Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbially induced hepatocarcinogenesis

Zhongming Ge,* Arlin B. Rogers, Yan Feng, Amy Lee, Shilu Xu, Nancy S. Taylor and James G. Fox
Division of Comparative Medicine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

Summary

Bacterial cytolethal distending toxins (CDTs) containing DNase I-like activity can induce limited host DNA damage that leads to activation of the DNA-damage repair responses in cultured cell lines. However, in vivo experimental evidence linking CDTs to carcinogenesis is lacking. In this study, infection of A/JCr mice with an isogenic mutant of Helicobacter hepaticus lacking CDT activity (CDT mutant) induced chronic hepatitis comparable to wild-type H. hepaticus (Hh) infection at both 4 and 10 months post inoculation (MPI); however, the CDT mutant-infected mice did not develop hepatic dysplastic nodules at 10 MPI, whereas those infected with Hh did. There was no significant difference in hepatic colonization levels between the CDT mutant and Hh at both time points (P > 0.05). At 4 MPI, mice infected with Hh had significantly enhanced hepatic transcription of proinflammatory TNF-α, IFN-γ and Cox-2, growth mediators IL-6 and TGF-α, anti-apoptotic Bcl-2 and Bcl-Xₐ, and increased hepatocyte proliferation (P < 0.05) compared with the control or the CDT mutant-infected mice. In addition, Hh infected male mice had upregulated hepatic mRNA levels of RelA (p65), p50, GADD45β and c-IAP1, components of the NF-κB pathway compared with the CDT mutant-infected mice. At 10 MPI, Hh infection was associated with significant upregulation of IL-6 mRNA. Activation of the inflammatory NF-κB pathway and upregulation of proinflammatory cytokines plus IL-6 in the Hh but not in the CDT mutant-infected mice suggest that Hh CDT plays a key role in promoting the dysplastic changes in Hh-infected mouse livers.

Introduction

Cytolethal distending toxins (CDTs) are produced by multiple pathogenic Gram-negative bacteria, including Escherichia coli, Campylobacter species, Shigella dysenteriae, Haemophilus ducreyi, Helicobacter species, Actinobacillus actinomycetemcomitans and Salmonella enterica serovar typhi (Haghjoo and Galan, 2004; Thelestam and Frisan, 2004). Bacterial CDTs are generally holotoxins containing three subunits A, B and C except for Salmonella enterica serovar typhi which has only CdtB (Haghjoo and Galan, 2004). CDT causes cell cycle arrest and subsequent cellular distension and eventual cell death in cultured mammalian cells (Smith and Bayles, 2006). CdtB, after delivery into the target cells with the aid of CdtA and CdtC, uses its DNase I-like activity to induce limited host DNA damage (such as double strand breaks), leading to activation of the DNA repair responses (Lara-Tejero and Galan, 2002). It has been speculated that bacterial CDTs could be a contributing factor for the development of cancer; however, in vivo experimental evidence linking CDTs to carcinogenesis is lacking.

Helicobacter hepaticus is a CDT-producing enterohelobacter helicobacter and is genetically closely related to human bacterial pathogens Helicobacter pylori and Campylobacter jejuni (Young et al., 2000; Suerbaum et al., 2003). H. hepaticus infection induces chronic active hepatitis, hepatocellular carcinoma (HCC), typhlocolitis and lower bowel cancer in susceptible strains of inbred and genetically engineered mice (Fox et al., 1994; 1996; Ihrig et al., 1999; Erdman et al., 2003; Rogers et al., 2004; Rao et al., 2006). Pathological features of H. hepaticus-induced lesions recapitulate in many respects those documented in human patients, thus providing a valuable rodent model for dissecting basic mechanisms underlying microbially induced diseases. It has been reported that infection with CDT-deficient H. hepaticus mutants when compared with wild-type (WT) H. hepaticus produces less severe typhlocolitis in C57BL/6 IL-10⁻⁻ mice (Young et al., 2004). This toxin is also essential for persistent...
H. hepaticus colonization in Swiss Webster and IL-10−/− mice (Ge et al., 2005; Pratt et al., 2006). In this study, the roles of CDT in the progression of H. hepaticus-induced liver disease were investigated in male and female A/JCr mice.

Results

Cytolethal distending toxin was essential for progression of inflammation to dysplasia but did not influence the severity of H. hepaticus-induced hepatic inflammation

As determined by histopathology, there was no significant difference in type or severity of hepatitis induced either by WT H. hepaticus or the CDT mutant in male mice at 4 or 10 months post infection (MPI) (Fig. 1A). In both infected groups, subacute hepatitis at 4 MPI was characterized by multifocal mixed mononuclear and polymorphonuclear cell lobular hepatitis with intraslesional hepatocyte coagulative necrosis, and mild to moderate lymphocyte-predominant portal and interface hepatitis (Fig. 1A, top row). At 10 MPI, infected mice in both groups displayed chronic hepatitis, with fewer lobular lesions and increased severity of portal and interface hepatitis, including lymphoid-like follicles (‘tertiary lymphoid tissue’) in some mice (Fig. 1A, middle row). However, 80% of the WT H. hepaticus-infected male mice (4/5) developed early dysplastic lesions including mixed foci of altered hepatocytes (Fig. 1A, bottom row), but none of the CDT mutant-infected group (0/5) developed hepatocellular dysplasia at 10 MPI (Fig. 1, P < 0.05). Within foci of altered hepatocytes, liver cells demonstrated moderate to marked variation in cell and nuclear size and shape, failure of linear organization with loss of hepatic architecture, and flocculent intracytoplasmic inclusions, when compared with normal hepatocytes (N) at left. (i) Cellular detail within FAH demonstrating absence of linear cord arrangement, marked cellular pleomorphism, atypical syncytia, altered staining patterns including subplasmalemmal radial magenta striations (tigroid cells), and variable cytoplasmic vacuolation. HandE, bar = 160 μm (a–f), 100 μm (g), 40 μm (h), 16 μm (i).

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd, Cellular Microbiology, 9, 2070–2080
Colonization levels of WT *H. hepaticus* or a CDT mutant in the infected livers and ceca. Boxed samples are also positive for *H. hepaticus* by culture. Numbers represent the copies of the *H. hepaticus* genomes per μg mouse DNA in the individual samples.

**Fig. 2.** Colonization levels of WT *H. hepaticus* or a CDT mutant in the infected livers and ceca. Boxed samples are also positive for *H. hepaticus* by culture. Numbers represent the copies of the *H. hepaticus* genomes per μg mouse DNA in the individual samples.

Cords, and aberrant syncytialization. Tinctorial changes were also evident, including clear vacuolation, hypereosinophilia, or aggregation of subplasmalemmal radial striations representing linearly arrayed rough endoplasmic reticulum (tigroid cells) (Rogers et al., 2004). In agreement with previous observations in this model, infected female mice developed only minimal hepatitis and sham-dosed controls of both genders were essentially lesion-free at both time points (data not shown). For this reason, molecular analyses described below were limited to male mice. Taken together, these data indicate that the presence or absence of CDT in *H. hepaticus* does not affect the severity of induced hepatitis; however, CDT-deficiency prevents the emergence of foci of altered hepatocytes, the early stage of HCC, at 10 MPI.

**Cytolethal distending toxin mutant had a similar hepatic colonization level in the liver at 4 MPI but tended to be lower at 10 MPI when compared with WT *H. hepaticus***

To determine whether CDT plays a role in *H. hepaticus* colonization in A/JCr mice and thereby influences hepatic disease, colonization levels of *H. hepaticus* in the livers from WT *H. hepaticus* or the CDT mutant-infected mice were determined by quantitative polymerase chain reaction (QPCR). All female mice infected with WT *H. hepaticus* or CDT mutant were negative for *H. hepaticus* in the livers (data not shown). At 4 MPI, all the CDT mutant-infected (*n* = 5) and 80% of the WT *H. hepaticus*-infected male mice (*n* = 5) were *H. hepaticus*-positive and there was no significant difference in hepatic bacterial colonization levels between these two groups (Fig. 2, *P* = 0.99). At 10 MPI, *H. hepaticus* was detected in 60% of the livers of the CDT mutant-infected (*n* = 5) and 80% of the WT *H. hepaticus*-infected male mice (*n* = 5); the level of the CDT mutant tended to be lower than WT *H. hepaticus* (Fig. 2, *P* = 0.09). Viability of *H. hepaticus* in the livers (boxed, Fig. 2) was confirmed by recovery in culture, whereas the lack of *H. hepaticus* by QPCR was confirmed by the nested PCR (data not shown). There were no detectable WT *H. hepaticus* in one out of five male mice at both time points by QPCR and culture. The WT *H. hepaticus*-negative mouse by 10 MPI developed less severe hepatitis and no dysplasia compared with the others in the group (Fig. 1B). Resistance of a subset to *H. hepaticus* colonization and hepatitis agrees with previous studies in A/JCr mice (Fox et al., 1996; Rogers et al., 2004).

Colonization levels of *H. hepaticus* were also measured in the ceca of the infected mice. WT *H. hepaticus* was detected in 80% of the ceca (*n* = 5) at both time points; three at 4 MPI and one at 10 MPI out of five mice were positive for the CDT mutant (Fig. 2). In infected female mice, cecal prevalence and levels of WT *H. hepaticus* were comparable to those in the males at 4 MPI but the female mice lost cecal WT *H. hepaticus* colonization by 10 MPI, whereas all female mice infected with the CDT mutant lacked *H. hepaticus* in the ceca at both time points (data not shown). There is no corelationship between cecal bacterial levels and severity of hepatic inflammation.

**Presence of CDT is associated with upregulation of a subset of proinflammatory mediators**

As *H. hepaticus*-induced inflammation is often associated with upregulation of proinflammatory mediators, mRNA levels of TNF-α, IL-1β, IFN-γ and Cox-2, were determined. At 4 MPI, infection with WT *H. hepaticus* significantly increased the mRNA levels of TNF-α (*P* = 0.03), IFN-γ (*P* = 0.002) and Cox-2 (*P* = 0.001) when compared with the CDT mutant in the livers of male mice; in contrast, there was no significant difference on the mRNA levels of IL-1β (*P* = 0.66) between the WT *H. hepaticus* and the CDT mutant-infected mice (Fig. 3A). At 10 MPI, there was no significant difference in levels of all these cytokines between the WT *H. hepaticus* and the CDT mutant-infected mice (Fig. 3A). When compared with the sham controls, the livers of mice infected with WT *H. hepaticus* or CDT-deficient mutant contained much higher mRNA levels of these measured proinflammatory cytokines at 4 MPI (all *P* < 0.001), whereas mRNA expression of TNF-α, IFN-γ and Cox-2 were significantly upregulated at 10 MPI (Fig. 3A), which is in consistent with the development of chronic active hepatitis in the infected male mice. These data suggest that this bacterial CDT enhances transcriptional upregulation of a subset of proinflammatory mediators.
Fig. 3. Relative mRNA levels of selected proinflammatory mediators (A) and the NF-κB subunits (B). In each sample, the target mRNA was normalized to that of Gapdh. Numbers on the left represent mean fold change of the individual mRNA levels in reference to the control group (defined as 0 meaning no change and its standard deviations represented by hatched boxes) for both 4 and 10 MPI unless otherwise denoted on the right for 10 MPI. Bars, standard deviations. \( P \)-values when compared with the sham controls: *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
cytokines during chronic inflammation. TNF-α but not IL-1β, likely activates the classical NF-κB (see below).

**Bacterial CDT contributes to upregulation of the RelA(p65) and p50 mRNA coding for the proteins of the classical NF-κB pathway**

Next we examined a host signalling pathway mediating the progression of chronic hepatitis to liver dysplasia in response to *H. hepaticus* infection. Activation of the classical IKK-β-dependent NF-κB pathway appears to be a crucial step in inflammation-induced tumour growth and progression of HCC (Pikarsky et al., 2004) and colon cancer (Greten et al., 2004). We measured mRNA levels of RelA (p65) and p50 using QPCR (Fig. 3B). WT *H. hepaticus* infection in the male mice significantly increased the mRNA levels of RelA (p65) (*P* = 0.006) and p50 (*P* = 0.001) when compared with the CDT mutant-infected counterparts at 4 MPI but not 10 MPI (Fig. 3B). In contrast, there were similar mRNA levels of RelB involved in the alternative NF-κB pathway between the WT *H. hepaticus* and the CDT mutant-infected groups (*P* = 0.7). The difference of the mRNA levels of RelA (p65) and p50 between the WT *H. hepaticus* and CDT mutant-infected groups should not be due to *H. hepaticus* colonization levels given they were comparable between these two groups at 4 MPI (Fig. 2). These results indicate that the *H. hepaticus* CDT was essential for the observed upregulation of the classical NF-κB pathway in the early stage of infection (by 4 MPI).

**Cytolethal distending toxin in *H. hepaticus* increases hepatocyte proliferation and mRNA expression of anti-apoptotic proteins**

Enhanced cell proliferation plays a critical role during cancer development (Hanahan and Weinberg, 2000). The index of hepatocyte proliferation was analysed by using immunochemical Ki-67 staining and compared between the WT *H. hepaticus* and the CDT mutant-infected livers. At 4 MPI, locally populated Ki-67-positive hepatocytes were visualized in the WT *H. hepaticus*-infected (Fig. 4C) but not the CDT mutant- or the sham dosed mice (Fig. 4A and B). Hepatocyte proliferation was significantly higher with WT *H. hepaticus* infection than the CDT-mutant infection at both time points (Fig. 4D, *P* < 0.05). In addition, the mRNA levels of two stimulatory growth factors IL-6 (*P* = 0.006) and TGF-α (*P* = 0.016) were significantly higher in the WT *H. hepaticus*-infected male mice compared with the CDT mutant-infected counterparts at 4MPI (Fig. 5A), whereas at 10 MPI only IL-6 was significantly upregulated (*P* = 0.03).

Because chronic inflammation-driven or chemically induced hepatocarcinogenesis involves enhancement of anti-apoptotic activity (Pikarsky et al., 2004; Sakurai et al., 2006), mRNA levels for anti-apoptotic proteins GADD45β, c-IAP1, Bcl-2 and Bcl-XL were measured. We found that transcription of GADD45β (*P* = 0.02) and c-IAP1 (*P* = 0.015) was upregulated by WT *H. hepaticus* when compared with the CDT mutant at 4 MPI (Fig. 5B). In addition, mRNA levels of anti-apoptotic Bcl-2 (*P* = 0.006 at 4 MPI and *P* = 0.019 at 10 MPI) and Bcl-XL (*P* = 0.0001 at 4 MPI and *P* = 0.015 at 10 MPI) were statistically upregulated by WT *H. hepaticus* infection when compared with their levels tabulated from the livers infected with the CDT mutant (Fig. 5B). Early onset upregulation of the NF-κB mRNA by WT *H. hepaticus* coincided with the enhanced expression of its upstream genes TNF-α and TGF-α, and its downstream genes IL-6, Cox-2, Bcl-XL, GADD45β and c-IAP1 (Papa et al., 2004; Pikarsky et al., 2004; Karin, 2006). There was sustained upregulation of IL-6 and Bcl-XL in WT *H. hepaticus*-infected livers by 10 MPI (Fig. 5); however, no significant differences in the mRNA levels of the NF-κB between WT *H. hepaticus* - and CDT mutant-infected groups was noted (Fig. 3B).

**Discussion**

In this study we have shown that *H. hepaticus* CDT is not necessary to induce chronic hepatitis in susceptible male A/JCr mice. However, CDT appears to play an essential role in inflammation-associated preneoplastic lesions typically noted in chronic *H. hepaticus* infection. It is unlikely that differences in liver colonization of *H. hepaticus* played a major role in the hepatic lesions noted between WT *H. hepaticus* and the CDT-mutant infections, because there was no significant difference in hepatic colonization levels between WT *H. hepaticus* and the CDT mutant at both 4 and 10 MPI (Fig. 2). Based on these histopathologic and molecular data, we have developed a working model to explain the role of bacterial CDT-induced signaling in the *H. hepaticus*-induced hepatocarcinogenesis (Fig. 6). In this proposed model, *H. hepaticus* infection causes chronic hepatitis; in the absence of CDT, the inflammation is not sufficient to progress to HCC, in part due to a relative downregulation of proinflammatory responses and the failure to upregulate the NF-κB pathway. In contrast, the presence of CDT elevates expression of a subset of proinflammatory mediators such as TNF-α, IL-6 and Cox-2. The classical NF-κB pathway is activated by these proinflammatory mediators, most likely TNF-α, but not IL-1 (Pikarsky et al., 2004). The activation of the classical NF-κB pathway leads to the increased production of downstream growth mediators IL-6 and TGF-α. These proinflammatory and growth mediators in combination with the CDT-mediated suppression of apoptosis could increase survival of 'initiated
cancer hepatocytes' in the early phase of the infection. During the course of bacterial infection, the sustained overproduction of IL-6 and anti-apoptotic proteins Bcl-2 and Bcl-X, facilitates proliferation of the initiated hepatocytes, thereby leading to premalignant dysplastic lesions, and eventual HCC (Fox et al., 1996; Rogers et al., 2004). IL-6, a pleiotropic cytokine, has various biological activities including growth stimulation, proinflammatory responses and development of autoimmunity (Hodge et al., 2005). It has been demonstrated that this cytokine is essential for hepatocyte regeneration (Cressman et al., 1996) and also plays an important role in compensatory proliferation in a chemically induced HCC mouse model (Maeda et al., 2005). In the current study, mRNA of IL-6 in livers was upregulated by WT H. hepaticus infection at both 4 and 10 MPI. These data suggest that IL-6 functions as a central growth factor during the development of the H. hepaticus-induced liver malignancy. The observation that the upregulation of IL-6 and Bcl-X was not correlated with the mRNA levels of genes in the NF-κB pathway in

Fig. 4. Hepatocyte proliferation visualized by immunostaining with Ki-67 antibody. Representative Ki-67-positive hepatocytes in the liver of the sham control (A), the CDT mutant (B) and WT H. hepaticus-infected (C) mice. The mean numbers of Ki-67-positive hepatocytes per cm² of liver for the individual groups (n = 4) are presented in D. *P < 0.05 when compared with the CDT mutant-infected group.
the WT *H. hepaticus*-infected livers by 10 MPI indicate that additional transcription activators also play regulatory roles in transcription of these two genes. Indeed, transcriptional involvement of AP-1 and C/EBP in the production of IL-6 as well as AP-1 and STATs in the expression of Bcl-X\textsubscript{L} has been reported (Sevilla et al., 2001; Baccam et al., 2003). Whether this is the case in our model will require further studies.

There are two major, functionally distinct NF-\(\kappa\)B-signalling pathways: classical pathway critical for activating inflammation and alternative pathway required for lymphoid organ development, B cell maturation and adaptive immunity (Bonizzi and Karin, 2004). RelA and p50 are the components of NF-\(\kappa\)B transcription activator for the classical pathway, whereas RelB is an essential component for the alternative pathway. In *Mdr2\textsuperscript{-/}ΔIxB\textsuperscript{amy*} mice, activation of the classical NF-\(\kappa\)B pathway plays a crucial role in promoting chronic inflammation which leads to HCC (Pikarsky et al., 2004). Mouse *Mdr2* encodes a P-glycoprotein apparently responsible for transporting phospholipids into bile; its disruption abolishes this process, leading to the development of chronic hepatitis shortly after birth, dysplasia by 7 months of age and HCC by 10 months of age (Mauad et al., 1994; Pikarsky et al., 2004). Activation of the NF-\(\kappa\)B pathway is required for progression of the dysplastic stage to HCC (at 7–10 months of age) but not for development of chronic inflammation (Pikarsky et al., 2004). In our study, the
finding that transcriptional upregulation of RelA(p65) and p50 for the classical NF-κB pathway but not RelB for the alternative NF-κB pathway was associated with the progression of dysplastic nodules in WT H. hepaticus-infected male mice indicates that this classical pathway could be important for the development of H. hepaticus CDT-induced liver lesions. It is worth noting that the CDT-induced NF-κB upregulation in our study occurred at 4 MPI (inflammation phase) but not at 10 MPI (dysplastic phase), which apparently differs from the timing for the activation of the NF-κB pathway in Mdr2−/−/Δ1xB(+/−) mice. In our model, H. hepaticus infection occurs via oral exposure with subsequent colonization of the lower bowel. Only after chronic infection, does liver colonization occur. Bacterial colonization and disease development are influenced by multiple factors, dynamic changes in the gastrointestinal environment, and complex host-pathogen interactions. Therefore, this difference in the activation of the NF-κB pathway may be, at least in part, due to differences in factors initiating liver damage in these two rodent models.

H. hepaticus infection evidently does not elevate promutagenic liver DNA damage via formation of N7-methylguanine and O6 methylguanine DNA adducts via nitric oxide release and nitrosation of endogenous amines (Canella et al., 1996). In addition, mutations in ras onco-genes or tumour-suppressor gene p53 in carcinomas and adenomas of the infected murine livers were not noted (Canella et al., 1996; Sipowicz et al., 1997). H. hepaticus infection does, however, increase incidence of liver tumours initiated by N-nitrosodimethylamine in male A/JCr mice (Diwan et al., 1997). These data suggest that H. hepaticus induces liver tumours via a promoter-like mechanism. In our study, enhanced expression of proinflammatory mediators, growth factors and the classical NF-κB subunits as well as hepatocyte proliferation by WT H. hepaticus infection compared with the CDT mutant are consistent with this proposed mechanism (Fox et al., 1996).

Colonization durability of H. hepaticus CDT-deficient mutants has been characterized in three mouse strains: disease-resistant Swiss Webster (SW) mice (Ge et al., 2005), inflammatory bowel disease-susceptible C57BL/6 IL-10−/− mice (Pratt et al., 2006), and liver disease-susceptible A/JCr mice (this study). Colonization with H. hepaticus in livers of SW or C57BL/6 IL-10−/− mice is undetectable by QPCR and culture at 2 MPI (Z. Ge and J.G. Fox, unpubl. data). The CDT mutant lose cecal colonization in female SW mice by 2 weeks postinoculation (WPI) or in female A/JCr mice by 16 WPI, and in male SW mice by 16 WPI or in 80% of male A/JCr mice (four out five) by approximately 43 WPI (10 MPI). In C57BL/6 IL-10−/− mice (likely females which were not specified by the authors), the CDT mutant showed progressive loss of colonization starting at 17 WPI (20% of cages) and at 32 WPI for 80% of cages as judged by nested PCR (Pratt et al., 2006). These data suggest that colonization durability of the CDT mutant in these mouse strains is host-dependent (immunity-dysregulated mouse strains is host-dependent (immunity-dysregulated C57BL/6 IL-10−/− mice > immunocompetent, inbred A/J mice > immunocompetent, outbred SW mice) and also sex-dependent (male > female). In addition, it has been demonstrated that CDT is essential for persistent colonization of C. jejuni in C57BL/6 mice (Fox et al., 2004). Furthermore, bacterial CDTs have the ability to arrest growth of various cultured cells including T and B lymphocytes (Heywood et al., 2005). These lines of evidence indicate the CDT could play a role in the ability of bacteria

© 2007 The Authors
Journal compilation © 2007 Blackwell Publishing Ltd, Cellular Microbiology, 9, 2070–2080
to evade host immune surveillance and establish chronic infection, which, in combination with the increased production of proinflammatory mediators, growth factors and anti-apoptotic proteins, facilitates proliferation of the initiated cancer cells and eventually promoting the tumorigenesis process. Long-term studies (> 15 MPI) using CDT mutants are required to establish whether CDT is essential for progression of dysplastic nodules to HCC.

Cytolethal distending toxin-producing bacteria, including E. coli, Campylobacter spp., A. actinomycetemcomitans, H. ducreyi, Shigella spp., S. enterica and Helicobacter spp., cause or are associated with various medical complications such as gastroenteritis, chancroid, IBD, periodontitis and diarrhea. Pathogenic roles of bacterial CDTs have been shown in several mouse models with chronic infection where bacterial infection was monitored for 2–10 months, including A/JCr mice (our study) and C57BL/6 IL10–/– mice for H. hepaticus CDT mutant (Pratt et al., 2006), and NF-κB-deficient mice infected with C. jejuni CDT mutants (mixed 129 × C57BL/6/ background) (Fox et al., 2004). It is clear that CDT plays a pathogenic role in chronic inflammation in the gastrointestinal tract. However, in vivo acute models, including scid mice infected with C. jejuni CDT mutant (Purdy et al., 2000), rabbits and human volunteers infected with H. ducreyi CDT mutants (Lewis et al., 2001; Young et al., 2001), failed to show a significant role of the respective CDTs in inducing acute inflammation nor did CDT play a role in establishing bacterial colonization. Further studies are required for elucidating the role of CDT in the pathogenesis of acute inflammatory diseases by CDT-producing bacterial species.

The role of bacterial CDTs in liver tumorigenesis and other gastrointestinal diseases highlights their pathogenic potential in comparable human diseases including HCC and other hepatobiliary cancers (Solnick and Schauer, 2001; Fox et al., 2004; Young et al., 2004). Given that CDT is essential for persistent bacterial colonization of H. hepaticus (Ge et al., 2005; Pratt et al., 2006) and C. jejuni (Fox et al., 2004) in immunocompetent and IL-10–/– mice, drugs targeting CDT could be beneficial in preventing or treating these bacterial infections. Recent advances in understanding the role of NF-κB in cancer development offers potential for the utility of targeting this transcription activator in preventing or curing cancers (Karim, 2006). It is also worth noting that the classical NF-κB pathway controls various genes involving survival, cell growth, angiogenesis and proinflammatory responses, some of which involve a protective role for hosts in combating microbial infections (Lavon et al., 2000; Erdman et al., 2001; Fox et al., 2004; Karim, 2006).

Thus, IL-6 could possibly be used as an alternative therapeutic target with fewer side-effects for cancer prevention and therapy in certain disease conditions.

Experimental procedures

Bacterial strains and growth conditions

Culture conditions for WT H. hepaticus 3B1 (ATCC51448) and a cdtB-deficient H. hepaticus mutant (CDT mutant) were previously described by our laboratory (Ge et al., 2005). The CDT mutant contains a minitransposon-mediated mutation within the cdtB gene coding for a catalytic subunit of CDT; the mutant lacks CDT activity (Ge et al., 2005).

Experimental design

Sixty 4- to 6-week-old A/JCr mice (30 male and 30 female) were obtained from the National Cancer Institute (Frederick, MD). The mice were free of known murine viruses, pathogenic bacteria including Helicobacter spp. and parasites. Mice were maintained in an Association for Accreditation and Assessment of Laboratory Animal Care, International-accredited facility in static microisolation cages and provided pelleted diet and H2O. Six groups of 10 mice (either male or female) were dosed with WT H. hepaticus, the CDT mutant or sham-dosed with Brucella broth as a control respectively. Mice received 0.2 ml of fresh inocula containing approximately 2 × 10⁷ organisms by gastric gavage every other day for three inoculations. Five male or female mice from each group were necropsied at 4 or 10 MPI. Immediately after CO₂ euthanasia, contents in the intestine were removed by rinsing with sterile saline. Two liver sections (one sample per liver lobe) and approximately 1-cm segments of cecum were collected for culture and RNA/DNA isolation. Tissues for RNA/DNA isolation were frozen in liquid nitrogen immediately after sampling and stored at −70°C prior to use. Representative tissue sections were fixed in 10% buffered formalin for histology.

Quantitative PCR, nested PCR and culture for
H. hepaticus

For real-time QPCR, chromosomal DNA from cultured bacteria was prepared using a High Pure PCR Template kit according to the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN). Total DNA and RNA from frozen liver and cecum were isolated using Trizol Reagents following the supplier’s procedure (Invitrogen, Carlsbad, CA). H. hepaticus in liver were measured by QPCR in the Prism Sequence Detection Systems 7700 (Applied Biosystems) as described elsewhere (Ge et al., 2001). The numbers of H. hepaticus were then normalized to μg of mouse chromosomal DNA whose quantities in the samples were measured by QPCR using the 18S rRNA gene-based primers and probe mixture (Applied Biosystems). In addition, nested PCR was performed for detecting low numbers of H. hepaticus in the livers as previously described (Rogers et al., 2004). In brief, an approximately 1.2 kb within the H. hepaticus 16S rRNA gene was amplified using the Helicobacter genus-specific primers for the first round PCR. In the second round, a 417 bp product within the first-round amplicon was nested using H. hepaticus-specific primers. Furthermore, viable H. hepaticus from the cecum and the liver was recovered using a published protocol (Fox et al., 1996).
Relative quantification of mRNA levels of selected genes

Total RNA from livers of AJCr mice was prepared using Trizol reagent according to the recommendations of the manufacturer (Invitrogen). For cytokine mRNA quantification, 5 μg of total RNA from samples were converted into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems). Levels of IFN-γ, Cox-2, IL-6, IL-1β, TNF-α, RelA (p65), p50, Bcl-2, Bcl-Xs, RelB, cIAP1 and GADD45β mRNA were measured by QPCR using commercial primers and probes (TaqMan Gene Expression Assays). QPCR was performed in the ABI Prism Sequence Detection System 7700 (Applied Biosystems). Transcript levels were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase mRNA (Gapdh), and expressed as fold change compared with sham-dosed control mice using the Comparative Ct method (Applied Biosystems User Bulletin no. 2).

Histopathology and histoimmunocytochemistry

As previously described, the ileocolic junction and two sagittal sections of each liver lobe were collected, processed, sectioned at 4 μm, stained with haematoxylin and eosin, and examined by a veterinary pathologist blinded to sample identity (Rogers et al., 2004). Selected formalin-fixed liver sections were stained with an antibody against the mitosis marker Ki-67 (BD Pharmingen, San Diego, CA). The number of Ki-67-positive hepatocytes was expressed per cm² of liver area.

Statistical analysis

Histopathological scores among the groups were compared using a Mann–Whitney non-parametric t-test. Data on the levels of H. hepaticus and cytokine mRNA in the tissues were analysed using two-tailed Student’s t-test. Values of P < 0.05 are considered significant.

Acknowledgements

This study was supported in part by NIH Grants R01 CA67529, R01 AI51404, P01 CA26731 and P30ES02109 to J.G.F. We thank Kathleen Cormier, Chakib Boussahamain, and Kate Rydstrom for help with histology and immunocytochemistry, Kristen Clapp and Juri Miyamae for bacterial inoculation and necropsy, and Elaine Robbins for assistance with preparation of the figures.

References

Baccam, M., Woo, S.Y., Vinson, C., and Bishop, G.A. (2003) CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-κB, AP-1, and C/EBP. J Immunol 170: 3099–3108.

Bonizzi, G., and Karin, M. (2004) The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25: 280–288.

Canella, K.A., Diwan, B.A., Gorelick, P.L., Donovan, P.J., Sipowicz, M.A., Kasprzak, K.S., et al. (1996) Liver tumorigenesis by Helicobacter hepaticus: considerations of mechanism. In Vivo 10: 285–292.

Cressman, D.E., Greenbaum, L.E., DeAngelis, R.A., Ciliberto, G., Furth, E.E., Poli, V., and Taub, R. (1996) Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 274: 1379–1383.

Diwan, B.A., Ward, J.M., Ramljuk, D., and Anderson, L.M. (1997) Promotion by Helicobacter hepaticus-induced hepatisis of hepatic tumors initiated by N-nitrosodimethylamine in male AJCr mice. Toxical Pathol 25: 597–605.

Erdman, S., Fox, J.G., Dangler, C.A., Feldman, D., and Horwitz, B.H. (2001) Typhlocolitis in NF-κB-deficient mice. J Immunol 166: 1443–14447.

Erdman, S.E., Poutahidis, T., Tomczak, M., Rogers, A.B., Cormier, K., Plank, B., et al. (2003) CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice. Am J Pathol 162: 691–702.

Fox, J.G., Dewhirst, F.E., Tully, J.G., Paster, B.J., Yan, L., Taylor, N.S., et al. (1994) Helicobacter hepaticus sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J Clin Microbiol 32: 1238–1245.

Fox, J.G., Li, X., Yan, L., Cahill, R.J., Hurley, R., Lewis, R., and Murphy, J.C. (1996) Chronic proliferative hepatitis in AJCr mice associated with persistent Helicobacter hepaticus infection: a model of helicobacter-induced carcinogenesis. Infect Immun 64: 1548–1558.

Fox, J.G., Rogers, A.B., Whary, M.T., Ge, Z., Taylor, N.S., Xu, S., et al. (2004) Gastroenteritis in NF-κB-deficient mice is produced with wild-type Campylobacter jejuni but not with C. jejuni lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun 72: 1116–1125.

Ge, Z., White, D.A., Whary, M.T., and Fox, J.G. (2001) Fluorogenic PCR-based quantitative detection of a murine pathogen, Helicobacter hepaticus. J Clin Microbiol 39: 2598–2602.

Ge, Z., Feng, Y., Whary, M.T., Nambiar, P.R., Xu, S., Ng, V., et al. (2005) Cytolethal distending toxin is essential for Helicobacter hepaticus colonization in outbred Swiss Webster mice. Infect Immun 73: 3559–3567.

Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., et al. (2004) IKKζ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118: 285–296.

Haghjoo, E., and Galan, J.E. (2004) Salmonella typhi encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proc Natl Acad Sci USA 101: 4614–4619.

Hanahan, D., and Weinberg, R.A. (2000) The hallmarks of cancer. Cell 100: 57–70.

Heywood, W., Henderson, B., and Nair, S.P. (2005) Cytolethal distending toxin: creating a gap in the cell cycle. J Med Microbiol 54: 207–216.

Hodge, D.R., Hurt, E.M., and Farrar, W.L. (2005) The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 41: 2502–2512.

Ilhig, M., Schrenzel, M.D., and Fox, J.G. (1999) Differential susceptibility to hepatic inflammation and proliferation in AXB recombinant inbred mice chronically infected with Helicobacter hepaticus. Am J Pathol 155: 571–582.

Karin, M. (2006) Nuclear factor-κB in cancer development and progression. Nature 441: 431–436.
Lara-Tejero, M., and Galan, J.E. (2002) Cytolethal distending toxin: limited damage as a strategy to modulate cellular functions. Trends Microbiol 10: 147–152.

Lavon, I., Goldberg, I., Amit, S., Landsman, L., Jung, S., Tsuberi, B.Z., et al. (2000) High susceptibility to bacterial infection, but no liver dysfunction, in mice compromised for hepatocyte NF-κB activation. Nat Med 6: 573–577.

Lewis, D.A., Stevens, M.K., Latimer, J.L., Ward, C.K., Deng, K., Blick, R., et al. (2001) Characterization of Haemophilus ducreyi cdtA, cdtB, and cdtC mutants in in vitro and in vivo systems. Infect Immun 69: 5626–5634.

Maeda, S., Kamata, H., Luo, J.L., Leffert, H., and Karin, M. (2005) IKKβ couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 121: 977–990.

Mauad, T.H., van Nieuwkerk, C.M., Dingemans, K.P., Smit, J.J., Schinkel, A.H., Notenboom, R.G., et al. (1994) Mice with homozygous disruption of the mdr2 P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis. Am J Pathol 145: 461–466.

Pratt, J.S., Sachen, K.L., Wood, H.D., Eaton, K.A., and Young, V.B. (2006) Modulation of host immune responses by the cytolethal distending toxin of Helicobacter hepaticus. Infect Immun 74: 4496–4504.

Purdy, D., Buswell, C.M., Hodgson, A.E., McAlpine, K., Henderson, I., and Leach, S.A. (2000) Characterisation of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni. J Med Microbiol 49: 473–479.

Rao, V.P., Poutahidis, T., Ge, Z., Nambiar, P.R., Boussahmain, C., Wang, Y.Y., et al. (2006) Innate immune inflammatory response against enteric bacteria Helicobacter hepaticus induces mammary adenocarcinoma in mice. Cancer Res 66: 7395–7400.

Rogers, A.B., Boutin, S.R., Whary, M.T., Sundina, N., Ge, Z., Cormier, K., and Fox, J.G. (2004) Progression of chronic hepatitis and preneoplasia in Helicobacter hepaticus-infected A/JCr mice. Toxicol Pathol 32: 668–677.

Sakurai, T., Maeda, S., Chang, L., and Karin, M. (2006) Loss of hepatic NF-κB activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. Proc Natl Acad Sci USA 103: 10544–10551.

Sevilla, L., Zaldumbide, A., Pognonec, P., and Boulikos, K.E. (2001) Transcriptional regulation of the bcl-2 gene encoding the anti-apoptotic Bcl-XL protein by Ets, Rel/NF-κB, STAT and AP1 transcription factor families. Histol Histopathol 16: 595–601.

Sipowicz, M.A., Weghorst, C.M., Shiao, Y.H., Buzard, G.S., Calvert, R.J., Anver, M.R., et al. (1997) Lack of p53 and ras mutations in Helicobacter hepaticus-induced liver tumors in A/JCr mice. Carcinogenesis 18: 233–236.

Smith, J.L., and Bayles, D.O. (2006) The contribution of cytolethal distending toxin to bacterial pathogenesis. Crit Rev Microbiol 32: 227–248.

Solnick, J.V., and Schauer, D.B. (2001) Emergence of diverse Helicobacter species in the pathogenesis of gastric and enterohepatic diseases. Clin Microbiol Rev 14: 59–97.

Suerbaum, S., Josenhans, C., Sterzenbach, T., Drescher, B., Brandt, P., Bell, M., et al. (2003) The complete genome sequence of the carcinogenic bacterium Helicobacter hepaticus. Proc Natl Acad Sci USA 100: 7901–7906.

Thelestam, M., and Frisan, T. (2004) Cytolethal distending toxins. Rev Physiol Biochem Pharmacol 152: 111–133.

Young, V.B., Knox, K.A., and Schauer, D.B. (2000) Cytolethal distending toxin sequence and activity in the enterohemepathogenic Helicobacter hepaticus. Infect Immun 68: 184–191.

Young, R.S., Fortney, K.R., Gelfanova, V., Phillips, C.L., Katz, B.P., Hood, A.F., et al. (2001) Expression of cytolethal distending toxin and hemolysin is not required for pustule formation by Haemophilus ducreyi in human volunteers. Infect Immun 69: 1938–1942.

Young, V.B., Knox, K.A., Pratt, J.S., Cortez, J.S., Mansfield, L.S., Rogers, A.B., et al. (2004) In vitro and in vivo characterization of Helicobacter hepaticus cytolethal distending toxin mutants. Infect Immun 72: 2521–2527.