Snykers S, Vinken M, Rogiers V. 2007. Differential role of epigenetic modulators in malignant and normal stem cells: a novel tool in preclinical in vitro toxicology and clinical therapy. Arch Toxicol 81:533–544.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblasts cultures by defined factors. Cell 126:663–676.

Talens-Visconti R, Bonora A, Jover R, Mirabet V, Carbonell F, Castell JV, Gomez-Lechon MJ. 2006. Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal cells. World J Gastroenterol 28:5834–5845.

Tanimizu N, Saito H, Mostov K, Miyajima A. 2004. Long-term culture of hepatic progenitors derived from mouse Dlk$^+$ hepatoblasts. J Cell Sci 117:6425–6434.

Tanimizu N, Miyajima A, Mostov KE. 2007. Liver progenitor cells develop cholangiocyte-type epithelial polarity in the three-dimensional culture. Mol Biol Cell 18:1472–1479.

Teramoto K, Asahina K, Kumashiro Y, Kakinuma S, Chinzei R, Shimizu-Saito K, Tanaka Y, Teraoka H, Arii S. 2005. Hepatocyte differentiation from embryonic stem cells and umbilical cord blood cells. J Hepatobil Pancreat Surg 12:196–202.

Teratani T, Yamamoto H, Aoyagi K, Sasaki H, Asari A, Quinn G, Sasaki H, Terada M, Ochiya T. 2005. Direct hepatic fate specification from mouse embryonic stem cells. Hepatology 41:836–846.

Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS. 2000a. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 31:235–240.

Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegariu O, Krause DS. 2000b. Liver from bone marrow in humans. Hepatology 32: 11–16.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.

Thorgeirsson SS, Grisham JW. 2006. Hematopoietic cells as hepatocyte stem cells: A critical review of evidence. Hepatology 43:2–8.

Ucelli A, Pistoia V, Moretta L. 2007. Mesenchymal stem cells: a new strategy for immunosuppression?Trends Immunol 28: 219–226.

Urs S, Smith C, Campbell B, Saxton AM, Taylor J, Zhang B, Snoddy J, Jones Voy B, Moustaid-Moussa N. 2004. Gene expression profiling in human preadipocytes and adipocytes by microarray analysis. J Nutr 134:762–770.

Vassilopoulos G, Wang PR, Russell DW. 2003. Transplanted bone marrow regenerates liver by cell fusion. Nature 422: 901–904.

Vig P, Russo FP, Edwards RJ, Tadrous PJ, Wright NA, Thomas HC, Alison MR, Forbes SJ. 2006. The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. Hepatology 43:316–324.

Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. 2005. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 33:1402–1416.

Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. 2003. The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci USA 1:11881–11888.

Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. 2003. Cell fusion in the principal source of bone marrow-derived hepatocytes. Nature 422:897–901.

Wang Y, Nan X, Li Y, Zhang R, Yue F, Yan F, Pei X. 2005. Induction of umbilical cord blood-derived beta2m-c-Met$^+$ cells into hepatocyte-like cells by coculture with CFSC/HGF cells. Liver Transpl 11: 635–643.

Watanabe N, Tanaka M, Suzuki K, Kumanogoh A, Kikutani H, Miyajima A. 2007. Tim2 is expressed in mouse fetal hepatocytes and regulates their differentiation. Hepatology 45:1240–1249.

Yamada T, Yoshikawa M, Kanda S, Kato Y, Nakajima Y, Ishizaka S, Tsunoda Y. 2002. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. Stem Cells 20:146–154.

Yamamoto H, Quinn G, Asari A, Yamanokuchi H, Teratani T, Terada M, Ochiya T. 2003. Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. Hepatology 37:983–993.

Yamamoto Y, Teratani T, Yamamoto H, Quinn G, Murata S, Ikeda R, Kinoshita K, Matsubara K, Kato T, Ochiya T. 2005. Recapitulation of in vivo gene expression during hepatic differentiation from embryonic stem cells. Hepatology 42:558–567.

Yin Y, Lim YK, Salto-Tellez M, Ng SC, Lin CS, Lim SK. 2002. AFP(+), ESC-derived cells engraft and differentiate into hepatocytes in vivo. Stem Cells 20:338–346.

Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD. 2007. Novel hepatic progenitor cell surface markers in the adult rat liver. Hepatology 45:139–149.

Zhao DC, Lei JX, Chen R, Yu WH, Zhang XM, Li SN, Xiang P. 2005. Bone marrow-derived mesenchymal stem cells protect against experimental liver fibrosis in rats. World J Gastroenterol 11:3431–3440.

Zhao R, Duncan SA. 2005. Embryonic development of the liver. Hepatology 41: 956–967.

Zhou H, Rogler LE, Teperman L, Morgan G, Rogler CE. 2007. Identification of hepatocytic and bile ductual cell lineages and candidate stem cells in bipolar ductual reactions in cirrhotic human liver. Hepatology 45:716–24.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz PH, Hedrick MH. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.

Oncogene (2007) 26, 5587–5595

www.nature.com/onc

ORIGINAL ARTICLE

Expression of activation-induced cytidine deaminase in human hepatocytes via NF-κB signaling

Y Endo[1], H Marusawa[1], K Kinoshita[2], T Morisawa[1], T Sakurai[1], I-M Okazaki[3], K Watashi[4], K Shimotohno[4], T Honjo[3] and T Chiba[1]

[1]Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [2]Evolutionary Medicine, Shiga Medical Center Research Institute, Moriyama, Japan; [3]Department of Immunology and Genomic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan and [4]Laboratory of Human Tumor Viruses, Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan

Activation-induced cytidine deaminase (AID) is involved in somatic DNA alterations of the immunoglobulin gene for amplification of immune diversity. The fact that constitutive expression of AID in mice causes tumors in various organs, including lymphoid tissues and lungs, suggests the important role of the aberrant editing activity of AID on various tumor-related genes for carcinogenesis. AID expression, however, is restricted to activated B cells under physiological conditions. We demonstrate here that ectopic AID expression is induced in response to tumor necrosis factor-α stimulation in cultured human hepatocytes. The proinflammatory cytokine-mediated expression of AID is achieved by IκB kinase-dependent nuclear factor (NF)-κB signaling pathways. Hepatitis C virus, one of the leading causes of hepatocellular carcinoma (HCC), enhanced AID expression via NF-κB activation through expression of viral core protein. The aberrant expression of AID in hepatoma-derived cells resulted in accumulation of genetic alterations in the *c-myc* and *pim1* genes, suggesting that inappropriate expression of AID acts as a DNA mutator that enhances the genetic susceptibility to mutagenesis in human hepatocytes. Our current findings indicate that the inappropriate expression of AID is induced by proinflammatory cytokine stimulation and may provide the link between hepatic inflammation and the development of HCC.
Oncogene (2007) **26**, 5587–5595; doi:10.1038/sj.onc.1210344; published online 2 April 2007

Keywords: activation-induced cytidine deaminase; TNF-α; NF-κB; hepatocellular carcinoma; hepatitis C virus; chronic hepatitis

Correspondence: Professor T Chiba, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: chiba@kuhp.kyoto-u.ac.jp
Received 13 October 2006; revised 8 January 2007; accepted 15 January 2007; published online 2 April 2007

Introduction

A causal association between inflammation and cancer has been proposed in a variety of chronic inflammatory diseases (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). One of the well-recognized models of inflammation-associated tumor development is human hepatocarcinogenesis, which is closely associated with hepatitis virus-associated chronic liver disease. In fact, epidemiologic studies demonstrate that most human hepatocellular carcinoma (HCC) develops during chronic hepatic inflammation with features of liver cirrhosis or chronic hepatitis (Tsukuma *et al.*, 1993). On the other hand, human cancer develops by a multistep process occurring through the accumulation of gene alterations that govern cell proliferation, regeneration and apoptosis (Lengauer *et al.*, 1998; Loeb *et al.*, 2003). Indeed, DNA mutations leading to the activation of oncogenes and/or inactivation of tumor suppressor genes have been reported in hepatoma cells (Thorgeirsson and Grisham, 2002). The mechanisms of how hepatocytes with underlying chronic inflammation acquire the genetic changes leading to malignant transformation, however, are unknown.

Activation-induced cytidine deaminase (AID) is a member of the cytidine deaminase family (Muramatsu *et al.*, 1999) and is closely related to apolipoprotein B RNA-editing cytidine deaminase 1, which converts cytosine nucleotides to uracils in RNA (Teng *et al.*, 1993). AID is expressed in activated B cells, especially in germinal centers and induces somatic hypermutation (SHM), untemplated point mutations, at a high frequency of 10^{-3}–10^{-4} per base pair, into the variable regions of immunoglobulin genes (Muramatsu *et al.*, 2000; Revy *et al.*, 2000; Kinoshita and Honjo, 2001; Honjo *et al.*, 2002). As overexpression of AID increases the rate of SHM in B-cell lines (Martin *et al.*, 2002) and AID can attack the non-immunoglobulin genes, tight control of this protein activity appears crucial under physiologic conditions (Martin and Scharff, 2002; Okazaki *et al.*, 2002, 2003; Yoshikawa *et al.*, 2002; Kinoshita and Nonaka, 2006). We demonstrated that aberrant AID expression can induce SHM in a non-immunoglobulin gene in nonlymphoid cells (Okazaki

et al., 2002; Yoshikawa *et al.*, 2002), and mice with constitutive expression of AID develop various tumors, including T-cell lymphomas and lung adenomas, suggesting that AID has direct oncogenic potential via the mutagenesis of inappropriate target genes (Okazaki *et al.*, 2003). Notably, we recently revealed that endogenous AID was significantly upregulated in both HCC and surrounding noncancerous liver tissues with underlying cirrhosis or chronic hepatitis, where only minute AID expression is detectable in normal liver (Kou *et al.*, 2007). Moreover, both tumor and non-tumor liver tissues with hepatitis C virus (HCV) infection exhibited significantly higher expression levels of the *AID* gene compared with liver tissues of normal control. These findings led to our hypothesis that aberrant expression of AID is present in hepatocytes and might be involved in the enhanced genetic susceptibility to mutagenesis under inflammatory conditions during hepatocarcinogenesis. We demonstrate here that expression of AID is transcriptionally regulated by nuclear factor (NF)-κB in human hepatocytes, allowing it to induce nucleotide alterations in tumor-related genes. The findings thus provide novel insights into the mechanism of genetic mutations during the process of hepatocarcinogenesis and suggest a link between hepatic inflammation and mutagenesis associated with cancer development.

Results

Development of HCC in AID Tg mice

We previously demonstrated that constitutive expression of AID in transgenic (Tg) mice caused both T-cell lymphoma and micro-adenoma in lung alveoli (Okazaki *et al.*, 2003). In this study, we focused on the impact of AID expression in the liver and thus analysed the liver phenotype of randomly selected AID Tg mice killed between 58 and 84 weeks after birth for pathologic analysis. We found that 4 of 16 (25.0%) Tg mice developed tumors in the liver. Histological examination revealed that those tumors showed a trabecular pattern of growth with focal steatosis, consistent with the morphological appearance of well-differentiated HCC (Figure 1). These findings suggested that aberrant expression of AID in the liver can be genotoxic, leading to the development of HCC.

Endogenous AID is induced in response to proinflammatory cytokines in human hepatocytes

To determine whether proinflammatory cytokines enhance *AID* expression in human hepatocytes, we first analysed the expression of endogenous *AID* transcripts in human hepatoma cell lines by quantitative reverse transcription–polymerase chain reaction (RT–PCR)

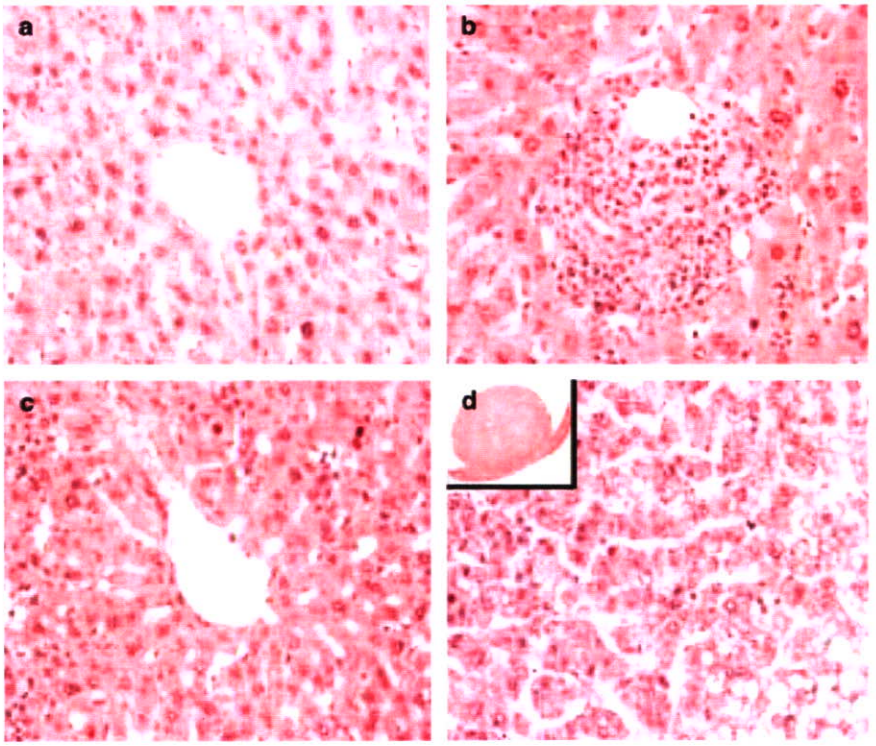

Figure 1 Development of HCC in AID Tg mice. (**a**) H&E-stained sections of the liver of a non-transgenic mouse. (**b**) Liver specimens of the AID Tg mouse with T-cell lymphoma development. Non-tumor region (**c**) and tumor (**d**) of the liver of AID Tg mice with HCC development (original magnification × 400). Insert: a large tumor with extrahepatic growth compressing normal liver tissue was found in a 58-week-old Tg mouse.

using primer sets specific for the human *AID* gene. The expression of *AID* transcripts was undetectable in untreated cells; however, expression of *AID* was induced after the treatment of the cells with tumor necrosis factor (TNF)-α for 10 h (Figure 2a, upper panel). RT–PCR analysis revealed that *AID* transcripts also increased in response to another pro–inflammatory cytokine, interleukin (IL)-1β, in HepG2 cells (Figure 2a, lower panel). To examine whether TNF-α enhances *AID* expression generally in human hepatocytes, we used other hepatoma-derived cell lines, Hep3B and Huh-6. Although both cells lacked the expression of endogenous *AID* transcripts in the resting state, marked upregulation of *AID* transcripts was observed 12–24 h after treatment with TNF-α (Figure 2b), as in HepG2 cells. To confirm the TNF-α-mediated induction of *AID* expression in human hepatocytes, we investigated whether *AID* expression is upregulated by TNF-α stimulation in primary human hepatocytes. Although only trace amounts of endogenous *AID* transcripts were detectable in primary hepatocytes without any stimulation, quantitative RT–PCR analysis revealed a marked upregulation of *AID* transcripts 12 h after treatment with TNF-α (Figure 2c).

To investigate the TNF-α-mediated induction of *AID* more precisely, we examined the time course of *AID* expression by incubating HepG2 cells with TNF-α. *AID* transcripts were promptly induced, and the peak level was observed 8–10 h after TNF-α treatment (Figure 2d). Moreover, TNF-α increased the amount of *AID* transcripts in a dose-dependent manner, whereas the expression of the internal control *18S ribosomal RNA (18S rRNA)* gene was constant under all conditions tested (Figure 2e).

Next, to study the expression of AID protein in hepatocytes, we performed immunoblotting analyses using antibodies specific for human AID (Ta *et al.*, 2003). To confirm the specificity of the antibodies, we used AID-specific small interference RNA (siRNA) to knock down the expression of endogenous AID. Only

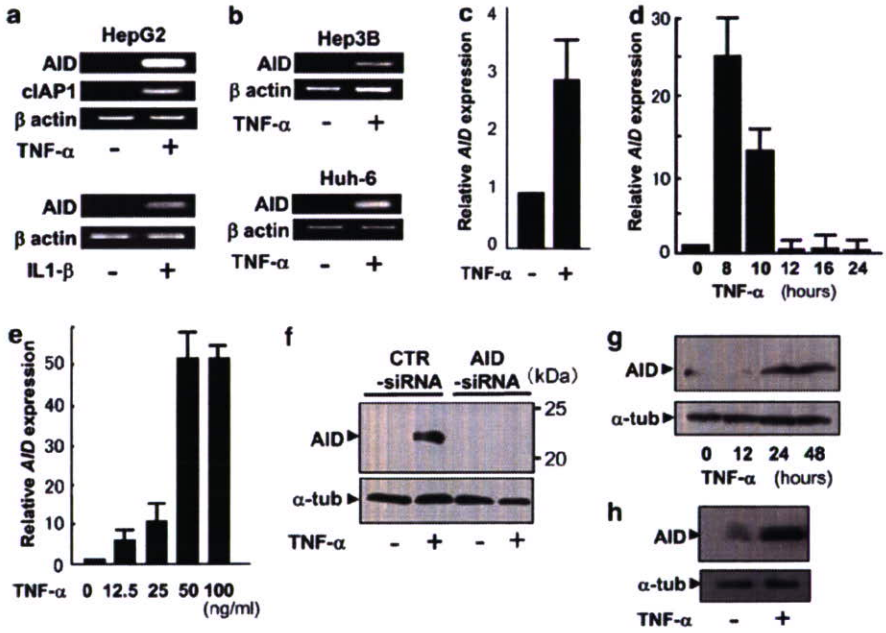

Figure 2 AID expression is induced in human hepatocytes in response to TNF-α and IL-1β stimulation. (**a**) Semiquantitative RT–PCR analyses for *AID* expression in HepG2 cells treated with TNF-α (upper panel) or IL-1β (lower panel). Total RNA was extracted from HepG2 cells after 10 h of treatment with TNF-α (100 ng/ml) or 12 h of treatment with IL-1β (25 ng/ml). Semiquantitative RT–PCR was performed using oligonucleotide primer sets specific for human *AID* and *β-actin*. The expression of *cIAP1*, another TNF-α-inducible gene, was also examined as a control. (**b**) Hep3B and Huh-6 cells were stimulated with TNF-α (100 ng/ml) for 12 h and semiquantitative RT–PCR was performed in each RNA sample using specific primers for *AID* and *β-actin*. (**c**) Human primary hepatocytes were established from surgical specimens of normal liver tissues of patients with metastatic liver tumors. *AID* transcripts were measured with or without TNF-α (12.5 ng/ml) stimulation for 12 h by quantitative real-time RT–PCR and the expression levels of *AID* were normalized to *18S rRNA* as an endogenous control. (**d** and **e**) Time-dependent and dose-dependent effects of TNF-α on *AID* expression. HepG2 cells were treated with TNF-α (100 ng/ml) for the indicated time points (**d**) or with various concentrations of TNF-α (0–100 ng/ml) for 8 h (**e**). Total RNA was extracted from each specimen and subjected to quantitative real-time RT–PCR analyses for *AID* expression. (**f**) HepG2 cells were transfected with siRNA targeting *AID* or control RNA and lysates were prepared from the siRNA-treated cells after stimulation with TNF-α (100 ng/ml) for 24 h. Protein samples were resolved by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting was performed using antibodies specific for human anti-AID (upper panel) or anti-α-tubulin antibody (α-tub, lower panel). (**g**) HepG2 cells were treated with TNF-α (100 ng/ml) for 0, 12, 24 or 48 h, followed by immunoblotting using anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel). (**h**) The human primary non-neoplastic hepatocyte cell line was treated with TNF-α (100 ng/ml) for 15 h. Immunoblotting was performed using anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel).

trace amounts of endogenous AID protein were detectable in HepG2 cells without any stimulation. AID protein expression was markedly increased after TNF-α treatment. In contrast, AID protein was not detected in cells treated with AID-targeting siRNA, irrespective of stimulation with TNF-α (Figure 2f). A time course study revealed that AID protein expression was detected after stimulation with TNF-α (Figure 2g). We further confirmed the proinflammatory cytokine-mediated expression of endogenous AID protein in a non-neoplastic primary hepatocyte cell line that retained primary hepatocyte characteristics (Figure 2h) (Aly *et al.*, 2007). Taken together, these data suggest that proinflammatory cytokine signaling induces *AID* gene expression in human hepatocytes.

AID expression in hepatocytes is achieved through NF-κB activation

Our findings that proinflammatory cytokines induced *AID* transcripts in hepatocytes led us to test whether AID expression is mediated by an NF-κB-dependent mechanism, because both TNF-α and IL-1β activate the transcription factor NF-κB and contribute to the expression and regulation of various genes. Activation of the classical NF-κB pathway converges on IκB kinase complex (IKK), a protein complex composed of two kinase subunits (IKK-α and IKK-β) and a non-catalytic subunit (IKK-γ/NF-κB expansion modulator (NEMO)). To clarify whether IKK is involved in TNF-α-mediated AID expression, we examined the effects of wild-type IKK-α and IKK-β on AID expression. The AID expression levels were significantly upregulated in the cells expressing IKK-α, IKK-β or NF-κB RelA protein itself (Figure 3a).

Next, we examined the TNF-α-mediated *AID* expression with NF-κB signaling inhibitors. We found that the NF-κB inhibitory reagents SN50 and MG132 significantly reduced TNF-α-induced NF-κB activation, and pretreatment with SN50 or MG132 almost completely suppressed the TNF-α-induced increase in *AID* transcripts (Figure 3b). Furthermore, TNF-α-induced AID protein expression was almost completely abolished by co-production of the super-repressor form of IκBα, a specific NF-κB inhibitor (Figure 3c). We then used siRNA to reduce the expression of endogenous IKK-γ/NEMO. Transfection of IKK-γ-specific siRNA, but not control siRNA, reduced endogenous IKK-γ protein levels in HepG2 cells and the lower levels were sustained for at least 3 days. Under these conditions, TNF-α stimulated AID expression in control cells. In contrast, TNF-α failed to elicit an increase in AID protein in cells in which endogenous IKK-γ was reduced by siRNA (Figure 3d). Taken together, these findings indicate that the induction of AID expression in human hepatocytes by TNF-α is achieved through the activation of NF-κB.

HCV core protein triggers AID expression via NF-κB

Previously, we demonstrated that the HCV core protein has the potential to induce NF-κB activation in human hepatocytes (Marusawa *et al.*, 1999). Thus, the expression

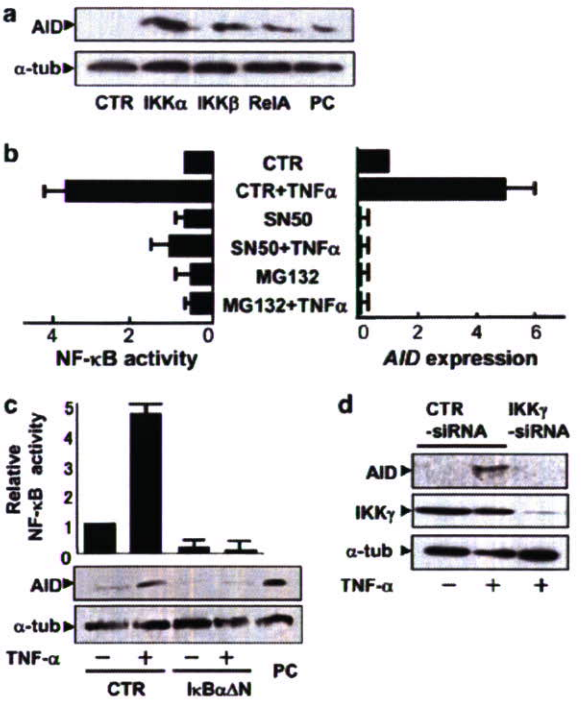

Figure 3 AID expression in human hepatocytes is regulated by NF-κB activity. (**a**) HepG2 cells were transfected with plasmid for the expression of IKK-α, IKK-β or RelA, or with a control vector (CTR). After 48 h, lysates of the transfected cells were immunoblotted with anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel). The lysate from the BL2-lymphoma cell line, which contains a high expression level of endogenous AID, was used as a positive control for AID expression (PC). (**b**) Effects of the NF-κB inhibitors on TNF-α-induced NF-κB activity and endogenous AID expression. HepG2 cells were transfected with pNF-κB-Luc, followed by the treatment with MG132 (2 μg/ml) or SN50 (50 μg/ml) for 2 h, and further subjected to TNF-α (100 ng/ml) stimulation for 12 h. Luciferase activities were monitored in each extract and normalized by the activity of *Renilla* luciferase (left graphs). Total RNA was also isolated from each cell and the expression levels of *AID* mRNA were measured by quantitative real-time RT–PCR (right graphs). The data present the means of the relative luciferase activity and *AID* expression in three independent experiments. (**c**) pcDNA3-IκBαΔN was transfected into Hep3B cells and then treated with TNF-α (100 ng/ml) for 24 h. Empty vector was used as a control (CTR). Each sample was harvested and luciferase activities were monitored to quantify the endogenous NF-κB activities. Cell lysates were also probed with anti-AID (upper panel) or anti-α-tubulin (α-tub, lower panel). Lysate from the BL2-lymphoma cell line was used as a positive control (PC). (**d**) HepG2 cells were transfected with siRNA targeting IKK-γ/NEMO for 48 h and then treated with TNF-α (100 ng/ml) for 24 h. Whole-cell lysates were probed by anti-AID antibody (upper panel), anti-IKK-γ/NEMO antibody (middle panel) or anti-α-tubulin antibody (α-tub, lower panel).

of AID protein was analysed by immunoblotting in the presence or absence of various HCV proteins. There was a significant upregulation of AID protein expression in HepG2 cells transfected with expression plasmid encoding the whole HCV genome (Figure 4a). Moreover, marked induction of AID was observed in core-producing cells. In contrast, the production of nonstructural viral protein resulted in little change in the expression

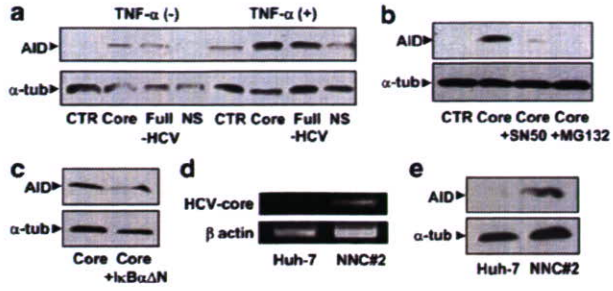

Figure 4 AID expression is enhanced by production of the HCV core protein. (**a**) HepG2 cells were transfected with plasmids encoding the whole HCV genome (Full-HCV), core protein (Core), non-structural protein (NS) or control vector (CTR). After 48 h, lysates were extracted from the cells with or without treatment with TNF-α (100 ng/ml) for 24 h. AID (upper panel) or α-tubulin (α-tub, lower panel) expression levels were analysed by immunoblotting. (**b**) HepG2 cells were transfected with plasmid encoding the core protein. After 24 h, the cells were treated with MG132 (2 μg/ml) or SN50 (50 μg/ml) for 2 h and stimulated with TNF-α (100 ng/ml) for 24 h. Lysates from these cells were collected and AID or α-tubulin (α-tub) expression levels were analysed by immunoblotting. (**c**) HepG2 cells were transfected with plasmids encoding the core protein with or without the super-repressor form of IκBα (IκBαΔN), a specific NF-κB inhibitor. After 48 h, lysates were extracted and AID (upper panel) or α-tubulin (α-tub, lower panel) expression levels were analysed by immunoblotting. (**d**) Total RNA was extracted from HCV full-genome replicon cells and control parental cells lacking HCV replication. Semiquantitative RT–PCR analyses were performed using specific primers for *HCV-core* (upper panel) or *β-actin* as an internal control (lower panel). (**e**) Whole-cell lysates were collected from the cells of HCV full genome replicon cells or control cells and subjected to immunoblotting analyses using anti-AID antibody (upper panel) or anti-α-tubulin antibody (α-tub, lower panel).

levels of endogenous AID protein. Consistent with the findings of the synergy of proinflammatory cytokine stimulation and viral protein on NF-κB activation (Marusawa *et al.*, 1999), the treatment of whole HCV protein- or core-producing cells with TNF-α further enhanced the expression of AID expression.

To determine whether HCV core-induced AID expression was achieved in an NF-κB-dependent manner, the effects of NF-κB inhibitors and the super-repressor form of IκBα on AID expression were examined in the viral core-expressing cells. We found that incubation of the cells with NF-κB inhibitors, SN50 and MG132, reduced the expression of AID in the cells expressing the viral protein (Figure 4b). Moreover, co-production of the super-repressor form of IκBα with HCV core protein resulted in the reduced expression of endogenous AID protein in HepG2 cells (Figure 4c).

To further confirm the aberrant upregulation of AID by HCV infection, we analysed the expression levels of endogenous AID in the full-genome HCV replicon cells producing whole HCV RNA and proteins (NNC#2 cells) (Ishii *et al.*, 2006). Consistent with a previous report, the expression of the *HCV core* in the replicon cells was confirmed by RT–PCR (Figure 4d). Immunoblotting analyses revealed that AID protein expression was dramatically enhanced in the full-genome replicon cells compared with that in the parental cells lacking HCV replication (Figure 4e). These results demonstrate that HCV core protein induces the expression of AID via NF-κB activation in human hepatocytes.

Expression of AID is sufficient to induce nucleotide alterations in the tumor-related genes in human hepatocytes

To determine whether aberrant AID expression could contribute to the accumulation of genomic mutations in hepatocytes, we investigated the mutation occurrence in the *c-myc* and *pim1*, both of which are thought to be common targets for abnormal editing in the lymphoma cells of AID Tg mice (Kotani *et al.*, 2005). For this purpose, we established a conditional system that allows for AID activation in the cells in response to an estrogen analog, 4-hydroxytamoxifen (OHT) (Doi *et al.*, 2003). The OHT treatment triggers a post-translational conformational change and thus prompt activation of AID in AID-estrogen receptor (ER)-expressing cells. When the AID ER-expressing HepG2 cells were treated with OHT for 8 days, we found the emergence of nucleotide alterations in the *c-myc* and *pim1* genes in the cells with AID activation, whereas the mutation frequencies in AID-activating cells did not show a statistically significant difference compared to the frequencies in the cells without AID activation (Figure 5a). In contrast, none of the nucleotide substitutions was detected in the control cells with OHT treatment. As the *c-myc* and *pim1* genes were found to be the possible targets for AID in HepG2 cells, we asked whether AID could induce genomic mutations in other tumor-related genes, *β-catenin* and *PTEN*, both of which are thought to be involved in human hepatocarcinogenesis (Thorgeirsson and Grisham, 2002). None of the nucleotide alterations, however, were observed in the *β-catenin* and *PTEN* genes after the OHT treatment. As it has been shown that the AID-mediated somatic mutation is induced in a transcription-dependent manner, we examined the expression levels of these four tumor-related genes in HepG2 cells. RT–PCR analyses revealed evidence showing that the transcription levels of *β-catenin* and *PTEN* genes were comparable with those of *c-myc* and *pim1* genes (Figure 5b).

Consistent with the findings observed in cultured human hepatoma-derived cells with AID activation, sequence analyses of HCC tissues of the AID Tg mice revealed that the *c-myc* gene, which was reported to accumulate high mutation frequencies in T-cell lymphoma, contained nucleotide alteration accompanied by upregulation of *c-myc* transcripts (Supplementary Figure). Taken together, these data suggest that the specificity for mutation of target genes is present in hepatocytes, as was observed in T-cell lymphoma cells (Kotani *et al.*, 2005).

Discussion

Deregulated expression of the *AID* gene is often observed in human lymphoid malignancies, including non-Hodgkin lymphoma and chronic lymphocytic leukemia (Greeve *et al.*, 2003; Heintel *et al.*, 2004).

a

Gene	Vector	Total clones analyzed	Mutations	Total bp sequenced	Mutation Frequencies
c-myc	AID	32	2	24830	0.8
	CTR	25	0	18562	0
pim1	AID	46	1	25484	0.4
	CTR	20	0	11080	0
β-catenin	AID	47	0	21537	0
	CTR	25	0	8085	0
PTEN	AID	56	0	25256	0
	CTR	15	0	6195	0

b

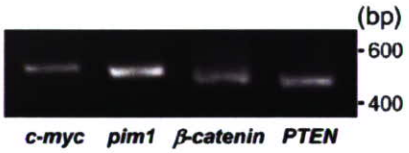

Figure 5 Mutation frequencies in various tumor-related genes in HepG2 cells with AID activation. (**a**) AID-expressing HepG2 cells and empty vector-expressing cells (CTR) were treated with OHT for 8 days. After the amplification of each tumor related gene, the PCR products were subcloned and sequence analyses were performed. Mutation frequencies were calculated per the total bases analysed $\times 10^{-4}$. (**b**) Total RNA was extracted from HepG2 cells and semiquantitative RT–PCR analyses were performed using specific primers for *c-myc*, *pim1*, *β-catenin* and *PTEN*.

Moreover, animal models demonstrate that aberrant expression of AID causes an accumulation of DNA mutations in various genes leading to tumorigenesis (Okazaki *et al.*, 2003; Kotani *et al.*, 2005). These findings suggest that inappropriate or deregulated AID expression increases the mutation rate of genes that are not normally attacked by AID and are associated with tumor development (Kinoshita and Nonaka, 2006). Under physiologic conditions, however, basal expression levels of AID are not high and the expression of AID is restricted to activated B cells located at the germinal centers. In this study, we clearly demonstrated for the first time that AID expression in hepatocytes is strongly induced in response to proinflammatory cytokine stimulation via NF-κB activation.

At present, little is known about how AID expression is regulated, except that it is under the control of B-cell activating stimulation. It was shown that CD40 ligation induces NF-κB binding to the two potential NF-κB sites located in the 5′ upstream region of the *AID* gene, suggesting that NF-κB participates in the regulatory process of the *AID* gene in B cells (Dedeoglu *et al.*, 2004). In this study, we examined the mechanisms of AID regulation by proinflammatory cytokines and demonstrated that NF-κB activation is involved in TNF-α- as well as IL-1β-induced *AID* gene transcription in hepatocytes. The induction of *AID* transcripts is regulated in an NF-κB-dependent manner in both human lymphoid and human epithelial cells, which suggests the presence of a common mechanism underlying the regulation of *AID* gene transcription.

Several proinflammatory cytokines, including TNF-α and IL-1β, play important roles in the pathophysiology of chronic inflammatory disease, including chronic hepatitis (Tilg *et al.*, 1992; Gonzalez-Amaro *et al.*, 1994). IκB kinase-dependent NF-κB signaling pathways are reported to link inflammation to tumorigenesis (Greten *et al.*, 2004; Pikarsky *et al.*, 2004; Karin and Greten, 2005). Activation of this pathway leads to increases in a variety of genes that encode mediators of inflammatory cytokines, chemokines, cell adhesion molecules, and apoptosis inhibitors (Pahl, 1999; Balkwill and Mantovani, 2001). In this study, we identified AID as a target gene of the IKK-β-dependent NF-κB activation pathway in human hepatocytes. A connection between inflammatory stimulation and AID expression led to the speculation that one possible mechanism for increased susceptibility to HCC in the liver during chronic inflammation is inappropriate expression of AID in hepatocytes via NF-κB activation.

An important finding in this study is that HCV infection, one of the major causes of HCC, might trigger

aberrant AID expression in hepatocytes. We previously demonstrated that among the HCV proteins, the core protein functioned in the activation of NF-κB and that NF-κB-dependent transcriptional activities induced by TNF-α were synergistically enhanced by the presence of a viral core protein (Marusawa *et al.*, 1999). Consistent with our previous findings, the TNF-α-induced AID expression was strongly enhanced by the presence of HCV core protein. Interestingly, HCV-associated HCC shows various mutations in proto-oncogenes, but non-viral HCC does not (Machida *et al.*, 2004). Taken together, these findings suggest that the upregulation of AID induced by HCV infection in chronically damaged liver might contribute to enhanced susceptibility to mutagenesis, resulting in the accumulation of somatic mutations leading to the production of sufficient genomic alterations and eventually to cancer development.

In this study, we showed that constitutive and ubiquitous expression of AID in Tg mice caused the development of HCC as well as T-cell lymphoma. Moreover, aberrant AID expression was capable of triggering accumulation of nucleotide alterations in the *c-myc* gene in cultured hepatoma-derived cells *in vitro*. It has been demonstrated that deregulation of c-myc has been implicated in the etiology of a wide variety of human cancers (Pelengaris *et al.*, 2002). The deregulating mutations impact either on the *c-myc* gene itself or on upstream regulatory sequences, and point mutations or gene amplification of *c-myc* was reported in human HCC (Feitelson, 2006). Moreover, several studies revealed that the mouse models with overexpression of c-myc developed liver cancers (Murakami *et al.*, 1993; Coulouarn *et al.*, 2006). Thus, it is tempting to speculate that increased AID expression might be responsible for the enhanced susceptibility of the hepatocytes to somatic gene alterations in tumor-related genes including *c-myc*, which might facilitate HCC development. In the current study, however, the *c-myc* mutation frequency in AID-activating HepG2 cells did not show a statistically significant difference compared with that in the cells without AID activation. It has been supposed that the AID-mediated somatic mutation is induced in a transcription-dependent manner and the preference of target genes is variable between the cells with AID expression (Kotani *et al.*, 2005). Thus, there is room for further investigation to identify the frequent target genes of AID in human hepatocytes.

In conclusion, our findings provide the first evidence that AID is induced in response to proinflammatory cytokines via the NF-κB/IKK-β signaling pathway and might be responsible for the development of HCC in the underlying chronic liver disease. Understanding the mechanisms of AID upregulation in human hepatocytes might provide a new strategy for preventing the development and progression of HCC in patients with chronic liver disease.

Materials and methods

AID Tg mice

Generation of AID Tg mice was described previously (Okazaki *et al.*, 2003). Tissue samples from the mice were removed and fixed in 4% (w/v) formaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined for histological abnormalities.

Cell culture and transfection

Human hepatoma-derived cell lines, Hep3B, Huh-6 and HepG2, were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Tokyo, Japan) containing 10% fetal bovine serum. For plasmid transfection, we used Trans-IT LT1 transfection reagent (Mirus Bio Corporation, Madison, WI, USA).

siRNA duplexes composed of 21-nucleotide sense and antisense strands used for targeting IKK-γ/NEMO and AID were obtained from Dharmacon Research (Lafayette, CO, USA). Transfection of siRNA was described previously (Matsumoto *et al.*, 2006).

Primary human hepatocytes were isolated from surgical specimens of patients with metastatic liver tumors undergoing partial hepatectomy (Tateno *et al.*, 2000; Tanaka *et al.*, 2006), after informed consent for the use of resected tissues was obtained in accordance with the Declaration of Helsinki Principle. An established non-neoplastic human primary hepatocyte cell line that retained primary hepatocyte characteristics was also used (Aly *et al.*, 2007).

A full genome HCV replicon system was established by transfecting to the cells with the NN strain (genotype 1b) (Ishii *et al.*, 2006).

A system that allows conditional expression of the active form of AID was established by stable transfection of the HepG2 cells with pAID-ER-BOSbsr vector encoding the active form of AID fused with the hormone-binding domain of the human ER designated as AID-ER (Doi *et al.*, 2003).

Reagents and antibodies

Recombinant human TNF-α and IL-1β were obtained from Peprotech EC (London, UK) and R&D Systems Inc. (Minneapolis, MN, USA), respectively. NF-κB inhibitors MG132 and SN50 were purchased from Biomol International LP (Plymouth Meeting, PA, USA). Polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen (Ta *et al.*, 2003). Mouse monoclonal antibodies against human IKK-γ/NEMO and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Calbiochem (San Diego, CA, USA), respectively.

Plasmids

pcDNA3-IκBαΔN, for expression of the super-repressor form of the IκB-α protein, was made by inserting the cDNA fragment of human IκB-α into the *Bam*HI-*Eco*RI sites of pcDNA3 (Invitrogen, Carlsbad, CA, USA). The cDNA fragment for pcDNA3-IκBαΔN (amino acids 37–317) was synthesized by RT–PCR with the oligonucleotide primers 5′-CGCGGATCCATGAAAGACGAGGAGTACGA-3′ (forward) and 5′-CCGGAATTCTCATAACGTCAGACGCTG GC-3′ (reverse). The expression plasmids pcDNA3-IKK-α and IKK-β were described previously (Marusawa *et al.*, 2001). The plasmids for the expression of various HCV proteins, including pCMV-3010 for the expression of full viral proteins, pCMV-core for the expression of the core, and pCMV-NS for the expression of the non-structural protein, were described previously (Marusawa *et al.*, 1999).

Semiquantitative and quantitative real-time RT-PCR

Total RNA was extracted from the cells using Sepasol-RNA 1 Super (Nacalai Tesque, Kyoto, Japan) according to the

manufacturer's protocol. For the RT reaction, 1 μg of total RNA was reverse transcribed into 20 μl of cDNA using the Superscript III first-strand synthesis system and oligo(dT)$_{12\ 18}$ primers (Invitrogen). PCR amplification was performed using Takara Ex Taq DNA polymerase (Takara, Tokyo, Japan). The oligonucleotide primers for the amplification of *AID*, *cIAP1* and *HCV-core* were as follows: *AID*, 5′-AAATGTCC GCTGGGCTAAGG-3′ (forward) and 5′-GGAGGAAGAGC AATTCCACGT-3′ (reverse); *cIAP1*, 5′-CTCTGAGGTTTAG CATTTCA-3′ (forward) and 5′-CTCCAGGTCCAAAATGA ATA-3′ (reverse); *HCV-core*, 5′-TCGTTGGTGGAGTTTAC CTG-3′ (forward) and 5′-GCGGAATGTACCCCATGA-3′ (reverse).

Quantification of gene expression was performed by quantitative real-time RT–PCR using the 7300 Real-Time PCR System (PE Applied Biosystems, Foster City, CA, USA) (Matsumoto *et al*., 2006). The 6-carboxyfluorescein-labeled probe specific for human *AID* was 5′-TCGGCGTGAGACC TACCTGTGCTAC-3′. Standard curves for *AID* were generated for every target using a 10-fold serial dilution series of five independent transcripts derived from BL2-lymphoma cells, which contain a high expression level of endogenous *AID*. To assess the quantity of isolated RNA as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene *18S rRNA* (Matsumoto *et al*., 2006). For simplicity, the ratios were represented as relative values compared with expression levels in a lysate from control cells.

Subcloning and sequencing of the tumor-related genes

Genomic DNA from the cultured cells and mouse liver tissues was prepared using the DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

The oligonucleotide primers for *c-myc* were designed to amplify the sequences between exon 1 and intron 1 of the human *c-myc* gene using 5′-GCCGAATTCTTGCAGCTGC TTAGACGCTG-3′ (forward) and 5′-ATCCTCGAGCCAA CACTCCTTTTGCCAGC-3′ (reverse). The primers for human exon 1 to exon 4 of the human *pim1* gene were as follows: 5′-GCCGAATTCGTCCAAAATCAACTCGCTT-3′ (forward) and 5′-ATCTCTAGACGAAGTCGATGAGCTT GA-3′ (reverse). The primers for the amplification of the human *β-catenin* and *PTEN* genes were as follows: 5′-G CCGAATTCCTGATTTGATGGAGTTGGAC-3′ (*β-catenin*-forward), 5′-ATCCTCGAGAACGCTGGACATTAGTGG GA-3′ (*β-catenin*-reverse), 5′-GCCGAATTCTCATCAAAG AGATCGTTAGCAG-3′ (*PTEN*-forward), and 5′-ATCCTC GAGTGTCTCTGGTCCTTACTTCC-3′ (*PTEN*-reverse). The primers for the amplification of the mouse *c-myc* gene were as follows: 5′-GTTAAGCTTGCGTTTTTTTCTGACT CGCTG-3′ (forward) and 5′-GTTGAATTCCCTACCCC AACTACTCTTGA-3′ (reverse). After the amplification of each gene using high-fidelity Phusion Taq polymerase (FINNZYMES), the PCR products were subcloned by insertion into the pcDNA3 vector and further subjected to sequence analyses as described previously (Marusawa *et al*., 2000).

Acknowledgements

We are grateful to Y Ueda for his useful suggestions and critical reading of the paper, Y Matsumoto for helpful advice and T Kou for help with the analyses on Tg mice. This study was supported by a grant in aid for Scientific Research (15209024, 16790378 and 17002015) from the Japan Society for the Promotion of Science (JSPS).

References

Aly HH, Watashi K, Hijikata M, Kaneko H, Takada Y, Egawa H *et al*. (2007). Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes. *J Hepatol* **46**: 26–36.

Balkwill F, Mantovani A. (2001). Inflammation and cancer: back to Virchow? *Lancet* **357**: 539–545.

Coulouarn C, Gomez-Quiroz LE, Lee JS, Kaposi-Novak P, Conner EA, Goldina TA *et al*. (2006). Oncogene-specific gene expression signatures at preneoplastic stage in mice define distinct mechanisms of hepatocarcinogenesis. *Hepatology* **44**: 1003–1011.

Coussens LM, Werb Z. (2002). Inflammation and cancer. *Nature* **420**: 860–867.

Dedeoglu F, Horwitz B, Chaudhuri J, Alt FW, Geha RS. (2004). Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkappaB. *Int Immunol* **16**: 395–404.

Doi T, Kinoshita K, Ikegawa M, Muramatsu M, Honjo T. (2003). *De novo* protein synthesis is required for the activation-induced cytidine deaminase function in class-switch recombination. *Proc Natl Acad Sci USA* **100**: 2634–2638.

Feitelson MA. (2006). Parallel epigenetic and genetic changes in the pathogenesis of hepatitis virus-associated hepatocellular carcinoma. *Cancer Lett* **239**: 10–20.

Gonzalez-Amaro R, Garcia-Monzon C, Garcia-Buey L, Moreno-Otero R, Alonso JL, Yague E *et al*. (1994). Induction of tumor necrosis factor alpha production by human hepatocytes in chronic viral hepatitis. *J Exp Med* **179**: 841–848.

Greeve J, Philipsen A, Krause K, Klapper W, Heidorn K, Castle BE *et al*. (2003). Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. *Blood* **101**: 3574–3580.

Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ *et al*. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* **118**: 285–296.

Heintel D, Kroemer E, Kienle D, Schwarzinger I, Gleiss A, Schwarzmeier J *et al*. (2004). High expression of activation-induced cytidine deaminase (AID) mRNA is associated with unmutated IGVH gene status and unfavourable cytogenetic aberrations in patients with chronic lymphocytic leukaemia. *Leukemia* **18**: 756–762.

Honjo T, Kinoshita K, Muramatsu M. (2002). Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol* **20**: 165–196.

Ishii N, Watashi K, Hishiki T, Goto K, Inoue D, Hijikata M *et al*. (2006). Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* **80**: 4510–4520.

Karin M, Greten FR. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* **5**: 749–759.

Kinoshita K, Honjo T. (2001). Linking class-switch recombination with somatic hypermutation. *Nat Rev Mol Cell Biol* **2**: 493–503.

Kinoshita K, Nonaka T. (2006). The dark side of activation-induced cytidine deaminase: relationship with leukemia and beyond. *Int J Hematol* **83**: 201–207.

Kotani A, Okazaki IM, Muramatsu M, Kinoshita K, Begum NA, Nakajima T *et al.* (2005). A target selection of somatic hypermutations is regulated similarly between T and B cells upon activation-induced cytidine deaminase expression. *Proc Natl Acad Sci USA* **102**: 4506–4511.

Kou T, Marusawa H, Kinoshita K, Endo Y, Okazaki IM, Ueda Y *et al.* (2007). Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int J Cancer* **120**: 469–476.

Lengauer C, Kinzler KW, Vogelstein B. (1998). Genetic instabilities in human cancers. *Nature* **396**: 643–649.

Loeb LA, Loeb KR, Anderson JP. (2003). Multiple mutations and cancer. *Proc Natl Acad Sci USA* **100**: 776–781.

Machida K, Cheng KT, Sung VM, Shimodaira S, Lindsay KL, Levine AM *et al.* (2004). Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. *Proc Natl Acad Sci USA* **101**: 4262–4267.

Martin A, Bardwell PD, Woo CJ, Fan M, Shulman MJ, Scharff MD. (2002). Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* **415**: 802–806.

Martin A, Scharff MD. (2002). Somatic hypermutation of the AID transgene in B and non-B cells. *Proc Natl Acad Sci USA* **99**: 12304–12308.

Marusawa H, Hijikata M, Chiba T, Shimotohno K. (1999). Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. *J Virol* **73**: 4713–4720.

Marusawa H, Hijikata M, Watashi K, Chiba T, Shimotohno K. (2001). Regulation of Fas-mediated apoptosis by NF-kappaB activity in human hepatocyte derived cell lines. *Microbiol Immunol* **45**: 483–489.

Marusawa H, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K *et al.* (2000). Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. *Hepatology* **31**: 488–495.

Matsumoto T, Marusawa H, Endo Y, Ueda Y, Matsumoto Y, Chiba T. (2006). Expression of APOBEC2 is transcriptionally regulated by NF-kappaB in human hepatocytes. *FEBS Lett* **580**: 731–735.

Murakami H, Sanderson ND, Nagy P, Marino PA, Merlino G, Thorgeirsson SS. (1993). Transgenic mouse model for synergistic growth factor and oncogene action in liver: cooperation between hepatocyte growth factor and myc in tumorigenesis: interaction of c-myc and transforming growth factor alpha in hepatic oncogenesis. *Cancer Res* **53**: 1719–1723.

Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**: 553–563.

Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO *et al.* (1999). Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* **274**: 18470–18476.

Okazaki IM, Hiai H, Kakazu N, Yamada S, Muramatsu M, Kinoshita K *et al.* (2003). Constitutive expression of AID leads to tumorigenesis. *J Exp Med* **197**: 1173–1181.

Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T. (2002). The AID enzyme induces class switch recombination in fibroblasts. *Nature* **416**: 340–345.

Pahl HL. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**: 6853–6866.

Pelengaris S, Khan M, Evan G. (2002). c-MYC: more than just a matter of life and death. *Nat Rev Cancer* **2**: 764–776.

Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S *et al.* (2004). NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* **431**: 461–466.

Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O *et al.* (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**: 565–575.

Ta VT, Nagaoka H, Catalan N, Durandy A, Fischer A, Imai K *et al.* (2003). AID mutant analyses indicate requirement for class-switch-specific cofactors. *Nat Immunol* **4**: 843–848.

Tanaka Y, Marusawa H, Seno H, Matsumoto Y, Ueda Y, Kodama Y *et al.* (2006). Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. *Biochem Biophys Res Commun* **341**: 314–319.

Tateno C, Takai-Kajihara K, Yamasaki C, Sato H, Yoshizato K. (2000). Heterogeneity of growth potential of adult rat hepatocytes *in vitro*. *Hepatology* **31**: 65–74.

Teng B, Burant CF, Davidson NO. (1993). Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* **260**: 1816–1819.

Thorgeirsson SS, Grisham JW. (2002). Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* **31**: 339–346.

Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G *et al.* (1992). Serum levels of cytokines in chronic liver diseases. *Gastroenterology* **103**: 264–274.

Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T *et al.* (1993). Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N Engl J Med* **328**: 1797–1801.

Yoshikawa K, Okazaki IM, Eto T, Kinoshita K, Muramatsu M, Nagaoka H *et al.* (2002). AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. *Science* **296**: 2033–2036.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

+Model
BC2637 1–4

ARTICLE IN PRESS

Available online at www.sciencedirect.com

ScienceDirect

IJBCB

ELSEVIER

The International Journal of Biochemistry & Cell Biology xxx (2008) xxx–xxx

www.elsevier.com/locate/biocel

Medicine in focus

Aberrant AID expression and human cancer development

Hiroyuki Marusawa *

Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Received 16 December 2007; received in revised form 17 January 2008; accepted 18 January 2008

Abstract

Cancer develops via a multistep process that occurs through the accumulation of somatic mutations of tumor-related genes that govern cell proliferation, regeneration, and apoptosis. The question how normal cells acquire the genetic changes that lead to malignant transformation is, however, unknown at present. Activation-induced cytidine deaminase (AID) produces immune-diversity by inducing somatic hypermutations and class-switch recombinations in human immunoglobulin genes. Unfortunately, this function of AID as a genome mutator could aim at the generation of somatic mutations in various host genes of non-lymphoid tissues and contribute to tumorgenesis. Notably, aberrant AID expression can be triggered by several pathogenic factors, including *Helicobacter pylori* infection and proinflammatory cytokine stimulation, in human epithelial cells, whereas AID expression is absent in those cells under physiologic conditions. Thus, aberrant AID activity in epithelial tissues may provide the critical link between inflammation, somatic mutations, and cancer development.

Keywords: Activation-induced cytidine deaminase; Mutation; Cancer

1. Introduction

In contrast to normal human cells that replicate their DNA with exceptional accuracy, most cancer cells arise from a stepwise accumulation of genetic changes. The genomes of incipient cancer cells acquire alterations in the nucleotide sequences of proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation, regeneration, and apoptosis (Hahn & Weinberg, 2002). Because normal mutation rates cannot account for the accumulation of multiple mutations in tumor cells (Loeb, Loeb, & Anderson, 2003), certain molecular mechanisms must be present to account for the nucleotide alterations observed in most human cancer cells. One mechanism that may account for the enhanced susceptibility to mutagenesis is a genetic defect in the DNA repair pathways. For example, impairments of the mismatch repair system result in a familial colorectal cancer syndrome, defects in nucleotide excision repair are associated with skin cancer, and defects in homologous recombination and double-strand break repair are associated with breast cancer and lymphoma (Hoeijmakers, 2001). The frequency of such defects in the DNA repair system, however, is generally low among human cancers. Thus, how a large number of genetic mutations arise during the course of cancer development remains a fundamental question. We provide an overview of the novel molecular mechanism

* Tel.: +81 75 751 4319; fax: +81 75 751 4303.
E-mail address: maru@kuhp.kyoto-u.ac.jp.

1357-2725/$ – see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.biocel.2008.01.018

Please cite this article in press as: Marusawa, H., Aberrant AID expression and human cancer development, Int J Biochem Cell Biol (2008), doi:10.1016/j.biocel.2008.01.018

by which normal epithelial cells acquire DNA mutations under pathologic conditions, including chronic inflammation, and pathogenic viral or bacterial infections.

2. Nucleotide-editing enzymes that can induce mutations in DNA and/or RNA

To maintain homeostasis and conserve genetic information, cells have several systems to prevent mutations, and repair any changes in nucleotide sequences, thus avoiding harmful sporadic nucleotide alterations, so-called "somatic mutations". In contrast to normal cells, however, cancer cells usually acquire a variety of somatic mutations during the transformation process. Recently, a novel enzyme family was highlighted in association with the mechanism of mutagenesis. Cytidine deaminases are enzymes involved in DNA and/or RNA editing by converting cytosine to uracil, resulting in nucleotide alterations in target sequences. Among them, the apolipoprotein B-editing catalytic polypeptide (APOBEC) represents a clustered family characterized by a zinc-binding catalytic domain with the consensus amino acid sequences (Cascalho, 2004). The human APOBEC family comprises a series of molecules, including APOBEC1, -2, -3A, -3B, -3C, -3DE, -3F, -3G, -3H -4 and activation-induced cytidine deaminase (AID) (Conticello, Thomas, Petersen-Mahrt, & Neuberger, 2005). APOBEC family member-induced cytidine deamination has a critical role in mediating subtle changes in the DNA or RNA sequences that can produce diverse physiologic functions of target genes (Pham, Bransteitter, & Goodman, 2005). For example, APOBEC1 induces a mutation in apoB mRNA at a specific site that results in the generation of a premature stop codon, thereby producing a truncated form of apoB (Chen et al., 1987; Powell et al., 1987). A full-length apoB mRNA product, apoB-100, is a component of very-low-density and low-density lipoprotein, whereas the truncated apoB is secreted in the triglyceride-rich chylomicrons that carry dietary fat (Chan, 1992). In contrast to APOBEC1, APOBEC-3G is involved in cellular defense against retroviruses by inducing mutations into the viral genome. It has been shown that deamination activity of APOBEC-3G contributes to its antiviral activity against human immunodeficiency virus (HIV)-1 and restricts viral growth through a massive deamination of cytosines in the viral-minus DNA strands (Goff, 2003; KewalRamani & Coffin, 2003). Interestingly, more recent study reported that a deamination-independent mechanism might also be involved in APOBEC-3G antiviral activity (Iwatani et al., 2007).

3. AID is capable of inducing mutations in DNA sequences

Among the APOBEC family, AID has a unique ability with favorable function. Antigen stimulation of activated B lymphocytes triggers somatic hypermutations, which diversifies the variable region of the immunoglobulin genes, and AID expression is essential for this process. The finding that AID induces the production of somatic hypermutations in the immunoglobulin gene indicates that AID can induce nucleotide alterations in human DNA sequences. The activity of AID as a genome mutator leads to the question of whether AID induces inappropriate mutations in non-immunoglobulin genes.

The link between AID expression and unfavorable consequences in various organs was first revealed by phenotypic analyses of a transgenic mouse model with AID expression. Constitutive and ubiquitous AID expression in transgenic mice induced the development of lymphomas (Okazaki et al., 2003). Moreover, point mutations are massively introduced in various non-immunoglobulin genes, including the proto-oncogene *c-myc* in lymphoma cells. Interestingly, those mice also develop epithelial tumors including micro-adenomas and dysgenetic lesions of the respiratory bronchioles in the lung. Further phenotypic analyses revealed that AID-transgenic mice develop neoplasia in other epithelial tissues, including liver and stomach (Endo et al., 2007; Matsumoto et al., 2007). These findings indicate that aberrant AID expression might cause tumorigenesis in both lymphoid and non-lymphoid organs, via the accumulation of somatic mutations in tumor-related genes. Fortunately, AID transcription is restricted to activated B lymphocytes and thus almost no AID expression is observed in most human tissues under physiologic conditions.

4. Aberrant AID expression in gastric epithelial cells in association with *Helicobacter pylori* infection

The majority of human gastric cancers arise in the stomach with clinical features of chronic gastritis (Aoi, Marusawa, Sato, Chiba, & Maruyama, 2006). The most important causative pathogen for chronic gastric inflammation and a class one carcinogen for human gastric cancer is *H. pylori* infection (Chiba, Seno, Marusawa, Wakatsuki, & Okazaki, 2006). The mechanisms that link *H. pylori*-induced chronic gastric inflammation and cancer development remain unclear, but it is thought to involve a multistep process of genetic alterations. Indeed, several studies have reported various nucleotide alter-

Please cite this article in press as: Marusawa, H., Aberrant AID expression and human cancer development, Int J Biochem Cell Biol (2008), doi:10.1016/j.biocel.2008.01.018

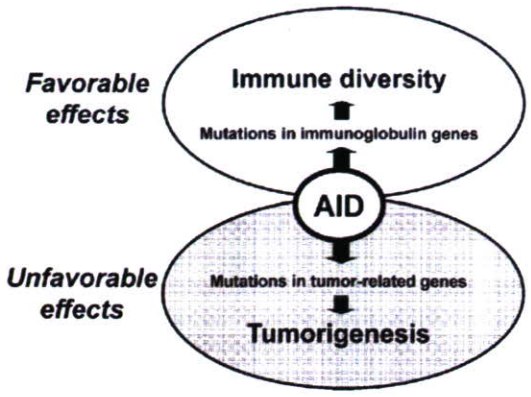

Fig. 1. Dual effects induced by AID activation. A schematic depicting the favorable and unfavorable effects of AID. AID acts as a cytidine deaminase that is capable of inducing nucleotide alterations in human DNA sequences. Under the physiological condition, AID is a protein indispensable for the diversification of immunoglobulin genes by somatic hypermutation and class-switch recombination. On the other hand, AID is able to induce genome-wide mutations in a variety of mammalian non-lymphoid cells, and thus can contribute to the production of unfavorable genetic changes in the tumor-related genes leading to carcinogenesis.

ations in tumor-related genes, including *TP53* in gastric cancer cells (Fenoglio-Preiser, Wang, Stemmermann, & Noffsinger, 2003). These somatic mutations are also observed in non-cancerous stomach tissues with *H. pylori* infection, suggesting that nucleotide alterations accumulate in gastric epithelial cells during the course of chronic inflammation. The *in vivo* findings that AID-transgenic mice develop gastric neoplasms suggest that aberrant AID expression in gastric epithelial cells contributes to cancer development via the accumulation of somatic mutations (Matsumoto et al., 2007). Surprising findings were obtained by analyses of AID expression in human stomach tissue specimens with *H. pylori*-related chronic gastritis and gastric cancers. In contrast to normal gastric mucosa, aberrant AID expression is present in gastric epithelial cells of the stomach tissues with *H. pylori*-positive chronic gastritis (Matsumoto et al., 2007). In addition, AID protein is expressed in neoplastic cells in approximately 80% of *H. pylori*-infected gastric cancer tissues. Because AID expression is specifically upregulated in human gastric epithelial cells and neoplastic cells with *H. pylori*-induced chronic inflammation, the relationship between *H. pylori* infection, proinflammatory cytokine stimulation, and AID expression was further investigated *in vitro*. Although AID expression is low in cultured human gastric epithelial cells, marked upregulation of AID is induced in response to either *H. pylori* infection or tumor necrosis factor (TNF)-α stimulation. AID expression in human gastric epithelial cells by *H. pylori* infection or TNF-α is induced by the activation of the transcription factor NF-κB, indicating that AID expression is regulated through an NF-κB activation pathway in human gastric epithelial cells. Notably, aberrant AID expression triggers the accumulation of nucleotide alterations in the *TP53* gene in human gastric epithelial cells. Taken together, these findings provide evidence that AID is induced in response to *H. pylori* infection or proinflammatory cytokine stimulation via the NF-κB signaling pathway and is capable of contributing to the generation of somatic mutations in tumor-related genes in gastric epithelial cells. Thus, inflammation-mediated AID expression might underlie the development of human gastric cancer via *H. pylori*-associated chronic gastritis.

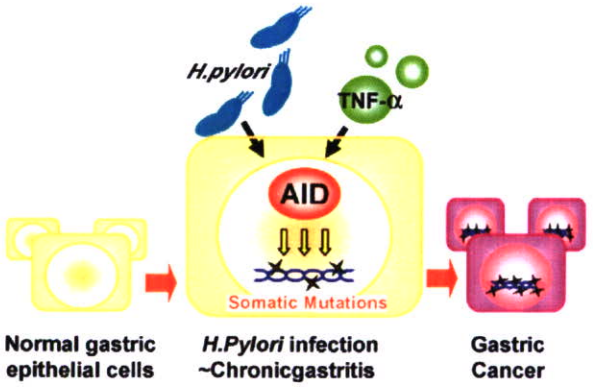

Fig. 2. AID links chronic inflammation to gastric cancer development via its mutagenic activity. This figure presents a model that depicts how AID plays a role in the development of human gastric cancer. Human gastric epithelium lacks endogenous AID expression under the physiological condition. *Helicobacter pylori* infection and the resultant inflammatory stimulation, however, trigger the aberrant AID expression in gastric epithelial cells. Constitutive AID activation in gastric epithelium results in the accumulation of somatic mutations in various target genes. If critical nucleotide changes in the tumor-related genes may be induced by AID activity, the resultant gastric epithelial cell can acquire the transformation, leading to the development of gastric cancer cells.

5. Conclusion and future aspects

Proinflammatory cytokine induction of AID expression via the NF-κB activation pathway is not limited to gastric epithelial cells. Indeed, AID expression is also mediated by TNF-α or interleukin-1β in human hepatocytes (Endo et al., 2007). More importantly, hepatitis C virus (HCV) strongly triggers AID expression in hepatocytes in collaboration with proinflammatory cytokines (Endo et al., 2007), and ectopic AID expression is observed in human liver specimens with chronic hepatic inflammation caused by HCV infection (Kou et al., 2007). Thus, AID possibly has a role in enhanc-

Please cite this article in press as: Marusawa, H., Aberrant AID expression and human cancer development, Int J Biochem Cell Biol (2008), doi:10.1016/j.biocel.2008.01.018

ing genetic susceptibility to mutagenesis, leading to the development of hepatocellular carcinoma in the setting of HCV-related chronic liver disease.

In conclusion, these recent findings support the idea that aberrant expression of the endogenous DNA mutator AID in epithelial cells provides a novel link between inflammation, mutagenesis, and cancer development. There might be more examples of human cancers that arise due to chronic inflammation that causes mutational accumulation mediated by AID activity (Figs. 1 and 2).

Acknowledgement

I am grateful to Dr. Yoko Endo for help of manuscript preparation.

References

Aoi, T., Marusawa, H., Sato, T., Chiba, T., & Maruyama, M. (2006). Risk of subsequent development of gastric cancer in patients with previous gastric epithelial neoplasia. *Gut*, *55*, 588–589.

Cascalho, M. (2004). Advantages and disadvantages of cytidine deamination. *J. Immunol.*, *172*, 6513–6518.

Chan, L. (1992). Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins. *J. Biol. Chem.*, *267*, 25621–25624.

Chen, S. H., Habib, G., Yang, C. Y., Gu, Z. W., Lee, B. R., Weng, S. A., et al. (1987). Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science*, *238*, 363–366.

Chiba, T., Seno, H., Marusawa, H., Wakatsuki, Y., & Okazaki, K. (2006). Host factors are important in determining clinical outcomes of *Helicobacter pylori* infection. *J. Gastroenterol.*, *41*, 1–9.

Conticello, S. G., Thomas, C. J., Petersen-Mahrt, S. K., & Neuberger, M. S. (2005). Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol. Biol. Evol.*, *22*, 367–377.

Endo, Y., Marusawa, H., Kinoshita, K., Morisawa, T., Sakurai, T., Okazaki, I. M., et al. (2007). Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene*, *26*, 5587–5595.

Fenoglio-Preiser, C. M., Wang, J., Stemmermann, G. N., & Noffsinger, A. (2003). TP53 and gastric carcinoma: a review. *Hum. Mutat.*, *21*, 258–270.

Goff, S. P. (2003). Death by deamination: a novel host restriction system for HIV-1. *Cell*, *114*, 281–283.

Hahn, W. C., & Weinberg, R. A. (2002). Rules for making human tumor cells. *N. Engl. J. Med.*, *347*, 1593–1603.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature*, *411*, 366–374.

Iwatani, Y., Chan, D. S., Wang, F., Maynard, K. S., Sugiura, W., Gronenborn, A. M., et al. (2007). Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G. *Nucleic Acids Res.*, *35*, 7096–7108.

KewalRamani, V. N., & Coffin, J. M. (2003). Virology. Weapons of mutational destruction. *Science*, *301*, 923–925.

Kou, T., Marusawa, H., Kinoshita, K., Endo, Y., Okazaki, I. M., Ueda, Y., et al. (2007). Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int. J. Cancer*, *120*, 469–476.

Loeb, L. A., Loeb, K. R., & Anderson, J. P. (2003). Multiple mutations and cancer. *Proc. Natl. Acad. Sci. U.S.A.*, *100*, 776–781.

Matsumoto, Y., Marusawa, H., Kinoshita, K., Endo, Y., Kou, T., Morisawa, T., et al. (2007). *Helicobacter pylori* infection triggers aberrant expression of activation-induced cytidine deaminase in gastric epithelium. *Nat. Med.*, *13*, 470–476.

Okazaki, I. M., Hiai, H., Kakazu, N., Yamada, S., Muramatsu, M., Kinoshita, K., et al. (2003). Constitutive expression of AID leads to tumorigenesis. *J. Exp. Med.*, *197*, 1173–1181.

Pham, P., Bransteitter, R., & Goodman, M. F. (2005). Reward versus risk: DNA cytidine deaminases triggering immunity and disease. *Biochemistry*, *44*, 2703–2715.

Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., & Scott, J. (1987). A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. *Cell*, *50*, 831–840.

Please cite this article in press as: Marusawa, H., Aberrant AID expression and human cancer development, Int J Biochem Cell Biol (2008), doi:10.1016/j.biocel.2008.01.018