Keratin-8 null mice have different gallbladder and liver susceptibility to lithogenic diet-induced injury

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Summary

Keratin transgenic mouse models and the association of human keratin mutations with liver disease highlight the importance of keratins in protecting the liver from environmental insults, but little is known regarding keratins and their function in the gallbladder. We characterized keratin expression pattern and filament organization in normal and keratin polypeptide-8 (K8)-null, K18-null and K19-null gallbladders, and examined susceptibility to liver and gallbladder injury induced by a high-fat lithogenic diet (LD) in K8-null mice. The major keratins of normal mouse gallbladder are K8>K19>K18 which become markedly depleted in K8-null mice with minor K18/K19 remnants and limited K7 over-expression. Compensatory K18/K20 protein and RNA overexpression occur in K19-null but not in K18-null gallbladders, probably because of the higher levels of K19 than K18 in normal gallbladder. LD challenge causes more severe liver injury in K8-null than wild-type mice without altering keratin protein levels. In contrast, wild-type and K8-null gallbladders are equally susceptible to LD-induced injury and stone formation, but wild-type gallbladders do over-express keratins upon LD challenge. LD-induced injury triggers keratin hyperphosphorylation in wild-type livers and gallbladders. Hence, mouse gallbladder K8/K18/K19 expression is induced in response to cholelithiasis injury. A high-fat LD increases the susceptibility of K8-null mice to liver but not gallbladder injury, which suggests that keratin mutations may increase the risk of liver damage in patients with steatohepatitis. Differences between K8-null mouse gallbladder and hepatocyte susceptibility to injury may be related to their minimal versus absent keratin expression, respectively.

Key words: Keratin, Gallbladder, Liver, Lithogenic diet, Gallstone, Tissue injury

Introduction

Intermediate filament (IF) proteins represent one of the three major cytoskeletal networks that are expressed in most mammalian cells. Unlike the two other major cytoskeletal families, actin microfilaments and tubulin microtubules, the five main subfamilies of IF proteins manifest significant tissue and differentiation-specific diversities (Fuchs and Weber, 1994; Ku et al., 1999). Based on their genomic structure and amino acid sequence homology, all IF proteins share the common structural features of N-terminal 'head' and C-terminal 'tail' non-α-helical domains, and a highly conserved central α-helical 'rod' domain (Herrmann and Aebi, 2000). Keratins, which consist of more than 20 unique polypeptides (K1-K20), are the largest subgroup of IF proteins and are preferentially expressed in epithelial cells (Coulombe and Omary, 2002; Moll et al., 1982). Most if not all epithelial cells express at least one type I (K9-K20) and one type II (K1-K8) keratins that form obligate non-covalent heteropolymers (Hatzfeld and Franke, 1985; Steinert et al., 1976). Keratin heteropolymers form extended cytoplasmic filament networks that radiate from the nuclear periphery to the cell surface. An important unique feature of keratins is their epithelial cell-type-specific expression. For example, hepatocytes express only K8/K18, enterocytes express primarily K8/K19 with low levels of K7/K18/K20 (Zhou et al., 2003), and keratinocytes express primarily K5/K14 basally and K1/K10 suprabasally (Moll et al., 1982).

The functional significance of epithelial cell-specific keratin expression remains unclear but mutations in specific keratins result in cell and tissue-specific diseases that parallel the expression profile of the involved keratin (Coulombe and Omary, 2002; Irvine and McLean, 1999). For example, mutations in K5 or K14 cause epidermolysis bullosa simplex, mutations in K1 or K10 cause epidermolytic hyperkeratosis, and mutations in K8 or K18 are associated with the development of cryptogenic and noncryptogenic forms of liver disease (Irvine and McLean, 1999; Ku et al., 2001; Ku et al., 2003b). The association of K8 and K18 mutations with liver disease meshes well with the clearly defined function of keratins, including K8 and K18, in protecting cells from mechanical and nonmechanical forms of injury (Coulombe and Omary, 2002; Marceau et al., 2001; Omary et al., 2002). The functional association of K8 and K18 with cytoprotection in hepatocytes is supported by numerous studies in transgenic

animal models that over-express a dominant-negative K18 mutation or that are null for K8 or K18 (Magin et al., 2000; Marceau et al., 2001; Omary et al., 2002). For example, hepatocytes isolated from K8- and K18-null mice lack any keratin filaments, and livers of K8-null mice are highly susceptible to liver injury as compared with their heterozygous or wild-type (WT) controls (Baribault et al., 1994; Caulin et al., 2000; Gilbert et al., 2001; Loranger et al., 1997; Magin et al., 1998; Toivola et al., 1998; Zatloukal et al., 2000). The importance of keratins as cytoprotective proteins in most epithelia is clearly evident from the growing list of diseases that are caused by mutations in the unique keratins that are expressed in such epithelia (Coulombe and Omary, 2002; Irvine and McLean, 1999; Marceau et al., 2001; Omary et al., 2002). This keratin-related cytoprotective function and their involvement in human diseases are also supported by the phenotypes of several keratin-null or dominant-negative animal models, involving epithelial cells of the skin, liver, cornea and oral cavity (Coulombe and Omary, 2002; Magin et al., 2000). One clear exception, however, appears to be epithelial cells of the exocrine pancreas. For example, although pancreatic acinar cells and hepatocytes lack keratin cytoplasmic proteins in K8-null mice, pancreata of K8-null mice are equally susceptible to injury as wild-type mice when tested using two injury models (Toivola et al., 2000). The apparent dispensability of keratins in the pancreas, in marked contrast to the liver, raises the possibility that identical keratins may serve different functions in different tissues and led us to test the gallbladder response to injury in the context of keratin absence or perturbation.

Little is known regarding keratin expression in the gallbladder, except for limited analysis in human (Moll et al., 1983) and mouse (Katayanagi et al., 1998) gallbladders, and the description of ectopic K20 expression in some human gallbladder adenocarcinomas (Duval et al., 2000). For example, human gallbladder epithelia express primarily K8 and K19 with lower, but easily detectable levels of K7 and K18 as determined by two-dimensional gel analysis (Moll et al., 1983). In addition, keratins have been described in cultures of mouse gallbladder epithelial cells using anti-pan-keratin antibodies but their specific individual keratin profile is not known (Katayanagi et al., 1998). In the present study, we first analyzed the keratin expression profile in normal mouse gallbladder and in gallbladders of mice null for K8, K18 or K19. We then compared the cytoprotective function of keratins in gallbladders and livers of K8-null, heterozygous and WT mice using a high-fat lithogenic diet injury model. We focused on K8-null mice, rather than K18-null or K19-null mice, since their hepatocytes and their gallbladder epithelial cells had the most dramatic cytoplasmic keratin filament perturbations.

Materials and Methods

Antibodies

The antibodies (Ab) used included monoclonal (m) rat anti-mouse K8 (mAb Troma I, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); rat anti-mouse K18 (mAb Troma II) and rat anti-mouse K19 (mAb Troma III) (Boller et al., 1987); mouse anti-K7 (mAb RCK 105; ICN, Aurora, OH); antibodies directed to K20 (K20.8), actin (ACTN05) and tubulin (Cocktail) (NeoMarkers; Fremont, CA). Other previously described antibodies: rabbit anti-human/mouse K8/K18 (Ab 8592), mAb LJ4, which recognizes human K8 phospho(p)-Serine(S)73 and the corresponding mouse phospho-epitope (K8 pS79), also mAb 5B3, which recognizes human K8 pS431 and mouse K8 pS436 (Ku and Omary, 1997; Liao et al., 1997).

Mice and the high-fat lithogenic diet

K8-null (Baribault et al., 1994), K18-null (Magin et al., 1998) and K19-null (Hesse et al., 2000; Tamai et al., 2000) mice were used. The K8-null and K19-null mice had an FVB/n genetic background while the K18-null mice had a mixed 129/Sv and MF-1 background. BALB/c and FVB/n mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were bred and housed with strict infection control, and received humane care in compliance with recommendations of the American Veterinary Medical Association. Genotyping to distinguish null (–/–), heterozygous (+/–), and wild-type (+/+) mice was carried out using polymerase chain reaction (PCR) analysis of mouse-tail DNA and specific primers. Breeding was done using heterozygous mice to generate –/–, +/– and +/+ progenies, and littermates of the same breedings were used for the described experiments. Male mice with genotypes of $K8^{-/-}$, $K8^{+/-}$ and $K8^{+/+}$ (n=10-13 in each group; 4- to 6-weeks old) were fed, for 5 weeks, a high-fat lithogenic diet (cat. no. 960393, ICN Biomedicals, Aurora, OH) containing 1.23 g cholesterol, 0.48 g sodium cholate, 17.84 g butter fat, 0.98 g corn oil, 48.33 g sucrose, 19.33 g casein, essential vitamins and minerals (Nishina et al., 1990; Wang et al., 1999). All mice were allowed free access to water, and normal mouse chow was purchased from Dean's Animal Feeds (Belmont, CA). Gallstone formation was assessed by visualization of the isolated gallbladders using a Prior™ dissection microscope (Prior Scientific Instrument Ltd, England). Of note, female FVB/n mice are resistant to gallstone formation (http://www.jax.org/index.html).

Keratin isolation and biochemical analysis

Tissues were homogenized with a Teflon homogenizer (4°C 200 strokes) in phosphate-buffered saline (PBS, pH 7.4) containing a protease inhibitor cocktail (0.1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 10 μM leupeptin, 10 μM pepstatin), and 5 mM EDTA. Homogenates were pelleted to remove any remaining tissue debris, and the protein concentration was measured using a BCA protein assay kit (Pierce; Rockford, IL). The protein was then mixed with an SDS-polyacrylamide gel electrophoresis (PAGE) Laemmli sample buffer (Laemmli et al., 1970). Equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes followed by immunoblot analysis (Towbin et al., 1979). Protein bands were visualized using enhanced chemiluminescence.

Keratins from mouse liver, gallbladder and small intestine were isolated by high-salt extraction (HSE) (Achtstaetter et al., 1986; Ku et al., 1995). Briefly, tissue fragments were disrupted with a Teflon homogenizer using PBS containing 1% Triton X-100, 5 mM EDTA and the protease inhibitor cocktail (Ku et al., 1995) followed by centrifugation. The resulting pellet was further homogenized with 200 strokes in HSE buffer (140 mM NaCl, 1.5 M KCl, 5 mM EDTA, protease inhibitor cocktail, in 10 mM Tris-HCl, pH 7.6) then incubated for 30 minutes (4°C). After repelleting, the remaining insoluble IF-enriched preparations were dissolved in SDS-containing sample buffer for analysis by one-dimensional SDS-PAGE or in urea-containing sample buffer for analysis by two-dimensional isoelectric focusing then SDS-PAGE.

Reverse transcription PCR (RT-PCR) and Real time PCR

RNA was isolated from wild-type or null K19 mice gallbladders using an RNeasy midi kit (Qiagen; Chatsworth, CA). Total RNA from three mice was used as template and first strand cDNA was synthesized with random primers and SuperScript II reverse transcriptase (Invitrogen; Carlsbad, CA). Standard PCR was performed using mouse-specific intron-spanning primers for each

Table 1. Sequences of primers used for RT-PCR and real time PCR

	Sense primers (5′ to 3′)	Antisense primers (5′ to 3′)	Product size (bp)
RT-PCR			
K8	TGCAGAACATGAGCATTC	CAGAGGATTAGGGCTGAT	342
K18	GACGCTGAGACCACACT	TCCATCTGTGCCTTGTAT	119
K19	GCTATGTCTTCCTTTGGGGG	TTCTTCAGGTAGGCCAGCTC	630
K20	GTTCAGTACGCATTGGGTCA	CTGAAGTCATCTGCAGCCAG	390
Real time PCR			
K8	GGACATCGAGATCACCACCT	TGAAGCCAGGGCTAGTGAGT	155
K18	ATTGCCAGCTCTGGATTGAC	GTCTCAGCGTCCCTGATTTC	200
K19	CGGTGGAAGTTTTAGTGGGA	AGTAGGAGGCGAGACGATCA	101
K20	CCCAGAAGAACCTGCAAGAG	ACGAGCCTTGACGTCCTCTA	210
L7	GAAAGGCAAGGAGGAAGCTCATCT	AATCTCAGTGCGGTACATCTGCCT	80

keratin (Table 1). 18S rRNA was used as an internal control for the RT-PCR and amplified PCR products were analyzed using 1% agarose gel electophoresis. Real time quantitative PCR was performed using an ABI Prism 7900HT Sequence Detection System (PE Biosystems, Foster city, CA) as recommended by the manufacturer (Lu et al., 2001). Primers are listed in Table 1 and the level of mRNA for L7 ribosomal protein were used as an internal reference. After total RNA isolation, samples were amplified by PCR using the cycling parameters: 95°C for 10 minutes; 95°C for 15 seconds and 60°C for 1 minute, 40 cycles. Each sample contained amplified RNA from three gallbladders and was analyzed in triplicates. After confirming that the amplification efficiency of the keratins and L7 were nearly equal, the amount of the transcripts for the specific keratin relative to the L7 transcript was determined.

Indirect immunofluorescence staining

Freshly resected liver or gallbladder pieces were snap-frozen in optimum cutting temperature compound (Miles, Elkhart, IN) then sectioned (6 μm slices). Sections were blocked with PBS containing 2.5% bovine serum albumin (BSA) and 2% normal goat serum for 5 minutes then incubated with the primary antibody in PBS containing 2.5% BSA (20 minutes, 22°C). After washing, sections were incubated with the blocking buffer then with Texas Red-conjugated or fluorescein isothiocyanate-conjugated second stage antibodies. Triple staining experiments included toto-3 iodide (Molecular Probes, Eugene, OR) for nuclear staining, after pretreatment with 0.5 mg/ml RNase A. Staining was visualized using a Bio-Rad laser-confocal laser scanner (Hercules, CA) and a Nikon Eclipse TE300 fluorescence microscope (Melville, NY).

Histology and serum enzyme activity assay

Mice were euthanized by CO_2 inhalation followed by isolation of the gallbladders and livers and fixation in 10% formalin (pH 7.4; Columbia Diagnostics, Springfield, VA). Fixed tissues were paraffin embedded, sectioned, then stained using Hematoxylin and Eosin (Histotec Laboratories; Hayward, CA). Histopathologic changes were assessed by assigning a relative score for the presence of steatosis, hemorrhage, inflammation, edema, and cell death (0, none; 1, moderate; 2, severe) by a pathologist (S.A.M.) who did not have prior information on the diet of the animals from which the tissue sections were obtained. Blood samples were collected by intracardiac puncture of euthanized mice. Serum enzyme activities (IU/l) of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (AP) were measured using a Bayer Express-Plus Chemistry Analyzer™ (Bayer Diagnostics, Tarrytown, NY). Data are presented as means±s.d. Student's *t*-test or Pearson's Chi-squared test was used, and statistical significance was defined as a two-tailed probability of less than 0.05.

Results

Expression profile of keratins in normal mouse gallbladder

Multiple studies have already demonstrated that K8 and K18 are the major keratin IF proteins in liver. In contrast, little is known, particularly in the mouse, regarding keratin expression in the gallbladder. Hence, we initially set out to characterize the expression profile of keratins in normal mouse gallbladder. As shown in Fig. 1A (upper panel), BALB/c mouse gallbladders express primarily K8 and K19 with moderate levels of K18, while mouse liver expresses K8 and K18 exclusively and mouse small intestine expresses primarily K8 and K19 with low levels of K18 [and K20, not shown (see Zhou et al., 2003)]. Assignment of the individual bands seen by one-dimensional gel analysis was confirmed by immunoblotting using keratin-specific antibodies (Fig. 1A, lower panel) and by two-dimensional gel analysis (Fig. 1B). Two dimensional gel analysis shows the characteristic separation of the relatively basic K8 isoforms and the relatively acidic K18 and K19 isoforms (Liao et al., 1996; Moll et al., 1982).

Keratin expression and distribution in gallbladders of mice null for K8, K18 or K19

In order to test the potential role of keratins in protection from gallbladder injury, we examined the expression profile of keratins in gallbladders of K8, K18 and K19 null mice as compared to WT mice. Keratins were isolated using a high salt extraction method that takes advantage of their relative insolubility in high salt buffers. The high-salt-insoluble extracts were analyzed by SDS-PAGE then Coomassie Blue staining, or by immunoblotting. As shown in Fig. 2A, keratins are nearly completely absent from $K8^{-/-}$ mice, with barely detectable remaining K19 (compare lane 1 with lanes 2 and 3) that is likely stabilized by partnering with K7 as also seen in biliary ductal elements of the liver (see Fig. 7A). The near absence of keratins in the gallbladder of K8-null mice, as compared with $K8^{+/-}$ and $K8^{+/+}$ mice, was also confirmed by immunoblotting of total gallbladder tissue homogenates with keratin-specific antibodies (Fig. 2B). The reason for this dramatic decrease of keratin protein levels in $K8^{-/-}$ mice is the well-established degradation of the remaining partner keratin via the proteasome, due to the interdependence of the type I and II keratins on heteropolymerization for their cellular protein stabilization (Coulombe and Omary, 2002). However, although it would be anticipated that ablation of K19 in K19-

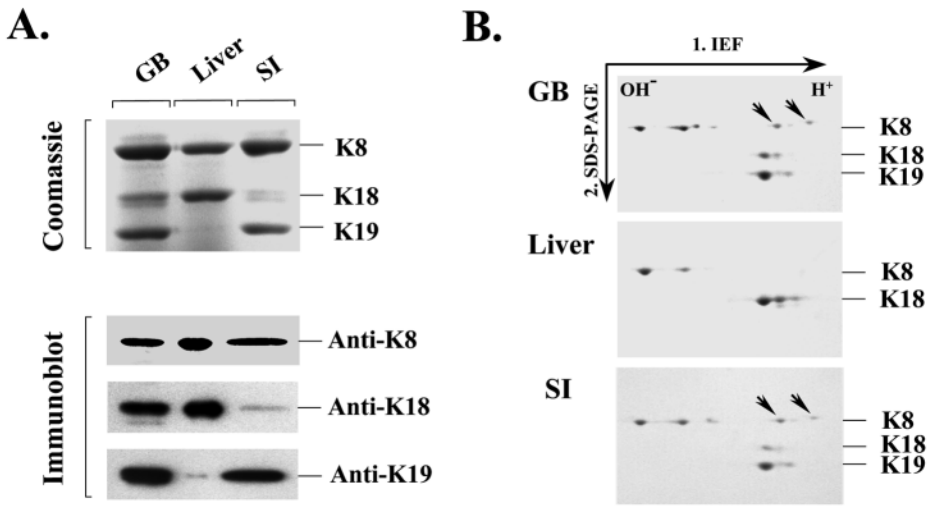

Fig. 1. Characterization of normal mouse gallbladder keratins and comparison with keratins of the liver and small intestine. (A) Keratins were isolated using high salt extraction from BALB/c mouse gallbladder (GB), liver and small intestine (SI), then analyzed by SDS-PAGE and Coomassie Blue staining (upper panel) or by immune blotting using antibodies specific to K8, K18, and K19 (lower panel). (B) Keratin high salt extract preparations were obtained as in A then analyzed using two-dimensional gels followed by Coomassie Blue staining. Separation in the first (horizontal) dimension was carried out using isoelectric focusing (IEF) followed by separation in the second (vertical) dimension using SDS-PAGE. Arrowheads highlight desmin isoforms that are derived from smooth muscle cells present in the gallbladder and intestine but not the liver.

null gallbladders would result in similar near-absence of keratins as noted for K8$^{-/-}$ mice, there is a significant compensatory over-expression of K18 and K20 in K19-null mouse gallbladder that helps stabilize K8 and consequently maintain essentially normal keratin levels (Fig. 2Ca). This K18/K20 overexpresssion is mediated, at least in part, by transcriptional activation as determined by an increase in K18 and K20 but not K8 mRNA (Fig. 2Cb; bar diagram shows mRNA quantification, while standard RT-PCR agarose gel analysis of the products is shown below the bar diagram).

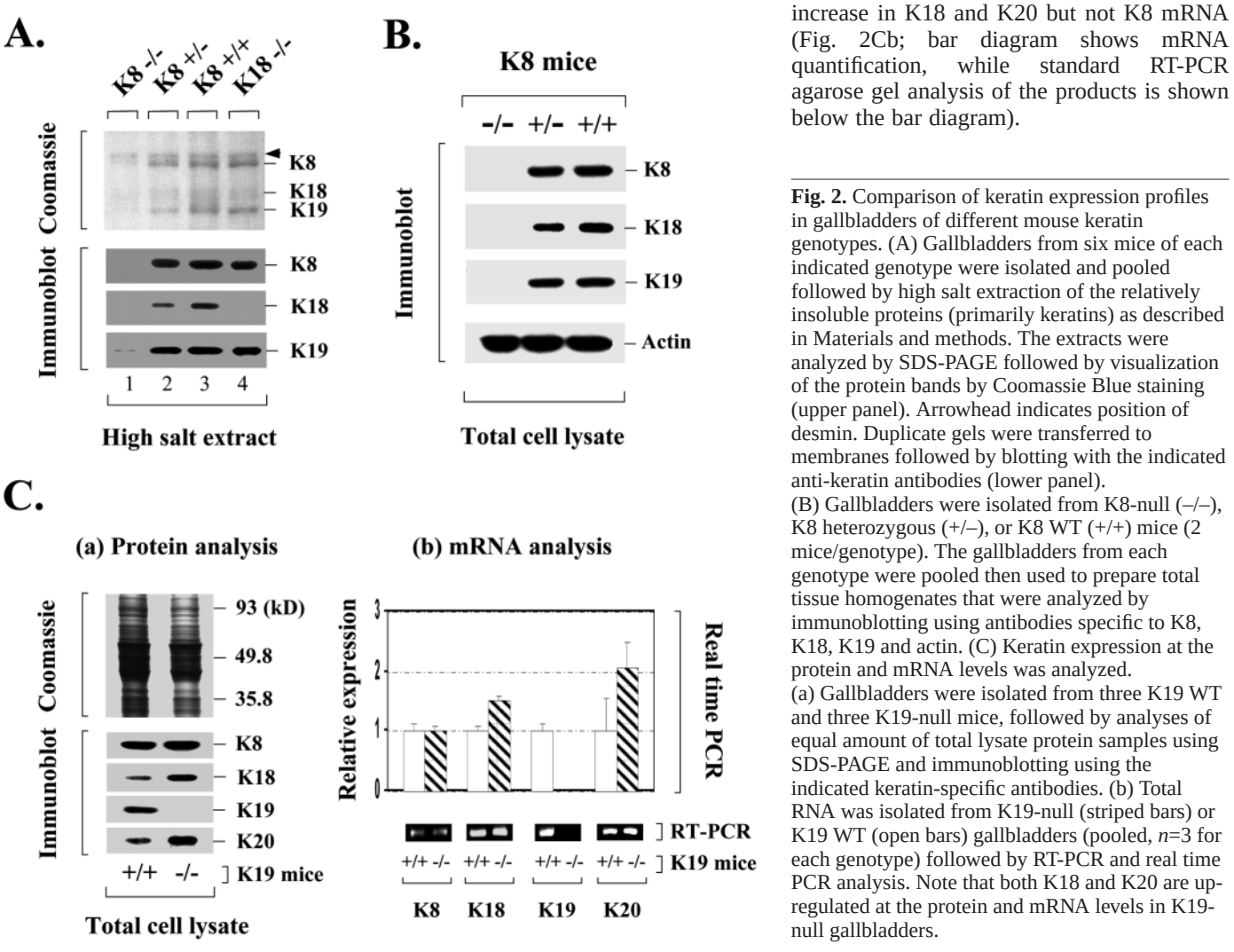

Fig. 2. Comparison of keratin expression profiles in gallbladders of different mouse keratin genotypes. (A) Gallbladders from six mice of each indicated genotype were isolated and pooled followed by high salt extraction of the relatively insoluble proteins (primarily keratins) as described in Materials and methods. The extracts were analyzed by SDS-PAGE followed by visualization of the protein bands by Coomassie Blue staining (upper panel). Arrowhead indicates position of desmin. Duplicate gels were transferred to membranes followed by blotting with the indicated anti-keratin antibodies (lower panel).
(B) Gallbladders were isolated from K8-null (–/–), K8 heterozygous (+/–), or K8 WT (+/+) mice (2 mice/genotype). The gallbladders from each genotype were pooled then used to prepare total tissue homogenates that were analyzed by immunoblotting using antibodies specific to K8, K18, K19 and actin. (C) Keratin expression at the protein and mRNA levels was analyzed.
(a) Gallbladders were isolated from three K19 WT and three K19-null mice, followed by analyses of equal amount of total lysate protein samples using SDS-PAGE and immunoblotting using the indicated keratin-specific antibodies. (b) Total RNA was isolated from K19-null (striped bars) or K19 WT (open bars) gallbladders (pooled, n=3 for each genotype) followed by RT-PCR and real time PCR analysis. Note that both K18 and K20 are up-regulated at the protein and mRNA levels in K19-null gallbladders.

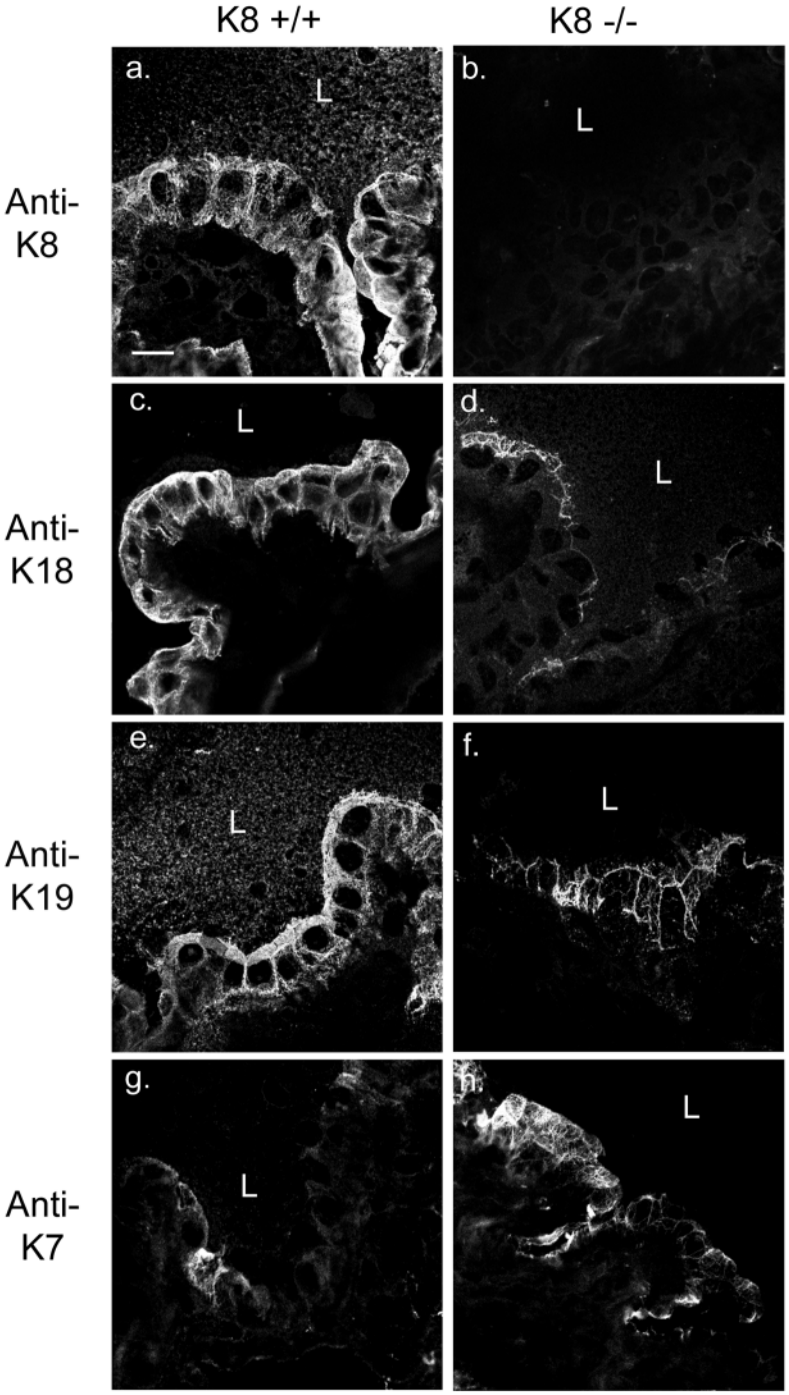

Fig. 3. Indirect immunofluorescence keratin staining of gallbladders of $K8^{+/+}$ and $K8^{-/-}$ mice. Gallbladders were removed from age and sex matched $K8^{+/+}$ or $K8^{-/-}$ mice, followed by fixing then staining using the indicated keratin-specific antibodies. L, lumen; scale bar (in a): 10 μm. Note the absence of K8 and the limited compensatory induction of K7 in $K8^{-/-}$ mouse gallbladder.

We also examined the distribution of keratins in WT and K8-null mouse gallbladders by indirect immunofluorescence staining. As shown in Fig. 3, epithelial cells stain with antibodies to K8, K18 and K19 in WT gallbladder while K8 staining is absent in K8-null mouse gallbladder with minimal remnant staining of K18 and K19 (compare Fig. 3a, c and e with b, d and f). These findings are consistent with the immunoblot results shown in Fig. 2. The remaining limited K18 and K19 staining in K8-null mouse gallbladder is probably the result of the stabilization of K18 and K19 by K7 overexpression, based on the increase in K7 staining (Fig. 3, compare g with h). K7 was not detected by immunoblotting in WT or K8-null mice, probably because of its low overall expression level (not shown). In addition to luminal epithelial cells, the K7 antibody also strongly stained the serosal layer of gallbladders (not shown).

Effect of a lithogenic diet on gallbladder and liver injury in K8-null and WT mice

We compared the effect of a lithogenic high fat diet on gallbladder and liver injury. This diet is known to cause gallstone formation and accumulation of fat in mouse liver (Alexander and Portman, 1987; Nishina et al., 1990). We focused on comparing K8-null with heterozygous and WT mice given that K8-null mice had the most significant keratin alterations in the gallbladder (Figs 2, 3) and liver (Baribault et al., 1994; Caulin et al., 2000; Gilbert et al., 2001; Loranger et al., 1997; Toivola et al., 1998; Toivola et al., 2001; Zatloukal et al., 2000). We used a feeding period of 5 weeks based on the known frequency of mouse gallstone formation (~50-60%) among different mouse strains (though there are significant strain and sex related differences) (Alexander and Portman, 1987; Nishina et al., 1990). As shown in Table 2, the incidence of gallstone formation is similar among the three K8 mouse genotypes, and hence is independent of gallbladder keratin expression level or filament organization. No gallstones were noted in any of the K8 mouse genotypes that were fed a normal control diet (not shown), and the histologic appearance of the gallbladders was similar in WT and K8-null mice (Fig. 4a,b). In addition, the extent of inflammation and edema were similar in WT with K8-null mice after feeding the lithogenic diet (Fig. 4c,d). The fasting cholesterol levels of K8-null mice are similar to their wild-type counterparts, whether they are fed the control or the high fat lithogenic diet (not shown). Therefore, the dramatic decrease in keratin protein levels and cytoplasmic filaments in K8-null mice have no apparent effect on LD-induced gallbladder injury.

In contrast to the findings in the gallbladder, K8-null mice manifested significant differences in susceptibility to LD-induced liver injury as compared with WT and heterozygous mice. For example, although the lithogenic diet increased the AST, ALT and AP levels in all three genotypes ($K8^{+/+}$, $K8^{+/-}$, $K8^{-/-}$), K8-null mice had significantly higher ALT and AP levels than WT and heterozygous K8 mice (Fig. 5; $P<0.05$). No differences were noted between the WT and heterozygous groups. In addition, the lithogenic diet caused more significant

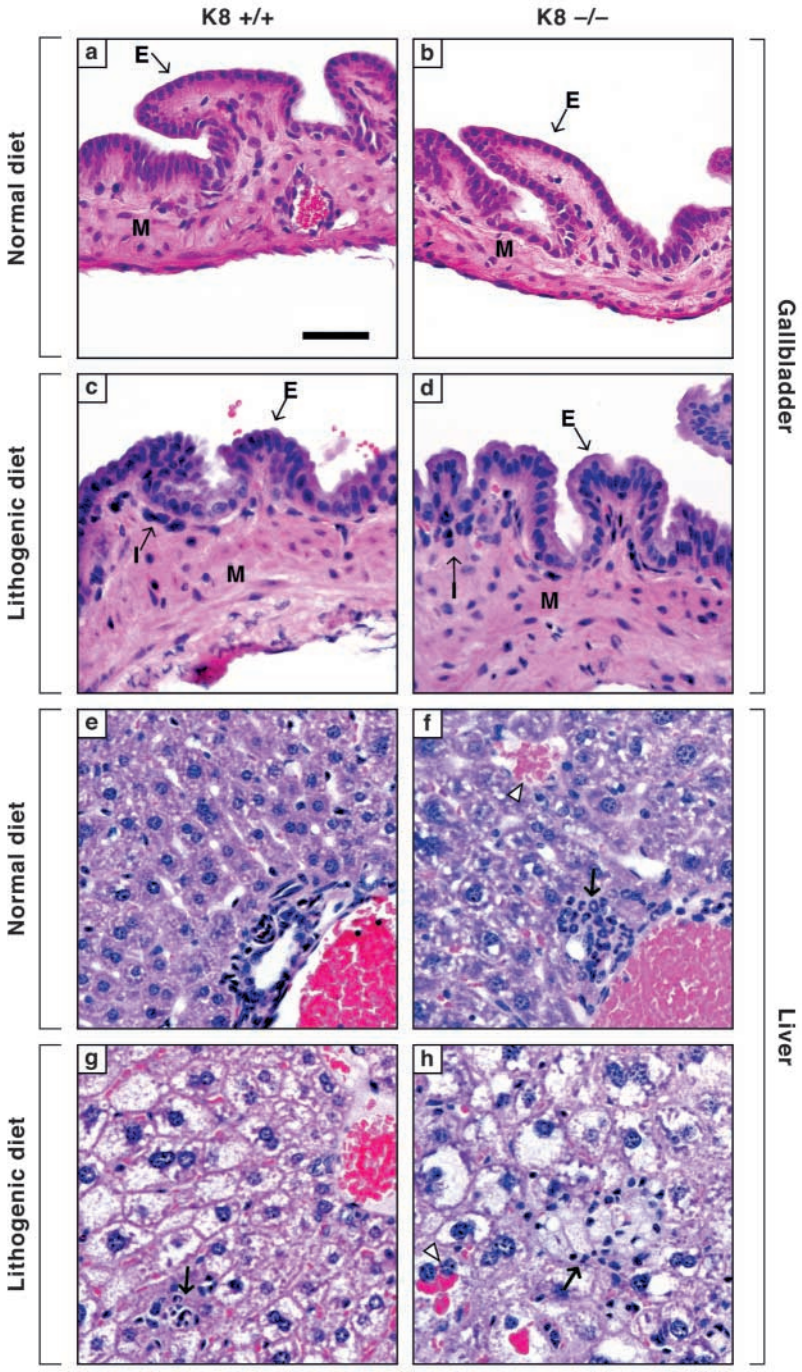

Fig. 4. Histologic analysis of LD-induced injury in WT and K8-null mouse gallbladder and liver. K8 mice (–/–, +/+) were fed a normal or lithogenic diet for 5 weeks. Gallbladders and livers were resected, fixed, paraffin embedded, sectioned, and then stained with Hematoxylin and Eosin. (a-d) Gallbladder sections from mice fed a normal (a,b) or a lithogenic (c,d) diet. E, single layer of cuboidal epithelial cells; M, muscularis; I, small foci of mononuclear inflammatory cells. (e-h) Liver sections from mice fed a normal (e,f) or a lithogenic (g,h) diet. (f) A focal area of inflammation (arrow) and a small area of hemorrhage (arrowhead). (g) A small area of inflammation and necrosis (arrow). Panel h shows a markedly vacuolated cytoplasm with displacement of the nuclei to the cell periphery, Kupffer cell nodule (arrow) and hepatocyte erythrophagocytosis (arrowhead). The histologic findings of the liver and gallbladder of $K8^{+/-}$ mice that were fed a normal diet or LD (not shown) were very similar to those shown for the corresponding $K8^{+/+}$ mice. Scale bar (in a): 100 μm for a-d and 200 μm for e-h.

histologic changes in K8-null mice, including steatosis, hemorrhage, inflammation and cell death (Fig. 4e-h; Table 3). The marked steatosis in the LD-fed K8-null mice was accompanied by prominent displacement of the nucleus into the cell periphery in many hepatocytes, a finding only occasionally seen in WT (Fig. 4g) or $K8^{+/-}$ mice (not shown).

LD-induced effects on keratin expression and phosphorylation in the gallbladder and liver

Previous studies demonstrated that K8/K18 are over-expressed (Denk et al., 2000) and hyperphosphorylated in response to hepatotoxins that induce liver injury (Omary et al., 2002) and in response to pancreatic injury (Toivola et al., 2000). We compared the effect of the lithogenic diet on K8, K18 and K19 expression and phosphorylation in the gallbladder and liver. As shown in Fig. 6, keratins are induced in WT mice upon LD-induced gallbladder (Fig. 6A) but not liver injury (Fig. 6B). This induction was not as evident in the heterozygous mice (Fig. 6A), and there was no evidence of new keratin expression (e.g. K18 or K19) in K8-null or WT mice livers (Fig. 6B and Fig. 7A). The limited keratin staining in K8-null mice livers was related to decoration of biliary tree cholangiocytes because of the presence of K18 (Fig. 7Ac), and K19 and K7 (not shown). In addition, the LD did not cause any significant changes in hepatocyte keratin filament organization (Fig. 7A; compare a with b and c with d), although an increase in K8 and K18 phosphorylation in the liver and gallbladder at several phosphorylation sites was noted (Fig. 7B,C). Hence, keratin over-expression also occurs in the gallbladder in response to injury, as noted previously in the liver and pancreas. However, the results with the LD indicate that not all liver injuries induce keratin over-expression and that the same injury (i.e. LD diet) can result in two different keratin over-expression responses in different tissues.

Discussion

Keratin expression profile in mouse gallbladder, and differential keratin overexpression in keratin-null mice depending on the null keratin and the tissue

Our results show that mouse gallbladder epithelial cells express K8, K18 and K19 (K8>K19>K18) with limited K7 levels (detected only by fluorescence staining). This keratin expression pattern is quite different from hepatocytes that express K8/K18 only, but is somewhat similar to mouse small intestine except that K18 is more prominent in the gallbladder

Table 2. Comparison of LD-induced gallstone formation in $K8^{+/+}$, $K8^{+/-}$ and $K8^{-/-}$ genotypes

Genotypes	$K8^{+/+}$	$K8^{+/-}$	$K8^{-/-}$
Total number of mice used	11	13	10
Number of mice with stones	8	6	6
Percentage with stones	73%	46%	60%

Age matched K8 mice (all male) were fed a lithogenic diet for 5 weeks. The gallbladders were resected and examined for stone formation using a dissection microscope. Presence of one or more stones was scored as positive. *P* values for differences in stone formation were: $K8^{+/+}$ vs $K8^{+/-}$, 0.19; $K8^{+/+}$ vs $K8^{-/-}$, 0.54; $K8^{+/-}$ vs $K8^{-/-}$, 0.51.

Table 3. Comparison of the histologic changes in livers of the three mouse K8 genotypes after lithogenic diet feeding

	K8 mouse genotype		
	$K8^{+/+}$ (*n*=4)	$K8^{-/+}$ (*n*=5)	$K8^{-/-}$ (*n*=5)
Histologic score	0.75±0.50	0.80±0.45	2.0±0**

Mice (numbers examined indicated in parentheses) were fed a lithogenic diet for 5 weeks followed by removal of the livers, fixation, then staining with Hematoxylin and Eosin. Histologic scoring included assessment of steatosis, hemorrhage, inflammation, and cell death (0=none, 1=moderate and 2=severe). The overall histologic score is shown as mean ± s.d. Double asterisks indicate significant differences ($P<0.01$) when comparing $K8^{-/-}$ with $K8^{+/-}$ or $K8^{+/+}$ scores.

than in the small intestine (Fig. 1A,B). Interestingly, different keratin-null mice have markedly different keratin expression compensatory responses in the absence of a given type I or type II keratin. This observation is clearly evident in the gallbladders of K8-, K18- and K19-null mice. For example, significant K20 over-expression is observed in type I keratin K19-null (Fig. 2) but not in K18-null (not shown) gallbladders. However, the type II keratin-null $K8^{-/-}$ mice manifest an increase in K7 expression, albeit minimal, in gallbladder epithelia (Fig. 3) but not in hepatocytes (not shown). Over-expression of a type II keratin in a type II keratin-null background also varies as we have shown here for type I keratins, which probably accounts for the marked differences in the observed mouse phenotypes. For example, esophageal epithelia of K4-null mice (where K4 is the dominant type II keratin) over-express significant levels of a K6-like protein (Ness et al., 1998) while K6-null mice do not appear to over-express K4 or any other compensatory type II keratin and also have a more dramatic phenotype than their K4-null counterparts (Wong et al., 2000). Compensatory keratin over-expression was also noted in patients with homozygous keratin-null mutations. For example, absence of K14 protein due to a mutation that causes premature termination was found to result in increased K15 levels in a kindred with recessive epidermolysis bullosa simplex (Jonkman et al., 1996). Therefore, the accumulating evidence indicates that keratin over-expression does occur for both type I and II keratins in a fashion that depends on the specific ablated keratin and the involved tissue. In that context, it appears that K20 and K6

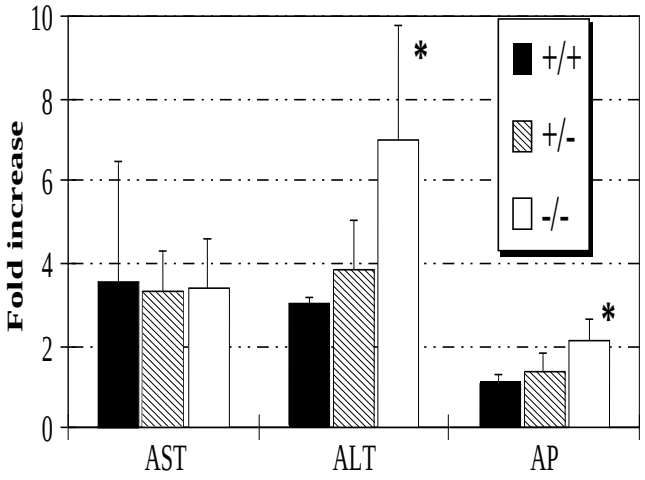

Fig. 5. Serologic assessment of liver injury among K8 mouse genotypes after a lithogenic diet. Blood was collected from K8 mice that were fed a lithogenic diet (the same mice as used for the experiments of Table 3) , or from five age-matched mice that were fed a normal diet. Sera were isolated and used to measure alkaline phosphatase (AP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The *y* axis shows the mean fold increase in serum enzymes compared with mice fed control diet. *$P<0.05$ when comparing $K8^{-/-}$ with $K8^{+/-}$ or $K8^{+/+}$.

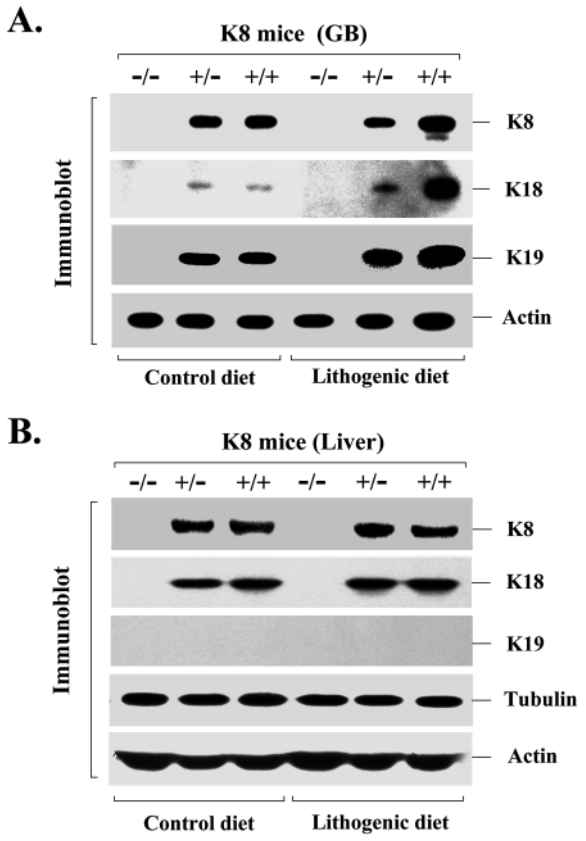

Fig. 6. Effect of a lithogenic diet on keratin expression levels in the gallbladder and liver. (A) Total tissue homogenates of gallbladders (GB) from $K8^{-/-}$, $K8^{+/-}$ and $K8^{+/+}$ mice that were fed a normal or lithogenic diet were analyzed by immunoblotting using antibodies to the indicated keratins and to actin. (B) Total tissue homogenates of livers from the same mice used in A were analyzed by immunoblotting using antibodies to actin, tubulin and the indicated keratins. Note that the LD induces overexpression of keratins in WT mouse gallbladder but not liver.

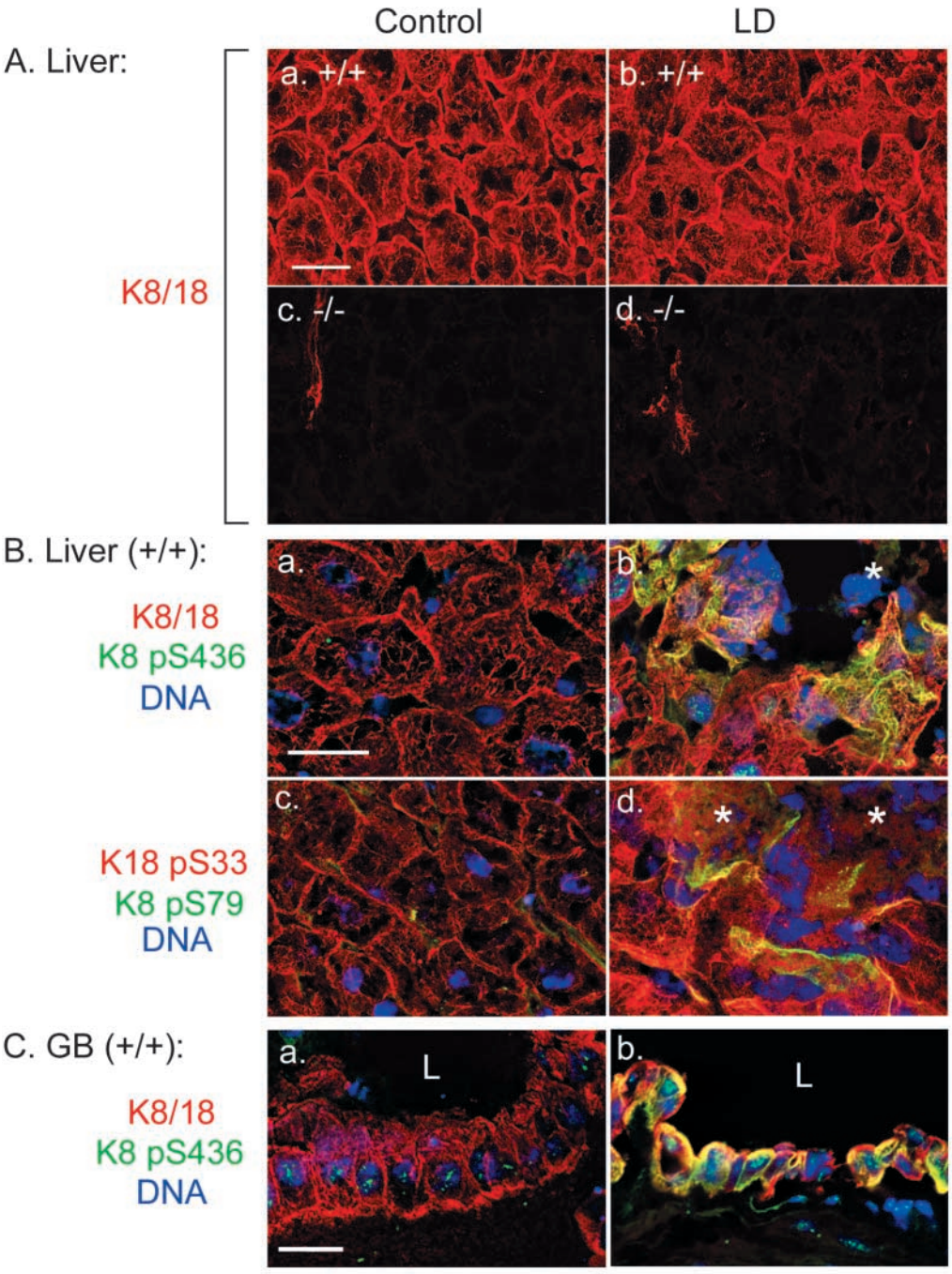

Fig. 7. Effect of a lithogenic diet on keratin phosphorylation in the gallbladder and liver. (A) Liver sections from $K8^{+/+}$ (a,b) or $K8^{-/-}$ (c,d) mice that were fed control diet (a,c) or LD (b,d) were stained by indirect immunofluorescence using anti-K8/18 antibodies. Note the absence of keratins in $K8^{-/-}$ liver, except for staining of biliary ductal cells owing to the presence of K7 and K19 in these cells. (B,C) Liver (B) and gallbladder (C) sections from $K8^{+/+}$ mice that were fed a control or lithogenic diet were triple stained using toto-3 (blue; nuclear DNA staining), anti-K8/K18 or anti-K18-pS33 (red), and anti-K8 pS436 or pS79 (green). L, lumen. Asterisks in b and d of B indicate areas of injury. Note the induction of liver and gallbladder keratin phosphorylation at the three different K8/K18 phosphorylation sites tested, after exposure to the lithogenic diet. Scale bars in A,B,C: 20 μm.

show significant plasticity and inducibility. This is consistent with the known properties of significant K6 induction upon wound healing (Coulombe and Omary, 2002) while the findings described herein for K20 are new. Further studies will determine how common K20 over-expression is in different type I keratin-null mice, and in different biologic contexts, and whether it occurs in tissues other than the gallbladder.

Tissue and context variability of keratin over-expression in response to injury

In addition to the compensatory over-expression of keratins described above, exposure to injury in vivo causes over-expression of endogenous keratins in the liver and pancreas, as previously described, and in the gallbladder as described herein. Such increase is despite the fact that K8/K18 are already highly abundant proteins [e.g. they make up ~5% of the total cellular protein in cultured human colonic tumor cell lines (Chou et al., 1993)]. For example, K8/K18 proteins increase approximately twofold in mouse liver upon exposure to the hepatotoxins griseofulvin (Cadrin et al., 2000), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Denk et al., 2000) or cholic acid (Fickert et al., 2002), or after common bile duct ligation (Fickert et al., 2002), and in the pancreas after hyperstimulation with caerulein [(Toivola et al., 2000) and unpublished observations]. Upon LD feeding, gallbladder K8/K18/K19 expression increased without any change in keratin expression in the liver despite being exposed to the same injury and despite the evidence of injury and a keratin hyperphosphorylation response in both tissues (Figs 4, 6, 7). In terms of the three major cytoskeletal protein families, the increase in protein expression appears to be specific to keratins and does not involve actin (Fig. 6A) or tubulin (not shown). The increase in keratin upon injury also occurs at the RNA level as noted in several injury models of the liver (Cadrin et al., 2000; Denk et

al., 2000; Fickert et al., 2002) and the pancreas (unpublished observations). Stress-induced keratin over-expression is reminiscent of the induction of stress proteins such as heat shock protein 70 upon injury and may serve a protective role given the known ATP-dependent association of heat shock protein 70 with K8/K18 (Liao et al., 1995). The overall functional significance of keratin over-expression upon injury is unclear, although such over-expression is probably essential for providing cytoprotection in some tissues such as the liver (see below). Our results show for the first time that keratin over-expression is dependent on the type of injury (in the case of the liver) and that it also occurs in the gallbladder.

Hyperphosphorylation of keratins as a cellular response to injury

Keratin phosphorylation is a major posttranslational modification and overall increases in K8/K18 phosphorylation have been uniformly associated with every stress model that has been examined (Coulombe and Omary, 2002). This generalization extends across all tissues studied to date including the liver and pancreas (Coulombe and Omary, 2002), atherosclerotic vascular lesions, which contain low levels of keratins that become hyperphosphorylated upon injury (Bar et al., 2001) and the gallbladder (Fig. 7). The role of keratins in cytoprotection (Coulombe and Omary, 2002; Marceau et al., 2001; Omary et al., 2002) is akin to keratins serving as a 'stress thermometer' of epithelial cells. As such, overall keratin phosphorylation reflects the 'cell temperature' (i.e. the higher the stress the higher the 'temperature' of keratin phosphorylation). The increase that we observed in liver and gallbladder keratin phosphorylation after LD-induced injury, as noted by immunofluorescence staining (Fig. 7), occurred in relatively few cells in proximity to the most affected injury areas thereby rendering analysis by immunoblotting below the level of detection (not shown). Although the increase in K8/K18 phosphorylation occurs uniformly in a global fashion there are examples of specific sites that become dephosphorylated in response to stress, as exemplified by K18 pS33 dephosphorylation after Fas-mediated apoptosis of mouse hepatocytes in vivo (Ku et al., 2003a).

What is the function of keratins in the gallbladder and liver?

The function of keratins as protectors of the liver from a variety of environmental stresses is well supported by several transgenic mouse studies and by the K8/K18 mutations that have been identified in patients with liver disease (Ku et al., 2003b; Magin et al., 1998; Marceau et al., 2001; Omary et al., 2002). The importance of keratins in the liver was further substantiated in this study by the increased susceptibility of K8-null mice to LD-induced liver injury. Of note, the prevalence of keratin mutations in patients with cryptogenic liver disease is 8.8% as compared with 2.8% in patients with noncryptogenic (primarily viral, alcohol, biliary and autoimmune) liver disease and 0.6% in blood-bank volunteer controls (Ku et al., 2003b). These differences are highly significant when comparing the prevalence of keratin mutations in patients with cryptogenic versus noncryptogenic liver disease (P value <0.03) or when comparing the prevalence of keratin mutations in patients with liver disease versus the blood-bank controls (P value <0.004). Given the emerging association of steatohepatitis with cirrhosis and cryptogenic liver disease (Falck-Ytter et al., 2001; Harrison and Diehl, 2002; Maher, 2002; Reid, 2001), our data raise the hypothesis that some patients with steatohepatitis may have K8 or K18 mutations that may accelerate their liver disease progression, and that patients with steatohepatitis may be a relevant group to screen for K8/K18 mutations.

In contrast to the liver, the function of keratins in the gallbladder is unclear. Our results with the LD model in K8-null mice suggest that keratins are not important in protecting the gallbladder from gallstone formation or gallstone-induced injury, despite the LD-induced keratin over-expression in wild-type mice. These data suggest that keratin over-expression per se is unlikely to account for the apparent dispensability of keratins in the gallbladder since K8-null mice do not overexpress any detectable keratins (e.g. K18, K19 or K20) upon LD feeding. However, K8-null mice do have a slight increase in K7 over-expression (Fig. 3) that may provide compensatory protection in the LD-induced gallbladder injury model. The apparent dispensability of keratins in the gallbladder of K8-null mice is similar to that seen in caerulein injection and choline/methionine-deficient ethionine-supplemented diet injury models of the pancreas (Toivola et al., 2000). This suggests that the same keratins can have different functions in different organs and/or that other cellular or cytoskeletal-related proteins may play a more important protective role in the pancreas and gallbladder but less so in the liver. One caveat to this interpretation is the inherent differences in the models used to stress the different tissues, owing to their tissue-specific effects.

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