

Keratin-dependent, Epithelial Resistance to Tumor Necrosis Factor-induced Apoptosis

Carlos Caulin,* Carl F. Ware,† Thomas M. Magin,§ and Robert G. Oshima*

*Cancer Research Center, The Burnham Institute, La Jolla, California 92037; †La Jolla Institute for Allergy and Immunology, San Diego, California 92121; §Institut fuer Genetik, Abteilung Molekulargenetik, Universitaet Bonn, Bonn, Germany

Abstract. Tumor necrosis factor (TNF) is a cytokine produced by macrophages and T lymphocytes that acts through two distinct receptors, TNFR1 (60 kD, CD120a) and TNFR2 (80 kD, CD120b), to affect cellular proliferation, differentiation, survival, and cell death. In addition to its proinflammatory actions in mucosal tissue, TNF is important for liver regeneration. Keratin 8 (K8) and keratin 18 (K18) form intermediate filaments characteristic of liver and other single cell layered, internal epithelia and their derivative cancers. K8-deficient ($K8^{-}$) mice, which escape embryonic lethality, develop inflammatory colorectal hyperplasia, mild liver abnormalities, and tolerate hepatectomy poorly. We show that normal and malignant epithelial cells deficient in K8 and K18 are ~ 100 times more sensitive to

TNF-induced death. K8 and K18 both bind the cytoplasmic domain of TNFR2 and moderate TNF-induced, Jun NH_2 -terminal kinase (JNK) intracellular signaling and $NF\kappa B$ activation. Furthermore, $K8^{-}$ and $K18^{-}$ mice are much more sensitive to TNF dependent, apoptotic liver damage induced by the injection of concanavalin A. This moderation of the effects of TNF may be the fundamental function of K8 and K18 common to liver regeneration, inflammatory bowel disease, hepatotoxin sensitivity, and the diagnostic, persistent expression of these keratins in many carcinomas.

Key words: cytoskeleton • intermediate filament • inflammatory bowel disease • tumor necrosis factor receptor 2

Introduction

Keratins are obligate heteropolymers composed of type I (acidic, K9 through K20) and type II (neutral-basic, K1 through K8) subunits, which form filaments intermediate in size between actin microfilaments and microtubules. Skin abnormalities associated with mutations of specific epidermal keratins have provided strong evidence for a mechanical function for the intermediate filaments of epidermis (Fuchs and Weber, 1994). However, evidence for a mechanical function for K8 and K18 is less compelling. K8 and K18 form intermediate filaments in trophectoderm, extraembryonic endoderm, and a variety of adult epithelial organs, including liver, lung, kidney, pancreas, gastrointestinal tract, mammary gland, and the cancers arising from these organs (Oshima et al., 1996). In certain genetic backgrounds, most K8-deficient ($K8^{-}$)¹ mice die well after the initial formation

of K8/K18 filaments in the trophectodermal layer of blastocysts (Baribault et al., 1993). However, some $K8^{-}$ and all $K18^{-}$ mice survive to adulthood (Baribault et al., 1994; Magin et al., 1998). $K8^{-}$ mice that escape embryonic lethality develop inflammatory colorectal hyperplasia similar to mice deficient for the T cell receptor (Baribault et al., 1994). $K8^{-}$ hepatocytes generally fail to survive explantation after partial hepatectomy (Loranger et al., 1997). Transgenic mice that overexpress a disrupting, mutant form of K18 develop mild chronic hepatitis and are more sensitive to the hepatotoxins acetaminophen and griseofulvin (Ku et al., 1995, 1996). K18 binds several different cytoplasmic proteins, however, the biological significance of these interactions are not yet clear. (Omary and Ku, 1997). The inflammatory bowel disease and liver abnormalities of mice with disrupted or no K8 may involve proinflammatory and apoptotic cytokines related to TNF.

Address correspondence to Robert G. Oshima, Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. Tel.: (858) 646-3147. Fax: (858) 713-6268. E-mail: rgoshima@burnham-inst.org

¹Abbreviations used in this paper: CHX, cycloheximide; ConA, concanavalin A; GST, glutathione S-transferase; JNK, Jun NH_2 -terminal ki-

nase; K8, keratin 8; K18, keratin 18; $K8^{-}$, homozygous null *Krt2-8* targeted mutation; $K18^{-}$, homozygous null *Krt1-18* targeted mutation; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TNFR2, tumor necrosis factor receptor 2; TRAF, TNF receptor associated factors.

Multiple members of the TNFR family of receptors (TNFR1, Fas, DR3, TRAIL-R1, and TRAIL-R2) contain a death domain that nucleates an apoptotic signaling complex with binding of the adapter protein, FADD, activation of caspase 8 and downstream caspases that results in cell death (Ashkenazi and Dixit, 1998). TNFR2 and other members of the TNF receptor family (LT β R, CD40, and CD30) lack a death domain, but also bind members of the TNF receptor-associated factors (TRAF) family. TRAF3 is proapoptotic (VanArsdale et al., 1997), whereas TRAF2 and TRAF5 may inhibit apoptosis. One mechanism of TNFR2 induction of apoptosis is through the induction of TNF, which then stimulates TNFR1 (Grell et al., 1999). TNF is also essential for liver regeneration (Yamada et al., 1997). Here, we present evidence for a key role for K8 and K18 in moderating the signaling and effects of TNF. This moderating effect provides epithelial cells with resistance to the apoptotic effects of TNF.

Materials and Methods

HR9 mouse parietal endodermal cells and the HR-1 and HR-7 clonal derivatives and their culture conditions have been described (Kulesh et al., 1989). A K8⁻ MDA MB435 human breast carcinoma cell line was received from Mary Hendrix (University of Iowa). After transfection with the LK444-K8 expression vector (Kulesh et al., 1989), multiple K8⁺ and K8⁻ clones resistant to 400 μ g/ml G418 were isolated. Expression of K8 mRNA in these clones restored K8/K18 filaments detected by immunostaining (data not shown). The K8⁻ epithelial cell line designated CMK8 was isolated from differentiated K8⁻ ES cells, subcloned, and rescued by cotransfection with the EndoA gene and PGK-neo. Multiple K8 cytoskeleton positive and negative clones were then isolated and compared. For drug sensitivity studies, the indicated concentrations of drugs were added to near confluent cultures in complete medium containing 10% FBS. Cell viability was determined by the MTT dye reduction assay (Green et al., 1984).

pGEX vectors containing the cytoplasmic domains of CD40, LT β R,

HVEM, Fas, TNFR1, and TNFR2 were used for expressing and purifying the corresponding glutathione *S*-transferase (GST) fusion proteins (Mosiakos et al., 1995). pGEX-Fas and pGEX-TNFR2 were gifts from Dr. Takaaki Sato (Columbia University, New York). GST fusion proteins were purified from *Escherichia coli* (A0202) bacteria after induction in 0.1 mM of isopropyl- β -D-thiogalactopyranoside for 3 h following the manufacturer's instructions (Pharmacia Biotech). GST fusion protein binding experiments were performed using 5 μ g of each protein (Sato et al., 1995). K8 and K18 proteins were synthesized and K18 was cleaved with caspase 6 after coupled transcription and translation reactions as described previously (Caulin et al., 1997).

HR9 cells on coverslips were fixed with cold methanol and were then processed for immunostaining using rabbit antiserum #18 for K18 (Oshima, 1981), anti-K18 mAb CK5 (Sigma Chemical Co.), monoclonal rat anti-human TNFR2 (Genzyme Diagnostics), FITC-labeled goat anti-rat IgG (Jackson ImmunoResearch), and rhodamine-labeled goat anti-rabbit (Sigma Chemical Co.). Cells were visualized with a BioRad MRC 1024 confocal microscope. Figures were generated with the use of Adobe Photoshop software. Single confocal sections were used to visualize colocalized proteins.

Jun N-terminal kinase (JNK) activity was assessed as described (Cavigelli et al., 1995). HR9 and HR-7 cells were transfected with 1 μ g of pCMV-M2-FLAG-JNK1 by the calcium phosphate precipitation method. 48 h after the addition of the DNA, the cells were treated with 10 ng/ml of TNF for the indicated times and assayed for JNK activity. Film signals were quantified using the NIH Image software. Reporter genes for NF κ B (NF κ B-luc) and Ets (E18-luc) were transfected and assayed as described (Galang et al., 1996). The human β -actin promoter-driven *LacZ* gene was included for normalization of transfection efficiency. The cells were treated with 10 ng/ml of TNF for 6 h, 48 h after the addition of DNA. Luciferase and β -galactosidase activity was determined with the Dual-Light commercial kit (Tropix) with an EGG Berthold luminometer. Relative luciferase activity was normalized to the β -galactosidase activity and expressed as a percentage of the maximum activity.

The K8⁻ mice were in an FVB/N genetic background (Baribault et al., 1993, 1994). The K18⁻ mice (Magin et al., 1998) had a mixed background (129/Sv,MF1,FVB/N). Littermates without targeted keratin alleles were used as controls. Male mice (12–14-wk-old) were fasted for 24 h before i.v. injection of concanavalin A (ConA; 30 mg/kg), dissolved in 200 μ l of pyrogen-free saline (Tiegs et al., 1992). 8 h after injection, blood was collected by cardiac puncture. Serum was frozen at -85°C. The activity of alanine aminotransferase and aspartate aminotransferase was measured with a commercial kit (Sigma Chemical Co.) according to the directions of the

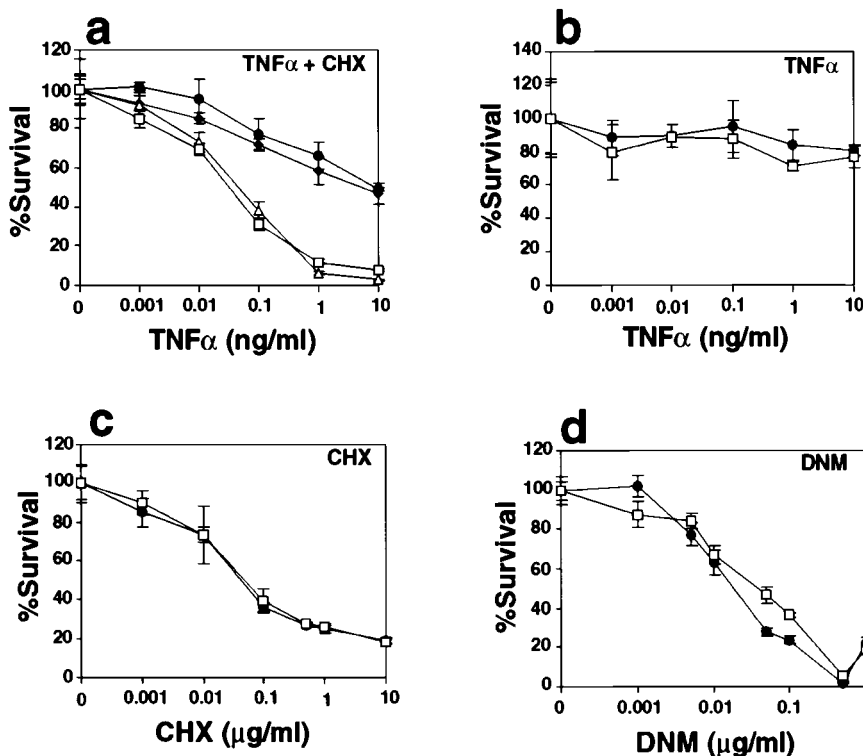


Figure 1. Keratin-dependent TNF sensitivity. Cells were incubated for 12 h with the indicated amounts of TNF in the presence of 5 μ g/ml CHX (a), or for 24 h with TNF alone (b), CHX alone (c), or daunomycin (DNM; d). Cell survival is expressed as the mean of triplicate samples \pm SD. Filled circles and diamonds represent HR9 and a stable HR9 transfectant expressing wild-type K18, respectively. Open triangles and squares are HR-1 and HR-7 clones, respectively, that express dominant negative K18 (Kulesh et al., 1989).

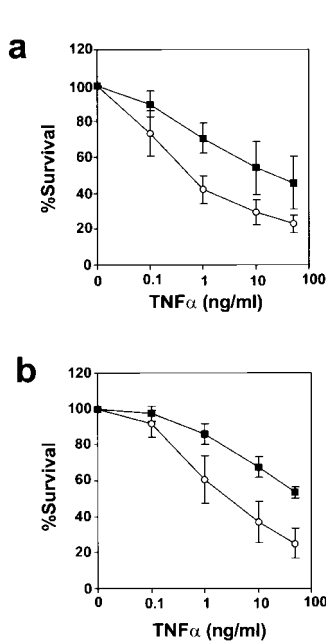


Figure 2. TNF sensitivity of MDA-MB-435 and CMK8 epithelial cells. **a**, K8-transfected MDA-MB-435 cells (filled squares) and K8 negative cells (open circles) were treated for 12 h with the indicated amounts of TNF in the presence of 15 μ g/ml of CHX. Cell survival was determined with the MTT assay. Data represent the mean \pm SD of three independent K8⁻ and three K8⁺ clones. **b**, the K8⁻ CMK8 epithelial cell line was transfected with the mouse K8 gene. TNF sensitivity was determined in the presence of 2.5 μ g/ml of CHX. Open circles represent the mean of three keratin negative clones. Filled squares represent the mean of three keratin positive clones.

manufacturer. Livers were fixed in 10% neutral-buffered formalin and embedded in paraffin. 5- μ m thick sections were stained with hematoxylin and eosin or for detection of apoptosis. Apoptosis was detected with the ApopTag kit (Oncor) according to the instructions of the manufacturer.

Results

We first determined the influence of K8 and K18 on the sensitivity of normal epithelial cells to TNF. HR-1 and HR-7 are two independent subclones of HR9, a mouse parietal endodermal cell line. Both subclones are deficient in K8 and K18, due to the expression of a disrupting, truncated form of K18 (Kulesh et al., 1989). Like many cultured cells, HR9 cells are resistant to killing by TNF unless the induction of protective proteins is also inhibited by treatment with cycloheximide (CHX). HR-1 and HR-7 cells were nearly 100 times more sensitive to the combination of TNF and CHX than either the parental cells or a control clone that expresses full-length K18 (Fig. 1 a). None of the cell lines were killed by TNF alone (Fig. 1 b), and all had similar sensitivity to CHX-induced cell death (Fig. 1 c). No difference was observed in the sensitivity of HR-7 cells to apoptosis induced by treatment with daunomycin (Fig. 1 d). Similar results were obtained with multiple clones of the human breast carcinoma cell line (MDA-MB-435) deficient in K8 RNA expression (data not shown) and stably transfected subclones which express K8 (Fig. 2 a). In addition, an epithelial cell line established from K8⁻ ES cells was found to be more sensitive to TNF than multiple isolates of the same line that uniformly expressed keratin filaments due to transfected K8 (Fig. 2 b and data not shown).

To determine if K8 and K18 might alter TNF sensitivity

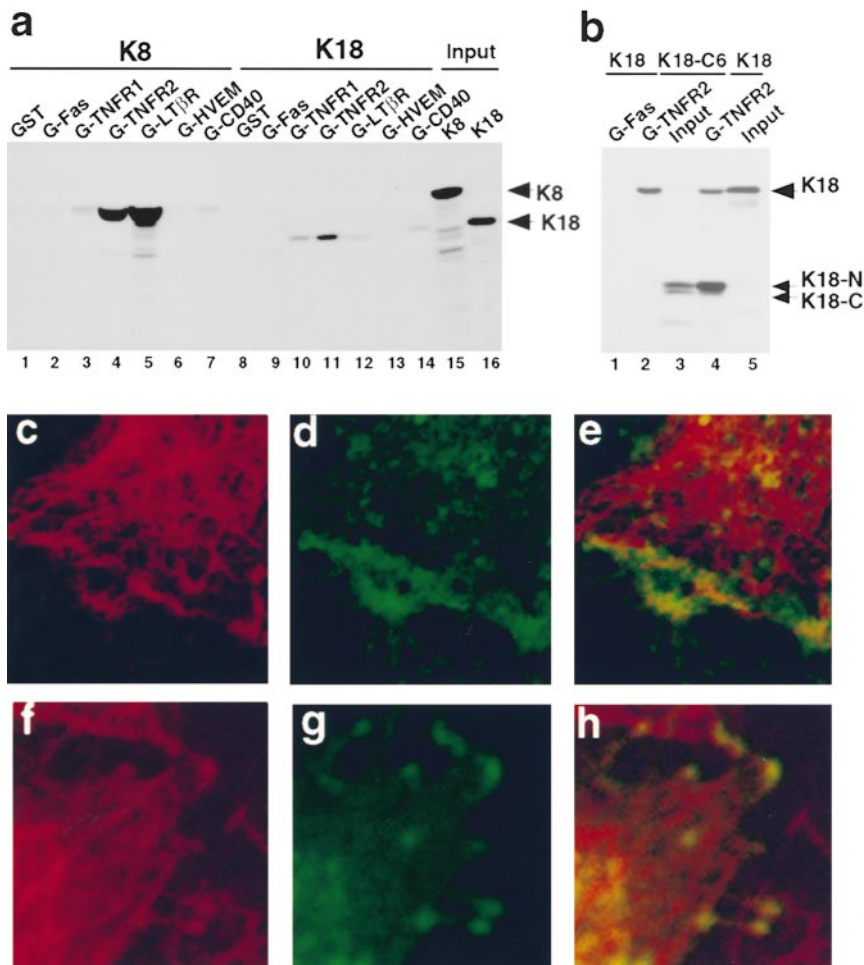


Figure 3. Binding of K8 and K18 to members of the TNFR family. **a**, GST fusion proteins (5 μ g) of the cytoplasmic domains of different members of the TNFR family were incubated with ³⁵S-methionine labeled, in vitro translated, K8 (lanes 1–7) or K18 (lanes 8–14). Input samples (lanes 15 and 16) represent 1/10 of the amount incubated with the fusion proteins. **b**, In vitro translated K18 (lanes 1 and 2) or caspase-6–cleaved K18 (lanes 3 and 4) was incubated with GST-TNFR2 (lanes 2 and 4) or GST-Fas (lane 1). Binding products were analyzed by SDS-PAGE and fluorography. **c–h**, Double immunofluorescent staining of HR9 (**c–e**) and SW13 cells stably transfected with K8 and K18 (**f–h**) were transiently transfected with TNFR2 and stained with polyclonal antiserum (**c**) or mAb for K18 (**f**) and mAb for TNFR2 (**d** and **g**). Colocalization of K18 and TNFR2 (**e** and **h**) was visualized in single confocal sections.

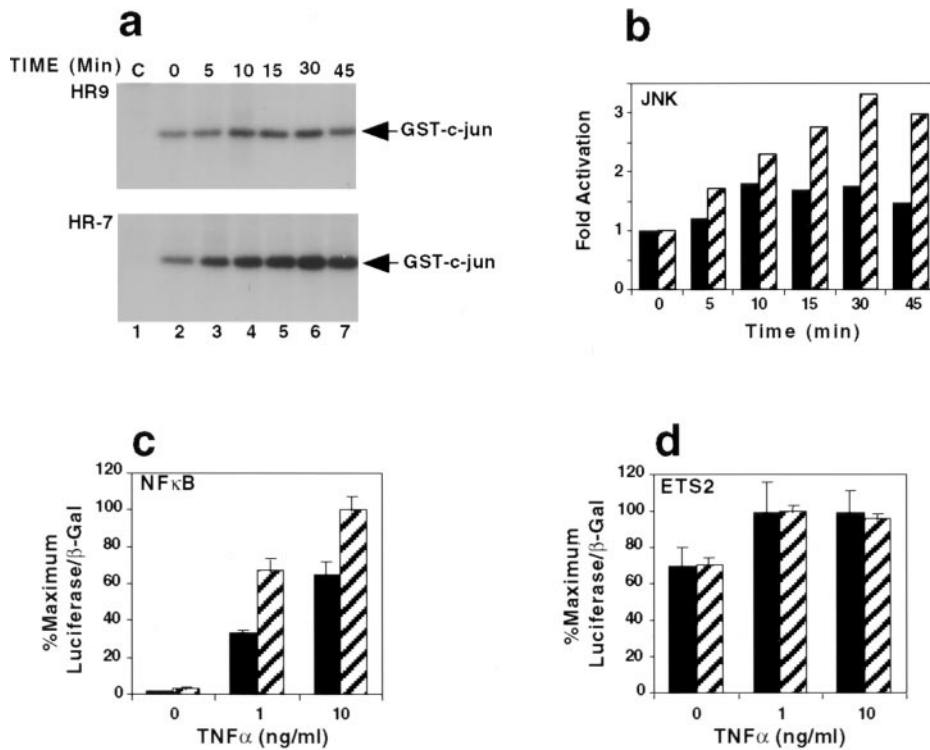


Figure 4. Downstream signaling induced by TNF. **a**, JNK activity after stimulation with TNF (10 ng/ml) in HR9 and HR-7 cells. Autoradiographic exposures of GST-c-jun after incubation with immunoprecipitated JNK and radioactive ATP are shown. **b**, Densitometric analysis of the films shown in **a**. Filled bars, HR9; hatched bars, HR-7. **c**, Normalized luciferase activity of NF κ B and Ets2 (**d**) reporter genes in HR9 (black bars) and HR-7 (hatched bars) after stimulation with 1 or 10 ng/ml of TNF. Results are represented as a percentage of the maximum activity.

by direct interaction with TNF receptors, we performed binding assays of in vitro translated K8 or K18 with GST fusion proteins of the cytoplasmic domains of different TNF receptor family members (Mosialos et al., 1995). K8 bound to the cytoplasmic domains of TNFR2 and LT β R (Fig. 3 a), which both lack death domains. K18 binds TNFR2, and more weakly with LT β R and inconsistently with TNFR1. No binding of K8 or K18 was detected with control GST protein alone, or with the Fas, or HVEM cytoplasmic domains. We localized the portion of K18 responsible for interacting with TNFR2 to the first 238 residues by performing binding studies with caspase 6-digested K18 (Caulin et al., 1997; Fig. 3 b). Caspase 6 cleaves K18 after residue 238, resulting in a 26-kD NH $_2$ -terminal fragment and a 22-kD COOH-terminal fragment. Incubation of GST-TNFR2 with K18 partially digested with caspase 6, resulted in enrichment for the larger 26-kD NH $_2$ -terminal fragment and the full-length, uncleaved K18 (Fig. 3 b, lane 4). Thus, the binding of TNFR2 appears specific for the NH $_2$ -terminal half of K18.

We analyzed the possible colocalization of K8 and K18 filaments with TNFR2 by expressing human TNFR2 (Weiss et al., 1997) in HR9 cells. Endogenous keratins and TNFR2 were detected by confocal microscopy of cells stained for both mouse K18 and TNFR2. TNFR2 was found in a diffuse pattern of membrane staining that was uniformly distributed over the cell surface and in granular, punctuate structures (Fig. 3 d). K8 and K18 intermediate filaments were colocalized with some of these granular forms of TNFR2, particularly at the flattened edges of the cells (Fig. 3, c and e). A similar staining distribution was obtained with transfected human adenocarcinoma cell line SW13, which is devoid of both keratins and vimentin (Sarría et al., 1990). SW13 cells stably transfected with K8 and

K18 genes were transiently transfected with the expression vector for TNFR2. Colocalization of K18 and TNFR2 was evident near the flat edges of the cell (Fig. 3, f, g, and h). These results are consistent with the view that K8 and K18 may bind to TNFR2 within cells. No colocalization of keratins with TNFR1 was detected.

If modulation of apoptosis is mediated by the direct interaction of K8 and K18 with TNF receptors, the presence of K8 and K18 might affect TNF signaling pathways. Treatment of HR9 cells with TNF (10 ng/ml) results in a modest increase in activity of JNK that reaches a maximum 10 min after ligand exposure and stays constant for up to 45 min (Fig. 4 a). However, HR-7 cells, with disrupted K8 and K18 filaments, responded to TNF by increasing the level and duration of JNK activity. The maximum induction was 30 min after TNF exposure. Quantitation indicated that TNF induced a maximum 1.5-fold activation of JNK in HR9 cells, whereas the activation in HR-7 cells was more than threefold (Fig. 4 b).

We transfected HR9 and HR-7 cells with luciferase reporter genes for NF κ B and Ets transcription factors. NF κ B activity was higher after TNF treatment in HR-7 than HR9 cells (Fig. 4 c). In comparison to HR9 cells treated with 10 ng/ml TNF, tenfold less TNF was needed to elicit similar NF κ B activity in HR-7 cells. In contrast to NF κ B activity, Ets transcriptional activities were similar in both cell types (Fig. 4 d). These results indicate that TNF-mediated NF κ B activation is moderated by the presence of K8 and K18. Both the JNK and NF κ B responses indicate that K8 and K18 may moderate TNF-initiated intracellular signaling.

To test the functional significance of K8 and K18 in cell death that is mediated by membrane receptors in vivo, we used the mouse model of ConA-induced liver damage

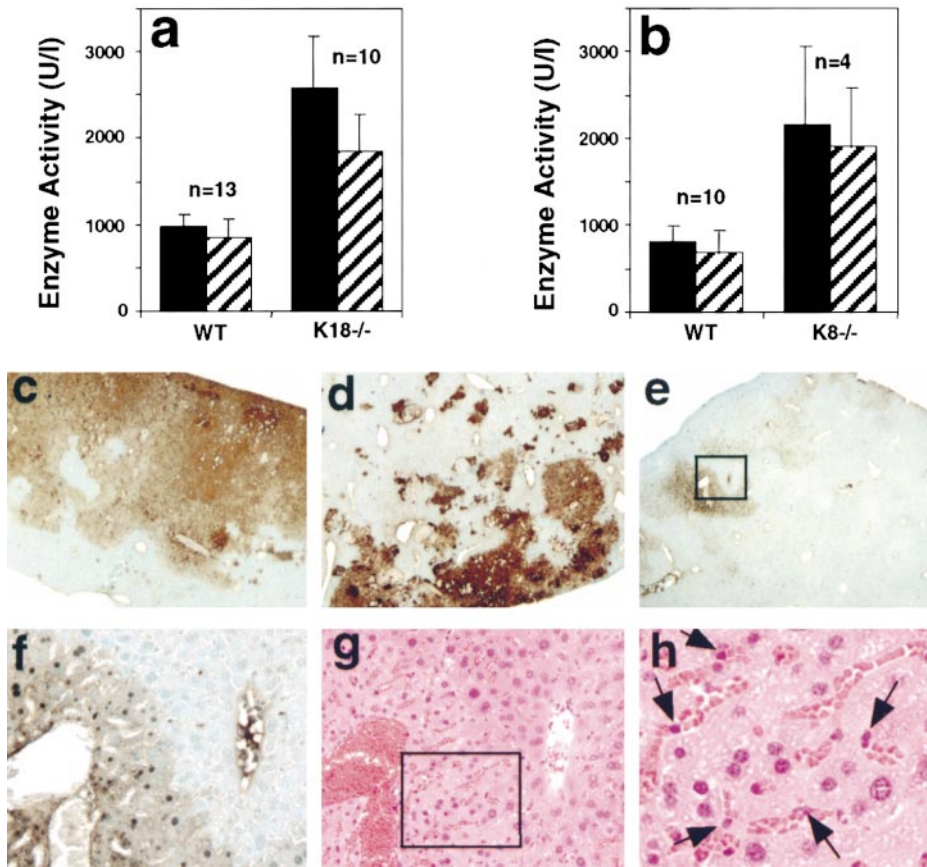


Figure 5. ConA-induced liver damage and apoptosis. a and b, The activity of liver transaminases in the serum of K18^{-/-} (a) or K8^{-/-} (b) mice. Serum activity of aspartate aminotransferase (black bars) and aspartate aminotransferase (hatched bars) was determined 8 h after i.v injection of 30 mg/kg ConA. c-h, Apoptosis in livers from K18^{-/-} mice (c), K8^{-/-} (d), and control mice (e and f) challenged with ConA was detected by terminal deoxynucleotidyl transferase TUNEL method. f, Represents a magnification of the insert area in e, g and h. Hematoxylin and eosin staining. f and g, Represent serial sections of the same liver. Note the presence of pyknotic nuclei in g, in areas that are TUNEL positive (f). Infiltrating lymphocytes in this area are indicated by arrows in h.

(Tiegs et al., 1992). ConA-induced liver apoptosis is mediated by activated T cells through TNF (Mizuhara et al., 1994). Both TNFR1 and TNFR2 are required for liver injury (Kusters et al., 1997). We injected ConA into wild-type, K8^{-/-}, and K18^{-/-} mice. Both strains of keratin-deficient mice were more sensitive to liver damage initiated by ConA (Fig. 5, a and b). Serum levels of both liver transaminases were two to three times higher in treated K8^{-/-} or K18^{-/-} mice than wild-type littermates ($P < 0.028$). Whereas K8^{-/-} mice have slightly elevated, but nonpathological, levels of these enzymes in untreated animals (Baribault et al., 1994), the levels caused by ConA were 50–100 times higher than untreated mice, reflective of the massive liver damage.

Hepatocyte apoptosis precedes liver failure in ConA-induced hepatitis (Leist et al., 1995). To evaluate apoptosis in the livers of K8 and K18 null mice, we examined sections of the livers of ConA-treated animals for degraded nuclear DNA. Livers from K8^{-/-} (Fig. 5 c) or K18^{-/-} (Fig. 5 d) mice treated with ConA had large apoptotic areas throughout the tissue. The livers of treated control mice had smaller focal areas of apoptotic cells (Fig. 5 e). Standard histology revealed massive hemorrhage and the presence of inflammatory lymphocytes in the three types of mice (Fig. 5, g and h). Apoptotic areas were also recognized by weakly staining cytoplasm and pyknotic nuclei (Fig. 5 f).

In summary, decreasing levels of K8 and K18 in cultured epithelial cells increases their cellular sensitivity to killing by TNF. K8 and K18 bind the cytoplasmic domains of TNFR2, colocalize with TNFR2 within cells, and moderate the TNF-dependent activation of JNK and the NF κ B

transcription factor. Mice without K8 or K18 are more sensitive to TNF-mediated apoptotic liver damage.

Discussion

Specialized epidermal keratins clearly serve a structural function (Fuchs and Weber, 1994). However, neither the midgestational developmental defects nor the inflammatory bowel disease that develops in adult K8^{-/-} mice appear to be consistent with a simple structural function for K8 and K18. Whereas other keratins may partially compensate for the absence of K8 or K18 in some tissues of null mice, the intermediate filaments of trophoblast at the blastocyst stage and of adult liver are composed exclusively of K8 and K18. The trophoblast of K8^{-/-} and K18^{-/-} blastocysts withstands the stress of normal implantation. K8^{-/-} embryos die at midgestation when placental function becomes crucial. Perhaps keratin-dependent resistance to maternally presented TNF may also be important for trophoblast derivative function beyond 12 d. The inflammatory bowel disease which develops in adult K8^{-/-} mice resembles the effects caused by the targeted mutation of the T cell receptor or other cytokines. These models indicate the participation of the immune system (Sartor, 1994). In humans, increased levels of TNF are found in patients with Crohn's disease (Braegger et al., 1992), a form of inflammatory bowel disease, and the use of TNF inhibitors has been successful for treating this disease (van Dullemen et al., 1995). We suggest the colorectal hyperplasia and inflammation of K8^{-/-} mice may stem from increased sensitivity to TNF. This phenotype is not found in K18^{-/-} mice, perhaps be-

cause of compensation with K19 or K20 from the intestine (Magin et al., 1998). The organization of K8 intermediate filaments, with either K18, K19, or K20 as a partner, may be sufficient to protect the colon from the effects of TNF.

The disruption or absence of keratins in the liver leads to mild hepatitis and increased sensitivity to the hepatotoxin acetaminophen (Baribault et al., 1994; Ku et al., 1996). Based on an increased sensitivity of K8⁻ hepatocytes to perfusion, microcystin exposure and partial hepatectomy, K8 and K18 have been suggested to be necessary for the structural integrity of hepatocytes (Ku et al., 1995; Loranger et al., 1997; Toivola et al., 1998). However, TNF acts as both a growth factor and apoptotic stimulus in liver. It is expressed in liver during acute and chronic liver damage (Czaja et al., 1989) and after exposure to high levels of acetaminophen (Blazka et al., 1995). Neutralizing anti-TNF antibodies significantly reduce liver injury caused by acetaminophen, but inhibit liver regeneration after partial hepatectomy (Akerman et al., 1992; Blazka et al., 1996). The increased sensitivity of K8⁻ liver cells to TNF may be the basis for poor tolerance of partial hepatectomy and poor survival of explanted hepatocytes. A distinctive feature of K8, K18, and K19 is persistent or induced expression in carcinomas in comparison to other keratins. The keratin-dependent resistance to TNF may be the selective advantage responsible for the persistence of K8 and K18 expression in carcinoma cells. Resistance of epithelia to apoptosis is likely important during common inflammatory responses.

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