Double-stranded RNA activates a p38 MAPK-dependent cell survival program in biliary epithelia

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Double-stranded RNA (dsRNA) molecules are not a major component of mammalian cells but are actively formed intracellularly during viral infection and genotoxic stress (9). Minute quantities of dsRNA can profoundly alter the cellular physiology, and dsRNA can induce several genes involved in diverse cellular processes including apoptosis, RNA synthesis, protein synthesis, cell metabolism, transport, and maintenance of cell structure (4). During viral replication, dsRNA may be produced as an essential replicative intermediate for RNA synthesis or as a by-product generated by annealing of complementary RNAs encoded by the opposite strands of a DNA virus genome (9). Host cellular response to dsRNA produced during viral infection involves the activation of antiviral responses that result in host cell apoptosis or promote survival of noninfected cells. Induction of apoptosis by dsRNA thus serves as a powerful mechanism to limit viral infection by the elimination of virally infected cells (10).

Several kinase signaling pathways involved in cellular responses to stress have been identified. These include JNK, p38 MAPK, and extracellularly regulated p44/p42 MAPK. Although p38 MAPK is a classical stress-activated protein kinase, the role of p38 MAPK signaling in the cellular response to dsRNA remains poorly understood. We have shown constitutive expression of the p38 MAPK signaling pathway in malignant cholangiocytes (18). Furthermore, p38 MAPK signaling maintains a transformed cell phenotype in malignant human cholangiocytes (19). These observations suggest a role for aberrant cellular stress-mediated signaling in maintaining the malignant phenotype in cholangiocytes.

Some of the cellular effects of dsRNA are mediated through the dsRNA-dependent protein kinase R (PKR), a 65- to 68-kDa serine-threonine kinase activated by binding to dsRNA (reviewed in Ref. 23). PKR is involved in several signaling pathways mediating stress responses and antiproliferative and apoptotic responses (20). In addition to mediating virally induced apoptosis, PKR has been found to be associated with STAT1, phosphorylate p53, and mediate NF-κB signaling (14, 25). Although PKR has been postulated as having tumor suppressor effects, this remains to be proven. However, PKR is expressed in proliferating cholangiocytes and in malignant cholangiocarcinoma (21). These suggest that cholangiocyte responses to dsRNA may be altered during cellular proliferation or carcinogenesis.

Although dsRNA can modulate the expression of a wide variety of genes with profound physiological consequences, the cellular responses of biliary epithelia to dsRNA are unknown. Knowledge of these responses is central to understanding the effects of cellular stress related to DNA damage or viral infection in biliary epithelia. Thus the aims of our study were to study the cellular response to dsRNA and, in particular, to assess the role of the stress-activated p38 MAPK signaling pathway in these responses. We therefore asked the following questions. Can dsRNA elicit a biologically
relevant response in biliary epithelia? Is apoptosis or proliferation perturbed in response to dsRNA? Are stress-activated protein kinases activated by dsRNA? Does activation of p38 MAPK signaling mediate the cellular response to dsRNA?

**EXPERIMENTAL PROCEDURES**

**Cell lines and culture.** Mz-Cha-1 cells, malignant human cholangiocytes, (kindly provided by Dr. J. G. Fitz, University of Colorado, Denver, CO) were used for these studies. The cells were cultured in CMRL 1066 media with 10% fetal bovine serum, 1% L-glutamine, and 1% antimycotic antibiotic mix. H69 cells (nonmalignant human cholangiocytes) and KMCH-1 cells (malignant human cholangiocytes) were obtained and cultured as previously described (17, 18).

**Cytokine gene expression.** Macroarray experiments were performed by using the GE Array Expression Array kit (SuperArray, Bethesda, MD) following the manufacturer’s instructions without any modifications. In brief, total RNA was isolated from cells incubated with or without dsRNA for 24 h. Biotinylated cDNA probes were synthesized by using MMLV reverse transcriptase and biotin-labeled dCTP (Boehringer Mannheim). The probes were then hybridized to nylon membranes containing cDNA fragments from cytokine genes (original series human common cytokine array). Membranes were then incubated with alkaline phosphatase-conjugated streptavidin. Gene expression was detected by chemiluminescence using the alkaline phosphatase-conjugated streptavidin. Gene expression was normalized to GAPDH expression and expressed as a ratio of expression in untreated controls. A cytokine gene expression was normalized to GAPDH expression and expressed as a ratio of expression in untreated controls. Cytokine gene expression was normalized to GAPDH expression and expressed as a ratio of expression in untreated controls. Cytokine gene expression was normalized to GAPDH expression and expressed as a ratio of expression in untreated controls.

**Proliferation assay.** Cells were seeded into 96-well plates (10,000 cells/well) and incubated in a final volume of 200 μl of medium. Cell proliferation was assessed as previously described by using a commercially available colorimetric cell proliferation assay (CellTiter 96 AQueous; Promega, Madison, WI) (19).

**Apoptosis assays.** Morphological changes indicative of cell death by apoptosis were identified and quantitated by flow cytometric analysis and the use of acridine orange as previously described (18). Fluorescence was visualized by using an upright fluorescence microscope (model BX40; Olympus America, Melville, NY). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation. At least 300 nuclei in four high-power fields were counted. Biochemical changes of apoptosis resulting in DNA fragmentation were assessed by using a cell death colorimetric enzyme immunoassay detection ELISA kit to quantitate cytoplastic histone-associated DNA fragments (Cell Death Detection ELISA; Roche Biochemicals, Indianapolis, IN). Cells were incubated for 24 h and assayed following the manufacturer’s instructions.

**Viability assay.** Cells were seeded into 96-well plates (10,000 cells/well) and incubated in a final volume of 200 μl of medium. For the kinase inhibitor studies, cells were preincubated with the inhibitor for 1 h. The kinase inhibitors were stored as concentrated stock solutions in DMSO. Controls contained equivalent volumes of DMSO to those used in the inhibitors. Cells were then incubated with varying concentrations of dsRNA for 24 h before the addition of either camptothecin or TNF-related apoptosis-inducing ligand (TRAIL). Cell viability was assessed by using a commercial tetrazolium bioreduction assay for viable cells and was expressed as a percentage of control (CellTiter 96 AQueous; Promega).

**Generation of stably transfected cell lines.** Stably transfected cell lines were generated from parental Mz-Cha-1 malignant human cholangiocytes. Cells transfected with pRc/ RSV-Flag MKK3 (Ala) (encoding a dominant interfering upstream activator of p38 MAPK with double-point mutations in Ser180 and Thr183 replaced by Ala) had decreased constitutively and stimulated p38 MAPK activity compared with control cells transfected with pRc/ RSV-Flag MKK3. Expression plasmids were kindly provided by Dr. Roger Davis (Howard Hughes Medical Institute, Worcester, MA). Plasmids were purified by using the Plasmid Midi Kit (Qiagen, Valencia, CA) and linearized by restriction enzyme digestion before transfection by using Trans-T (Panvera, Madison, WI). After 48 h, the media were replaced with media containing blasticidin. Stable transfected cells were identified by expression of the green fluorescent protein by the incorporation of G418. Stable transfection was confirmed after 3 wk by fluorescence microscopy and the use of acridine orange as previously described.

**Measurement of cytosolic caspase activity.** Cells were washed with PBS, and lysed with 1 ml of a hypotonic buffer containing (in mM) 25 HEPES, 5 MgCl₂, 1 EGTA, and freshly added 0.5 PM Sulf, plus 2 μg/ml pepstatin and 2 μg/ml leupeptin. Cells were then homogenized by 20 strokes by using a Tissue Tearor (Biospec, Bartlesville, OK). The homogenate was centrifuged at 21,000 g for 45 min at 4°C by using a Microfuge R centrifuge (Beckman Instruments, Palo Alto, CA). The protein content in the supernatant cytosolic fraction was measured by using the Bradford reagent. Caspase activity was assayed by adding 50 μl of cytosolic protein to 0.45 ml of buffer containing (in mM) 25 HEPES (pH 7.5), 10 dithiothreitol, 0.5 PM SFSF, plus 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 2 μM alprostin, and 20 μM fluorogenic substrate. The substrates used were Ile-Glu-Thr-Asp (IETD)-7-amino-4-trifluoromethylcoumarin (-AFC), Leu-Glu-His-Asp (LEHD)-7-amino-4-methylcoumarin (-AMC) and Asp-Glu-Val-Asp (DEVD)-AMC for caspase 8, 9, and 3-like activities, respectively. After incubation at 37°C for 30 min, 1 ml of diH₂O was added. The change in fluorescence intensity was measured over 30 min by using a fluorometer (model TD700; Turner Designs, Mountain View, CA) with excitation and emission wavelengths of 360 and 460 nm. With each experiment, standard curves were generated with AMC or AFC, and caspase activity was expressed as picomole AMC or AFC per milligram protein per minute.

**Immunoblot analysis.** Confluent cells in culture were trypsinized and sonicated for 20 s at 4°C (Sonic dismembrator; Fisher Scientific, Pittsburgh, PA) in a lysis buffer containing (in mM) 50 Tris base, 2 EDTA, 100 NaCl, plus 1% NP-40 and one miniprotease inhibitor cocktail tablet. Protein content was determined by the Bradford assay. Protein samples were separated on 4–12% gradient polyacrylamide gels (Noven, San Diego, CA) under reducing conditions and electroblotted to positively charged 0.45 μm nitrocellulose membrane (Millipore, Bedford, MA). Membranes were soaked for 5 min in transfer buffer (13.4 mM Tris, pH 8.3, 20% methanol, 108 mM glycine). Blots were preblocked in 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk, for 3–4 h or overnight at 4°C. Kinase expression levels were assessed by using phosphorylation state-independent polyclonal
rabbit antibodies, and the presence of phosphorylated (activated) kinase was measured by using phosphorylation state-specific antibodies to PKR, p38 MAPK, MEK1/2, or JNK as previously described (19). Membranes were incubated overnight at 4°C with the respective anti-human primary antibody used at a 1:1,000 dilution. Primary antibodies were diluted in a solution containing 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk. Membranes were washed three times for 10 min with 20 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (TTBS) and then incubated with the secondary antibody, a polyclonal goat anti-rabbit immunoglobulin-peroxidase conjugate (Zymed, San Francisco, CA) at a 1:5,000 dilution for 60 min at 4°C. The secondary antibody was diluted in TTBS buffer. For all immunoblots, membranes were washed three times for 10 min with TTBS and were then visualized by using an enhanced chemiluminescence kit (ECL plus; Amersham Biosciences, Piscataway, NJ) following the manufacturer's directions. The relative activity of phosphorylated to total kinase expression was determined by densitometry by using a CCD camera-based image analyzer (MultiImager; Alpha Innotech).

NF-κB activation assay. Activation of NF-κB was determined with the use of TransAM assay (Active Motif, Carlsbad, CA) following the manufacturer's instructions. Briefly, cells cultured in 35-mm dishes were washed with ice-cold PBS and removed by trypsinization. The cells were centrifuged for 10 min at 1,000 rpm at 4°C and resuspended in 100 μl of lysis buffer at 4°C. Lysates (5 μg of total protein) were incubated in 96-well dishes containing immobilized oligonucleotides containing the NF-κB consensus DNA-binding site (5’-GGGACTTTCC-3’) for 1 h at room temperature. Wells were then washed three times, and 100 μl of p65 subunit monoclonal antibody (1:1,000 dilution) were added to each well for 1 h at room temperature. Wells were washed three times, and 100 μl of horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution) then were added to each well for 1 h at room temperature. Wells were washed four times, and 100 μl of developing solution were added to each well for 10 min at room temperature. Stop solution (100 μl) was added to each well and the absorbance at 450 nm was determined by using a VersaMax plate spectrophotometer. Specificity of binding was determined with the use of 200-fold excess wild-type and mutated NF-κB oligonucleotides. Extracts from HeLa cells treated with TNF-α were used as positive controls.

Materials. Polynosinic-polycytidylic acid (poly[dI-dC]), fetal bovine serum, Bradford reagent, and camptothecin were obtained from Sigma (St. Louis, MO). CMRL 1066 media, l-glutamine, and antibiotic-antimycotic mix were from Gibco-BRL (Grand Island, New York). P38 MAPK, JNK-, and MEK1/2-specific antibodies were obtained from Cell Signaling (Beverly, MA). PKR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and phosphospecific PKR antibodies were obtained from Biosource (Camarillo, CA). Kinase inhibitors and cycloheximide were obtained from Calbiochem-Novabiochem (San Diego, CA). Protease inhibitor cocktail tablets were obtained from Roche Molecular Biochemicals (Indianapolis, IN). All other reagents were of analytical grade from the usual commercial sources. Soluble, recombinant human TRAIL (Killer TRAIL) was obtained from Alexis Biochemicals (Carlsbad, CA). Monoclonal anti-human IL-6R antibody was from R&D Systems (Minneapolis, MN).

Statistical analysis. Data are expressed as the means ± SD from at least three separate experiments performed in triplicate, unless otherwise noted. The differences between groups were analyzed by using a double-sided Student’s t-test when only two groups were present. For repeated measures among multiple groups, analysis was performed by using ANOVA with a post hoc Bonferroni test to correct for multiple comparisons. Statistical significance was considered as P < 0.05. Statistical analyses were performed with the GB-STAT statistical software program (Dynamic Microsystems, Silver Spring, MD).

RESULTS
dsRNA elicits a cellular response in malignant human cholangiocytes. We began by first determining whether a biologically relevant cellular response could be elicited in human Mz-ChA-1 cells in response to incubation with dsRNA. For our studies, we used poly(dI-dC), a synthetic dsRNA molecule that is not virus specific. At the concentrations used for these studies, dsRNA was found to be nontoxic by using a viable cell assay and did not significantly alter cell proliferation. Expression of proinflammatory cytokines is commonly observed in infected epithelial cells. Thus we assessed the effect of incubation with this dsRNA molecule on cytokine gene expression in cholangiocytes by using a membrane-based cDNA hybridization macroarray. Incubation with dsRNA (5 μg/ml) for 24 h resulted in altered gene expression of several cytokines that are important mediators of inflammation. The most prominent changes observed included alterations in interferon-γ, IL-6, and transforming growth factor β-1 (TGF-β) (Fig. 1). Thus incubation with dsRNA elicits biologically relevant cellular responses in Mz-ChA-1 cells.

dsRNA does not induce apoptosis in malignant cholangiocytes. Apoptosis is a common response to virally infected cells, and can be induced by dsRNA in several cell types, including hepatocytes (3). However, the lack of toxicity observed during incubation with
dsRNA prompted an assessment of whether or not dsRNA could induce apoptosis in Mz-ChA-1 human cholangiocytes. Cells were incubated with varying concentrations of dsRNA, and the occurrence of apoptotic cell death was assessed by using both morphological and biochemical criteria. First, cells demonstrating characteristic nuclear morphological changes of apoptosis were quantitated (Fig. 2A). We then used an ELISA-based assay for the identification of histone-bound DNA (Fig. 2B). In support of these observations, we were also able to demonstrate cleavage of poly-(ADP-ribose) polymerase by Western blot analysis of protein extracts from cells incubated with dsRNA (data not shown). These complementary assays thus showed that incubation with dsRNA does not induce apoptosis in Mz-ChA-1 human cholangiocytes. Similar results (using morphological assays) were observed with both nonmalignant (H69) and malignant (KMCH-1) human cholangiocyte cells (data not shown). Thus incubation with dsRNA does not induce cellular apoptosis in human cholangiocytes.

dsRNA decreases toxin or death receptor-mediated cytotoxicity in Mz-ChA-1 human cholangiocytes. Cytoprotective responses may be elicited in response to viral infection or stress to limit intracellular damage. Therefore, we studied whether dsRNA was cytoprotective. Endogenous inducers of apoptosis include death receptor ligands as well as chemotherapeutic agents. To assess whether dsRNA could protect against apoptosis, we assessed cytotoxicity in response to the death receptor ligand TRAIL or the chemotherapeutic agent camptothecin. In preliminary studies, we established the concentration dependency of cytotoxicity in response to these agents. For our next studies, we chose concentrations of these agents that resulted in 40–60% cytotoxicity after 24 h. Cells were preincubated with dsRNA for 24 h before incubation with TRAIL or camptothecin at these concentrations, and toxicity was assessed after 24 h (Fig. 3). Incubation with dsRNA decreased toxicity due to either camptothecin or TRAIL. Because dsRNA increases the expression of IL-6, we assessed the role of IL-6-mediated signaling on the cellular response to dsRNA. Incubation with neutralizing antibodies for human IL-6 did not alter dsRNA cytoprotection from camptothecin cytotoxicity. Cell viability in cells treated for 24 h with camptothecin (100 nM) was 85 ± 8% in cells preincubated with 5 μg/ml dsRNA alone compared with 82 ± 7% in cells preincubated with dsRNA and anti-IL-6 (1 μg/ml). Thus indirect effects through IL-6 signaling are unlikely to contribute to the cell survival observed in response to dsRNA. These findings suggested that dsRNA might preferentially activate a mechanism that protects against apoptosis. We thus postulated that cytoprotection by dsRNA may represent a mechanism to limit cell death during genotoxic stress or viral infection in vivo.

dsRNA decreases basal caspase activity in Mz-ChA-1 human cholangiocytes. Activation of caspases represents a common intracellular mechanism culminating in apoptosis. We therefore assessed the effect of dsRNA on activation of intracellular caspases assessed fluorometrically by using peptide aminomethyl or trifluoromethyl coumarin substrates. Caspase-3-like activity was increased during incubation with camptothecin. However, the increased caspase-3-like activity was decreased in cells preincubated with dsRNA for 24 h before incubation with camptothecin (Fig. 4). Unexpectedly, basal levels of caspase-3-like activity were decreased in the presence of dsRNA alone. Furthermore, incubation with dsRNA decreased the hydrolytic activity of caspase-3, -8, and -9-like activity under basal conditions (Fig. 5). Preincubation with the protein synthesis inhibitor cycloheximide (100 μg/ml)
inhibited the effect of dsRNA on caspase-3-like activity. Thus the protective effect of dsRNA is likely to be mediated by altered expression of genes or proteins that act upstream of caspase activation and are effective under basal conditions in the absence of signaling pathways inducing apoptosis.

**Differential activation of intracellular protein kinases by dsRNA.** Because protein kinase activation participates in multiple cellular responses to external stress, such as infection, we assessed the role of several stress-activated protein kinases. First, kinase activation was assessed by the use of phosphorylation state-specific antibodies to p38 MAPK, MEK1/2, and JNK. Incubation of Mz-ChA-1 cells with dsRNA increased active site phosphorylation of the p38 MAPK as well as the JNK but not MEK1/2, which resulted in activation of the p44/p42 MAPK (Fig. 6). The differential activation of kinases with selective activation of the stress-associated protein kinase signaling pathways indicates that the cellular response to dsRNA is similar to that in response to exposure to bacterial products or inflammatory cytokines, such as IL-1 and TNF-α. Similar to our previous observations with serum stimulation (19), we did not detect an increase in p38 MAPK activation in H69 nonmalignant human cholangiocytes in response to incubation with 5 μg/ml dsRNA, p38 MAPK activity was not increased during incubation with camptothecin (100 nM) or TRAIL 200 ng/ml for 24 h.

We then assessed the role of the p38 MAPK by using complementary pharmacological and genetic approaches to inhibit p38 MAPK activation. Pharmacological inhibitors were used at concentrations previously shown to have selective effects of kinase activation in malignant cholangiocytes (19). Preincubation with 25 μM SB-203580, an inhibitor of p38 MAPK activity, decreased dsRNA inhibition of basal caspase activity, whereas 25 μM PD-098059, an inhibitor of ERK1/2 activation did not significantly alter the response of Mz-ChA-1 cells to dsRNA (Fig. 7A). In addition, the inhibition of basal caspase-3-like activity by dsRNA was almost completely prevented in cells stably transfected with a mutated MKK3 that prevents p38 MAPK activation (Fig. 7B). Although preincubation with the p38 MAPK inhibitor SB-203580 did not in-

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Fig. 3. dsRNA decreases toxin or death receptor-mediated cytotoxicity in Mz-ChA-1 human cholangiocytes. Cells were preincubated with 0, 1, or 5 μg/ml dsRNA for 24 h before treatment with the cytotoxic agent camptothecin (100 nM) or the TNF-related apoptosis inducing ligand (TRAIL; 200 ng/ml) for 24 h. Cytotoxicity was then assessed by using the MTS assay for viable cells. Preincubation with dsRNA decreased toxicity due to either agent, *P < 0.05 compared with controls without dsRNA. Results represent the means ± SD from 3 separate experiments.

Fig. 4. dsRNA decreases caspase-3 activation by camptothecin (CPT). Mz-ChA-1 cells were preincubated with 0 or 5 μg/ml dsRNA for 24 h, before treatment with camptothecin (100 nM) for 24 h. Caspase-3-like activity was then assessed by using the fluorogenic substrate DEVD-AMC. CPT increased caspase-3-like activity. This increase was significantly inhibited by preincubation with dsRNA. Results represent means ± SD from 3 separate experiments.
dsRNA-induced apoptosis, the cytoprotective effect of dsRNA on camptothecin cytotoxicity was inhibited. Cells were preincubated with 0, 1, or 5 μg/ml dsRNA, in the presence or absence of 25 μM SB-203580, before induction of cytotoxicity by 100 nM camptothecin. Cytoprotection by dsRNA was decreased by 73 ± 12% in cells pretreated with SB-203580. These observations indicated that the cellular response to dsRNA was mediated by a p38 MAPK-dependent mechanism.

dsRNA-dependent kinase PKR is not involved in dsRNA-mediated cell survival in malignant human cholangiocytes. The dsRNA-dependent protein kinase PKR plays a fundamental role in limiting viral replication and inhibiting protein synthesis and is an important mediator of the cellular response to dsRNA. Because this molecule participates in a variety of cell-signaling pathways involved in induction or inhibition of apoptosis, we assessed PKR expression and activa-

Fig. 5. dsRNA decreases basal caspase activity in Mz-ChA-1 human cholangiocytes. Cholangiocytes were incubated with 0, 1, or 5 μg/ml dsRNA. After 24 h, cells were lysed, and basal caspase activity was determined by using fluorogenic substrates DEVD-AMC (for caspase-3-like activity) (A), IETD-AMC (for caspase-8-like activity) (B), or LEHD-AMC (for caspase-9-like activity) (C). Incubation with dsRNA decreased basal caspase activity. Results represent the means ± SD for 3 separate experiments. *P < 0.05 compared with controls without dsRNA.

Fig. 6. Differential activation of cellular protein kinases by dsRNA. Cell lysates were prepared from cells incubated with 1 or 5 μg/ml dsRNA for 0, 1, or 24 h. Protein (20 μg) was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with phosphospecific antibodies for p38 MAPK, MEK1/2, or JNK. Blots were stripped and reprobed with the respective phosphorylation state-independent antibodies to determine total kinase expression. Subsequently, blots were reprobed with antibodies to β-actin. Phosphorylated and total kinase band intensities were quantitated by using scanning densitometry normalized for equal protein loading. Relative activity represents the ratio of the phosphorylated to total expression relative to basal conditions. Results represent the means ± SD from 3 separate experiments. An illustrative immunoblot is also shown. *P < 0.05 compared with basal values.
PKR did not phosphorylate dsRNA in cholangiocytes. Indeed, PKR was constitutively expressed. However, phosphorylation of dsRNA was not increased by dsRNA (Fig. 8). Furthermore, basal caspase-3-like activity was not altered by the use of the PKR inhibitor 1 mM 2-AP (Fig. 9). Thus neither PKR nor NF-κB are involved in the cellular response to dsRNA. Although PKR can induce apoptosis in response to diverse stimuli, such as serum deprivation and viral infection in other cell types (i.e., hepatocytes), the resistance to apoptosis in the setting of PKR expression suggests that PKR apoptosis pathways are being circumvented in human cholangiocytes.

DISCUSSION

These studies emphasize the cell-type specificity of the cellular response to dsRNA. In contrast to several other epithelial cell types, malignant human cholangiocytes respond to dsRNA by the stimulation of a cytoprotective response. dsRNA was not toxic and did not alter cellular apoptosis or proliferation. However,

![Fig. 7. p38 MAPK inhibition decreases dsRNA-mediated inhibition of basal caspase-3-like activity. A: cells were preincubated with the p38 MAPK inhibitor 25 μM SB-203580, or the p44/p42 MAPK inhibitor 25 μM PD-098059 for 30 min before treatment with 0, 1, or 5 μg/ml dsRNA for 24 h. Basal caspase-3-like activity was assessed in cell lysates by using the fluorogenic substrate DEVD-AMC. Preincubation with SB-203580 but not PD-098059 decreased dsRNA inhibition of basal caspase activity. B: cells were stably transfected with a mutated MKK3-Ala, which inhibits p38 MAPK activation, or with wild-type MKK3 (control). Caspase-3-like activity was assessed after incubation with 0 or 5 μg/ml dsRNA for 24 h. Results represent the means ± SD of 3 separate experiments. *P < 0.05 compared with controls without dsRNA.

![Fig. 8. PKR is constitutively expressed but is not phosphorylated by dsRNA in cholangiocytes. Cell lysates were prepared from cells incubated with 5 μg/ml dsRNA for varying periods of time up to 24 h. Protein (20 μg) was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies specific for the Thr451 phosphorylated form of PKR. Blots were stripped and reprobed twice, first with phosphorylation state-independent antibodies to PKR to determine total PKR expression and subsequently with antibodies to α-actin to allow for normalization of protein loading. The phosphorylated and total PKR band intensities were quantitated by using scanning densitometry normalized for equal protein loading. Relative activity represents the ratio of the phosphorylated to total expressed relative to the ratio under basal conditions. Results represent the means ± SD from 3 separate experiments. An illustrative immunoblot is also shown.

![Fig. 9. The dsRNA inhibitor 2-aminopurine (2-AP) does not alter basal caspase-3-like activity in dsRNA-treated cells. Cells were pre-treated with the PKR inhibitor 1 mM 2-AP for 30 min before incubation with 5 μg/ml dsRNA. Caspase-3-like activity was assessed after 24 h by using the fluorogenic substrate DEVD-AMC. 2-AP did not significantly alter the inhibition of basal caspase activity by dsRNA. Results represent the means ± SD of 3 separate experiments. *P = not significant compared with dsRNA alone.](http://ajpgi.physiology.org/Content/Full/284/4/4930.F7A.jpg)
dsRNA activated a cell survival program that suppressed the caspase effector machinery and inhibited cytotoxicity due to camptothecin or to TRAIL death receptor ligation. These cell type-specific effects on intracellular processes mediating cell survival are relevant in understanding viral infections affecting the liver. Furthermore, these observations may have implications for the use of sequence-specific dsRNA for the functional ablation of specific genes, an approach that is gaining increasing importance as a strategy for the manipulation of selective gene expression in eukaryotic cells (2).

The cell survival program initiated by dsRNA involves phosphorylation of the p38 MAPK, but not the p44/p42 MAPK or the dsRNA-dependent PKR. Activation of p38 MAPK has been shown to occur in response to dsRNA in fibroblasts and HeLa cells via a PKR-independent mechanism (8). PKR is a potent inducer of apoptosis and negative regulator of epithelial cell growth in response to dsRNA (reviewed in Ref. 5). However, PKR expression increases in proliferating cholangiocytes, and PKR is overexpressed in cholangiocarcinoma (21). The lack of an apoptotic response to PKR in malignant cholangiocytes may be explained by the presence of cellular mechanisms that override growth inhibition and apoptosis induced by PKR activation. Although our observations do not address the important question of whether the cellular responses to dsRNA are a cause or consequence of malignant transformation, these studies support the presence of a dominant protective effect mediated by p38 MAPK over apoptotic signaling in response to dsRNA in malignant cholangiocytes.

Several cellular modulators of the response to dsRNA have been identified in uninfected cells. These include p58, a member of the tetratricopeptide family that includes many regulators of cell cycle activity, such as cdc23 and cdc16. Interestingly, overexpression of p58 results in malignant transformation of cells in vitro, presumably by inhibiting endogenous PKR (1). Other modulators of PKR function have also been identified, including the c-Myc-associated glycoprotein p67 and the Tar RNA-binding protein (16, 24). We speculate that p38 MAPK may alter the expression of endogenous inhibitors of PKR function and that additional study to ascertain these relationships is warranted.

We (19) have previously shown the involvement of p38 MAPK signaling in transformed growth of malignant cholangiocytes. Aberrant p38 MAPK signaling in response to stress therefore represents a potential mechanism by which carcinogenesis is promoted. Indeed, the failure of dsRNA to initiate apoptosis despite constitutive expression of PKR emphasizes a mechanism by which carcinogenesis is promoted independently of viral infection. dsRNA is produced within cells as a result of direct perturbations to cellular RNA by genotoxic agents or as a secondary effect resulting from aberrant transcription of damaged DNA. The activation of stress-associated protein kinases (p38 MAPK and JNK) in response to dsRNA provides additional support of the hypothesis that dsRNA may function as an intracellular stress sensor or signal during genotoxic stress (7, 22). Failure to mount an apoptotic response in these situations may predispose to malignant transformation by allowing the persistence of cells that have sustained genotoxic damage and allowing for inheritable genomic damage.

Cholangiocytes are exposed to and are susceptible to virus infection during acute or chronic viral hepatitis (6, 11, 15). Cellular response to exogenous dsRNA mimics that of dsRNA produced during replicative viral infection, and many of the genes induced are also stimulated by viral infection or interferon. Prominent changes were observed in expression of inflammatory cytokines that can stimulate (e.g., IL-6) or inhibit (e.g., TGF-β) cholangiocyte proliferation. Host cellular responses prevent viral spread by inducing apoptosis in infected cells or by acting in a paracrine manner to protect surrounding cells. The cellular responses involve both dsRNA- and interferon-induced cytoprotective and cytotoxic mechanisms to promote host sur-
vival. Many viruses have evolved mechanisms to modulate host cell responses and thus the specific cellular response elicited is virus specific.

Recent studies (12) have demonstrated an association between chronic hepatitis C virus (HCV) infection and biliary tract malignancies. In hepatocytes, HCV activates PKR, which subsequently plays a fundamental role in the regulation of apoptosis. Evasion of host apoptosis is an important mechanism by which viruses maintain persistent infection. Disruption of PKR-dependent apoptosis is associated with the interferon-resistant phenotype of HCV and with persistence of viral HCV infection (13). Indeed, the HCV nonstructural 5A protein from interferon-resistant HCV can disrupt dsRNA-induced host apoptotic signaling by inhibiting PKR and contribute to tumor formation (3). Although PKR expression is also increased in cholangiocytes during chronic HCV infection, cholangiocyte death is infrequently observed, and viral persistence occurs in biliary epithelia. Abrogation of intracellular apoptotic signaling in response to dsRNA therefore represents a potential mechanism by which cholangiocytes may escape cell death during viral infection. We speculate that these aberrant responses to dsRNA contribute to persistent viral infection and the establishment of a reservoir in the biliary tract during infection with chronic HCV infection. Thus further study is warranted to ascertain the specific responses of nontransformed human biliary epithelia to dsRNA during infection with hepatotropic viruses such as hepatitis C.

REFERENCES


