主論文の要旨

Growth arrest and DNA damage-inducible protein (GADD34) enhanced liver inflammation and tumorigenesis in a diethylnitrosamine (DEN)-treated murine model

（GADD34 はジエチルニトロサミンによる
マウス肝障害及び腫瘍形成を促進する）

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Introduction

Hepatocellular carcinoma (HCC), which is a malignant tumor of liver parenchyma cells, is associated with hepatocellular dysfunction and mutant cellular pathways. Chronic inflammation is associated with the initiation of cancer, indicating a strong link between inflammation and carcinogenesis. Growth arrest and DNA damage-inducible protein (GADD34/Ppp1r15a) was induced by various stimuli including DNA damage and ER stress. Increased expression of GADD34 is correlated with apoptosis. Apoptosis-induced proliferation contributes to regeneration and cancer initiation. Recently, our laboratory showed that GADD34 protein was highly expressed in myeloid lineage cells. It has been shown that myeloid-derived cells participate in the progression of tumor development and contribute to the promotion of tumor growth and metastasis. However, it is not clear whether GADD34 involvement contributes to the pathogenesis of HCC. Therefore, we suggest that understanding the mechanism(s) of GADD34 in inflammation-mediated hepatocarcinogenesis is essential for the treatment and prevention of liver tumorigenesis.

Methods

In this study, we employed GADD34 KO mice to dissect the role of GADD34 and relative signal pathways in liver inflammation and tumorigenesis. Diethylnitrosamine (DEN) was employed to induce acute liver injury, chronic liver inflammation and HCC tumorigenesis in wild-type (WT) and GADD34 KO mice. Alanine transaminase (ALT) activity in serum was measured after treatment by DEN. H&E staining was used to evaluate pathological damage of liver. Flow cytometry and immunofluorescent staining were applied to examine the infiltration of immune cells and ROS production in the liver. Real-time PCR was performed to check the expression levels of chemokines and cytokines in the liver. Moreover, the immunohistochemistry (IHC) staining was used to evaluate the infiltration of macrophages/Kupffer cells and the compensatory proliferation of hepatocytes. Finally, Western blot was applied to analyze the expression level of proteins in related signal pathways.

Results

We initially investigated the effects of GADD34 in the acute liver injury model, using 6-week old male WT and GADD34-/- mice that were given a single intraperitoneal injection of high dose DEN (100 mg/kg) and sacrificed at 2, 4, 8, 12 and 24 h (Fig. 1a). H&E staining showed visible necrosis around the central vein area at 8, 12 and 24 h, histological scores revealed significant differences between WT and GADD34-/- mice (Fig. 1b). Similarly, ALT activity was increased in WT mice from 4 h to 24 h compared to those in GADD34-/- mice (Fig. 1c). These differences indicated that WT mice were subjected to greater liver injury and functional loss than GADD34-/- mice after DEN treatment. GADD34 mRNA

expression was significantly increased from 4 h to 24 h when compared to 0 h (Fig. 1d). Moreover, the WT mice showed higher expression levels of IL-6 and c-Myc than did GADD34-/- mice at 4 h (Fig. 1e). These results suggested that GADD34 enhanced pro-inflammatory cytokine production and oncogene activation. The expression of pH_2AX was increased after DEN treatment in both WT and GADD34-/- mice (Fig. 1f). Phosphorylated-p53 showed significantly higher expression levels in WT mice than GADD34-/- mice (Fig.1f, 1g), which suggested that phospho-p53 may work as downstream of GADD34. Consistent with the production of pro-inflammatory cytokine IL-6, the phospho-NF-κB p65 and phospho-STAT3 displayed significantly higher expression levels in WT than GADD34-/- mice from 12 h to 24 h (Fig. 1f, 1g). Taken together, these results clearly showed that DEN induced more severe liver damage in WT mice than in GADD34-/- mice.

We next examined the function of GADD34 in chronic liver inflammation. The mice were subjected to chronic DEN treatment (Fig. 1a), we found that mRNA level of GADD34 in WT mice was significantly increased (Fig. 2a). Moreover, c-Myc, phosphorylated p53, caspase1 p10 and cleaved caspase-3 showed higher expression levels in the livers of WT mice than in GADD34-/- mice (Fig. 2b, 2c). These results illustrated that GADD34 enhanced DNA damage, oncogene expression and hepatic damage after chronic exposure to DEN. Histological analysis showed that WT mice were subjected to significantly higher hepatic inflammation cells infiltration than GADD34-/- mice (Fig. 2d). Among the dissociated immune cells, the neutrophils ($Ly6C^{high}$ $Ly6G^{high}$) and Kupffer cells/macrophages ($F4/80^{high}CD11b^{high}$) were significantly increased in WT mice than were GADD34-/- mice (Fig. 2e, 2f). This observation indicated an immune dysfunction in mice after DEN treatment. Moreover, WT livers showed significantly elevated level of ROS production when compared to GADD34-/- following repeated treatment with 25 mg/kg DEN (Fig. 2g, 2h). These data indicated that ROS production by activated immune cells in WT mice may have contributed to higher levels of pro-tumorigenic cytokines production. As expected, we found that the mRNA expression of IL-1β, MMP9, MCP1, IL-6 and TNF-α was significantly increased in the livers of WT mice than that in GADD34-/- mice (Fig. 3a). Furthermore, the immunofluorescent staining showed that IL-6 was expressed in F4/80-positive Kupffer cells/macrophages, the expression of IL-6 in these cells presented significantly higher immunofluorescence intensity in WT than GADD34-/- mice (Fig 3b, 3c). These results suggested that GADD34 deletion led to reduced production of IL-6 by Kupffer cells. Correspondingly, significantly higher activation levels of NF-κB p50 and pSTAT3 were observed in the livers of WT mice compared to those in GADD34-/- mice (Fig. 3d). These results suggested that enhanced activation of Kupffer cells/macrophages may provide signals that contribute to hepatic compensatory proliferation in the presence of GADD34. Accordingly, IHC staining showed that higher numbers of Ki67 positive hepatocytes were present in the livers of WT mice than that in GADD34-/- mice following

repeated treatments with 25 mg/kg DEN (Fig. 3e). Based on these results, it appears that exposure to DEN upregulates GADD34, which can increase hepatic damage and compensatory proliferation of mutant hepatocytes.

After confirming that GADD34 played an important role in chronic inflammation, we next aimed at investigating whether GADD34 was involved in hepatocarcinogenesis by using a previously described protocol to generate HCC (Fig.1a). The sizes of the hepatocellular carcinoma and the liver/body weight ratios in GADD34-/- mice were significantly lower than WT mice (Fig. 4a, 4b and 4c). The non-tumor area in WT mice showed more dysplasia around central vein areas than in GADD34-/- mice and the borders of localized tumor areas were larger in WT mice than in GADD34-/- mice (Fig. 4d). These results indicated that an increased tumor burden was associated with severe dysplasia in WT mice. Interestingly, we found that the expression of GADD34 protein level was strongly increased in WT mice after HCC development (Fig. 4e). The expression of AFP, c-Myc and pAkt was significantly higher with the presence of GADD34 (Fig. 4e), which provided further evidence of higher tumor burden in WT mice than in GADD34-/- mice. Next, we tried to delineate which factor played a dominant role in HCC progression. IHC staining showed higher numbers of F4/80-positive Kupffer cells/ macrophages in both non-tumor areas and tumor areas of WT livers than in GADD34-/- (Fig. 5a, 5b). Consistently, the MCP1 and CCR2 showed significantly higher expression levels in the livers of WT mice than GADD34-/- mice (Fig. 5c). Meantime, c-Myc and TNF-α were significantly increased in WT livers relative to GADD34-/- livers (Fig. 5d, 5e). Accordingly, Ki67 positive cells were significantly higher within the tumor area in the presence of GADD34 (Fig.5f, 5g), indicating that GADD34 promoted hepatocarcinogenesis.

Discussion and conclusion

The effects of GADD34 in DEN-induced HCC proceed as follows. First, the carcinogen DEN damages hepatocytes DNA, which leads to elevated expression of GADD34 in the liver. The increased expression of GADD34 augments hepatic necrosis followed by release of IL-1β and MCP1. This process promotes Kupffer cell activation and macrophage infiltration followed by the production of ROS and pro-tumorigenic cytokines such as IL-6 and TNF-α. These pro-tumorigenic cytokines stimulate compensatory proliferation of surviving and mutant hepatocytes. Finally, the abnormal compensatory proliferation leads to HCC progression (Fig.6). In conclusion, GADD34 contributes to hepatic damage, inflammation and compensatory proliferation during HCC. However, it is still necessary to explore the detailed mechanisms underlying GADD34 regulation of signal pathways during liver tumorigenesis and to seek therapeutic opportunities for HCC by targeting associated pathways.