The quest for the function of simple epithelial keratins

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Summary

Simple epithelial keratins K8 and K18 are components of the intracellular cytoskeleton in the cells of the single-layered sheet tissues inside the body. As members of the intermediate filament family of proteins, their function has been a matter for debate since they were first discovered. Whilst there is an indisputable case for a structural cell-reinforcing function for keratins in the mutilayered squamous epithelia of external barrier tissues, some very different stress-protective features now seem to be emerging for the simple epithelial keratins. Even the emerging evidence of pathological mutations in K8/K18 looks very different from mutations in stratified epithelial keratins. K8/K18-like keratins were probably the first to evolve and, whilst stratified epithelial (keratinocyte) keratins have diversified into a large group of keratins highly specialised for providing mechanical stability, the simple epithelial keratins have retained early features that may protect the internal epithelia from a broader range of stresses, including osmotic stress and chemical toxicity. ***BioEssays*** **25:748–758, 2003.**

Introduction

Epithelia are avascular sheets of closely packed, highly polarised cells that line and surround organs throughout the body. By definition, they lie at the interface between two dissimilar environments and provide a barrier that maintains these differences. The major stratified epithelia of the body (such as the epidermis of skin) have multiple cell layers, whereas simple epithelia have only one layer. Different types of epithelia are defined by their distinct profiles of keratin intermediate filament proteins, and simple epithelia are characterised by the expression of simple epithelial keratins, K8 and K18, as their major structural proteins.

Studies of human diseases caused by keratin mutations provide robust evidence for a mechanical function of keratins in stratified epithelia, but the data are less convincing for simple epithelial keratins. But if the primary function of simple epithelial keratins is not mechanical reinforcement, then what selective pressures could have driven their evolution? This question is the focus of this review, as it holds the key to our understanding of the function of a major group of structural proteins that probably define epithelial function.

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Simple epithelium: a widespread and fundamental tissue structure

Most stratified epithelia are located close to or at external surfaces, while simple epithelia (such as glandular and intestinal epithelia) are exclusively internal (Fig. 1). Simple epithelial cells all have a free apical surface and are all in contact with the basal lamina of extracellular matrix; in stratified epithelia, only the basal cells maintain basal lamina contact and these cells have no free surface. Unlike stratified epithelia, which primarily function as a physically robust barrier to the free movement of water and solutes, the simple epithelia combine functions of selective uptake, permeability and secretion in a barrier that is more physiological and less physical. Simple epithelia form the lining of secretory and absorptive organs such as the intestine, pancreas, kidney, liver and the many different types of glands. This tissue type is the first recognisable structure to appear in embryogenesis, forming the hollow sphere of cells that constitutes the preimplantation blastocyst. This develops when the first cluster of dividing cells compact and the cells become polarised. An epithelial sphere is likely to have been the first multicellular structure to form in the evolution of organisms (Fig. 2): an epithelial sphere of tightly adhering cells would allow a distinction between ''inside'' and ''outside'' environments, much like the preimplantation embryo, providing a context for further cell differentiation.

The position and polarity of epithelial cells, whether simple or stratified, is maintained by cell–cell and cell–substrate junctions. In simple epithelia, the basolateral and apical domains of the plasma membranes are separated by tight junctions, which restrict movement within the lipid bilayer and simultaneously locally occlude the intercellular space between cells. However, polarity can persist after the loss of tight junctions.[1] Other junction types provide mechanical continuity in both simple and stratified epithelia—the adherens junctions and desmosomes link the actin and keratin cytoskeleton, respectively, through cadherins in the membrane, to

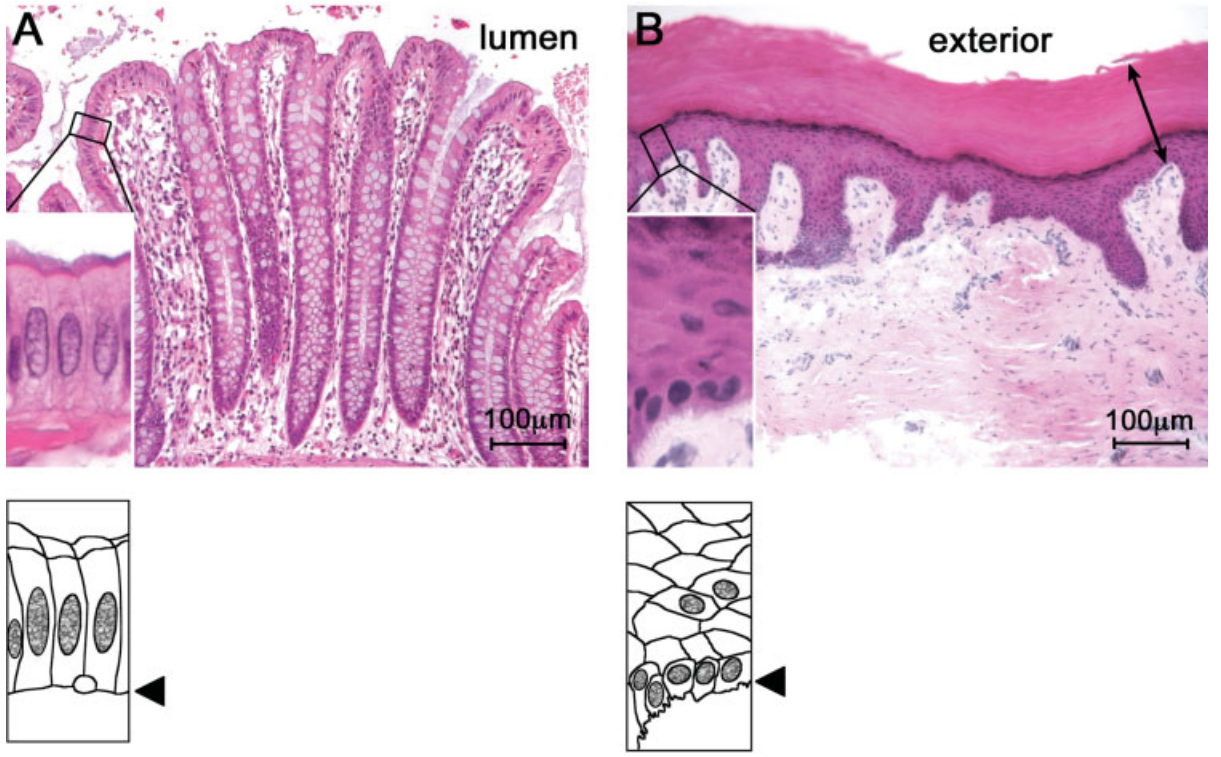

Figure 1. Simple versus stratified epithelial tissue structure. **A:** A typical simple epithelium, here from human colon, consists of a single layer of polarised cells (full thickness epithelium shown in inset). Apical surfaces of each cell are exposed to the (gut) lumen and the basal surfaces are in contact with the basal lamina (arrowhead on drawing) that separates the epithelium from the underlying connective tissue. **B:** In a stratified epithelium with multiple layers of cells, such as human epidermis shown here, the basal (proliferative) layer is in contact with the basal lamina (arrowhead on drawing). The rest of the tissue consists of postmitotic keratinocytes undergoing progressive terminal differentiation as they move towards the surface. Double headed arrow indicates full thickness of epidermis. Sections stained with haematoxylin and eosin.

provide an integrated system of mechanical support that holds the cells together.[2] Simple epithelial cells and the basal cells of stratified epithelia also interact with the basement membrane (basal lamina), through the actin-linked focal adhesions and keratin-linked hemidesmosomes, ultimately linking the cytoskeleton to the extracellular matrix through integrins, the heterodimeric transmembrane receptors for ligands in the extracellular matrix.[3]

Keratin expression and epithelial differentiation

Keratin intermediate filament proteins lie at the heart of epithelial differentiation. They are one of the first epithelial-specific structural proteins to be synthesised in a differentiation programme and are the most persistent. There are at least 49 keratin genes in the human genome[4] divided between two gene families (type I and type II) whose expression is inextricably linked. Keratin filaments will only assemble from type I–type II heterodimers so an epithelial cell must express at least one type I protein and one type II protein in order to form filaments. Epithelial tissues all show characteristic expression of specific pairs or subsets of keratins, in the same way as non-keratin intermediate filaments are differentiation-specific in non-epithelial tissues.

In differentiating epithelia, keratin expression proceeds through the initial (and persistent) expression of a pair of "primary" keratins, supplemented later by a pair of "secondary" keratins. Simple epithelia either express solely their "primary" keratins, K8 and K18, as in preimplantation embryos and adult hepatocytes, or they may acquire expression of additional simple epithelial keratins (K7, K19, K20) as their differentiation progresses. K7 is expressed in many gland ducts and internal epithelia (see Smith et al. Ref. 5). K20, a typical type I keratin, is expressed in gastrointestinal epithelia, urothelium and neuroendocrine cells.[6] K19 is an unusual keratin, lacking a proper tail domain[7] and this probably compromises its filament-forming ability.[8] Its expression in many incompletely differentiated cell types, including pluripotent

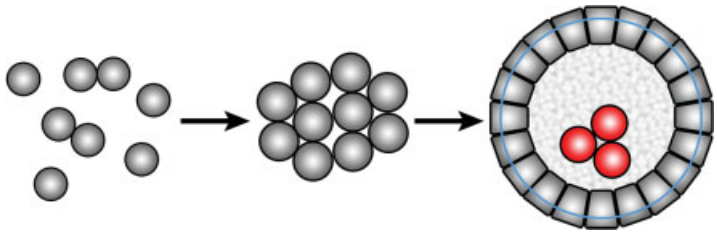

Figure 2. Model for simple epithelium as an early tissue in evolution. Failure of (solitary) cells to separate after division, due to increasing cell–cell adhesion mechanisms, would favour aggregates or clusters. Reinforced cell adhesion and cytoskeletal mechanisms (blue line) to stabilise the position of cell–cell contacts would facilitate the establishment of a persistent spherical structure. Distinction between "inside" and "outside" would be reinforced by any subsequent directionality in secretory and absorption processes. A regulated interior milieu would then provide a context for the subsequent evolution of non-epithelial differentiation pathways (red cells).

regions of hair follicle and mammary gland,[9–11] suggests that it might function as a transitional keratin in uncommitted cells and may help to identify stem-cell-containing regions.[11] An additional candidate simple epithelial keratin ("K23"), was recently reported in pancreatic epithelial cells,[12] but its protein expression has yet to be documented.

Although these keratins are all expressed in simple epithelia, their sequences clearly divide them into two groups. The primary simple epithelial keratins K8 and K18 are distinct in amino acid sequence from keratins of stratifying epithelia. K7 is closely related to K8, except for a very divergent head domain sequence.[5] K19 and K20, however, resemble epidermal type I keratins in their sequence[11,13] and are only distantly related to K18.

In contrast to simple epithelia, the primary keratins of stratified squamous epithelia are K5 (type II) and K14 (type I). Differentiating stratified squamous epithelia diversify more than simple epithelia, acquiring expression of at least two of 10 or more secondary keratins: K1/K10 in epidermis, K4/K13 in non-cornified epithelia (buccal or orogenital mucosa), K3/K12 in corneal epithelium, to name a few. Thus, secondary keratins are never seen as the first-expressed keratins but their expression is characteristic of the differentiated functional state of the epithelium. The rigorously tissue-specific pair-wise expression of keratins seen during differentiation strongly implies that each different epithelium, or subcompartment of an epithelium, needs the specific properties of a particular set of keratins in order to fulfil its differentiated function.

Conservation of K8/K18 genes and implications for evolution

Intermediate filament-like proteins have evidently been around for a long time, judging by the similarities that exist between vertebrate and invertebrate filament genes.[14] Of the modern intermediate filament genes, lamins were first proposed as the closest in structure to an ancestral filament gene,[15] although more evidence of keratin-like genes in some invertebrates, and even in lower chordates, is now emerging. Two evolutionary streams of intermediate filament genes have been recognised: lamin-like "long" rod proteins and keratin-like "short" rod proteins, which may have evolved in parallel after an early prechordate divergence.[14] Intermediate filament-like proteins with keratin characteristics have been identified in the tunicate *Styela*,[16] and in the worm *Caenorhabditis elegans*. In simpler organisms with fewer filament genes, like *C. elegans*, loss of some of these genes is lethal.[17]

Modern keratins account for about 75% of all intermediate filament genes and are encoded by two gene families that probably co-evolved. Each single keratin gene encodes a single keratin protein. The chromosomal distribution of keratin genes may have implications for their evolution. In humans, type I keratin genes (all but one on chromosome 17) and type II genes (all on chromosome 12) occur in tight clusters that could have arisen by local gene duplication. The gene for K18, *HKRT18*, is however not on chromosome 17 but on chromosome 12, where it is situated close to the K8 gene, *HKRT8*, in the type II cluster.[18] This observed juxtaposition supports the earlier interpretation by Blumenberg that K8 and K18 may have an ancient and common origin and may be the ancestral precursors of other keratin genes.[19] The gene cluster on chromosome 17 probably arose by a duplication and transposition of an ancestral K18 gene, followed by concerted duplication of keratin genes (and other gene families) in both locations. Subsequent divergent evolution would have given rise to keratins with different functional specialisations.

In contrast to the divergent evolution of more recent keratins such as the species-specific hair keratins in mammals,[19,20] the primary simple epithelial keratins K8 and K18 are strikingly conserved across a wide range of species. Comparisons of teleosts and mammals provide more evidence of this.[21,22] Sequence changes in K8 and K18 have been selected against, suggesting that these ancient keratins are indispensable.[19] One explanation for their high fidelity conservation through evolution may be that their expression is required very early in embryogenesis. During development,

K8 and K18 expression precedes that of all other cytoplasmic intermediate filaments and can be detected as early as the 4-cell stage in the developing mouse embryo.[23,24] Embryonic K8/K18 expression is well established in the preimplantation embryo long before more complex epithelial structures form.[25]

Keratin filament assembly

Polymerisation is an inherent property of the keratin proteins and does not require any catalysts or cofactors in vitro. In vitro, the initial stage is the formation of an in-register parallel type I–type II heterodimer (e.g., K18 + K8, or K14 + K5), triggered by coiled-coil interactions between the α-helical rod domains of the two proteins[26] (see Fig. 3 for protein domain structure). Coiled-coil type I–type II heterodimers then assemble into 10 nm filaments through a series of partially defined intermediates.[27] The degree of sequence conservation between keratin rod domains, particularly within the helix initiation and termination motifs at either end of the rod domain, indicates that the structural constraints of forming functional filaments tolerate little if any sequence variation.

Within cells, filaments tend to bundle along their length, the bundles merging and separating to generate a dense meshwork of anastomosing fibres that run through the cytoplasm and are linked peripherally into desmosomes and hemidesmosomes. The filament networks formed by some keratins of stratified epithelia, e.g., K1/K10, K4/K13 and the hair keratins, are further stabilised by disulphide bonding.[28,29] Disulphide bonding does not occur in simple epithelial keratins as they contain no cysteines in their sequence.[30]

Although there are substantial similarities between any two keratin pairs, the primary keratins of simple (K8/K18) and stratified epithelia (K5/K14) are probably the most divergent. K14 and K18 share only 48% sequence identity at the amino acid level.[31] Whilst K8/K18 form the most easily dissociated keratins, K5/K14 complexes are the least soluble in urea.[32] Despite these differences, studies using purified proteins have revealed that any type I keratin can probably make filaments in vitro with any type II keratin.[33] Measurements of the biophysical properties of different keratin polymers have shown relatively subtle differences,[34] but functional differences become much more apparent in vivo. For example, expression of K18 in the epidermis of K14-null mice revealed that K5/K18 filaments are not functionally equivalent to K5/K14 filaments[31] and cannot adequately rescue the phenotype. The full extent of keratin properties and functions will probably only emerge through careful in vivo studies.

How the epithelium remodels its keratins

Remodelling of intermediate filaments was first observed as the focal cleavage of perinuclear interphase rings of vimentin in mitotic endothelial cells.[35] Many epithelial cells show transient keratin disassembly during mitosis[36,37] and lamin dissociation is easily seen in all mitotic cells. Whereas actin and tubulin cytoskeleton components remodel their filament arrays by cycles of ATP/GTP hydrolysis, intermediate filaments use cycles of phosphorylation/dephosphorylation instead. Hyperphosphorylation of intermediate filaments drives the assembly equilibrium towards the depolymerised state[38] and phosphorylation is probably involved in reversible mitotic remodelling of all intermediate filament proteins. Phosphorylation may also protect the keratins against ubiquitination and degradation.[39]

The major phosphorylation sites in K8 and K18 are all serine residues located in the non-helical head and tail domains, as summarised in Fig. 3. Phosphorylation of these serine residues is increased in mitotic or stressed cells. Phosphorylation sites homologous to the K8 S74 (S73 if the initiating methionine is not numbered) also occur in K4, K5 and K6, but on threonine instead of serine.[40] The role of tyrosine phosphorylation of K8 is predicted to be minor in comparison with serine phosphorylation.[41]

K8 is also glycosylated,[42] but no other post-translational modifications have been documented for simple epithelial keratins. K8/K18 networks are, however, affected by interactions with other cytoplasmic proteins, which functionally fall

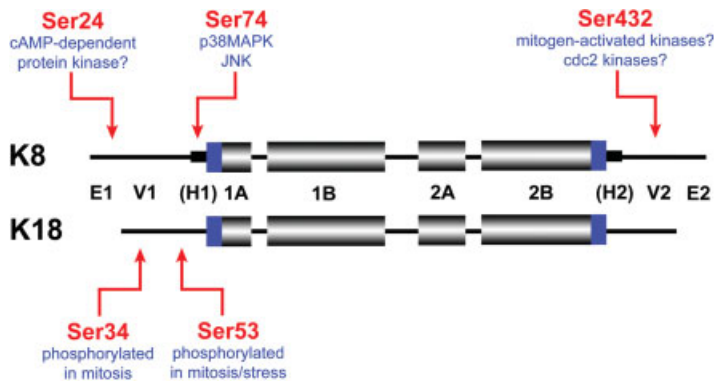

Figure 3. Structure of simple epithelial keratins K8 and K18. Schematic representations of the K8 (top) and K18 (bottom) protein molecules. The α-helical rod domain is flanked by non-helical N-terminal and C-terminal head and tail domains. Rod domains are subdivided into 1A, 1B, 2A and 2B, separated by non-helical linker regions. The highly conserved helix initiation and termination motifs at the rod domain ends are shaded purple. Head and tail domains are further subdivided into E1/E2 and V1/V2 regions, plus H1 and H2 segments that are absent from type I keratins. Phosphorylation sites (only found in the head and tail domains) are indicated along with the kinases known to act at these sites.

into two groups: (i) those with a structural role, including chaperones (that aid keratin folding and localisation) and cytolinkers (e.g., plakins, that link keratins to other cytoskeletal networks), and (ii) those involved in stress and apoptosis signalling. The interaction sites within the keratins for these various molecules are diverse, suggesting that multiple simultaneous interactions are possible (reviewed by Coulombe and Omary, Ref. 43).

Keratin aberrations and disease

Structural proteins such as keratins could give rise to disease either by expression of the "wrong" protein, i.e., a mutated version with incorrect function, or by expression of the right protein in the wrong place or time.

Expression of keratins in cancer

One of the strongest drivers of intermediate filament research has been cancer research and diagnosis. Epithelia are the origin of most human cancers (carcinomas), because epithelia are located at interfaces where trauma is highest and their cells must divide frequently so as to be able to shed and replace damaged cells. Such stressed, proliferative cells are vulnerable targets for carcinogenic transformation and account for over 90% of human cancers.

Most of these carcinomas arise on external skin surfaces where they are likely to be detected early and can be surgically removed, but carcinomas arising in the internal simple epithelia are less accessible and more difficult to diagnose and treat. Identification of tumour-specific antigens has therefore been a major challenge of cancer research for many years. Keratin proteins are stable, effective immunogens for generating monoclonal antibodies and keratin antibodies were among the earliest markers of simple epithelia[44,45] and simple epithelial cancers.[46,47] Several simple epithelial keratin antibodies have given rise to diagnostic kits in use today (TPA, CYFRA-21, Ref. 48 M30, Ref. 49), mostly based on the detection of protease-resistant keratin fragments released from necrotic (and apoptotic) cancer cells and shed into the circulation.

On the whole, keratin expression relates well to the tissue of origin, can identify the source of metastases and in some cases can contribute to prognoses.[50] However, K8 and K18 are often expressed ectopically in squamous cell carcinomas arising from stratified epithelia, especially in cancers with a less differentiated, more invasive phenotype and reduced expression of differentiation-specific keratins.[51] Aberrant expression of K8/K18 in squamous carcinomas may simply be a passive indicator of loss of differentiation, or a positively selected change in differentiation that favours tumour progression. There is evidence from cell cultures that co-expression of K8/K18 with the type III intermediate filament vimentin is correlated with increased migration (reviewed in Hendrix et al., Ref. 52). This raises the possibility that, as well as the diagnostic and prognostic value of simple epithelial keratins, K8 and K18 may also be potential targets for "anti-invasion" therapies.

Genetic skin disorders reveal a mechanical function for epidermal keratins

In the stratified epithelia of external barrier tissues, a major function of the keratin cytoskeleton is physical reinforcement of the cells in which they are expressed. This has been repeatedly demonstrated by the effect of dominant negative mutations in epidermal keratins K5 or K14 in the blistering epidermis of *epidermolysis bullosa simplex* (EBS), or in most other keratin pairs in a diverse range of disorders now recognised as related to EBS (reviewed by Irvine & McLean, Ref. 53). These diverse epidermal "keratinopathies", with phenotypes ranging from twisted hair, thick nails, corneal blisters and white plaques in the mouth to blistering skin all over the body, have the common feature of fragile epithelial cells and cell breakdown (cytolysis) upon physical trauma, and are mostly caused by missense point mutations in one of the keratins expressed in the affected cell population. The severity of the disease is influenced by the location and nature of the amino acid substitution within the protein (Fig. 4), with mutations affecting the boundaries of the rod domain being particularly disruptive.

Whether simple epithelial keratins perform the same structural reinforcing role in simple epithelia is less clear, as there are still no simple epithelial fragility disorders definitively proven to be caused by keratin mutations (but see below). The genes for K8 and K18 must be subject to the same mutation rate as the rest of the genome, so why, after twelve years of

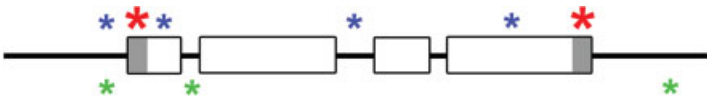

Figure 4. Positions of known mutation clusters in keratins. Locations of the mutation clusters seen in stratified epithelial keratins (above, red and blue asterisks) are shown in comparison with location of mutations found so far in simple epithelial keratins K8 and K18 (below, green asterisks). Hotspots associated with severe disease affect the ends of the rod domain (large red asterisks; cluster sites linked to milder phenotypes are shown by small blue asterisks. None of the mutations identified in simple epithelial keratins lie in the α-helical rod domains. By analogy with the mutations in stratified epithelial keratins, the K8/K18 sequence changes are predicted to have a mild effect.

studying keratin disorders, is the evidence for pathogenic mutations in K8 or K18 still so thin? Three explanations come to mind. Firstly, mutations in these keratins may be harmless as internal epithelia are not under the same physical stress as the skin and its appendages. Secondly, mutations in K8 or K18 may be lethal, as the expression of these keratins is required at the earliest stages of development. Thirdly, such mutations may be pathogenic but clinically cryptic: fragility in the internal tissue may not be detected until the patient is suffering from the downstream consequences, which may be a more serious pathology in which the role of a fragile epithelium is no longer obvious. Evaluation of mouse models of simple epithelial keratin defects may shed some light on this issue.

Effects of simple epithelial keratin defects as seen in mice

Several mouse strains have been generated in which simple epithelial keratin expression has been modified or ablated, but these have not always been easy to interpret because of the overlap in keratin expression between K18/K19 and K8/K7. The possibility that K8/K18 deficiencies might be lethal seemed to be borne out by the first report of mice lacking K8, which mostly failed to survive beyond mid-gestation.[54] On a different genetic background (FVB/N) about 55% of $K8^{-/-}$ offspring were viable but developed colorectal hyperplasia, whilst $K8^{-/-}$ females, although able to produce fertile eggs and to generate a decidual response, could not carry a pregnancy to term.[55] The current favoured hypothesis to account for this difference is that another closely related type II keratin, K7, can substitute for K8[56] if expressed early enough, but that in some mouse strains K7 is not expressed in time to rescue the $K8^{-/-}$ phenotype.

$K18^{-/-}$ mice had a milder phenotype than their $K8^{-/-}$ counterparts, being viable, fertile, having a normal lifespan and generally showing no difference from their wild-type littermates for the first 4 months of life.[56] Normal keratin filaments were observed in internal tissues, indicating that the other type I keratins expressed in these tissues, K19 and/or K20, could functionally substitute for K18. Although ablation of K19 expression alone has no particular phenotypic consequences while K18 is present,[57] K18/K19 double knockout mouse embryos (i.e., expressing no simple epithelial keratins in early development) were smaller than normal and only developed until ~E9.5, when they died due to placental failure, possibly trophoblast cell rupture.[58] A K8/K19 double knockout was also lethal, even in the FVB/N background.[57]

Curiously, loss of simple epithelial keratins is also associated with increased susceptibility to toxic liver damage - a phenomenon that is not obviously attributable to physical stress at first sight. In $K18^{-/-}$ mice, hepatocytes are devoid of keratin intermediate filaments. By 18 months, keratin aggregates called Mallory bodies (normally associated with alcoholic liver cirrhosis) have formed in these cells from the K8 synthesised in the absence of any type I partner, i.e., when filament formation cannot take place. Without K8, or where there is a relative excess of K18 over K8, Mallory bodies do not form during experimental hepatotoxicity regimes; toxic liver damage is then dramatically increased. Thus simple epithelial keratins seem to have a protective role in the liver.[59,60]

Expression of a dominant K18 mutant (hK18 R89C) in mice produced disruption of the endogenous K8/18 filaments,[61] particularly in the pancreas and liver. No pancreatic pathology was seen even under conditions of stress, but these mice developed chronic hepatitis as they aged. Younger animals were also more susceptible to the hepatotoxins griseofulvin and acetaminophen than wild-type mice.[62]

Thus, deleterious mutation or absence of a single simple epithelial keratin appears to predispose to hepatotoxicity, infertility or colorectal hyperplasia, rather than causing lethal multisystem failure. Simple epithelial keratin defects can predispose to tissue damage without apparently disrupting the architecture of the intracellular filament network, although histological results from snapshot sampling may of course fail to detect more subtle effects, such as delayed filament reassembly after mitosis. The clearest conclusions from the mouse studies come from combination knock-outs in which keratin filaments have been totally ablated, by deleting all possible genes of one type: no filaments are formed and "unpaired" keratins are rapidly degraded. Genotypes that totally fail to form early embryonic keratin filaments are not viable, due to fragility in their invasive placental trophoblast cells leading to placental insufficiency and failure of embryonic growth. Functional distinctions between different type I keratins, or between different type IIs, have not emerged from this work. The implicit ability for simple epithelial keratins to functionally substitute for one another, plus the lethal effect of lack of filaments, suggests that persistence of multiple simple epithelial keratin expression in one cell is more of an evolutionary insurance policy than true redundancy in the system.

K8/K18 mutations in human pathology

The mouse models raised the possibility that there might be human diseases affecting liver or colon caused by mutations in the simple epithelial keratins, analogous to the keratinopathies of the epidermis. This hypothesis was supported by the finding of cryptogenic cirrhosis patients carrying mutations in K8 or K18.[63,64] We have recently observed keratin 8 mutations in a subset of patients with inflammatory bowel disease (Owens et al., unpublished observations). Significantly, the mutations found in these two groups of patients fall outside the rod domains and do not affect the helix boundary peptides (Fig. 4). By comparison with mutations affecting the keratins of stratified epithelia, these would be predicted to be "mild" mutations, with only minor effect on the filament network. However, if cells expressing mild keratin mutations are induced to reorganise

the network in response to stress, then defects in the properties of these keratins do become apparent.[65] A significant finding is that very similar or even identical mutations can predispose to diseases in other tissues. For example, the mutation K8(G62C) has been identified in cirrhosis patients,[64] as well as patients with Crohn disease or ulcerative colitis (Owens et al., unpublished observations).

Thus there is as yet no direct relationship between simple epithelial keratin mutations and a human disease, unlike the keratin mutations in the keratins of stratified epithelia. "Severe" (rod end) mutations as seen in epidermal keratins could well be lethal in humans when occurring in K8/K18, whereas the predicted "mild" mutations that have been found in K8/K18 may not generate any pathology by themselves. Inflammatory bowel diseases are well known to be polygenic,[66] so that K8/K18 sequence changes are only likely to represent one predisposing factor to disease. Whether a patient succumbs to disease might then depend on the presence of sufficient other genetic, or environmental, risk factors.

Possible functions of simple epithelial keratins

So is there sufficient information available yet, to explain the function and heterogeneity of simple epithelial keratins? Three emerging models for intermediate filament function suggest: (i) that keratin filaments are essential for maintaining epithelial polarity, (ii) that simple epithelial keratins contribute to the apoptotic pathway, and/or (iii) that they do play a significant role in protection of cells against mechanical stress, as the epidermal keratins appear to do. These are discussed below.

K8/K18 may help to maintain cell polarity

Cell polarisation is a key defining feature of epithelial cells and polarity must be maintained at all times, and especially in simple epithelia, which perform specialised directional secretory and absorptive functions that are dependent on the correct subcellular distribution of key components. Polarised functions such as secretion are specific to simple epithelial cells that express K8 and K18, and active secretion may depend on how flexible the cytoplasmic keratin networks are. For example, a highly stable keratin network like K1/K10 could impede membrane trafficking.

The transcytoplasmic network of keratin filaments is intercalated into desmosomes at cell boundaries to produce a three-dimensional web through the tissue. Keratin distribution is often observed to be asymmetric in simple epithelia, with filaments usually concentrated towards the apical surface[67] and there is growing evidence that keratins can directly contribute to the maintenance of cell polarity. In enterocytes, keratin 19 was shown to be concentrated at the apical cortical region of the cell[68] and, when depleted, the polarity of several enterocyte components was altered.[68] In mouse intestine, K7 is restricted to the crypts and goblet cells, and $K8^{-/-}$ mice lose keratins from villus enterocytes, concomitant with a loss of apical membrane markers and disorganisation of microtubules.[69]

In a single-layered tissue containing proliferating cells, mechanisms must also exist to ensure the correct placement of daughter cells without breaching the epithelial barrier during mitosis. Specifying daughter cell location may determine whether an epithelium extends laterally as a monolayer or becomes a multilayered stratifying epithelium, and this may be influenced by the properties of keratins. Desmosomes are retained through mitosis but keratin networks are transiently interrupted[36,37] by phosphorylation, allowing desmosome adjustment and daughter cell repositioning. As phosphorylation sites differ between keratins, some keratins will be remodelled differently from others during mitosis. It is thus possible that the keratin expression profile could determine the ability of an epithelium to stratify. Simple epithelial keratins have several options for phosphorylation (Fig. 3) whilst, at the other extreme, some keratins in stratifying epithelia may even become secondarily refractory to the solubilising effects of phosphorylation, due to irreversible modifications like disulphide cross-links (e.g., hair keratins, K1/K10). This would probably inhibit the cell's ability to divide, and evidence for mitotic inhibition by K10 has indeed been reported.[70,71]

The position and distribution of simple epithelial keratin filaments reflects an intimate relationship with cell polarity, which existing filament remodelling mechanisms would allow the cell to maintain, through mitosis and postmitotic function. Whether this relationship is unique and fundamental enough to maintain the evolutionary conservation of K8 and K18 genes that we observe today, remains to be seen.

K8/K18 may contribute to apoptosis

It has been shown that many simple epithelial cells will die by apoptosis if they become detached from extracellular matrix.[72] This is distinct from keratinocytes of stratified epithelia, which remain metabolically active for several days after becoming detached from the basal lamina, although keratinocytes may eventually undergo a modified form of apoptosis.[73] Simple epithelial keratins K8 and K18 appear to interact with the process of apoptosis in two ways.

Simple epithelial keratins as targets in apoptosis. The type I simple epithelial keratins are early targets of caspase activity in apoptosis. Cleavage of one keratin will destabilise the filament network due to the heteropolymeric nature of the keratins. K18 and K19, but not keratin 8, are hyperphosphorylated and then cleaved into two stable fragments: 29kDa/23kDa for K18 and 28kDa/20kDa for K19.[39,74] These fragments remain associated with the K8 in the insoluble fraction. This fragmentation has been studied most thoroughly for K18 and it is now known that there are two

caspase cleavage sites in this keratin as well as in K19. Caspase 3 and/or caspase 7 first acts at the second aspartate in the sequence "DALD" in the tail of K18, very early in the process. This is followed by cleavage in the L12 domain at the sequence "VEVD" by caspase 8. The caspase 3/7 cleavage precedes, but is not required for, cleavage at the caspase 8 site. Interestingly, the second cleavage, but not the first, is inhibited by K18 hyperphosphorylation.[75] The early cleavage by caspase 7 creates a neo-epitope in K18, which is recognised by a monoclonal antibody M30, and M30 reactivity provides a useful tool for monitoring early apoptosis in carcinoma cells in the analysis of tumour cell response to anticancer drugs.[49] The role of keratin 18 fragmentation by caspases may facilitate apoptotic cell clearing by allowing the keratin network to be dismantled.

K8/K18 as modulators of apoptosis. Recent studies have suggested that K8/18 are intimately involved in modulating and attenuating cellular responses to pro-apoptotic stimuli. The K8/K18 keratin pair can desensitise cells to pro-apoptotic signalling mediated by tumour necrosis factor-α (TNFα)[76] or by Fas ligand,[77] by binding to their receptors. K18 also binds a downstream TNFR effector, TRADD.[78] Sequestration of signalling components by binding them to the cytoplasmic keratin network could interrupt the signalling cascade, as has been shown for Fas.[77] This reveals an entirely novel function for the simple epithelial keratins.

This inhibition of proapoptotic signalling provides some potential explanation for the K8/K18 knockout mouse phenotypes. For example, it is possible that the bowel phenotype observed in K8-null mice is triggered by TNF-α-mediated epithelial apoptotic damage. Involvement of TNF-α in human inflammatory bowel disease is suggested by the increased frequency of TNF-α promoter polymorphisms predicted to increase TNF-α production in IBD patients.[79] This is further supported by the efficacy of humanised anti-TNF-α antibodies in the treatment of Crohn disease (reviewed by van Deventer, Ref. 80).

The evidence that Fas/Fas ligand are involved in hepatotoxin-induced apoptosis[81] provides a possible explanation for the sensitivity of $K8^{-/-}$ and $K18^{-/-}$ hepatocytes to certain hepatotoxins. Stimulation of the Fas receptor of intestinal epithelial cells induces activation of the stress kinase JNK and phosphorylation of K8 on serine-73.[82] This coincides with the association of some JNK with K8 and a decreased ability of JNK to phosphorylate c-jun. Thus, the presence or absence of K8/K18 could also modulate signalling from Fas at this stage in the pathway, as well as by interfering with receptor targeting.

Involvement in signalling pathways, particularly those involved in apoptosis and responses to cell stress, may be an important emerging aspect of simple epithelial keratin function. An ability to attenuate or control the timing of apoptotic cell destruction may be important in epithelial tissues, especially single-layered simple epithelial, where the maintenance of an intact barrier at all times is paramount.

K8/K18 may protect against mechanical stress

From the compelling results of studies on human genetic skin disorders, the most likely function of intermediate filaments in simple epithelia would be to provide mechanical reinforcement to cells in tissues. The placental defects observed in mice lacking simple epithelial keratins, and possibly the colonic hyperplasia phenotype, could be interpreted as supporting this. However, it is harder to see how a mechanical weakness would predispose towards hepatotoxicity. Internal tissues are not subject to the same immense shear, compressive, abrasive and tensile stresses as the epidermis. If simple epithelial keratins are providing any physical reinforcement, against what forces are they protecting the cells?

One form of physical stress that must be very common in internal epithelia is that caused by osmotic stress. Local or widespread fluctuations in osmotic conditions must commonly occur at the free surface of the epithelial cells, or ischaemic trauma could lead to osmotic imbalance if membrane ion pumps falter. A persistent keratin cytoskeleton would help a cell maintain its tissue position during osmotic swelling or shrinkage and provide a stress-resistant framework upon which to recover its structure and shape after regulatory volume recovery. There is evidence that cells with deficient intermediate filaments are less resistant to osmotic stress.[83,84] We recently suggested that intermediate filaments may have evolved to preserve tissue structure during osmotic shock, because both the actin and tubulin filament systems of the cell are transiently disrupted at this time.[84] Toxic insults to cells may also result in osmotic imbalance as anything that slows or shuts down a cell's metabolism will result in a drop in energy and a shutting down of membrane ion pumps. It is conceivable that osmotic protection is the key feature of the chemoprotective effect of keratin filaments in liver, although the effect of Mallory bodies probably requires another explanation.

Osmotic stress must be a hazard as old as multicellularity itself, and may have been a more important driving force in the early evolution of intermediate filaments than the extreme mechanical pressure we see today operating on the external tissues of large terrestrial vertebrates.

Conclusions

Different keratin genes are specifically expressed in subsets of a wide range of different epithelia. Just as these different tissues perform different functions, so their keratins have presumably evolved to provide tissue-specific properties. Simple epithelial keratins sit at one extreme of the keratin spectrum: remodelled by phosphorylation, highly dynamic, expressed in undifferentiated cells, and with no evidence of reinforcing cross-linking potential. At the other end of the spectrum would be the hair keratins that mature into disulphide

cross-linked polymers—structures so mechanically robust that they have persisted from prehistoric times until today. The keratins of stratified epithelia fit in between these two extremes.

K8 and K18 may contribute to cellular reinforcement, but their function does not appear to be entirely mechanical. Since these are almost certainly the oldest keratins, and the most highly conserved, one must conclude that keratin evolution was not initially driven by the need for resistance to shear or compression stresses of the type that later drove the evolution of the epidermal and hair keratins. It seems more likely that intermediate filament precursors may have evolved to provide resistance against osmotic stress, for early organisms in an aqueous environment. As these organisms became more complex and acquired motility, progressively more extreme physical properties of such filaments would have been selected for, to provide mechanical strength and protection against increasing levels of stress on cells, such as torsion and flexing. Further divergent evolution might then have provided a varied intracellular scaffold or framework for tissue differentiation, allowing the development of larger body size and greater tissue forces concomitant with the loss of hydrostatic support and increase in friction of life on land. Throughout this evolutionary process, as the integument became impermeable and highly specialised, internal epithelia remained more like ancestral epithelia, multifunctional and still subject to osmotic challenge. Thus the ancient simple epithelial keratins K8 and K18 have remained conserved.

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