Organ-specific stress induces mouse pancreatic keratin overexpression in association with NF-κB activation

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Accepted 27 November 2003
Journal of Cell Science 117, 1709-1718 Published by The Company of Biologists 2004
doi:10.1242/jcs.01016

Summary

Keratin polypeptides 8 and 18 (K8/K18) are the major intermediate filament proteins of pancreatic acinar cells and hepatocytes. Pancreatic keratin function is unknown, whereas hepatocyte keratins protect from mechanical and non-mechanical forms of stress. We characterized steady-state pancreatic keratin expression in Balb/c mice after caerulein and choline-deficient ethionine-supplemented diet (CDD), or on exposure to the generalized stresses of heat and water immersion. Keratins were studied at the protein, RNA and organizational levels. Isolated acini were used to study the role of nuclear factor (NF)-κB using selective inhibitors. Keratins were found to be abundant proteins making up 0.2%, 0.3% and 0.5% of the total cellular protein of pancreas, liver and small intestine, respectively. Caerulein and CDD caused a threefold transcription-mediated overall increase in K8/K18/K19/K20 proteins. Keratin overexpression begins on tissue recovery, peaks 2 days after caerulein injection, or 1 day after CDD discontinuation, and returns to basal levels after 10 days. K19/K20-containing cytoplasmic filaments are nearly absent pre-injury but form post-injury then return to their original membrane-proximal distribution after 10 days. By contrast, generalized stresses of heat or water-immersion stress do not alter keratin expression levels. Caerulein-induced keratin overexpression is associated with NF-κB activation when tested using ex vivo acinar cell cultures. In conclusion, keratins are abundant proteins that can behave as stress proteins in response to tissue-specific but not generalized forms of injury. Pancreatic keratin overexpression is associated with NF-κB activation and may serve unique functions in acinar or ductal cell response to injury.

Key words: Pancreatitis, Keratins, Heat stress, Caerulein, Intermediate filaments, Choline-deficient diet, Water immersion

Introduction

Keratins are the largest subgroup of intermediate filaments (IF) proteins (Coulombe and Omary, 2002; Moll et al., 1982). They are expressed in an epithelial cell-specific fashion and include keratins 9-20 (K9-K20, type I) and keratins 1-8 (K1-K8, type II). Given the obligate heteropolymeric nature of keratins, all epithelial cells express at least one type I and one type II keratin in an overall 1:1 stoichiometry. For example, mouse hepatocytes express K8 and K18, exclusively, in a 1:1 molar ratio, whereas mouse pancreatic acinar cells express K8/K18/K19 such that K8=K18+K19 at the protein level. Keratins are among the most abundant cytoskeletal proteins, as reported in the skin and in one cultured cell line. For example, keratins make up ~30% of the total cellular protein in keratinocytes (Sun et al., 1979), whereas 5% of the cellular protein in cultured human colonic HT29 tumor cells consists of K8/K18 (Chou et al., 1993). The role of keratins in several human liver, skin, hair, ocular and oral diseases is well established (Coulombe and Omary, 2002; Irvine and Mclean, 1999).

Although the predicted multiple functions of keratins remain to be fully elucidated, an important well-defined function they serve is to protect epithelial cells from mechanical and non-mechanical forms of injury (Coulombe and Omary, 2002; Fuchs and Cleveland, 1998). This function is well-substantiated in the skin, hair, eye and liver on the basis of keratin mutation phenotypes in keratin-related diseases that target these organs or appendages, and is also supported by several keratin-related animal models (Irvine and Mclean, 1999; Magin et al., 2000). However, this function is not universal in that mouse pancreas injury does not appear to be modulated by keratin absence or by a keratin mutation that disrupts acinar cell cytoplasmic filaments (Toivola et al., 2000a; Toivola et al., 2000b). This is based on findings in the mouse injury models of caerulein-induced edematous pancreatitis (Jensen et al., 1980) or feeding with a choline deficient ethionine-supplemented diet (CDD), which induces hemorrhagic necrotizing and age-dependent lethal pancreatitis (Lombardi et al., 1975).

The mechanism whereby keratins protect hepatocytes from

liver injury may involve protection from transmittal of apoptotic stimuli when keratins are present or not mutated (Caulin et al., 2000; Gilbert et al., 2001; Ku et al., 2003), the stress-associated hyperphosphorylation of keratins (Coulombe and Omary, 2002; Ku et al., 1998), or possibly stress-induced overexpression of keratins as noted in mouse liver and gallbladder injury models (Cadrin et al., 2000; Denk et al., 2000; Fickert et al., 2002; Tao et al., 2003). Overexpression of keratins in response to liver or gallbladder injury suggests that these proteins may behave as stress proteins, similar to heat stress proteins (hsp), which is supported by the known physical and ATP-dependent association of hsp70 with K8/K18 (Liao et al., 1995). With regard to hsp expression and the pancreas, whole-body hyperthermia or water immersion induced hsp70 or hsp60 expression, respectively, and protected against pancreatic tissue injury in both edematous and necrotizing pancreatitis models (Beckmann et al., 1992; Bhagat et al., 2000; Grise et al., 2000; Hietaranta et al., 2001b; Wagner et al., 1996). The protective mechanism of hsp70/60 induction is poorly understood, but it may be related to preventing intracellular trypsinogen activation (Beckmann et al., 1992; Lee et al., 2000). As a first step towards understanding keratin function in the pancreas, we investigated three aspects: keratin quantitative levels in the pancreas, liver and small intestine; keratin expression in the pancreas on exposure to general (i.e. heat and water immersion) and pancreas-specific stresses (i.e. caerulein and CDD); and mechanisms underlying keratin expression alterations in pancreatic acinar cells on injury.

Materials and Methods

Animals, antibodies and stress models

Balb/c mice aged 4 to 8 months were utilized. The antibodies (Ab) used were: rat anti-mouse (m) K8 (Troma I, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); rat anti-mK19 (Troma III) (Boller et al., 1987); rabbit anti-human/mouse K8/18 (Ab 8592); rabbit anti-human/mouse K18 (Ab 4668); antibodies that recognize K20 (K20.8), actin, tubulin and Hsp60/70/90 (NeoMarkers; Fremont, CA). Four experimental mouse models that cause pancreas-specific or general stress were utilized. (1) Caerulein-induced pancreatitis: age- and sex-matched mice were fasted from solid food for 12-16 hours (but allowed water ad libitum) then received seven hourly intraperitoneal (ip) injections of saline (control group) or 50 μg/kg caerulein (Research Plus; Bayonne, NJ). (2) Choline/methionine-deficient diet supplemented with ethionine (CDD) induced pancreatitis: female mice weighing 14-19 g (typically ~3-4 weeks old) were fasted as above then fed CDD (Harlan Teklad, Madison WI) supplemented with 0.5% DL-ethionine (Sigma; St Louis, MO) or normal chow (control group) for 3 days, then switched to normal diet for 1, 2, 3, 5 or 7 days. (3) Hyperthermia stress: mice were placed on a warming blanket with a heating lamp 45 cm above their cages. Body temperature (monitored rectally every 2 minutes) gradually increased to 42°C and was maintained at 42°C for 20 minutes (Metzler et al., 1999). (4) Water-immersion stress: mice were placed in a mouse warming restrainer (Kent Scientific Corp; Torrington, CT) then immersed vertically to neck level in a water bath (23°C, 3-12 hours) (Lee et al., 2000). After each stress, animals were euthanized using CO_2 inhalation, after which pancreata were rapidly removed and divided into 3-4 pieces. Individual pancreas fragments were immediately fixed in 10% formalin, embedded in optimum cutting temperature compound (Miles; Elkhart, IN) or snap-frozen in liquid N_2 for subsequent protein and RNA analysis. Fixed tissues were sectioned then stained using hematoxylin and eosin (performed by Histo-tec Laboratory; Hayward, CA). Immunofluorescence staining was as described previously (Toivola et al., 2000a) and images were analyzed using confocal microscopy.

Isolation and culture of acini

Dispersed mouse pancreatic acini were prepared using collagenase (Worthington; Lakewood, NJ) digestion (Menozzi et al., 1990; Toivola et al., 2000a). Acini were suspended in 24.5 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 14 mM glucose and 1% bovine serum albumin (BSA). Acini were plated in tissue culture flasks then placed in a pre-warmed (37°C) sealed flask holder purged with 100% O_2 in a humidified incubator (37°C). For the time-course experiments, acini were incubated in buffer alone or were stimulated with 0.1 μM caerulein for 1-4 hours (Hietaranta et al., 2001a). Alternatively, acini were preincubated (30 minutes) with 0, 10, 25 and 50 μM of the proteasome inhibitor MG-132 (Calbiochem; San Diego, CA); or with 10, 25, 50 μM of the IκB-ubiquitin ligase inhibitor pyrrolidine dithiocarbamate (PDTC) (Sigma) or 0.5 or 2 μg/ml actinomycin D (Act-D, Sigma) followed by the addition of 0.1 μM caerulein. Acini were collected and washed twice with PBS, then processed for nuclear or total protein extraction and RNA isolation.

Protein extraction and quantification of keratins in tissues

Total tissue lysates were prepared by homogenization in Laemmli sample buffer, and enriched keratin fractions were prepared by high salt extraction (HSE) as described previously (Toivola et al., 2000a). Total tissue lysates or high salt extracts were analyzed by SDS-PAGE then stained with Coomassie blue, or were transferred to polyvinylidene difluoride membranes for western blotting and visualization of specific immunoblotted proteins using enhanced chemiluminescence. Measurement of keratins in tissues was done as follows. Two pieces of each organ were weighed then one was used to generate a total tissue homogenate and the second to prepare a HSE of keratins. The HSE method provides nearly 95% recovery of the K8/K18/K19 keratin pool (Chou et al., 1993). The micrograms of K8, K18 and K19 in the HSE were calculated as compared with serial dilutions of a BSA standard separated on the same gel; this was followed by Coomassie blue staining then densitometry scanning. The percent of keratin protein was calculated: [μg of K8+K18+K19 protein/μg of tissue protein] × 100, after using keratin-specific antibodies to blot serial dilutions of total tissue homogenates and HSE that are analyzed on the same gel (e.g. Fig. 1B). The peak fold-increase in keratin expression was determined using two independent methods: (1) HSE of pancreata isolated from mice injected with saline or caerulein, separation of the extracts by SDS-PAGE, Coomassie staining then densitometric scanning (after normalization based on the protein content of the lysate obtained after the first step of the HSE method) (Chou et al., 1993). (2) Blotting of total pancreas lysates isolated from saline or caerulein-treated mice, with inclusion in the same gel of serial dilutions of caerulein-treated lysates (which have increased keratin expression) then quantification by densitometric scanning.

Qualitative and real-time reverse transcription (RT)-PCR

Total RNA was isolated from pancreatic tissue and acini using an RNeasy midi kit (Qiagen; Chatsworth, CA) and quantified spectrophotometrically. First strand cDNA was synthesized using random primers and SuperScript II reverse transcriptase (Invitrogen; Carlsbad, CA). cDNA products were amplified using mouse-specific intron-spanning primers (Table 1), and amplified products were analyzed using 1% agarose gels. Real-time quantitative PCR was performed using an ABI Prism 7900 Sequence Detection System (PE Biosystems, Foster City, CA) as recommended by the manufacturer

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	GTTCAGTACGCATTGGGTCA	CTGAAGTCATCTGCAGCCAG	440
K20	CAACAATGTCAACGTGGAGG	GCGTTCTGTGTCACTCCTGA	385
β-Actin	TAAGATCATTGCTCCCCCTG	TGCTCACAGAGATCCACCAG	484
Nested PCR			
K20	CCCAGAAGAACCTGCAAGAG	ACGAGCCTTGACGTCCTCTA	210
Real time PCR			
K8	GGACATCGAGATCACCACCT	TGAAGCCAGGGCTAGTGAGT	155
K18	ATTGCCAGCTCTGGATTGAC	GTCTCAGCGTCCCTGATTTC	200
K19	CGGTGGAAGTTTTAGTGGGA	AGTAGGAGGCGAGACGATCA	101
L7	AAAGGCAAGGAGGAAGCTCATCT	AATCTCAGTGCGGTACATCTGCCT	80

(using the primers in Table 1) (Lu et al., 2001). Primers to the L7 ribosomal protein were used as an internal control reference. Following total RNA isolation, samples were amplified by PCR using the following cycling parameters: 95°C for 10 minutes; 95°C, 15 seconds to 60°C for 1 minute, 40 cycles. Each sample was analyzed three times, each in triplicate, and every experiment was performed at least twice. After confirming that the amplification efficiency of the gene of interest and L7 were approximately equal, the amount of the transcripts for the specific gene relative to the L7 transcript was determined. Relative RNA expression is reported as mean ± s.d., and experimental data from different groups were compared by analysis of variance (ANOVA).

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Nuclear protein extraction was carried out as previously described (Steinle et al., 1999). The binding reaction (Chen et al., 2002) was initiated by mixing 10,000 cpm of the [γ-^{32}P] ATP-labeled NF-κB binding oligonucleotide 5′-AGCTTGGGGACTTTCCACTAGTACG-3′ with 10 μg of nuclear extract, 5 μg poly (dI-dC) in binding buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 4% glycerol (30 minutes, 4°C). Samples were analyzed using 5% nondenaturing polyacrylamide gels and a PhosphoImager™ (Amersham Biosciences Corp; Piscataway, NJ). Specific binding was determined by adding a 100-fold molar excess of the unlabeled NF-κB binding oligonucleotide to the reaction mixture.

Results

Keratin expression levels in mouse pancreas, liver and intestine

We quantified the total simple epithelial keratin protein content in the pancreas, liver and intestine. Such determinations have not been done in simple epithelial organs, although K8/K18/K19 are known to make up ~5% of the total cellular protein in cultured human colonic HT29 cells (Chou et al., 1993). In mouse digestive organs, the major keratins are K8/K18 in the liver (K8=K18), K8/K18/K19 in the pancreas (K8>K18⩾K19) and K8/K18/K19/K20 in the intestine (K8>K19⩾K18≈K20) (Fig. 1A). The keratin protein content in these tissues was determined and found to range between 0.2 to 0.5% of the total tissue protein content (Fig. 1B,C; see Materials and Methods for experimental details).

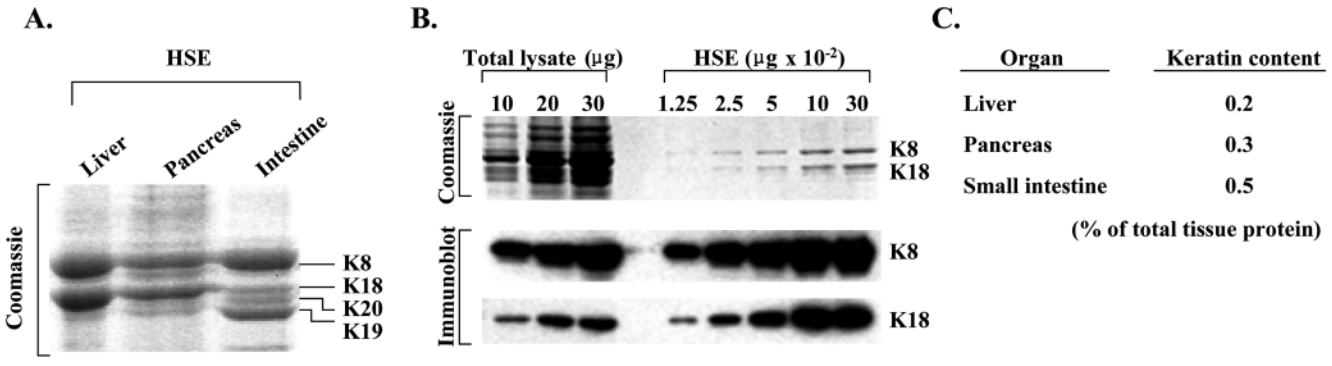

Fig. 1. Keratin content in mouse pancreas, liver and small intestine. (A) Liver, pancreas and small intestine were removed from Balb/c mice, and an enriched keratin fraction was obtained using high salt extraction (HSE) as described in Materials and Methods. Proteins were separated by SDS-PAGE, followed by Coomassie blue staining. Assignment of individual keratins was confirmed by two-dimensional gel analysis and immunoblotting using keratin-specific antibodies (not shown) (Zhou et al., 2003). (B) The indicated amounts (in μg) of serially diluted protein represent pancreatic keratins isolated by HSE or total pancreas lysates separated by SDS-PAGE. Resolved proteins were visualized by Coomassie staining or were analyzed by immunoblotting using keratin-specific antibodies. Similar analysis was done for keratins isolated from Balb/c mouse liver and distal ileum (not shown). (C) A summary of percent keratin protein contents in mouse liver, pancreas and small intestine (determined as described in Materials and Methods).

Keratin expression and dynamics in response to caerulein-induced pancreatitis

We investigated pancreatic keratin expression and dynamics in response to acute pancreatitis that is induced by caerulein – an established rodent pancreatitis model (Grise et al., 2000; Jensen et al., 1980; Metzler et al., 1999; Wagner et al., 1996). In this model, vacuoles, edema, cell death and infiltrating inflammatory cells are observed, with peak pancreas damage noted after 6 hours and gradual near complete histologic recovery 2 days after the first of seven hourly caerulein injections (Fig. 2A). Qualitative analysis of keratin expression, followed over a time-course, showed that K8/K18/K19 expression peaks 2 days after initiation of the injections, while actin levels remain minimally altered (Fig. 2B). A more refined time-course (2, 3, 4 and 5 days after caerulein injections) indicated that the peak of keratin expression is 2 days after the initiation of caerulein injections (not shown). The 2-day K8/K18/K19 peak expression is threefold more than basal keratin expression levels (Fig. 2C), as determined after Coomassie blue staining (Fig. 2C) or by immunoblotting (not shown, but see Materials and Methods). The increases in keratin proteins probably reflect transcriptional upregulation (or mRNA stabilization), given that K8/K18/K19 mRNA levels also increase significantly after caerulein treatment, while actin mRNA levels appear unchanged (Fig. 2D; see also Fig. 6B for ex vivo experiments). Notably, K20, a hitherto-undescribed keratin in mouse acinar cell pancreas is also induced at the protein and RNA levels after caerulein-induced pancreatitis (Fig. 2E). Both K19 and K20 form extended cytoplasmic filaments in association with their overexpression, which revert

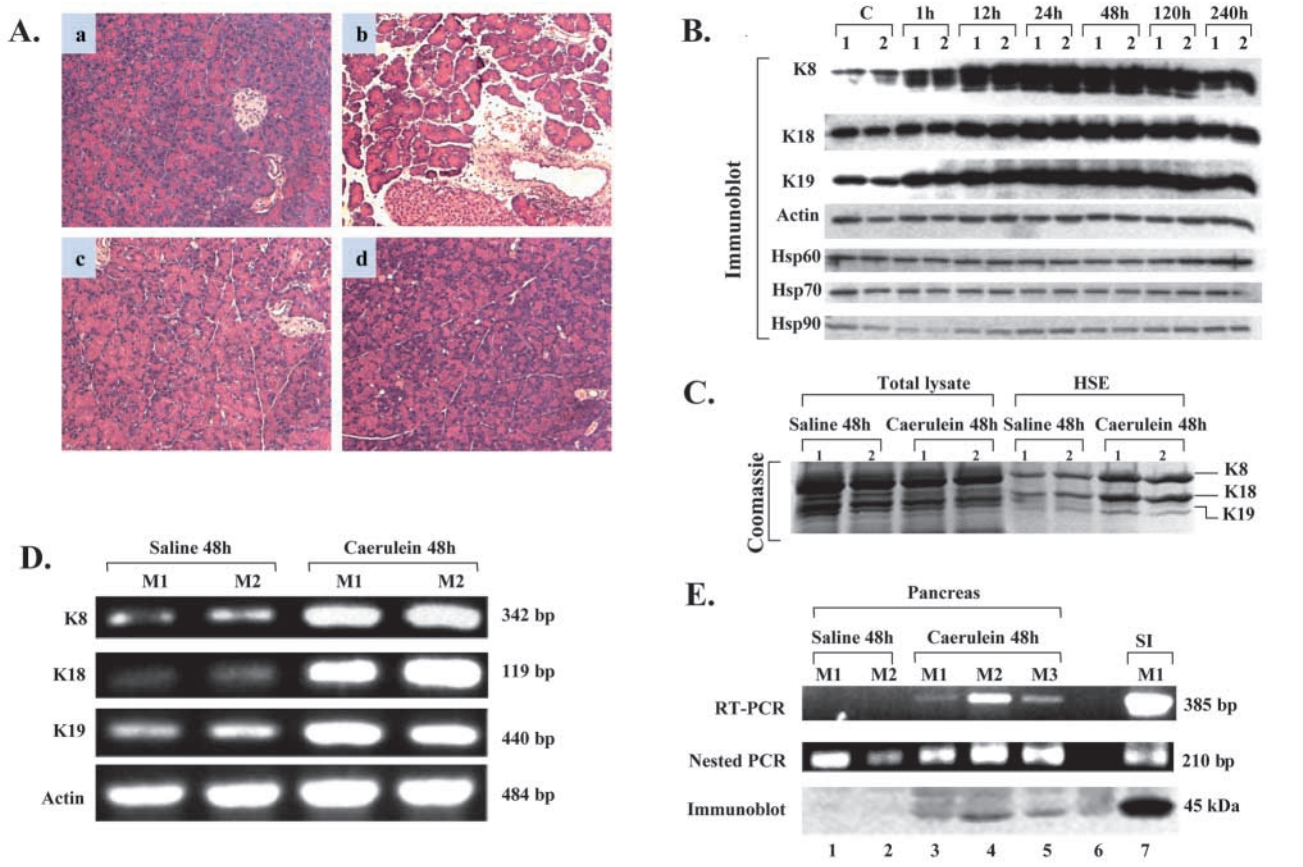

Fig. 2. Histologic, K8/K18/K19/K20 protein and mRNA analysis in caerulein-induced pancreatitis. (A) Mice were injected with caerulein (50 μg/kg, ip) seven times at hourly intervals, and euthanized at different time points (1-240 hours), after which pancreata were fixed then stained with hematoxylin and eosin. All time-points are given as hour after the first injection, and control mice were euthanized 48 hours after vehicle-alone administration (7 saline injections). a=control; b=6 hours after injection of caerulein 6× hourly; c=48 hours after injection of caeruelin 7× hourly; and d=240 hours after injection of caeruelin 7× hourly. (B) After caerulein injections (see Materials and Methods) pancreata were collected at the indicated time-points (two mice (1, 2) per time-point) then homogenized. Protein homogenates were separated by SDS-PAGE (20 μg/lane) then blotted using antibodies to the indicated antigens. Control mice 'C' were euthanized 48 hours after seven saline injections. (C) HSE or pancreatic tissue homogenates were prepared from mice injected with caerulein or saline (48 hours, two mice/condition). The amount of keratin isolated by HSE was normalized to the content of the detergent-soluble supernatant protein that was generated during the first step of the HSE procedure (see Materials and Methods), then analyzed by SDS-PAGE and densitometric scanning (to quantify fold-increase in keratins upon caerulein exposure). (D) Total RNA was isolated from pancreata 48 hours after caerulein or saline injection (two mice (M1, M2) per condition) followed by RT-PCR amplification of actin or the indicated keratin cDNAs. (E) Total pancreas RNA was isolated 48 hours after saline (2 mice) or caerulein (3 mice) treatment, or from the small intestine (SI, used as a control for K20 expression) of Balb/c mice (M1). The RNA was reverse transcribed using K20 primers (upper panel), followed by nested PCR using internal K20 primers (middle panel). HSE from the indicated tissues were also obtained and blotted using K20-specific Ab (lower panel). No sample was loaded in lane 6 in order to separate the small intestinal control from the remaining samples.

back to their baseline membrane-proximal apicolateral distribution 10 days after injury (Fig. 3).

Keratin expression and dynamics in response to CDD-induced pancreatitis

We tested the effect of the CDD pancreatitis model on keratin protein levels and dynamics. Three days of CDD feeding caused extensive pancreatic damage including inflammation, massive necrosis, hemorrhage and edema (Fig. 4A; compare a and b) in association with significant protein degradation (Fig. 4B, see profile of 3 day time-point (3d)). Tissue recovery is rapid and is clearly evident 1 day after discontinuation of CDD feeding (Fig. 4A,c); it is nearly complete 7 days after discontinuation of the CDD (Fig. 4A,d). As in the caerulein model, keratin overexpression is also noted and peaks 1-2 days after discontinuation of CDD feeding (i.e. days 4 and 5 after initiation feeding, Fig. 4B). Keratin levels revert to baseline 7 days after cessation of CDD feeding (Fig. 4B, 10d). K8/K18/K19/K20 overexpression also occurs at the mRNA level and is associated with extensive K19/K20 cytoplasmic filament formation (not shown), similar to what was observed in the caerulein model (Figs 2, 3). CDD feeding had no significant effect on heat stress hsp60/70/90 protein expression, except for the degradation that occured on day 3 (Fig. 4B).

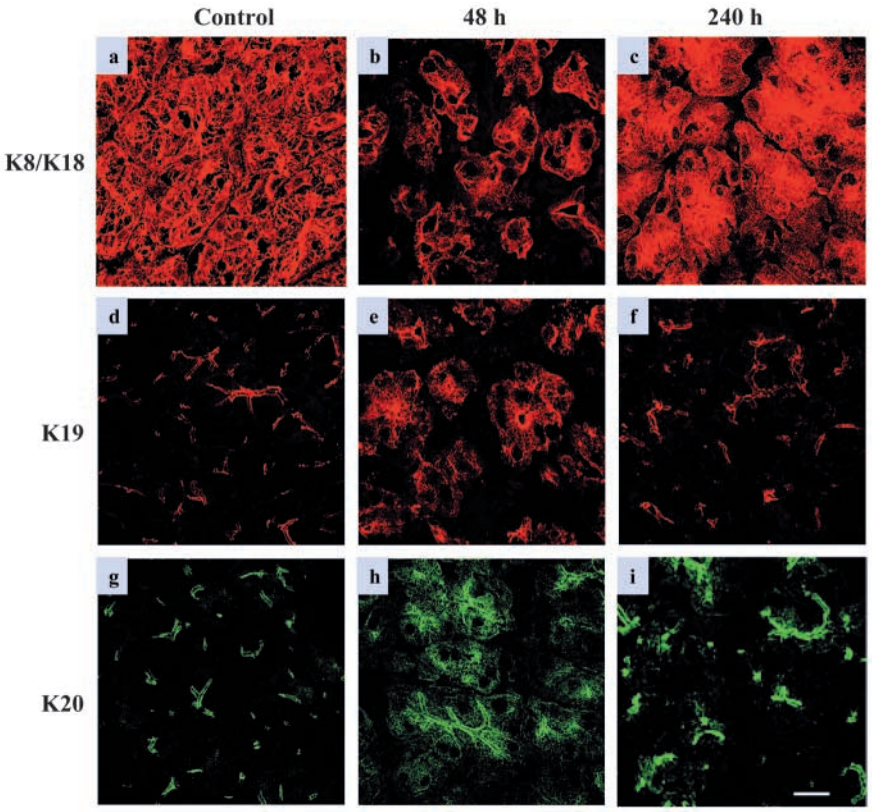

Fig. 3. Keratin filament dynamics after caerulein-induced pancreatitis. Pancreata from control (saline-injected) and caerulein-injected (48 hours and 240 days) mice were processed for immunofluorescence staining with antibodies to K8/K18 (a-c), K19 (d-f) and K20 (g-i). Bar, 20 μm.

Pancreatic keratin expression in response to the generalized stresses of hyperthermia and water immersion

The heat stress proteins hsp60 and hsp70 are expressed in mouse pancreas (Schafer and Williams, 2000). Hyperthermia or water immersion induces hsp70 or hsp60 expression, respectively, in rat pancreata, and pre-induction of these stress proteins protects from caerulein-induced pancreatic injury (Bhagat et al., 2000; Hietaranta et al., 2001b; Lee et al., 2000; Wagner et al., 1996). Given the known association of K8/K18 with hsp70 (Liao et al., 1995) and the increase in K8/K18/K19 protein levels in response to pancreatic injury, we asked if generalized stress, such as thermal or water-immersion stress, can affect keratin expression. As shown in Fig. 5A, hyperthermic stress induced pancreatic hsp70 expression, beginning 3 hours after the hyperthermic exposure, but no effect was noted in K8/K18/K19 expression. The hyperthermia-associated increase in hsp70 expression did not alter hsp60 or hsp90 levels, as expected (Fig. 5A). Similarly, water immersion had no effect on keratin protein levels when analyzed up to 12 hours (Fig. 5B) or up to 96 hours after the water immersion (not shown). Notably, and in contrast to rats (Lee et al., 2000; Otaka et al., 1994), Balb/c mouse hsp60 is not induced after water immersion stress. Therefore, heat and water-immersion regulation of hsp70 and hsp60 expression, respectively, appears to be similar in rat, and mouse in the case of hsp70 but not hsp60. Pancreatic K8/K18/K19 expression is induced in response to tissue-specific pancreatic stress but not in response to generalized forms of stress such as heat or water immersion.

Role of NF-κB in keratin overexpression in response to caerulein-induced pancreatitis

We investigated the mechanism of keratin overexpression in response to caerulein-induced pancreatitis. For this, we focused on NF-κB, given its known important role in regulating gene expression during inflammation (Grisham, 1999) and its reported activation in the pancreas in response to caerulein-induced injury (Frossard et al., 2001; Hietaranta et al., 2001b; Steinle et al., 1999; Tando et al., 1999; Wagner et al., 1996). We carried out our analysis using ex vivo cultured pancreatic acini. First, we assessed peak NF-κB activation in our experimental conditions on exposure of acini to caerulein. As shown in Fig. 6A, peak NF-κB activation occured after 2 hours of exposure to 0.1 μM caerulein (lanes 1-6), and this activation is inhibited in the presence of the NF-κB inhibitor MG-132 (a proteasome inhibitor (Jensen et al., 1995)) in a dose-dependent fashion (lanes 7-12). Second, we showed, using real-time RT-PCR amplification, that K8/K18/K19 mRNA levels do indeed increase (1.5-2 fold, $P<0.01$) in

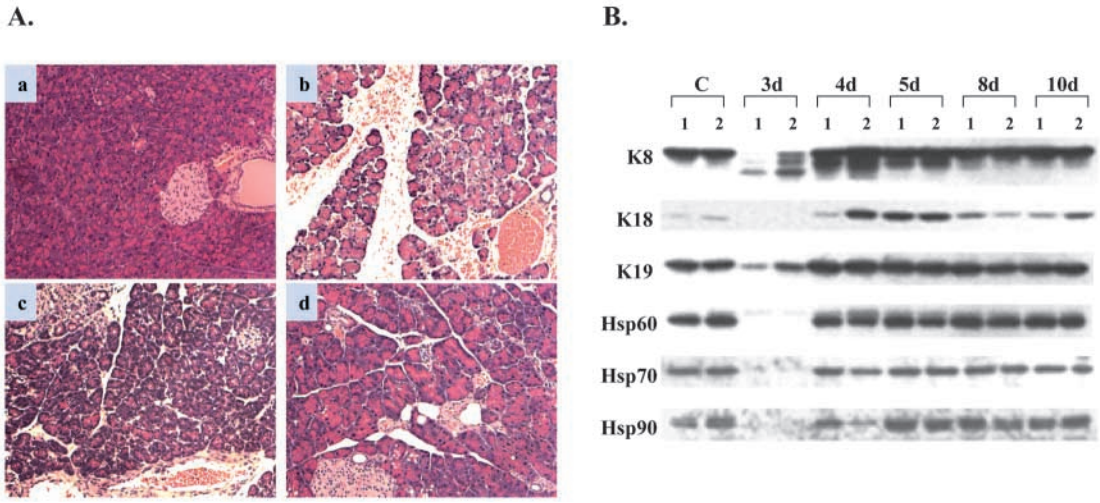

Fig. 4. Keratin expression on CDD-induced pancreatitis. (A) Mice were fed a CDD for 3 days, followed by harvesting of pancreata from CDD-fed or from normal-diet-fed mice at various time-points then fixing and staining with hematoxylin and eosin. a=control diet; b=3 days of CDD feeding; c=3 days of CDD feeding then 1 day or normal diet; d=3 days of CDD feeding then 7 days or normal diet. (B) Pancreata were isolated from control-diet-fed (C) or CDD-fed mice. Feeding was done for 3 days followed by switching to a normal diet for 1 day (4d), 2 days (5d), 5 days (8d) or 7 days (10d). Pancreas fragments were solubilized in sample buffer, then equal amounts of protein (20 μg/lane) were analyzed (two separate mice/time-point) by blotting with antibodies to the indicated proteins.

response to caerulein (Fig. 6B), thereby mimicking the overexpression of these keratins observed in vivo (Fig. 2). Notably, caerulein-mediated K8/K18/K19 mRNA induction was blocked by 1 hour pretreatment of the cultured acini with MG-132 ($P<0.01$, Fig. 6B), while MG-132 alone had no significant effect on keratin mRNA levels (Fig. 6B). Similar results were obtained on pretreatment with the NF-κB inhibitor PDTC (Fig. 7A). PDTC inhibits NF-κB by inhibiting the IκB-ubiquitin ligase activity (Hayakawa et al., 2003). The caerulein-mediated increase in keratin mRNA is likely to be related, at least in part, to an increase in transcription, as pretreatment with actinomycin D (Fig. 7B) blocked the observed increase. Taken together, these results indicate that caerulein-induced overexpression of K8/K18/K19 is mediated by increased transcription and translation ex vivo, and thereby probably in vivo, in association with NF-κB activation.

Discussion

The important findings of our study are: first, quantification of the keratin content in mouse pancreas, liver and small intestine (Fig. 1); second, keratin expression is induced nearly threefold in response to two independent pancreas injury models (Figs 2, 4), reminiscent of a stress-protein response. This induction is dramatic, given how abundant the keratins are to begin with, and does not involve other major cytoskeletal elements such as actin microfilaments and tubulin microtubules. Keratin induction occurs, at least in part, via increased transcription of keratin mRNA (Fig. 7B); third, K20 is expressed in the pancreas at low levels (in a membrane-proximal distribution) but is reversibly overexpressed after pancreatic injury with the formation of new extended cytoplasmic filaments similar to those that include K19 (Figs 2, 3); fourth, generalized forms of stress, such as heat and water immersion, do not induce keratin overexpression (Fig. 5); and fifth, pancreatic keratin

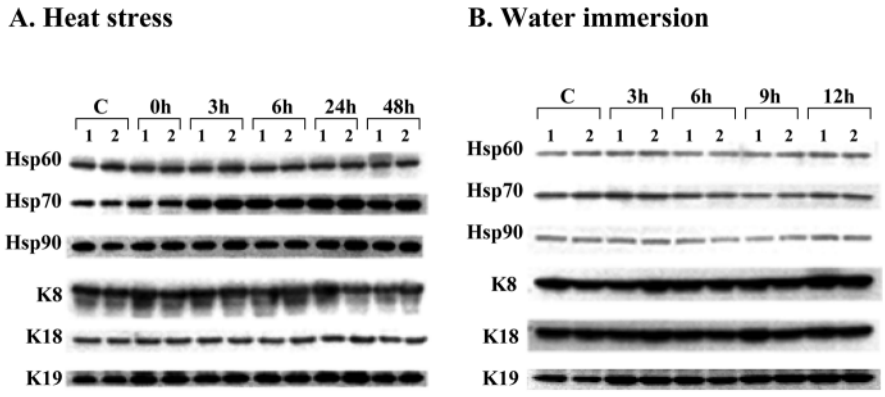

Fig. 5. Keratin expression after hyperthermia and water-immersion stresses. (A) Mouse core body temperature was increased to 42°C and maintained at this temperature for 20 minutes. Mice were then returned to their normal environment and allowed to maintain their usual activity and food/water intake. Pancreata were harvested from control non-heat exposed mice (C) or from the heat-stressed mice 0, 3, 6, 24 and 48 hours after the heat exposure. Two mice were used for each condition. Pancreatic total tissue homogenates were prepared and immunoblotted with antibodies to the indicated antigens. (B) Mice were immersed in a water bath (23°C) for 6 hours as described in Materials and Methods, then returned to their cages and allowed to maintain their usual activity and food/water intake. Pancreata were harvested from control non-water-immersed mice (C) or from the water-immersed mice 3, 6, 9 and 12 hours after removal from the water immersion. Total pancreatic protein extracts were prepared then analyzed by blotting as in A. Two mice were used for each condition.

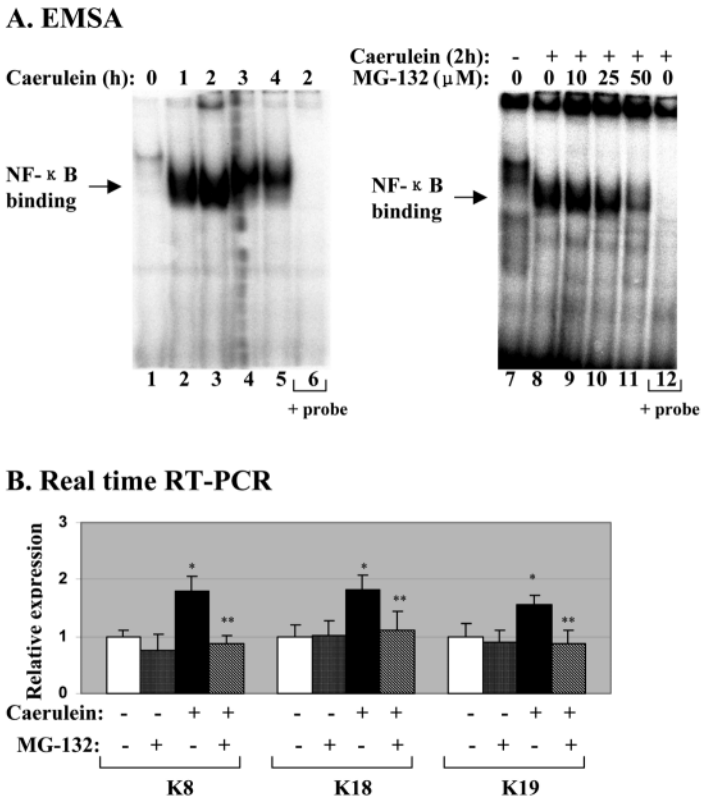

Fig. 6. NF-κB activity and keratin mRNA expression after ex vivo treatment of isolated acini with caerulein +/–MG-132. (A) Lanes 1-6: freshly isolated mouse pancreatic acini were treated with caerulein for 1-4 hours (37°C) followed by nuclear protein extraction and electrophoretic mobility shift assay (EMSA) as described in Materials and Methods. Binding in the presence of excess unlabeled NF-κB-binding oligonucleotide (+probe, lane 6) is included as a specificity control. Lanes 7-12: acini were incubated in the presence or absence of caerulein for 2 hours (±MG-132 for 1 hour before the caerulein incubation) followed by nuclear protein extraction and EMSA as in lanes 1-6. (B) Pancreatic acini were incubated with caerulein (0.1 μM, 4 hours) with or without pretreatment with MG-132 (50 μM, 1 hour). Total RNA was isolated followed by real-time RT-PCR analysis of K8, K18 and K19 mRNA as described in Materials and Methods. Expression of each keratin mRNA was normalized to expression of the L7 gene (not shown). Results represent the mean±s.d. of three independent experiments. *P<0.01 vs. control; **P<0.01 vs. caerulein.

overexpression in response to injury is associated with, and is probably related to, NF-κB activation (Figs 6 and 7).

Keratins are abundant and dynamic proteins in simple epithelia

Our results show that keratins make up 0.2%, 0.3% and 0.5% of the total protein content of mouse liver, pancreas and small intestine, respectively. Given that keratins are selective markers of epithelial cells, it is likely that K8/K18/K19 expression in epithelial cells of these tissues is somewhat higher than our estimates because our quantification does not take into account extracellular proteins and other resident nonepithelial cells. However, the estimates are unlikely to be higher than the 5% that we measured for K8/K18 in the cultured human colonic cell line HT29 (Chou et al., 1993). One important finding in this study is the reversible threefold increase in keratin expression in response to injury, which is remarkable given the high basal keratin expression level. The return of keratin protein from

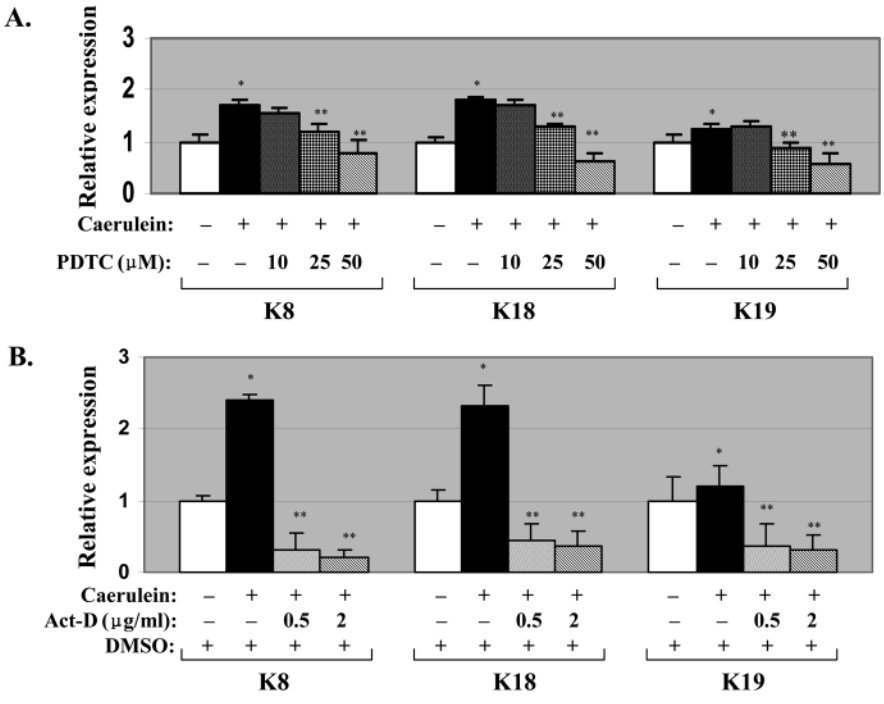

Fig. 7. Ex vivo acinar keratin mRNA expression after pretreatment with PDTC or Act-D. Isolated acini were incubated with medium±caerulein. The acini that were stimulated with caerulein were pre-incubated with 10, 25 or 50 μM of PDTC (A) or 0.5 or 2 μg/ml of Act-D (B) for 30 minutes followed by adding caerulein for 4 hours. The acinar cultures in B that did not include Act-D also included 0.1% DMSO (the solvent used for Act-D). Acini were then harvested and washed twice with PBS, followed by processing for real-time RT-PCR. L7 was used as internal control. The results represent mean±s.d. of three independent experiments. *P<0.01 vs. control; **P<0.01 vs. caerulein

post-injury to pre-injury levels suggests that excess keratin protein is likely to turn over by activation of a degradation pathway, as basal keratin turnover is slow as noted in cultured mouse hepatocytes and human colonic HT29 cells (Chou et al., 1992; Denk et al., 1987). Such degradation may be mediated by ubiquitination and the proteasome as shown for K8/K18 in HT29 cells and cell transfection systems (Ku and Omary, 2000).

Another potentially important aspect of keratin response to injury is the dramatic change in K19/K20-containing filament organization. As such, K19 and K20 protein levels increase commensurate with the formation of K19/K20-containing extended cytoplasmic filaments, which are normally located nearly exclusively in a band-like fashion at apico-lateral membrane proximal domains. These newly formed K19/K20-containing filaments colocalize with the normally resident K18-containing cytoplasmic filaments. Additional studies will help to clarify whether the newly formed K19/K20-containing filaments exhibit a cytoplasmic localization simply as part of de novo synthesis that is followed ultimately by degradation or transport to their typical plasma membrane-proximal localization.

Do keratins behave as stress proteins?

The induction of keratin overexpression in response to stress is highly reminiscent of the classic heat stress protein response to injury. Support for an essential role of keratins in protection from mechanical and non-mechanical forms of stress is found in tissues where their tissue-associated diseases are caused by, or predisposed to, keratin mutations (Coulombe and Omary, 2002; Fuchs and Cleveland, 1998; Irvine and Mclean, 1999), and in a variety of well-studied animal models that either lack or overexpress a mutant keratin (Coulombe and Omary, 2002; Magin et al., 2000). The disease-related tissues that are associated with keratin mutations include skin, cornea, oral cavity and liver. Keratin overexpression in response to injury has already been described in livers of mice fed the toxins DDC, GF or cholic acid; or subjected to bile duct ligation (Fig. 8) (Cadrin et al., 2000; Denk et al., 2000; Fickert et al., 2002). Our results, coupled with those of others, clearly indicate that the nature of the stress determines whether heat stress protein induction or keratin induction occurs (Fig. 8). For example, generalized stresses such as heat or water immersion upregulate hsp protein expression in several internal epithelia but do not impact on keratin expression in the pancreas. By contrast, tissue-targeted stress such as caerulein or CDD cause pancreatitis and increase pancreatic keratin expression without having a significant impact on hsp60/70/90 expression.

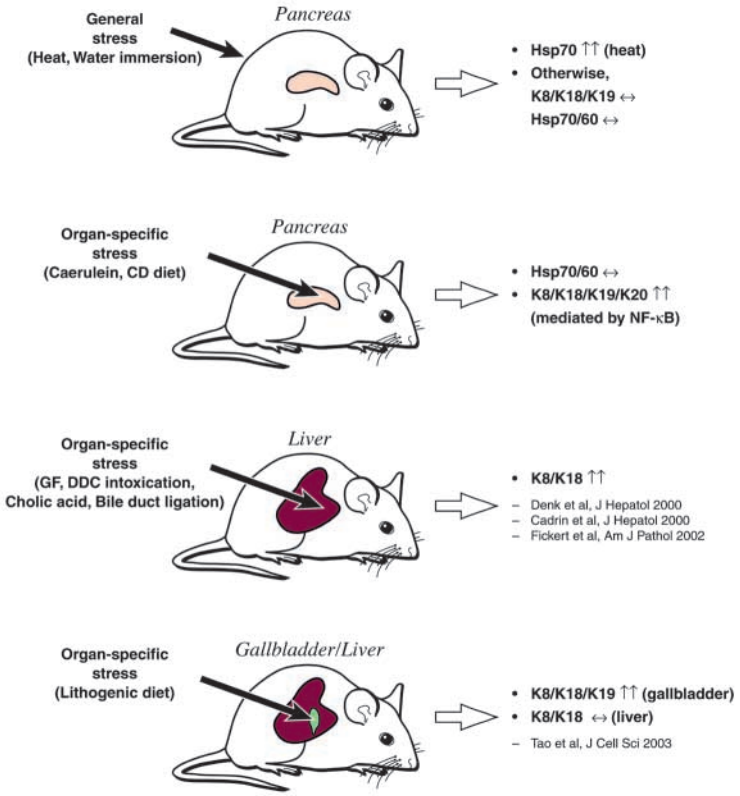

Fig. 8. Summary of generalized versus organ-specific stress on keratin expression. Generalized forms of stress, such as heat, which upregulates hsp70, do not have an impact on keratin expression levels in Balb/c mouse pancreas. By contrast, organ-specific stress that targets the pancreas, such as caerulein or CDD, increase pancreatic keratin levels without having a significant effect on hsp70/60 levels. The increased keratin expression in response to caerulein occurs in association with NF-κB activation. Similar findings, in terms of organ-specific effects on keratin overexpression, have also been noted in the liver (Cadrin et al., 2000; Denk et al., 2000; Fickert et al., 2002). For example, feeding mice the hepatotoxins griseofulvin (GF) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) also results in keratin overexpression. However, not all forms of liver injury induce keratin overexpression, as feeding mice a lithogenic diet causes liver injury without affecting keratin expression levels. The lithogenic diet also results in cholelithiasis and gall bladder injury, with concurrent overexpression of gallbladder keratins (Tao et al., 2003).

Our working model is that keratins can behave as stress-related genes that are induced in response to unique stresses in a highly dynamic fashion. Although keratin overexpression in response to stress appears to be a general phenomenon, this response is not universal. For example, keratin overexpression occurs in the pancreas (work herein), liver (Cadrin et al., 2000; Denk et al., 2000; Fickert et al., 2002) and gallbladder in response to injury, but such overexpression is not seen in the liver after feeding mice a lithogenic diet that induces steatohepatitis (Tao et al., 2003). K8/K18 undergo hyperphosphorylation in response to stress in every tissue context and injury model that has been examined to date, and indeed, the unique LLS/TPL phosphorylation motif that is found exclusively in several type II keratins (Toivola et al., 2002) is phosphorylated in K8 by p38 or Jun kinases in response to a variety of stresses (He et al., 2002; Ku et al., 2002) including caerulein and CCD-induced pancreatitis (Toivola et al., 2000a). In contrast to the situation in the liver, where the overexpression of keratins is likely to be important, given their well-established protective role (Omary et al., 2002), the significance of keratin overexpression in the pancreas is unclear because the absence of keratins in K8-null mice does not increase their susceptibility to pancreatic injury induced by caerulein or CDD (Toivola et al., 2000a). Furthermore, rechallenge with caerulein 2 days after the initial challenge (i.e.

at peak keratin overexpression and when recovery begins) does not appear to be protective and, if anything, manifests more severe injury than in mice that are challenged only once (not shown). Hence, we propose that injury-induced keratin overexpression in the pancreas may reflect a fine-tuning or bystander effect which suggests a different function for these proteins in the pancreas as compared with the liver. Furthermore, keratins are overexpressed in response to injury in several organs, akin to heat stress proteins, but at least in the case of the pancreas their overexpression does not appear to provide protection from pancreatic injury, as appears to be the case for hsp70 or hsp60 after heat stress or water immersion, respectively (Bhagat et al., 2000; Grise et al., 2000; Hietaranta et al., 2001b; Lee et al., 2000; Wagner et al., 1996).

Mechanism of keratin overexpression in the pancreas

NF-κB is a ubiquitous transcription factor and pleiotropic regulator of numerous inflammatory processes. Once activated, NF-κB translocates from the cytosol to the nucleus where it activates the transcription of a spectrum of pro-inflammatory genes (Grisham, 1999). Although NF-κB is a key regulator of cytokine expression in different cell types (Barnes and Karin, 1997; Blinman et al., 2000), there are only a few reports that link keratin transcription with NF-κB. For example, K17 gene expression is induced after respiratory syncytial virus infection of epithelial cells in an NF-κB-associated fashion (Domachowske et al., 2000). Similarly, NF-κB and C/EBPβ are necessary for tumor necrosis factor-induced K6b expression in the epidermis (Komine et al., 2000). The only potential linkage of K8/K18/K19 overexpression with NF-κB is the observation that caerulein activates pancreatic acinar cellular NF-κB both in vivo and vitro (Frossard et al., 2001; Hietaranta et al., 2001b; Steinle et al., 1999; Tando et al., 1999; Wagner et al., 1996). Our ex vivo results show that inhibition of NF-κB, using two independent inhibitors, also inhibited caerulein-induced upregulation of K8/K18/K19 mRNA, thereby providing evidence that in vivo induction of keratins in the pancreas on injury may be mediated, at least in part, by NF-κB. Our ex vivo results also suggest that caerulein-induced NF-κB activation and subsequent keratin upregulation may occur independently of cytokine release from activated mononuclear cells, unless such activation involves resident nonepithelial cells. It remains to be determined if NF-κB plays a major role in injury-induced keratin overexpression in the liver, and whether other transcription factors such as those belonging to the families of AP-1 and ETS may also play a role in such overexpression. For example, AP-1 and ETS are implicated in the persistence of K18 expression in epithelial tumors where, typically, the expression of such differentiation markers is suppressed (Oshima et al., 1996).

We are grateful to Drs. Ningguo Feng, Caroline Heckman Hsienming Hu and Tao Li for technical advice. We also thank Steve Avolicino (Histo-tec Laboratory, Hayward, CA) for histology staining. This work was supported by NIH grant DK52951, a VA Merit Award and by NIH Digestive Disease Center grant DK56339.

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