium did not appreciably change the IF pattern (lane 2).

To determine whether the addition of retinoids was sufficient to restore the expression of keratins and the effects of EGF, LP-9 cells were cultured in the presence of medium containing delipidized serum and 1×10^{-8} to 1×10^{-6} M retinoic acid. Even at 1×10^{-8} M retinoic acid, appreciable levels of all four simple epithelial keratins were synthesized

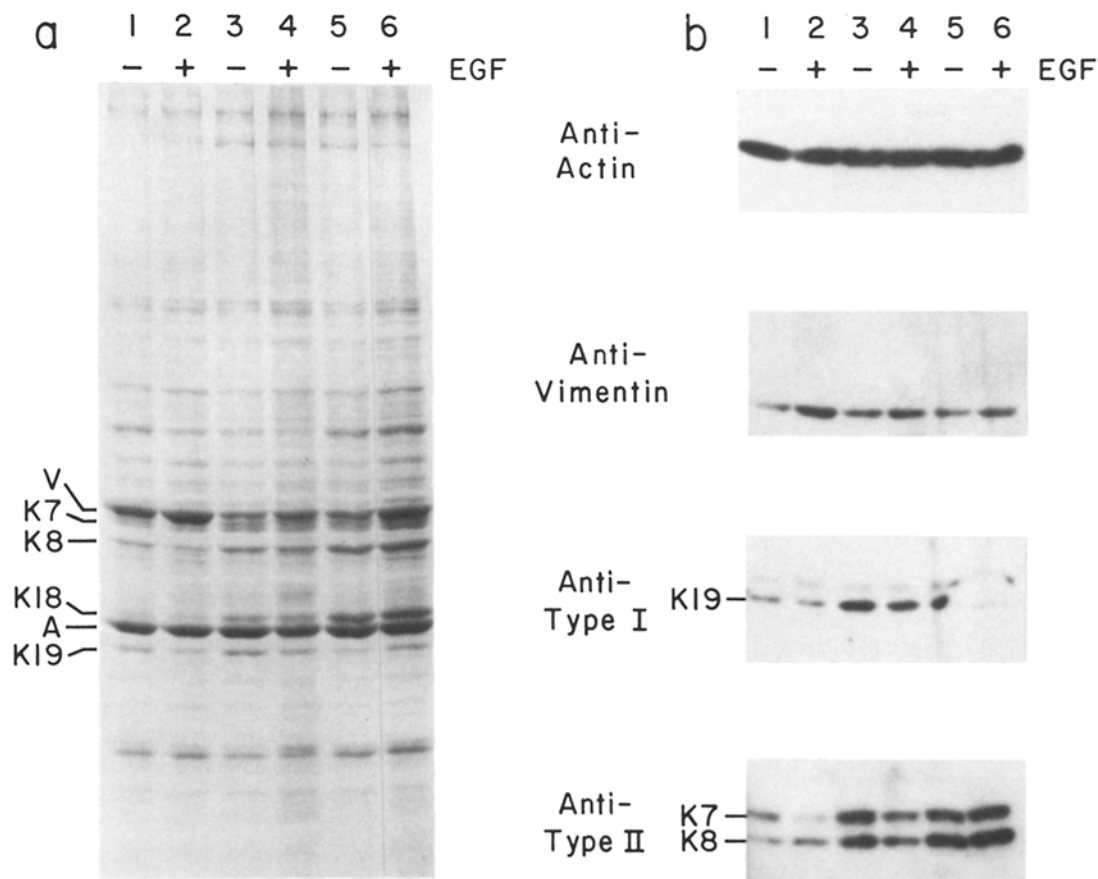


Figure 3. Effects of vitamin A and EGF on the absolute levels of IF proteins and actin in LP-9 cells. LP-9 cells were cultured in medium containing either delipidized serum, normal serum, or delipidized serum supplemented with 1×10^{-6} M retinoic acid. EGF was either added or omitted. At three-quarters confluence, total cellular proteins were extracted from the cultures, and samples were resolved by SDS polyacrylamide (8.5%) gel electrophoresis. (a) To visualize the proteins, one gel was stained with Coomassie Blue. (b) To examine actin, vimentin, and keratins in detail, four gels were subjected to immunoblot analyses with a polyclonal antiserum against actin, a polyclonal antisera against vimentin, a polyclonal antisera raised against gel-purified K19 (but cross-reactive with many type I keratins; Kim et al., 1984) and a polyclonal antisera that is broadly cross-reactive with most type II keratins (Fuchs and Marchuk, 1983), respectively. For all gels, total protein extracts were from cells cultured in medium containing: lane 1, delipidized serum; lane 2, delipidized serum + EGF; lane 3, normal serum; lane 4, normal serum + EGF; lane 5, delipidized serum + 1×10^{-6} M RA; lane 6, delipidized serum + 1×10^{-6} M retinoic acid + EGF. Keratins are indicated at left according to the numbering system of Moll et al. (1982a). V, vimentin; A, actin. Note that the presence of an air pocket interfered with the transfer of protein in the anti-type I immunoblot (b, lanes 5 and 6).

(lane 3). In this case, EGF showed a pronounced antagonistic effect on the expression of keratins (lane 4). Raising the level of retinoic acid from 1×10^{-8} to 1×10^{-6} M caused a slight additional increase in the overall level of keratin expression, and this effect was still suppressible by EGF treatment (lanes 5 and 6). Although retinoic acid showed a marked ability to upregulate keratin expression in LP-9 cells grown in delipidized medium, the amount of keratin synthesized by these cells was never quite as high as in LP-9 cells cultivated in normal medium (lane 7). Interestingly, whereas the expression of keratins was highest in cells grown in normal medium (lane 7), the antagonistic effects of EGF seemed to be highest in cells grown in retinoic acid-supplemented media (lanes 3–6). These data were consistent with our morphologic studies and suggested that delipidization of the serum might remove certain growth stimulatory factors that counteract the negative effects of EGF on keratin expression and epithelioid morphology. Retinoic acid only partially restores this function.

Although radiolabeled IF extracts enabled us to examine changes in the ratio of keratin/vimentin synthesis, they did not allow us to discern how the levels of IF proteins varied in comparison to other cellular proteins. Although actin has sometimes been used to gauge the levels of vimentin and keratin in IF extracts (Connell and Rheinwald, 1983; Ben-Ze'ev, 1984), it is not a reliable standard, because it is a contaminant in IF extracts and accounts for <1% of the total actin protein (see Fig. 2, lane 9). To determine the absolute levels of vimentin and keratin, we repeated the above experiment, only this time we extracted total proteins, and resolved them by SDS polyacrylamide gel electrophoresis. Gels were either stained with Coomassie Blue (Fig. 3 a) or subjected to immunoblot analysis using antibodies specific for actin, vimentin, type I keratins, and type II keratins (Fig. 3 b). Under all conditions, a number of cellular proteins including actin (43 kD, marked A in Fig. 3 a) showed no significant variation in response to retinoids or EGF (Fig. 3 a, lanes 1–6). In that the expression of many proteins is dependent upon cell den-

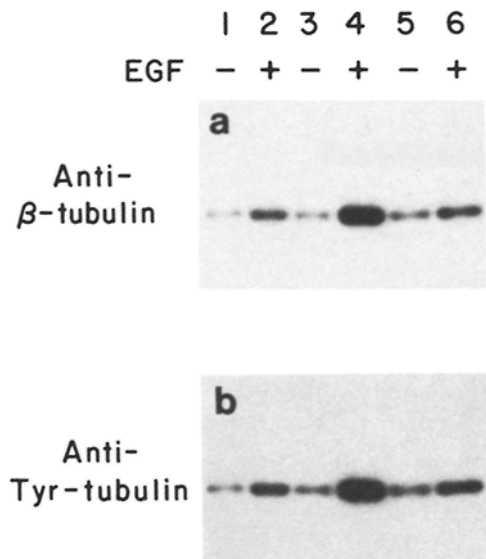


Figure 4. Delipidization of the serum lowers the levels of tubulin and EGF raises the levels of tubulin in LP-9 cells. Cells were grown under the conditions described in the legend to Fig. 3, and total proteins were extracted and resolved by SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose by blotting and the blots were incubated with antibodies against either tyrosinated (a) β -tubulin or (b) α -tubulin. ^{125}I -labeled *S. aureus* protein A was used to identify the bound antibody. Loadings on the gels were the same as in Fig. 3 a.

sity, cells were always examined at comparable states of confluence. However, as long as this condition was met, actin (or any number of other cellular proteins) could be used as an internal standard to measure fluctuations in vimentin and keratin.

When the expression of IF proteins was compared relative to actin, keratin levels were clearly higher in the presence of retinoic acid. This was particularly evident for K19, K7, and K8 in Fig. 3 b (compare lanes 1 and 2, proteins from retinoid-depleted cultures, with lanes 3-6, proteins from retinoid-containing cultures). Conversely, vimentin levels increased in retinoid-depleted cultures. This was most evident in Fig. 3 a (compare lanes 1 and 2 with lanes 3 and 4). EGF caused an additional increase in vimentin expression as seen in Fig. 3, a and b (compare odd and even numbered lanes). Similar results were obtained with radiolabeled extracts (not shown). Thus in all cases, the effects of retinoids and EGF are opposing on LP-9 morphology, and on both the absolute levels and the synthesis of keratin and vimentin.

Tubulin Levels Are Markedly Increased in the Presence of EGF and Slightly Increased in the Presence of Retinoids

To examine the effects of EGF and vitamin A on the remaining cytoskeletal element, namely tubulin, we prepared two additional immunoblots of the unlabeled protein extracts from the samples described in the previous section. A monoclonal antibody (DM1B) against β -tubulin (Blose et al., 1984) and a polyclonal antiserum against tyrosinated α -tubulin (Gundersen et al., 1984) were used to measure the total (unpolymerized and polymerized) levels of β - and α -tubulins

(Fig. 4, a and b). Unexpectedly, we discovered that both β - and α -tubulin levels were markedly sensitive to EGF. When EGF was present, tubulin levels were consistently higher (lanes 2, 4, and 6) than when EGF was absent (lanes 1, 3, and 5). When cells were cultured in the presence of regular medium containing EGF, the level was maximal (lane 4). Delipidization resulted in a decrease in tubulin levels (lanes 1 and 2), which was only slightly restored by the presence of retinoic acid (lanes 5 and 6). Thus, whereas the effects of EGF and retinoids on vimentin and keratin synthesis are opposing, their effects seem to be complementary with respect to tubulin synthesis.

As judged by Northern blot analysis using a specific human α -tubulin cDNA probe (Cowan et al., 1983), the EGF-mediated changes in tubulin levels were reflected at the mRNA level (not shown). The levels of tubulin and tubulin mRNAs correlated well with the growth rate of LP-9 cells, but it was not determined whether growth rate alone accounts for the dramatic EGF-mediated increase in tubulin levels in normal medium.

Construction and Identification of Cloned Human cDNAs Encoding Keratin K18 and Vimentin

To investigate whether the retinoid-mediated changes in intermediate filament protein synthesis were at the level of mRNA, we needed specific cloned human cDNA probes complementary to the simple epithelial keratins and to vimentin. We prepared two human cDNA libraries: one to the mRNAs expressed in human breast carcinoma cells (line MCF-7), and one to the mRNAs expressed in cultured human mesothelial cells (strain LP-9). As a source of simple epithelial keratin mRNAs, MCF-7 cells were preferred over LP-9 because they express keratins K18 and K8 in greater abundance.

To obtain a cloned human K18 cDNA, we initially screened the MCF-7 library with a ^{32}P -labeled cDNA to KB-2, a 1.4-kb cDNA insert encoding a human type I epidermal keratin K14 (Grunstein and Hogness, 1975; Hanukoglu and Fuchs, 1982). We later screened these clones with radiolabeled probe to pUC9B7, a 1.5-kb cDNA insert encoding the mouse endo B keratin, whose human counterpart is K18 (Oshima et al., 1986; Singer et al., 1986). One of the positive clones, λ KD-1 contained a 1,000-bp insert which hybridized strongly with the mouse K18 probe, and weakly with the human K14 probe. Fig. 5 a depicts the mapped restriction endonuclease sites of this insert. This restriction map agreed with recently published data on other human K18 cDNAs (Oshima et al., 1986; Romano et al., 1986). Partial sequence analysis confirmed the identification of this clone.

To obtain a cloned human vimentin cDNA, we screened the LP-9 library with a ^{32}P -labeled cDNA to pVim-2, a 1.2-kb insert encoding hamster vimentin (Quax-Jeuken et al., 1983). One of the positive clones, pVH-1 encompassed 1.0-kb of the human vimentin mRNA, and was selected for use in the studies described here. Fig. 5 b depicts the mapped restriction endonuclease sites of this clone. This restriction map agreed with recently published data on a human vimentin cDNA (Ferrari et al., 1986).

To unequivocally assign the identity of these clones, we conducted positive hybridization and translation analyses (Ricciardi et al., 1979). When total poly (A)+ RNAs from

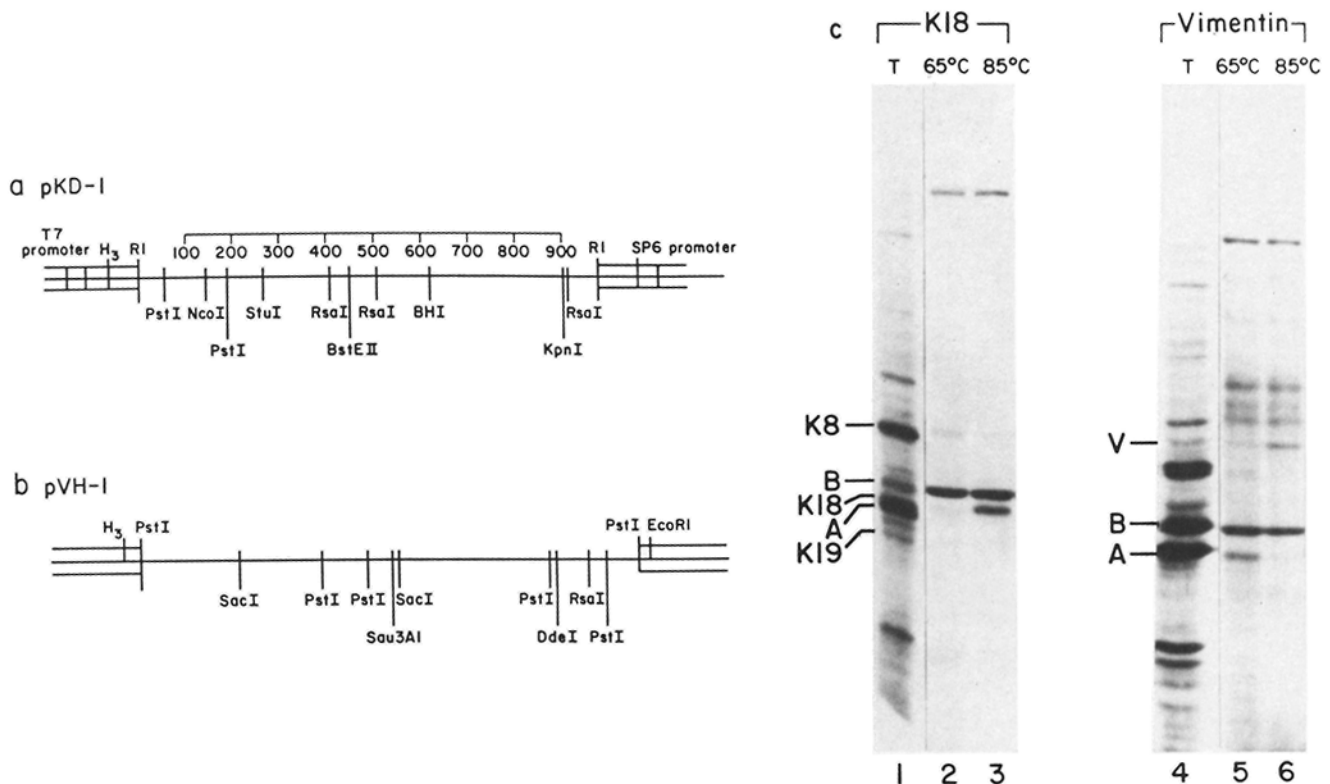


Figure 5. Characterization of cloned human cDNAs encoding keratin K18 and vimentin. (a and b) The restriction maps of two isolated cloned cDNAs are drawn to scale. (a) The keratin K18 insert KD-1 and (b) the vimentin insert VH-1 are contained in plasmid pGEM-4 blue and pUC8, respectively (thick bars). The size scale is shown in base pairs. (c) Positive hybridization-translation analyses with the inserts contained in plasmids pKD-1 (K18 cDNA) and pVH-1 (vimentin cDNA). cDNA fragments were isolated by restriction endonuclease digestion, agarose gel electrophoresis, and electroelution. Isolated fragments were denatured and immobilized on nitrocellulose filters. The filter containing KD-1 cDNA was hybridized with polyadenylated RNAs from cultured human breast carcinoma (MCF-7) cells (lanes 2 and 3), while the filter containing VH-1 cDNA was hybridized with polyadenylated RNAs from cultured human diploid fibroblast (IMR90) cells (lanes 5 and 6). Messenger RNAs were eluted sequentially at 65°C (lanes 2 and 5) and at 85°C (lanes 3 and 6). After translation of the eluted mRNAs in a reticulocyte lysate system, the [³⁵S]methionine-labeled translation products were subjected to SDS polyacrylamide gel electrophoresis, fluorography, and autoradiography. Lane 1, total translation products from MCF-7 mRNAs; lane 4, total translation products from IMR90 mRNAs. Keratins are indicated at left according to the numbering system of Moll et al. (1982a). V, vimentin; A, actin; B, mRNA independent artifact band of the translation system. Note that for the vimentin positive hybrid-translation, a number of background bands can be seen at both high and low stringency (lanes 5 and 6). The specific vimentin translation product shown in lane 6 is relatively weak due to the low abundance of vimentin RNA in the fibroblast cells.

MCF-7 cells were hybridized with pKD-1 DNA (denatured and immobilized on nitrocellulose filters), an mRNA encoding a 44-kD protein was specifically selected (Fig. 5 c, lane 3). When total poly (A)+ RNAs from human diploid fibroblasts (IMR90) were hybridized with pVH-1, one mRNA was predominantly eluted at 85°C (lane 6), but not at 65°C (lane 5). This RNA encoded a 57-kD protein corresponding to vimentin.

Retinoic Acid- and EGF-mediated Changes in IF Expression Are at the Level of mRNA

Total poly (A)+ RNAs were isolated from LP-9 cells grown under varying conditions of EGF and retinoic acid and were subsequently fractionated by formaldehyde agarose gel electrophoresis. After transferring by blotting to nitrocellulose paper (Thomas, 1980), the mRNAs were hybridized with a mixture of the two radiolabeled probes complementary to K18 and vimentin mRNAs (Fig. 6). By itself, the K18 probe detected a single RNA of 1.5 kb (lane 1) and the vimentin

probe detected a single RNA of 1.9 kb (lane 2). Quantitation of the relative levels of different mRNAs was difficult, so we examined the ratio of keratin and vimentin mRNAs. Our data show that the ratio of K18 mRNA to vimentin mRNA was high when cells were cultured in the presence of normal serum-containing medium (lanes 5 and 6), and it was low when cells were grown in medium containing delipidized serum (lanes 3 and 4). In normal medium, addition of EGF significantly decreased the ratio of K18 to vimentin mRNA (lane 6). These results indicate that the retinoid- and EGF-mediated changes in keratin and vimentin patterns arise at least in part from changes in the corresponding levels of IF mRNAs.

The suppression of keratin mRNA levels caused by growth of the LP-9 cells in medium containing delipidized serum was confirmed by in vitro translation of poly (A)+ RNAs (Fig. 7). Poly (A)+ RNAs isolated from LP-9 cells cultured in normal serum-containing medium contained abundant levels of mRNAs encoding K7, K8, K18, and K19 (Fig. 7 a),

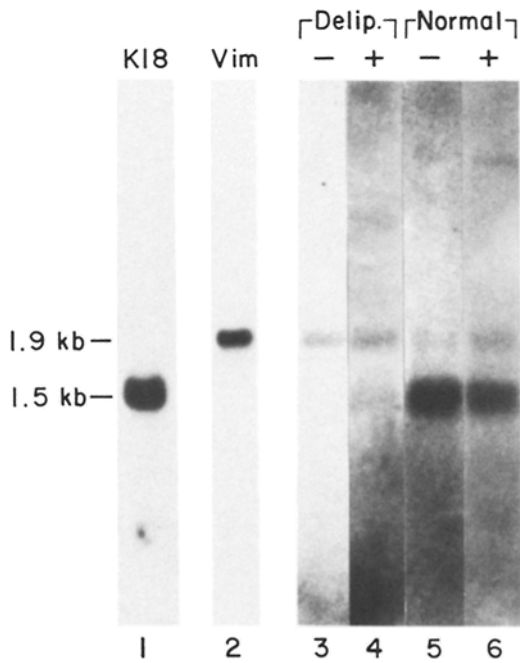


Figure 6. Ratio of keratin to vimentin mRNAs changes in response to retinoids and EGF. Poly(A)⁺ RNAs were isolated from human mesothelial cells grown in medium containing either delipidized or normal serum \pm EGF. RNAs were resolved by formaldehyde agarose gel electrophoresis and transferred to nitrocellulose paper by blotting (Thomas, 1980). The blot (lanes 3–6) was hybridized with a mixture of ³²P-labeled probes transcribed from KD-1 (K18 cDNA) and VH-1 (vimentin cDNA). After hybridization, blots were treated with RNase, washed, and exposed to X-ray film. Messenger RNAs were from LP-9 cells cultured in medium containing: lane 3, delipidized serum; lane 4, delipidized serum + EGF; lane 5, normal serum; lane 6, normal serum + EGF. Lane 1, LP-9 mRNA hybridized with radiolabeled K18 probe; lane 2, LP-9 mRNA hybridized with radiolabeled vimentin probe. Sizes shown at left were determined by rRNA markers from rabbit and *E. coli*.

whereas RNAs isolated from cells cultured in medium containing delipidized serum showed markedly reduced levels of all four of these keratin mRNAs (Fig. 7 *b*). In these reticulocyte translation extracts, the levels of vimentin were low relative to keratin and actin and thus precluded quantitation.

Retinoic Acid-mediated Changes in Intermediate Filament Protein Expression Are Reversible, but the Kinetics of the Response Are Slow

To investigate whether the retinoic acid-induced changes in the morphology and expression of IF proteins are reversible, we assessed the ability of the spindle-shaped, vitamin A-deprived LP-9 cells to revert to their normal morphology and biochemistry when returned to regular medium. Cells were grown to one-half confluence in medium containing delipidized serum. At $t = 0$, the retinoid-depleted media was replaced with normal medium. At $t = 24$ and 72 h after media switching, cells were examined microscopically and then radiolabeled by the addition of [³⁵S]methionine to the culture medium. Radiolabeled IF proteins were extracted 8 h later and resolved by gel electrophoresis.

An autoradiogram of the gel is shown in Fig. 8. It is clear from the results that the suppression of keratins by retinoid-

depleted medium (lane 1) was largely if not fully reversible (lane 3). However, the kinetics of the reversion were slow: it took between 48 and 72 h for keratin synthesis to return to its optimal level. The changes in morphology paralleled the changes in keratin synthesis (data not shown).

Intercellular Interactions and Retinoids Are Both Essential for Keratin Expression in LP-9 Cells

When densely populated (5×10^5 cells per 35-mm plate) kidney epithelial (MDBK) cells are grown on a plastic substratum, they express high levels of keratin and low levels of vimentin (Ben-Ze'ev, 1984). In contrast, sparsely populated MDBK cells (10^4 cells per 35-mm dish) grown on a plastic substratum express low levels of keratin and high levels of vimentin, whereas the same cultures grown as a single-cell suspension show low levels of both keratin and vimentin (Ben-Ze'ev, 1984). From these data, it has been suggested that keratin synthesis is enhanced by intercellular interactions (Ben-Ze'ev, 1984).

It was already apparent from our initial studies that in the absence of fat-soluble serum nutrients, dense populations of LP-9 cells do not express much keratin. There are several explanations which might account for the seemingly disparate results of Ben-Ze'ev and our own. It could be that for keratin expression to occur, both intercellular interactions and the presence of retinoids are necessary prerequisites. Furthermore, although keratin synthesis might be able to occur in epithelioid-shaped epithelial cells growing on a plastic substratum, a spindle-shaped morphology might be incompatible with keratin expression. Thus, a priori, our initial results were not necessarily in opposition to those obtained previously by Ben-Ze'ev (1984).

To assess whether intercellular interactions play a role in keratin expression in human mesothelial cells, we conducted several experiments. In the first study, we plated out cells at densities ranging from 50/cm² to 500/cm² in the presence of regular (vitamin A-containing) medium. After 5 d in culture, cells were radiolabeled for 4 h before extracting the IF proteins. Fig. 9 *a* shows an autoradiogram of the gel-resolved IF proteins. Our results indicate that sparsely grown LP-9 cells synthesize greater amounts of vimentin relative to keratin (lane 1), whereas dense cultures express greater amounts of keratin relative to vimentin (lane 4). As mentioned previously, no changes in cell shape accompanied these changes in IF expression. These data are consistent with the findings of Ben-Ze'ev (1984), and they further indicate that both vitamin A and intercellular interactions seem to be important in regulating keratin synthesis.

In the second experiment, we tested the ability of LP-9 cells to continue to express keratins when transferred from a plastic substratum to methyl cellulose suspension. At a density of 2.5×10^5 cells/ml of methyl cellulose-containing medium, human mesothelial cells remained as isolated cells and showed no tendency to aggregate (not shown). Cells grown in regular medium were transferred to medium containing 1.35% methyl cellulose and either (a) normal serum, (b) delipidized serum, or (c) delipidized serum and 1×10^{-6} M retinoic acid. After 72 h in suspension, 100 μ Ci/ml was added to the medium and cells were labeled for 2 h. Total protein extracts were processed to enable us to examine the levels of keratin and vimentin synthesis relative to other cellular proteins. Fig. 9 *b* shows an autoradiogram of the pro-

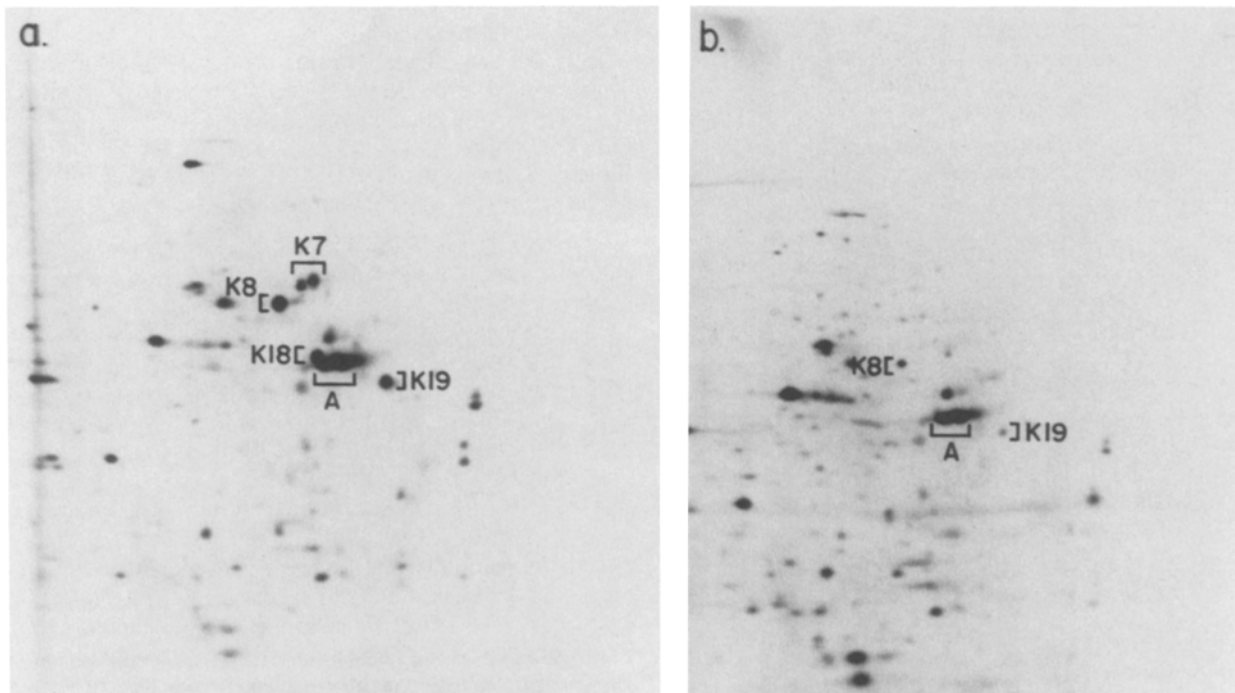


Figure 7. Two-dimensional gel electrophoresis of the translation products of mRNAs from LP-9 cells grown in the presence of normal or delipidized serum. LP-9 cells were grown to one half confluence in medium containing either (a) normal serum or (b) delipidized serum. Poly (A)⁺ RNAs were isolated and 0.5 μ g of each sample were translated in a rabbit reticulocyte cell-free translation system in the presence of [³⁵S]methionine. Radiolabeled translation products were subjected to two-dimensional (isoelectric focusing) gel electrophoresis (O'Farrell, et al., 1977). Gels were subjected to fluorography and autoradiography and were exposed to x-ray film at exposures such that the radiolabeled actin spots in the two gels were of approximately equal intensity. Keratin spots were identified and assigned numbers according to the nomenclature of Moll et al. (1982a). A, actin.

teins after polyacrylamide gel electrophoresis. Although cells expressed abundant levels of keratin and vimentin before suspension (lane 1), keratin synthesis appeared to be significantly suppressed after suspension (lanes 2–4). The presence of vitamin A did not appreciably enhance keratin synthesis (lanes 3 and 4). In contrast to keratin synthesis, vimentin synthesis seemed to be largely unaffected by this artificially induced change in cell morphology. With the exception of this finding, our results are in agreement with those of Ben-Ze'ev for MDBK cells, and further support the notion that when LP-9 cells are rounded and isolated in suspension, the presence of vitamin A is not sufficient to sustain keratin synthesis.

Discussion

Retinoids as Regulators of Mesothelial Differentiation

Several factors have been implicated in controlling differentiation of cultured simple epithelial cells. These include growth rates (Connell and Rheinwald, 1983), cell shape, and cell-cell interactions (Ben-Ze'ev, 1984). In this article, we have explored the possibility that retinoids may also regulate differentiation in simple epithelial cells. We were particularly interested in examining the effects of vitamin A on simple epithelial differentiation because a number of early *in vivo* studies have indicated that vitamin A promotes the features characteristic of simple, mucus-secreting epithelial tis-

sues (Fell and Mellanby, 1953; Beckingham-Smith, 1973; Yuspa and Harris, 1974; Sporn et al., 1975). In addition, we had previously shown that when the levels of retinoids in the medium are increased, cultured human epidermal cells are induced to express one of the simple epithelial keratins, namely K19 (Fuchs and Green, 1981).

Our results on LP-9 cells have clearly shown that retinoids have a profound influence on mesothelial cell differentiation. In contrast to the negative effects of retinoids on the expression of terminal differentiation-specific epidermal keratins, all four mesothelial keratins K7, K8, K18, and K19 are positively regulated by retinoids. In fact, the presence of retinoids seems to be essential for their expression; in retinoid-depleted medium, almost no keratins are expressed. The effects of retinoids go considerably beyond regulation of keratin synthesis: in the presence of medium containing delipidized serum, the morphology of the LP-9 cells is dramatically altered, showing a highly elongated, spindle-shaped configuration, and an increase in vimentin synthesis. Although other factors may also contribute to these shape changes, the addition of retinoic acid largely restores the epithelioid morphology of the cells, suppresses vimentin synthesis, and increases keratin synthesis. Hence, retinoids seem to participate in a positive fashion in regulating both epithelioid morphology and keratin expression, and in a negative fashion in regulating fibroblastic morphology and vimentin expression. Both their positive and negative regulatory powers are significantly stronger than those previously ascribed to EGF (Connell and Rheinwald, 1983).

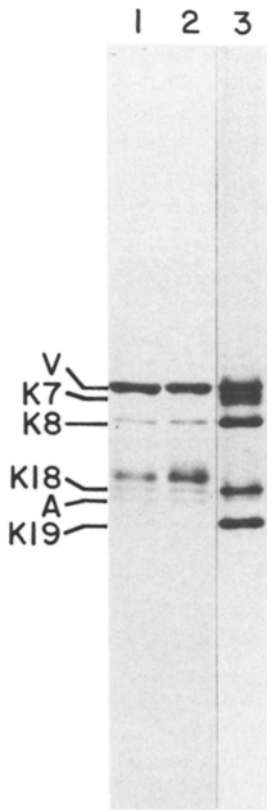


Figure 8. Recovery kinetics of LP-9 cells in response to retinoids are slow. Three 60-mm dishes of LP-9 cells were grown to one-half confluence in medium containing delipidized serum. This medium was then replaced with normal serum-containing medium. At increasing time intervals thereafter [^{35}S]methionine was added to one dish at 100 $\mu\text{Ci/ml}$, and IF proteins were extracted 8 h later. Radiolabeled proteins were resolved by SDS polyacrylamide gel electrophoresis, and subjected to fluorography and autoradiography. Extracts were from the following times: lane 1, $t = 0$; lane 2, $t = 24$ h; lane 3, $t = 72$ h. Keratins were identified according to the numbering system of Moll et al. (1982). V, vimentin; A, actin.

Possible Mechanisms for Retinoid Action: Indirect Versus Direct

It was realized more than 60 years ago that retinoids influence epithelial growth and differentiation (Wolbach and Howe, 1925). In the early 1950s, it was discovered that chick epidermis in organ culture could be converted from a keratinized to mucus-secreting epithelium by treatment with vitamin A (Fell and Mellanby, 1953). A number of studies on a number of different *in vitro* systems followed in an attempt to elucidate the molecular mechanisms underlying the vitamin A-mediated regulation of differentiation. Originally, it was thought that cell surface alterations via a retinyl phosphate mannose intermediate may play an important role in this process (Adamo et al., 1979; DeLuca et al., 1979; Wolf, 1980). Later, it was discovered that even though retinol can participate in sugar transfer reactions, a number of other potent and active retinoid analogues, including retinoic acid, cannot (for a review, see Sporn and Roberts, 1983). We cannot rule out the possibility that retinoid-induced cell surface changes might play a role in eliciting some of the biochemical and morphological effects on LP-9 cells: these changes need not necessarily be exerted at the level of sugar transfer.

Another hypothesis for retinoid action suggested that retinoids might act in a manner analogous to steroid hormones (Chytil and Ong, 1979; Liau et al., 1981). Indeed, several cytosolic binding proteins have been identified: two for retinol and one for retinoic acid (Chytil and Ong, 1983). However, the sequences of the two cellular retinol-binding proteins have recently been determined (Sundelin et al., 1985; Li et al., 1986), and there is no resemblance to other steroid hormone receptors. Our finding that the kinetics of

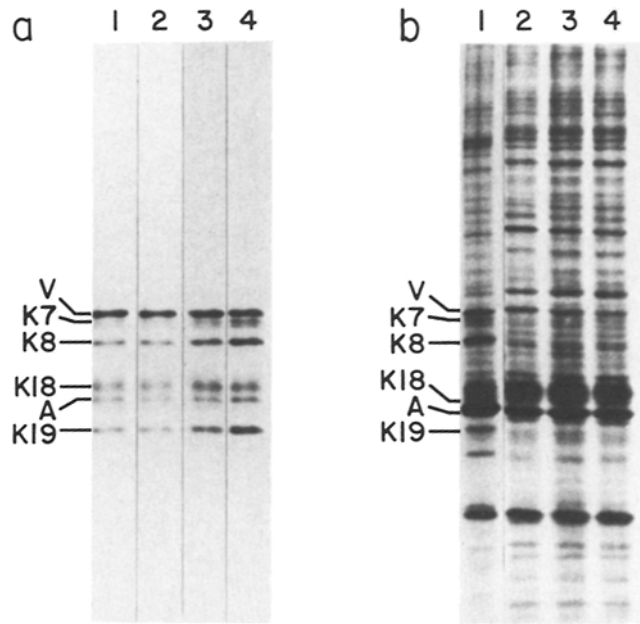


Figure 9. Effects of intercellular interactions on keratin and vimentin synthesis. (a) Effects of cell density on IF expression. LP-9 cells were plated at densities ranging from 50 to 500/cm 2 in the presence of normal serum-containing medium. After 5 d in culture, cells were radiolabeled for 4 h prior to extracting the intermediate filament proteins. At this point, cells seeded at 50/cm 2 were still exceedingly sparse, while cells seeded at 500/cm 2 were at three-quarters confluence. Radiolabeled proteins were resolved by SDS polyacrylamide gel electrophoresis and subjected to fluorography and autoradiography. IF extracts were from cells plated at original densities of: lane 1, 50/cm 2 ; lane 2, 100/cm 2 ; lane 3, 250/cm 2 ; lane 4, 500/cm 2 . IF proteins are indicated at left. V, vimentin; A, actin. (b) Effects of suspension in methyl cellulose on IF expression. Cells were cultured to one-half confluence in normal serum-containing medium. Cells were then trypsinized and transferred to medium containing 1.35% methyl cellulose and either delipidized serum, normal serum, or delipidized serum + 1×10^{-6} M retinoic acid. The cells were returned to the incubator for 72 h and allowed to adjust to suspension culture. [^{35}S]methionine was added, and total proteins were extracted 2 h later. Proteins were resolved by SDS polyacrylamide gel electrophoresis, and the gel was subjected to fluorography and autoradiography. Proteins were from: lane 1, cells before suspension; lane 2, cells switched to medium containing methyl cellulose and delipidized serum; lane 3, cells switched to medium containing methyl cellulose and normal serum; lane 4, cells switched to medium containing methyl cellulose, delipidized serum and 1×10^{-6} M retinoic acid.

the response of LP-9 cells to retinoids is relatively slow casts further doubt as to whether retinoid-protein complexes act directly on chromatin in mediating changes in the expression of the simple epithelial keratin genes.

Perhaps the best evidence to suggest that these cytoplasmic binding proteins play some role in potentiating the effects of retinoids is the characterization of a mutant in F9 teratocarcinoma cells that does not differentiate in response to retinoids and which is missing cellular retinoic acid-binding protein, a constituent of wild-type F9 cells (Schindler et al., 1981). Even so, cellular retinol- and retinoic acid-binding proteins have not been detected in all cell types that respond to retinoids (Douer and Koeffler, 1982; Libby and Bertram, 1982).

Hence, if these proteins are important in mediating the effects of retinoids, they do not appear to be universal in their control of retinoid-induced differentiation processes.

Among the plausible models for the action of retinoids is that they are involved in the comprehensive program of protein phosphorylation and growth regulation. Retinoids have been shown to increase cAMP-dependent protein kinase activity in several systems (Ludwig et al., 1980; Plet et al., 1982; 1986; Rogelj et al., 1984; Elias and Stewart, 1984). In F9 teratocarcinoma cells, relatively early events in retinoic acid-mediated differentiation include an increase in EGF receptor levels (Adamson and Hogan, 1984), the induction of the cellular proto-oncogene product c-fos (Griep and DeLuca, 1986), and the reduction of c-myc (Griep and DeLuca, 1986) and c-mos (Ogiso et al., 1986). Growth factors can have profound effects not only on cell growth, but also on differentiation (Carpenter and Cohen, 1979; Green and Shooter, 1980; Zullo et al., 1985). There are also a number of cases where the expression of growth factor-induced nuclear proteins, e.g., c-fos and c-myc, fluctuate in response to differentiation (Greenberg and Ziff, 1984; Greenberg et al., 1985; Campisi et al., 1984; Muller and Wagner, 1984). Whether any of these changes occur in mesothelial cells, and if so, to what extent they may represent principal and ecumenical cellular responses to retinoids has yet to be examined.

Roles of Growth Rate, EGF, Cell Shape, and Intercellular Interactions on Mesothelial Differentiation

The finding that retinoids are implicated in control of keratin expression and epithelioid morphology led us to reevaluate to what extent other factors, e.g., growth, EGF, cell shape, and cell-cell interactions, are important in regulating the differentiative processes of simple epithelial cells. Previously, it had been suggested that the increase in growth rate arising from EGF treatment of LP-9 cells may be directly responsible for the fibroblast morphology and preferential synthesis of vimentin (Connell and Rheinwald, 1983). Our results certainly confirm that EGF has a marked effect on suppressing keratin synthesis and enhancing vimentin synthesis. However, we find that medium containing delipidized serum is more proficient than EGF in promoting a highly elongated cell shape, an increase in vimentin synthesis, and a decrease in keratin synthesis. Since this medium substantially retards the growth rate of LP-9 cells, growth rate per se is clearly not sufficient to dictate either morphology or IF expression in these cells. That growth rate is relatively unimportant in defining the levels of vimentin and keratin is further supported by studies of Ben-Ze'ev (1984), who demonstrated that arresting cell growth in sparse and dense cultures of kidney epithelial cells does not influence the ratio of vimentin and keratin synthesis. Thus, even though EGF can suppress keratin levels and increase vimentin expression, the mechanism of its action may not be mediated solely by changing cellular growth rates.

A number of studies have previously suggested that vimentin synthesis is influenced by cell shape (Connell and Rheinwald, 1983; Ben-Ze'ev, 1983; Spiegelman and Farmer, 1982). To some extent, our studies are in agreement: the profound morphologic changes induced by culturing LP-9 cells

on medium containing delipidized serum are associated with a substantial increase in vimentin synthesis, which is even greater than that induced by EGF. Thus, whether LP-9 cells assume a fibroblastic morphology as a consequence of EGF treatment or as a result of growth in medium containing delipidized serum, both situations give rise to an increase in vimentin synthesis. However, although vimentin synthesis seems to prefer a fibroblast morphology, vimentin is still made even when LP-9 cells are epithelioid in normal medium, or when they are rounded in methyl cellulose suspension.

In contrast to vimentin synthesis, keratin synthesis seems to be dependent upon intercellular interactions. Even in normal media, neither sparse cultures of LP9 cells nor LP9 cells placed in single-cell suspension can maintain keratin synthesis. Ben-Ze'ev (1984) has also demonstrated that intercellular interactions are important for keratin synthesis in MDBK cells. However, cell-cell interactions alone do not seem to be sufficient for keratin expression: when LP-9 cells are cultured densely on dishes coated with poly-2-hydroxyethylmethacrylate (polyHEMA), which allows the formation of rounded, multicellular aggregates (Folkman and Moscona, 1978; Ben-Ze'ev, 1984), they still cannot synthesize abundant levels of keratins in medium containing delipidized serum (Kim and Fuchs, unpublished results).

In summary, we have discovered that both retinoids and EGF have profound and opposing roles in IF expression and differentiation in secondary cultures of human mesothelial cells. To sustain keratin expression, but not necessarily epithelioid morphology, intercellular interactions are also important. At present, the molecular mechanisms by which these factors influence human mesothelial differentiation, and the functional significance of the differential expression of keratin and vimentin in certain simple epithelial cells are still unknown. However, further investigations into the biochemical nature of these responses, and the points at which these processes go awry during malignant transformation, should lead to an increased understanding of the intricate pathways involved in epithelial differentiation.

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