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Original Article

Abrogation of ALK5 in hepatic stellate cells decreases hepatic fibrosis and ameliorates liver damage in mice following treatment with thioacetamide

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Abstract

While transforming growth factor-β (TGF-β) is known to be a key inducer of hepatic stellate cell (HSC) activation during liver fibrosis but it is unclear which TGF-β receptor is required for this HSC-mediated fibrogenesis. Here, we report that abrogation of TGF-β type I receptor ALK5 in HSC activation led to reduced collagen deposition and a decreased number of myofibroblasts in livers of mutant mice lacking ALK5 in HSC (*Alk5/GFAP-Cre* mice) following thioacetamide (TAA) exposure. The reduced fibrosis was accompanied by decreased expression of HSC activation markers in livers. In addition, *Alk5/GFAP-Cre* mice exhibited decreased immune cell infiltration and reduced production of inflammatory cytokines. Associated with reduced fibrosis and inflammation, amelioration of liver injury was observed in *Alk5/GFAP-Cre* mice after TAA treatment. In conclusion, our results indicated that TGF-β signaling via ALK5 in HSC enhanced liver fibrogenesis and inflammation led to amplification of hepatic injury in mice exposed to TAA.

Keywords: TGF-β, ALK5, hepatic stellate cells, fibrosis, liver injury

1. Introduction

Liver fibrogenesis is sustained by a heterogenous population of ECM-producing cells that includes α-smooth muscle actin (α-SMA) positive myofibroblasts (MFs) (Parola *et al.*, 2008). MFs are the major cell type contributing to the accumulation of fibrillar matrix in the fibrous scar (Kisseleva & Brenner, 2011). MFs also promote synthesis and release of growth factors which sustain and perpetuate chronic inflammatory response and neo-angiogenesis in liver cirrhosis (Forbes & Parola, 2011; Friedman, 2008b). In recent decades, various types of studies suggested that MFs could originate from hepatic stellate cells (HSCs) (Friedman, 2008a), portal fibroblasts (Knittel *et al.*, 1999), bone marrow-derived fibrocytes (Asawa *et al.*, 2007), or through an epithelial to mesenchymal transition (EMT) of hepatic epithelium (Zeisberg *et al.*, 2007). Among these cellular sources, MFs were assumed to be mainly derived from HSCs (Bataller & Brenner, 2005) since they expressed common fibroblast markers, such as, FSP-1, vimentin, desmin, and nestin (Brenner *et al.*, 2012; Iwaisako *et al.*, 2012; Niki *et al.*, 1999; Strutz *et al.*, 1995). However, these markers are not only expressed by the HSCs but also by portal fibroblasts, bone marrow-derived cells, and epithelial cells (Dave & Bayless, 2014; Eckes *et al.*, 2000; Mendez-Ferrer *et al.*, 2010; Osterreicher *et al.*, 2011; Zhao & Burt, 2007). Therefore, it has yet to be further elucidated whether the majority of MFs are derived from HSCs (Park, 2012). Additional experiments using cell specific gene targeting developed in mice can help us determining the contribution of HSC-derived MFs during the progression of hepatic fibrosis (Forbes & Parola, 2011).

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Upon liver damage, HSCs respond to various liver-damage factors and undergo cellular activation that enables them to acquire biological function that are pivotal for organ repair (Forbes & Parola, 2011). Among the compelling pathways of HSC activation, TGF-β remains a classical family of fibrogenic cytokines that are derived from both paracrine and autocrine sources (Breitkopf *et al.*, 2006; Inagaki & Okazaki, 2007). TGF-β elicits its effects through TGF-β type II (TβR II) and type I (TβR I) cell surface receptors that signal via cytoplasmic serine/theronine kinase domain (Attisano & Wrana, 2002). In HSCs, TGF-β ligand binding to TβR II leads to recruitment of two subclasses of TβR I (ALK5 and ALK1) that tranduce divergent intracellular signaling to regulate gene expression (Goumans *et al.*, 2003). While ALK1 phosphorylation induces the activation of Smad 1/5/8 pathway, stimulation of ALK5 results in activation of the Smad2/3 cascade (ten Dijke & Hill, 2004). TGF-β signaling has been known to control collagen gene expression in HSCs (Garcia-Trevijano *et al.*, 1999). Although previous studies using mouse models have shown that TGF-β is sufficient to drive HSC activation *in vivo* (Hellerbrand *et al.*, 1999; Kanzler *et al.*, 1999; Ueberham *et al.*, 2003), it has not been demonstrated which type of TβR I is required in HSCs during liver fibrosis. In this study, we show that abrogation of ALK5 signaling in HSCs inhibited collagen production in thioacetamide-induced fibrosis mouse model. Furthermore, deficient TGF-β signaling in HSCs led to a reduced inflammation and attenuated liver damage induced by toxic stimulus. Our results indicate that ALK5-mediated response in HSCs is required for the progression of liver fibrogenesis following liver injury.

2. Materials and Methods

2.1 Experimental mice

To generate mutant mice lacking ALK5 in HSCs, *Alk5*$^{fx/fx}$ mice (Larsson *et al.*, 2001) were crossed with *GFAP-Cre* mice which were also heterozygous for the *Alk5*^{fx} allele. The resulting homozygotes for *Alk5*^{fx} alleles which also carried *GFAP-Cre* trangene (*Alk5*$^{fx/fx}$/*GFAP-Cre*$^{+}$) had ALK5 specifically deleted in the HSCs (herein termed *Alk5/GFAP-Cre*), whereas littermates with incomplete combination of these alleles served as the control. Oligonucleotides for PCR-genetyping of the *Alk5*^{fx} alleles were previously described (Dudas *et al.*, 2006). *GFAP-Cre* mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).

2.2 Liver injury model

All animal studies were conducted with the approval of the Animal Ethics Committee on Use and Care of Animals at the Faculty of Science, Mahidol University, Thailand (Protocol number 257). Liver injury was induced by intraperitoneal thioacetamide (TAA) injection three times a week for 12 weeks (100 μg/g body weight for one week, 150 μg/g body weight for one week and 200 μg/g body weight for ten weeks).

2.3 Histological analyses and immunostaining

For histology, liver tissues were fixed with Bouin solution (Bio-Optica) for 24 h, dehydrated and embedded in paraffin. Liver sections (6 μm) were stained with hematoxylin and eosin (H&E). To analyze liver fibrosis, sections were stained with Sirius Red (saturated picric acid containing 0.1% direct red and 0.1% Fast Green FCF, Sigma). Five Sirius Red-stained slides per mouse were generated, with six 10x pictures taken randomly per slide for a total of 30 images per mouse. The images were analyzed for Sirius Red-positive areas using ImageJ program. Five mice per experimental group were used for collagen quantification. For immunohistochemistry, sections were stained with antibodies for α-SMA (M085, Dako), myeloperoxidase (PA5-16672, Thermo Fisher Sci.), CD3 (A0452, Dako), and cleaved caspase-3 (#9664, Cell Signaling Tech.).

2.4 Real-time PCR analyses

RNAs from livers were extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). The QuantiNova RT kit (Qiagen Inc., Valencia, CA, USA) was used to synthesize cDNA from 1 μg of RNA. Quantitative RT-PCRs were carried out with the Applied Biosystems real-time PCR 7500 using iTaq Universal SYBR Green Supermix (BioRad Laboratories Inc., Hercules, CA, USA). Data were represented as the relative expression of the genes after normalizing to β-actin. All primer pairs have been previously described (Basciani *et al.*, 2004; Bopp *et al.*, 2013; De Filippo *et al.*, 2008; Stewart *et al.*, 2014; Yang *et al.*, 2013).

2.5 Statistical analysis

All experiments were performed with five animals per experimental group and representative data were presented as mean±SD. Comparisons were performed by student's *t*-test and differences were considered significant if $P<0.05$.

3. Results and Discussion

3.1 Thioacetamide-induced fibrosis is reduced in *Alk5/GFAP-Cre* livers

Since TGF-β is known to promote HSC activation giving rise to collagen-producing myofibroblasts (Hellerbrand *et al.*, 1999), we hypothesized that abrogation of ALK5 in HSC would attenuate liver fibrogenesis. To test this idea, we analyzed the extent of liver fibrosis in Sirius red-stained liver sections obtained from the TAA-treated control and TAA-treated *Alk5/GFAP-Cre* mice (Figure 1). While control livers displayed intense Sirius red-positive staining in fibrotic lesions that were connected among perivenular areas to form pseudolobules (Figures 1A and B), fibrotic lesions in *Alk5/GFAP-Cre* livers were weakly stained by Sirius Red staining (Figures 1C and D). Morphometric analysis of Sirius red-stained sections showed that collagen deposits were signi-

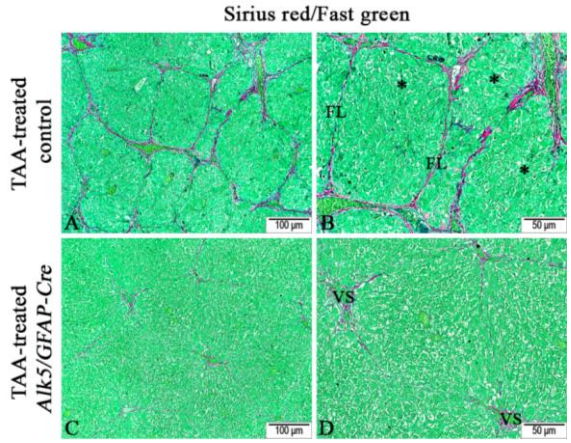

Figure 1. Representative images of Sirius red/fast green-stained liver sections obtained from TAA-treated control and TAA-treated *Alk5/GFAP-Cre* mice. In TAA-treated control livers, Sirius red-stained collagen fibers were seen in fibrotic lesions that separated liver parenchyma into pseudolobules (A and B). While developing fibrous bridgings were seen in livers of TAA-treated *Alk5/GFAP-Cre* mice, Sirius red-positivity was greatly reduced in fibrotic lesions and perivenular areas compared to the TAA-treated control mice (C and D).
FL, fibrotic lesions; VS, vascular space; *, pseudolobule.

ficantly reduced in livers of TAA-treated *Alk5/GFAP-Cre* mice compared to those of TAA-treated control mice (Figure 2). To determine whether the amount of myofibroblasts coincides with decreased fibrosis in *Alk5/GFAP-Cre* mice, we stained liver sections with α-SMA antibody. Our results showed that a number of α-SMA-positive cells were observed in the perivenular areas and fibrotic lesions of TAA-treated control livers (Figure 3A) whereas fewer α-SMA-positive cells were seen in livers of *Alk5/GFAP-Cre* mice (Figure 3B). Consistent with this finding, quantitative RT-PCR (qRT-PCR) showed that mRNA expression of α-SMA was greater in the livers of the TAA-treated control compared to those of TAA-treated *Alk5/GFAP-Cre* mice (Figure 3C). Additionally, we assessed whether hepatic expression of key HSC activation markers (Czochra *et al.*, 2006; Mann & Marra, 2010; Thieringer *et al.*, 2008) was reduced in *Alk5/GFAP-Cre* mice following TAA treatment. Figure 4 shows that PDGF-A, PDGF-B, and TGF-β mRNA levels were decreased in livers of TAA-treated *Alk5/GFAP-Cre* mice compared to those of TAA-treated control mice.

Although previous *in vitro* studies showed that both type I receptors ALK5 and ALK1 are used by HSCs (Khimji *et al.*, 2008), the requirements of these receptors in HSCs during TGF-β–mediated liver fibrosis has not been elucidated. Here, we report on a new HSC-specific, knockout mouse model that clarified a role of TGF-β signaling via ALK5 in HSCs during liver fibrosis progression. The results from our work demonstrated that genetic ablation of ALK5 in HSCs led to a decrease in collagen gene expression in the livers of mice exposed to TAA. These data suggested that ALK5 signaling is

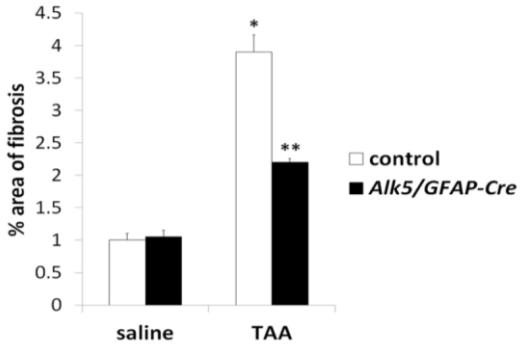

Figure 2. Morphometric analysis of Sirius red-stained sections of TAA-treated control and TAA-treated *Alk5/GFAP-Cre* mice. Data are mean±SD of N=5 per experimental group. *P<0.05, significantly different compared with saline-treated control group. There were significant differences compared with the TAA-treated control group (**P<0.05).

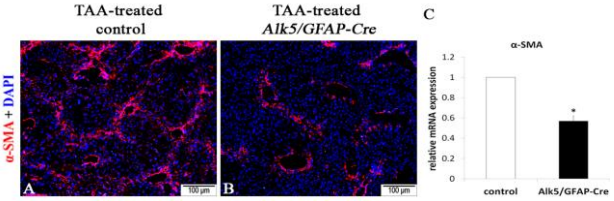

Figure 3. Representative images of immunohistochemical staining of α-SMA on liver tissue sections. After TAA exposure, control livers showed intense α-SMA-immunoreactivity (red) around perivenular areas as well as in fibrotic lesions (A). In livers of TAA-treated *Alk5/GFAP-Cre* mice, less α-SMA-positivity was detected around portal and peritotal areas (B). Quantitative RT-PCR (qRT-PCR) analysis showed a lower hepatic α-SMA mRNA expression in TAA-treated *Alk5/GFAP-Cre* mice, compared to those of TAA-treated control mice (C). Data were normalized to β-actin mRNA level. *, significantly different from TAA-treated control at $P<0.05$ (student's *t*-test).

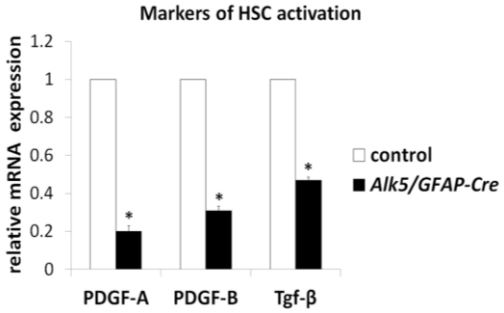

Figure 4. Relative mRNA expression of HSC activation marker: PDGF-A, PDGF-B and Tgf-β. Expression levels were normalized to β-actin mRNA level. Five mice per experimental group were used for the analysis. *, significantly different from TAA-treated control at P<0.05 (student's *t*-test).

necessary for HSC-dependent collagen synthesis during liver fibrogenesis. We also found that the loss of ALK5 in HSC inhibited TAA-induced myofibroblast production in the livers. These findings delineated an essential role of ALK5 during the transformation of quiescent HSCs to activated HSCs *in vivo*.

3.2 Reduced hepatic inflammatory response in *Alk5/GFAP-Cre* mice after TAA exposure

Because activated HSCs/myofibroblasts play important roles not only in promoting fibrosis but also influencing liver inflammation (Novo *et al.*, 2009), it is possible that a reduced number of myofibroblasts in *Alk5/GFAP-Cre* livers may lead to attenuation of inflammatory response to TAA-induced injury. To investigate this possibility, myeloperoxidase staining and CD3 immunostaining were performed to assess liver infiltration of neutrophils and T lymphocytes, respectively (Figure 5). Immunohistochemistry for myeloperoxidase showed a higher number of myeloperoxidase-positive cells in sinusoids of control livers (Figures 5A and B) than those in *Alk5/GFAP-Cre* livers (Figures 5E and F). Similar results were obtained from CD3 immunostaining. While plenty of CD3-positive cells were concentrated around perivascular areas of TAA-treated control livers (Figures 5C and D), fewer CD3-positive cells were found in livers of *Alk5/GFAP-Cre* mice (Figures 5G and H). In accordance with the reduced immune cell infiltration, hepatic expression of the inflammatory cytokines TNF-α, IL-6, MIP, and MCP were decreased in TAA-treated *Alk5/GFAP-Cre* mice compared to those of TAA-treated control mice (Figure 6). Since many inflammatory cytokines are known to be regulated by TGF-β signaling (Chen *et al.*, 2008; Park *et al.*, 2003; Zhang *et al.*, 2009), and HSCs were shown to modulate immunological response in livers by secreting several chemokines (Friedman, 2008a; Hernandez-Gea & Friedman, 2011), it is tempting to speculate that reduced immune infiltration in mutant livers may be attributed to reduced expression of inflammatory factors by HSCs. Further studies are needed to clarify whether

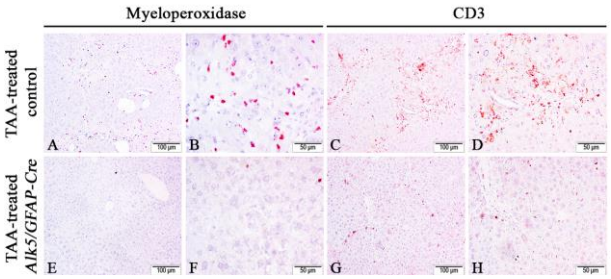

Figure 5. Immunohistochemistry for neutrophil: myeoloperoxidase and for T lymphocyte: CD3 in liver of TAA-treated mice. While a number of myeoloperoxidase-postive cells were evenly distributed throughout liver parenchyma of TAA-treated control mice (A and B), myeoloperoxidase-postivity was hardly detected in TAA-treated *Alk5/GFAP-Cre* livers (E and F). Immunostaining for CD3 showed that CD3-positive cells were concentrated around fibrotic lesions and perivascular areas in control livers (C and D) whereas CD3-immunoreactivity was less seen in *Alk5/GFAP-Cre* livers (G and H). Immunohistochemistry with NovaRED substrate, counterstained with hematoxylin.

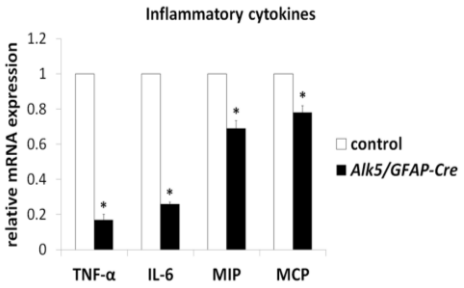

Figure 6. Hepatic mRNA expression of inflammatory cytokines in TAA-treated mice. qRT-PCR analysis of TNF-α, IL-6, MIP and MCP expression was performed by cDNA obtained from livers TAA-treated mice. Expression levels were normalized to β-actin mRNA level. *, significantly different from TAA-treated control at $P<0.05$ (student's *t*-test). Five mice per experimental group were used for the analysis.

TGF-β/ALK5 signaling in HSCs could directly mediate liver inflammation via the production of these cytokines and chemokines.

3.3 *Alk5/GFAP-Cre* mice showed amelioration of TAA-induced liver damage

Having found decreased liver fibrosis and inflammation in TAA-treated *Alk5/GFAP-Cre* mice, we speculated that inhibition of TGF-β/ALK5 signaling in HSCs is associated with reduced liver injury. To investigate the extent of liver injury, we analyzed H&E stained liver sections of the TAA-treated control (Figures 7A-C) and TAA-treated *Alk5/GFAP-Cre* mice (Figures 7D-F). While apoptotic bodies were often found in the perivenular (Figure 7B) and periportal areas (Figure 7C) in the livers of TAA-treated control mice, this degenerative change was rarely seen in TAA-treated *Alk5/GFAP-Cre* livers (Figures 7E and F). In line with this finding, immunohistochemistry for cleaved caspase 3 showed a lower number of cleaved caspase 3-positive cells in the livers of TAA-treated *Alk5/GFAP-Cre* mice than the TAA-treated control mice (Figure 8). Additionally, serum asparate transaminase (AST) and alanine transaminase (ALT) valves were significantly reduced in TAA-treated *Alk5/GFAP-Cre* mice compared to those of TAA-treated control mice (Figure 9).

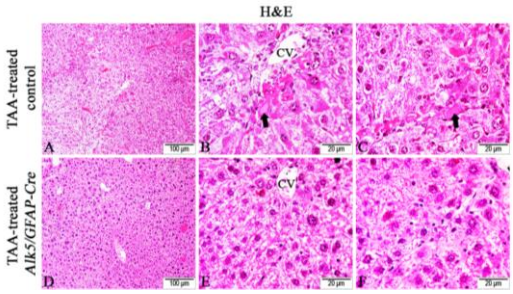

Figure 7. Histological changes of TAA-induced injury in control (A-C) and *Alk5/GFAP-Cre* mice (D-F). Apoptotic bodies were frequently seen around central veins (B, arrow) and in periportal areas (C, arrow) in TAA-treated control livers. This degenerative change was less seen in livers of *Alk5/GFAP-Cre* mice (D-F). Liver samples were stained for hematoxylin and eosin. CV, central vein.

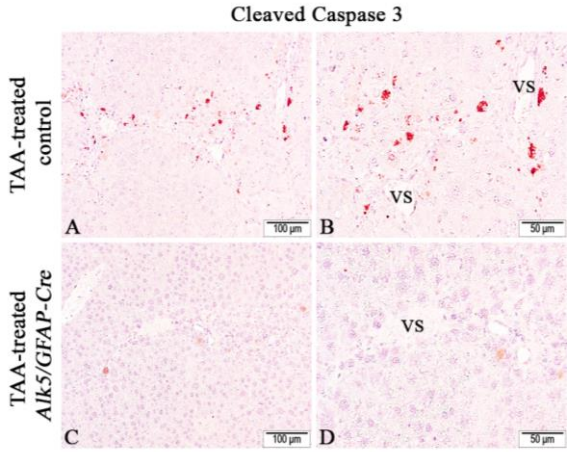

Figure 8. Immunohistochemistry for cleaved caspase 3 in livers of TAA-treated mice. Cleaved caspase3-postive cells (brown) were often found in perivenular areas of TAA-treated control mice (A and B). A decreased number of cleaved caspase3-postive cell was observed in livers of TAA-treated Alk5/*GFAP-Cre* mice (C and D). Immunohistochemistry with NovaRED substrate, counterstained with hematoxylin. VS, vascular space.

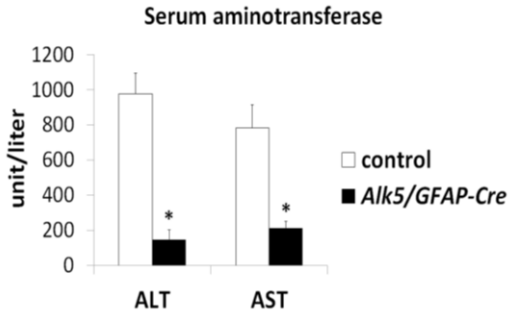

Figure 9. Analysis of serum liver enzyme level. The level of serum asparate transaminase (AST) and alanine transaminase (ALT) in TAA-treated Alk5/*GFAP-Cre* mice was lower than that of TAA-treated control mice. Five mice from each experimental group were used for the analysis. There was significant differences compared with TAA-treated control group (*P<0.05).

It is becoming clearer that HSCs mediate the amplification of fibrosis–associated liver damage. Works by Puche *et al.* (Puche *et al.*, 2013) and Stewart *et al.* (Stewart *et al.*, 2014) have demonstrated that HSCs contribute to hepatocyte death and exacerbate liver injury caused by a variety of toxic stimuli. The data from our studies further suggested that the pivotal functions of HSCs are mediated by TGF-β/ALK5 signaling. We have shown that fibrosis and liver damage was reduced because TGF-β signaling in HSCs was blocked by genetic ablation of ALK5. Although the precise mechanism of how TGF-β mediates HSC-dependent liver damage remains to be elucidated, our findings point to ALK5 signaling as a fundamental pathway directing liver damage. Therefore, an abrogation of ALK5 signaling in HSCs which amplifies chronic liver injury has potential therapeutic benefit for fibrosis-associated liver diseases.

4. Conclusions

In summary, we showed that Tgf-β type I receptor ALK5 was essential for HSC-dependent myofibroblast production during liver fibrosis. In addition, abrogation of ALK5 led to attenuation of liver inflammation and hepatic injury following TAA exposure. While further studies are still needed to investigate the mechanism in which HSC-specific TGF-β signaling influences inflammatory response and hepatocellular damage, our findings supported the beneficial effect of HSC-specific ALK5 inhibition as a promising treatment for chronic liver injury.

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