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Ⅲ. 研究成果の刊行物・別刷

Oxidative stress induces p53-dependent apoptosis in hepatoblastoma cell through its nuclear translocation

Hideki Yamamoto[1,2], Toshinori Ozaki[1], Mitsuru Nakanishi[1], Hironobu Kikuchi[1], Kaori Yoshida[1], Hiroshi Horie[3], Hiroyuki Kuwano[2] and Akira Nakagawara[1,*]

[1]*Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba 260-8717, Japan*
[2]*Department of General Surgical Science (Surgery 1), Gunma University, Graduate School of Medicine, Maebashi 371-8511, Japan*
[3]*Chiba Children's Hospital, Chiba 266-0007, Japan*

Hepatoblastoma (HBL) is the most common malignant liver tumor in children. Since tumor suppressor p53 is rarely mutated in HBL, it remains unknown whether p53 could contribute to the hepatocarcinogenesis. In the present study, we have found for the first time that, like neuroblastoma (NBL), wild-type p53 was abnormally accumulated in the cytoplasm of the human HBL-derived Huh6 cells. In accordance with this notion, immunohistochemical analysis demonstrated that p53 is largely expressed in cytoplasm of human primary HBLs. In response to the oxidative stress, Huh6 cells underwent apoptotic cell death in association with the nuclear translocation of p53 and the transactivation of its target gene implicated in apoptotic cell death. siRNA-mediated knockdown of the endogenous p53 conferred the resistance of Huh6 cells to oxidative stress. Intriguingly, histone deacetylase inhibitor (nicotinamide) treatment strongly inhibited the oxidative stress-induced nuclear translocation of p53 as well as the p53-dependent apoptosis in Huh6 cells. In contrast to the previous observations, the cytoplasmic anchor protein for p53 termed Parc had undetectable effect on the cytoplasmic retention of p53. Collectively, our present results suggest that the abnormal cytoplasmic localization of p53 might contribute at least in part to the development of HBL.

Introduction

Hepatoblastoma (HBL) is one of the most frequent malignant liver tumors of childhood. Indeed, its incidence is higher than that of hepatocellular carcinoma (HCC) in children. HBL arises from the hepatic precursor cells and displays a morphological similarity to the immature hepatocytes of the developing liver. In a sharp contrast to HCC, which is associated with hepatitis virus infection (Llovet *et al.* 2003), it has been shown that the incidence of HBL is highly elevated in patients with familial adenomatous polyposis (FAP), which carry germ-line mutations in the APC (adenomatous polyposis coli) tumor suppressor gene (Hughes & Michels 1992; Nagase & Nakamura 1993). APC protein forms a cytoplasmic multiprotein complex involved in the Wnt signaling pathway, which regulates the stability of β-catenin (Henderson & Fagotto 2002). Although APC is rarely mutated in sporadic HBL, accumulating evidence demonstrated that the frequent mutations or deletions of β-catenin at hot-spot regions within the exon 3 encoding its degradation targeting box are detectable in HBL, suggesting that the abnormal nuclear accumulation of the stabilized β-catenin which collaborates with Tcf/Lef complex plays a central role in the genesis of HBL (Koch *et al.* 1999). Consistent with this notion, Takayasu *et al.* (2001) revealed that β-catenin mutation is significantly correlated with the up-regulation of its target genes, including cyclin D1 and fibronectin. However, Harada *et al.* (2002, 2004) described that β-catenin mutation alone is not sufficient for the hepatocarcinogenesis, indicating that the additional mutations or epigenetic changes might be required for the genesis of HBL. The detailed molecular mechanism(s) behind the pathogenesis and development of HBL remains unknown.

The p53 tumor suppressor is a nuclear transcription factor, which has an ability to transactivate various p53-target genes implicated in the regulation of G1 cell cycle arrest and/or apoptosis such as $p21^{WAF1}$, MDM2, Bax and NOXA (Prives & Hall 1999; Sionov & Haupt 1999; Vousden & Lu 2002). The importance of p53 in the tumorigenesis has been emphasized by the observations

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**Correspondence*: E-mail: akiranak@chiba-cc.jp

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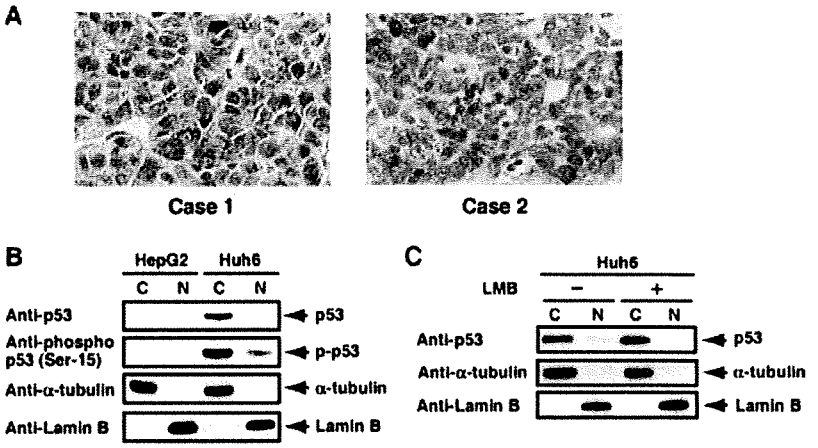

Figure 1 Cytoplasmic localization of p53 in HBL cells. (A) Immunohistochemical analysis. Sections (4 μm thick) of two primary hepatoblastoma tissues (case 1 and 2) were stained with the anti-p53 antibody. Note the positive signals in the cytoplasm of most tumor cells. (B) p53 is abundantly expressed in cytoplasm of Huh6 cells. Huh6 and HepG2 cells were biochemically fractionated into cytoplasmic (C) and nuclear (N) fractions as described under Experimental procedures. Equal amounts of cytoplasmic and nuclear extracts were subjected to Western blotting with the anti-p53 or with the anti-phosphorylated form of p53 at Ser-15. α-tubulin and Lamin B were used for the cytoplasmic and nuclear markers, respectively. (C) Leptomycin B has undetectable effect on the subcellular localization of p53. Huh6 cells were treated with or without 20 ng/mL of Leptomycin B (LMB). Six hours after the treatment, cells were fractionated into cytoplasmic (C) and nuclear (N) fractions, and subjected to Western blotting with the indicated antibodies.

showing that p53 mutation is detected in more than half of all human tumors (Hollstein *et al.* 1991; Vogelstein *et al.* 2000). The tumor-suppressive activity of p53 is dependent on its sequence-specific transactivation function. Indeed, the vast majority of p53 mutations are found within its central sequence-specific DNA-binding domain. Under normal conditions, p53 is a short-lived protein whose expression levels are kept extremely low. MDM2 acts as an E3 ubiquitin protein ligase for p53, and promotes its ubiquitination followed by degradation by 26S proteasome (Haupt *et al.* 1997; Honda *et al.* 1997; Kubbutat *et al.* 1997). Recently, it has been demonstrated that, like MDM2, Pirh2 and COP1 target p53 for degradation by 26S proteasome in an ubiquitin-dependent manner (Leng *et al.* 2003; Dornan *et al.* 2004). In response to genotoxic stresses, p53 is induced to be accumulated in cell nucleus through its phosphorylation at multiple sites, including Ser-15, Ser-20 and Ser-46, and exerts its pro-apoptotic activity (Sionov & Haupt 1999; Vousden & Lu 2002). In addition to the NH_2-terminal phosphorylation of p53, p300/CBP (CREB-binding protein) with the histone acetyltransferase (HAT) activity binds to the NH_2-terminal region of p53, mediates the acetylation of its COOH-terminal region and thereby enhances its activity (Gu & Roeder 1997). Thus, the post-translational modifications of p53 enhance its transcriptional as well as pro-apoptotic ability.

In contrast to other human tumors, p53 is infrequently mutated in certain human tumors such as neuroblastoma (NBL) and HBL (Vogan *et al.* 1993; Chen *et al.* 1995; Ohnishi *et al.* 1996; Kusafuka *et al.* 1997), indicating that p53 plays no role in the genesis and development of these tumors. However, this viewpoint has been challenged by the observations that the wild-type p53 is abnormally accumulated in the cytoplasm of NBLs (Moll *et al.* 1995). These findings strongly suggest that the nuclear exclusion of wild-type p53 might represent one non-mutational mechanism of p53 inactivation. In addition to NBL, wild-type p53 is abnormally sequestered in the cytoplasm in certain human tumors, including breast and colon cancers (Moll *et al.* 1992; Bosari *et al.* 1995). Although the detailed molecular mechanism(s) of the cytoplasmic accumulation of wild-type p53 remains unclear, Nikolaev *et al.* (2003) described that Parc (p53-associated parkin-like cytoplasmic protein) interacts with p53 in cytoplasm and inhibits its nuclear translocation.

In the present study, we have found that wild-type p53 is abundantly expressed in human primary HBLs and HBL-derived Huh6 cells. In response to oxidative stress, p53 was induced to be translocated into cell nucleus of Huh6 cells, and Huh6 cells underwent apoptotic cell death. Furthermore, nicotinamide treatment abolished the oxidative stress-induced nuclear translocation of p53, thereby inhibiting the p53-dependent apoptotic cell death.

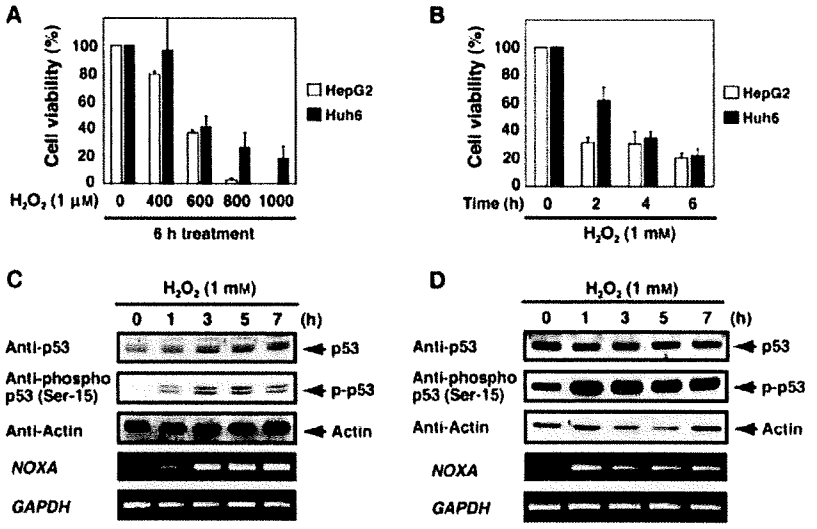

Figure 2 Effect of H_2O_2 treatment on Huh6 and HepG2 cell lines. (A, B) MTT cell survival assays. Huh6 (filled boxes) and HepG2 cells (open boxes) were exposed to H_2O_2 at the indicated concentrations for 6 h. After the treatment with H_2O_2, their cell viability was assessed by MTT assays (A). Similarly, Huh6 and HepG2 cells were treated with 1 mM of H_2O_2 for the indicated time periods, and their cell viability was examined by MTT assays (B). (C, D) Western blot analysis. HepG2 (C) and Huh6 cells (D) were treated with 1 mM of H_2O_2 for the indicated periods of time. Thereafter, whole cell lysates were prepared, and subjected to Western blotting with the anti-p53 (1st panel) or with anti-phospho-p53 at Ser-15 (2nd panel). Expression of actin was used to control equal protein loading (3rd panel). Alternatively, total RNA was extracted from cells treated with H_2O_2, and analyzed by RT-PCR for the expression of NOXA (4th panel). GAPDH was used to normalize (5th panel).

Results

Cytoplasmic expression of p53 in human HBLs

As previously described (Vogan *et al.* 1993; Moll *et al.* 1995), p53 is rarely mutated in human primary NBLs, and predominantly expressed in cytoplasm. Similar to NBLs, it has been shown that p53 is infrequently mutated in human primary HBLs (Chen *et al.* 1995; Ohnishi *et al.* 1996); however, its subcellular localization remains unclear. Then, we sought to examine the subcellular localization of p53 in surgically resected specimens of primary HBLs by immunohistochemistry. As shown in Fig. 1A, p53 immunoreactivity was detectable largely in cytoplasm of tumor cells, suggesting that, like NBLs, p53 might lack its intact function due to its abnormal cytoplasmic localization in HBLs. To further confirm these observations, we examined the subcellular distribution of p53 in HBL-derived Huh6 (Doi 1976) and HCC-derived HepG2 cells (Aden *et al.* 1979). As described (Bressac *et al.* 1990; Hsu *et al.* 1993), HepG2 cells carry wild-type p53. Our sequence analysis revealed that p53 expressed in Huh6 cells has a wild-type structure (data not shown). Huh6 and HepG2 cells were biochemically fractionated into cytoplasmic and nuclear fractions, and subjected to Western blotting with the anti-p53 antibody. α-tubulin and Lamin B were used as cytoplasmic and nuclear markers, respectively. Under our experimental conditions, E-cadherin, which is one of the membrane marker, was detected in the cytoplasmic fraction (data not shown). As shown in Fig. 1B, p53 was undetectable in each fraction of HepG2 cells, whereas p53 was largely expressed in cytoplasm of Huh6 cells, which was consistent with our immunohistochemical analysis of primary HBLs. It is worth noting that p53 is constitutively phosphorylated at Ser-15 in Huh6 cells in the absence of DNA damage. To rule out a possibility that the subcellular localization of p53 could be regulated by active nuclear export in Huh6 cells, Huh6 cells were treated with the nuclear export inhibitor leptomycin B (LMB). As shown in Fig. 1C, LMB had undetectable effect on the subcellular distribution of p53 in Huh6 cells.

Induction of apoptosis by oxidative stress in HBL-derived and HCC-derived cell lines

As described (Lluis *et al.* 2005), oxidative stress induced apoptotic cell death in hepatocytes. To examine a possible effect of the oxidative stress on Huh6 and HepG2 cells, these cells were treated with H_2O_2, and their cell viability was assessed by MTT cell survival assay. As shown in Fig. 2 A,B, Huh6 and HepG2 cells underwent apoptosis

Figure 3 siRNA-mediated knockdown of p53 in Huh6 cells. Huh6 cells were stably transfected with the empty plasmid (V1–V3) or with the expression plasmid encoding siRNA against p53 (P1–P13), and cultured in the presence of G418 (at a final concentration of 400 μg/mL) for 2 weeks. Whole cell lysates prepared from the indicated cell clones and the parental Huh6 cells (Control) were analyzed by Western blotting for the expression levels of the endogenous p53 and actin.

in response to H_2O_2 in a dose-dependent and a time-dependent manner. Since p53 plays a central role in the DNA damage-induced apoptosis (Prives & Hall 1999; Sionov & Haupt 1999), we examined the changes in endogenous p53 protein levels following treatment with H_2O_2. As shown in Fig. 2C, p53 was expressed at low levels in HepG2 cells without H_2O_2. Following exposure to H_2O_2, p53 was induced to be accumulated in association with a remarkable increase in the amounts of p53 phosphorylated at Ser-15. RT-PCR analysis revealed that the transcription levels of pro-apoptotic NOXA, which is one of the p53-target genes, are elevated in response to H_2O_2 treatment. In contrast, the amounts of total p53 remained almost constant and p53 was constitutively phosphorylated at Ser-15 in Huh6 cells regardless of H_2O_2 treatment (Fig. 2D). Under our experimental conditions, however, the expression levels of NOXA were increased in Huh6 cells exposed to H_2O_2, suggesting that p53 might contribute to the oxidative stress-mediated apoptotic cell death in Huh6 cells.

p53 plays a critical role in the H_2O_2-mediated apoptosis in Huh6 cells

To examine whether p53 could play an important role in the regulation of H_2O_2-dependent apoptosis in Huh6 cells, Huh6 cells were stably transfected with the expression plasmid encoding siRNA against p53 or with its control plasmid. Two weeks after the selection with G418 (at a final concentration of 400 μg/mL), we finally established several p53-knockdown cell clones as well as control cell clones (Fig. 3). We then investigated their sensitivity to H_2O_2 by TUNEL staining. Five hours after the treatment with H_2O_2 at a final concentration of 1 mM, V-2, V-3, P-9 and P-10 cells were subjected to TUNEL staining to identify the apoptotic cells. Cell nuclei were stained with DAPI. As shown in Fig. 4, exposure of V-2 and V-3 cells to H_2O_2 resulted in a significant increase in a number of TUNEL-positive cells, whereas two to threefold decrease in a number of cells with apoptotic nuclei was observed in P-9 and P-10 cells in response to H_2O_2. Similar results were also obtained in the other cell clones (data not shown). These results strongly suggest that p53 contributes at least in part to the H_2O_2-mediated apoptotic cell death in Huh6 cells.

H_2O_2-mediated nuclear translocation of p53

It is well documented that p53 is induced to be accumulated in cell nucleus in response to various DNA damaging agents, including cisplatin (CDDP) (Fritsche *et al.* 1993). In accordance with this notion, CDDP treatment stimulated the nuclear accumulation of p53 in Huh6 cells in a time-dependent manner, whereas the amounts of cytoplasmic p53 remained unchanged regardless of CDDP treatment (Fig. 5A). Additionally, Huh6 cells underwent apoptotic cell death in response to CDDP (Fig. 5B). Intriguingly, there existed an inverse relationship between the amounts of cytoplasmic and nuclear p53 in response to H_2O_2 (Fig. 5C). Indirect immunofluorescent staining indicated that p53 is largely expressed in cytoplasm of Huh6 cells, whereas p53 accumulates in cell nucleus in response to H_2O_2 (Fig. 5D). Thus, it is likely that the H_2O_2-mediated nuclear translocation of p53 might be one of the molecular mechanisms underlying the H_2O_2-dependent apoptosis in Huh6 cells.

Parc has an undetectable effect on the cytoplasmic retention of p53

The nuclear localization of p53 is critical for its transcriptional activity as well as apoptosis-inducing function. Recently, Nikolaev *et al.* (2003) have found that a Parkin-like ubiquitin ligase termed Parc acts as cytoplasmic anchor protein to block nuclear localization of p53. To ask whether Parc could be involved in the cytoplasmic retention of p53 in Huh6 cells, we examined the interaction between p53 and Parc by immunoprecipitation experiments. Whole cell lysates prepared from Huh6 cells were immunoprecipitated with normal mouse serum (NMS) or with the anti-p53 antibody, and the immunoprecipitates were analyzed by Western blotting with the anti-Parc antibody. Consistent with the previous results (Nikolaev *et al.* 2003), the anti-p53 immunoprecipitates contained the endogenous Parc (Fig. 6A). We then examined a possible effect of Parc on the subcellular distribution of p53. For this purpose, Huh6 cells were transiently transfected with siRNA against Parc or with the control siRNA. Twenty-four hours after transfection, total RNA and