A New Therapeutic Strategy for Hepatocellular Carcinoma by Molecular Targeting Agents via Inhibition of Cellular Stress Defense Mechanisms

Yuichi Honma* and Masaru Harada

Abstract: The prognosis of advanced hepatocellular carcinoma (HCC) has remained very poor. It has recently been reported that the molecular targeting agent sorafenib can improve the prognosis of patients with advanced HCC. However, the detailed mechanisms of sorafenib, especially its direct effects on hepatoma and hepatocyte cells, are poorly understood, making a more detailed investigation about the molecular mechanism of sorafenib necessary. Endoplasmic reticulum (ER) stress is related to the pathophysiology of various liver diseases, including chronic viral hepatitis, alcoholic and nonalcoholic steatohepatitis and HCC. In this regard, our recent data examining the molecular effects of sorafenib focused on the cellular defense mechanisms from ER stress, the unfolded protein response (UPR) and keratin phosphorylation, demonstrated that sorafenib inhibited both important cytoprotective mechanisms, UPR and keratin phosphorylation, and enhances the anti-tumor effect in combination with proteasome inhibitors. This review summarizes the cytoprotective mechanisms from ER stress and our results about the direct effect of sorafenib on the cytoprotective mechanisms.

Keywords: sorafenib, endoplasmic reticulum stress, unfolded protein response, keratin, autophagy.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common solid tumor and the third leading cause of cancer-related death [1]. The curative treatments for HCC are surgical resection, percutaneous local ablation, and liver transplantation. However, most patients with advanced stages of HCC cannot receive these effective treatments. For such patients with unresectable, large and/or multifocal HCC, transarterial chemoembolization (TACE) or systemic pharmacologic treatment is the final and main therapy. Advances in the treatment of HCC have been made with sorafenib. Sorafenib is a multi-kinase inhibitor which targets the Raf serine/threonine kinases, including Raf-1 and B-Raf, and tyrosine kinases such as vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) [2]. Two landmark phase III trials demonstrated that sorafenib significantly improved overall survival in patients with advanced HCC and established sorafenib as the standard for care in advanced HCC patients [3, 4]. However, the detailed mechanisms of the anti-tumor effect of sorafenib have remained unclear, especially in the direct molecular effects on the HCC cells. Although there is no doubt about the clinical efficacy of sorafenib, there are some problems in that the improvement of survival is considered to remain insufficient and severe side effects,
such as liver dysfunction, can occur. Therefore, the elucidation of the direct molecular effects of sorafenib on hepatoma cells and normal hepatocytes is necessary.

Endoplasmic reticulum (ER) stress is related to the pathophysiology of various chronic hepatic diseases, including chronic hepatitis C, alcoholic liver disease, and nonalcoholic steatohepatitis (NASH) [5]. Because hepatocytes perform abundant metabolic functions, such as protein synthesis and lipid metabolism, hepatocytes have cellular defense mechanisms against ER stress. The unfolded protein response (UPR) has been described as a response to the accumulation of unfolded abnormal proteins in the ER [5, 6]. The ubiquitin-proteasome pathway is involved in the degradation of misfolded proteins via the UPR. We previously reported that proteasome inhibitors (PIs) induced the accumulation of misfolded abnormal proteins in cytoplasm and subsequently induced the formation of protein aggregates and apoptosis [7, 8]. In addition, autophagy, another protein degradation system, eliminates cytoplasmic proteins and organelles. Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, induced autophagy and decreased PIs-mediated abnormal protein accumulation and apoptosis [7, 8].

Keratin 8 (K8) and K18 are intermediate filament-forming proteins of hepatocyte. Previous reports have shown that keratins protect hepatocyte not only from mechanical stresses by forming cytoskeleton but also from various other stresses by its unique functions [9]. Keratins are also reported to be very important in Mallory-Denk body (MDB) formation.

This review describes the cytoprotective mechanisms against ER stress and the protein degradation pathways in hepatocyte. Our perspective of HCC treatment is from our examinations which demonstrated that sorafenib inhibited the cellular defense mechanisms and enhanced the anti-tumor effects in combination with PIs.

**Endoplasmic reticulum stress and unfolded protein response**

The ER is an important intracellular organelle responsible for protein folding, modification and trafficking. Newly synthesized proteins are folded and processed properly in the ER, although nearly 30% of them are not folded properly [10]. The unfolded abnormal proteins are ubiquitinated and directed to the degradation pathway (ubiquitin-proteasome system). Various cellular stresses cause the accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved cellular defense mechanism which is termed UPR [5]. When unfolded proteins accumulate in the ER, ER stress is induced and sensed by the critical transmembrane ER stress signaling proteins, protein kinase activated by double stranded RNA (PKR)-like ER kinase (PERK), inositol requiring (IRE) 1α and activating transcription factor (ATF) 6α [6]. Among these stress sensors, IRE1α and PERK are activated by its phosphorylation. IRE1α is a type I transmembrane protein with serine/threonine protein kinase that activates X-box binding protein 1 (XBP1) protein, resulting in ER protein folding and ER-associated protein degradation. PERK is a type I ER resident protein kinase that phosphorylates eukaryotic translation initiation factor 2α (eIF2α), resulting in global mRNA translation attenuation. Functions of the UPR include enhancing protein proper folding, misfolded abnormal protein degradation, and down-regulating overall protein synthesis to decrease the burden of proteins in the ER. Thus, the UPR fundamentally acts against cellular stress-mediated cell death, but sustained ER stress leads to apoptosis. The transcriptional factor CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), the stress activated protein kinase c-jun N-terminal kinase (JNK), B-cell lymphoma 2 (Bcl-2) family proteins, calcium homeostasis and consequent caspase activation are considered to be implicated [5].

**Protein degradation pathways and therapeutic potential in cancer**

There are two major protein degradation systems in mammalian cells: the ubiquitin proteasome system and the autophagy. 26S proteasome is an adenosine triphosphate (ATP)-dependent multi-catalytic protease and is involved in protein degradation via the ubiquitin-proteasome system. PIs are noticed as a new class of chemotherapeutic drugs [11]. Bortezomib has been clinically approved for the treatment of hematologic malignant diseases and is expected to have an anti-
tumor effect in HCC [12]. Our reports have demonstrated that PIs induced the accumulation of abnormal proteins and apoptosis [7]. The molecular mechanism of the PIs-mediated anti-cancer effect has been considered to be due to down regulation of the nuclear factor-κ B (NFκB) and the Akt activity, accumulation of proapoptotic Bel-2 family members, and degradation of p53 and p27 [13, 14].

Autophagy is another protein degradative process which delivers cytoplasmic materials to the lysosome for degradation. Autophagy has been reported to play critical roles in adaptive responses to not only starvation but also other stresses, and to maintain cellular homeostasis, and cellular differentiation and development [15]. Autophagy is also involved in eliminating abnormal proteins [7, 16]. In addition, the relation between dysregulated autophagy and various kinds of diseases, including cancer, neurodegenerative diseases, infectious diseases, and metabolic diseases has been reported [17]. Autophagy induced by rapamycin has been shown to rescue hepatoma culture cells from PIs-mediated apoptosis via decreasing abnormal proteins [7]. In addition, sorafenib has been reported to induce autophagy and affect tumor cell viability [18]. Thus, modulation of autophagy may be an attractive therapeutic strategy for cancer.

Keratin phosphorylation by stress activated protein kinases

Keratins are intermediate filament-forming proteins and epithelial cells express type I and type II keratins that form obligate non-covalent heteropolymers [19, 20]. Adult hepatocytes express the K8 and K18 pair which protects hepatocytes from a variety of stresses, not only mechanical stresses but also non-mechanical stresses [19]. The effect of K8/K18 in protecting hepatocytes from apoptosis has been demonstrated in several keratin-related genetic mouse models. The important keratin phosphorylation sites, such as K8 Ser23/Ser73/Ser431 and K18 Ser33/Ser52, have been demonstrated [21]. In particular, phosphorylation of K8 Ser73 is considered to be associated with several functions, including keratin filament reorganization and cellular stress reduction serving as a phosphate sponge. Under stress conditions, stress-activated protein kinases, JNK and p38, are activated and induce apoptosis. Phosphorylation of K8 Ser73 is beneficial to protect hepatocytes from apoptosis, serving as a stress-activated protein kinase substrate [9].

Cellular stress and Mallory-Denk body formation

Mallory body was first described by Prof. Frank B. Mallory as cytoplasmic hepatocyte hyaline inclusions, and then renamed ”Mallory-Denk body” to honor the contributions of Prof. Helmut Denk [22]. Mallory-Denk bodies (MDBs) are found in several liver diseases, including alcoholic liver disease, NASH, primary biliary cirrhosis, Wilson disease and HCC. Accumulation of misfolded proteins in cytoplasm induces MDB-like inclusion body formation composed of K8/K18, ubiquitin, heat shock proteins and p62 [7]. PIs induce MDB formation via the accumulation of ubiquitinated abnormal proteins in cytoplasm [7, 8]. Recent studies demonstrated that MDB formation requires K8 phosphorylation [23], K8 overexpression state [24, 25] and keratin cross-linking by transglutaminase-2 (TG2) [26]. Although the significance of MDB formation has not been fully elucidated, MDB formation is considered to protect hepatocytes from cellular stress by sequestering abnormal proteins [22]. MDBs are related to disease severity in alcoholic liver diseases and NASH [27] and clinical decompensation and progression to cirrhosis in chronic hepatitis C [28].

How sorafenib affects the cytoprotective mechanisms

Our study demonstrated that sorafenib inhibited protein ubiquitination, UPR and keratin phosphorylation in hepatoma cells, and also inhibited enhanced cell death in combination with PIs (Fig. 1) [29, 30]. Because previous studies have reported that phosphorylation by some kinases is associated with protein ubiquitination [31], we considered that sorafenib inhibited the kinases related to protein ubiquitination. Activation of the ER stress sensors, PERK and IRE1α, and the UPR associated proteins, elf2α which is downstream of PERK and JNK which is downstream of IRE1α, requires phosphorylation, so sorafenib was
considered to inhibit the UPR by inactivating the UPR sensors and associated proteins. In addition, our data demonstrated that sorafenib induced autophagy. Because autophagy induced by rapamycin decreases abnormal proteins and apoptosis induced by PIs [7, 8], we examined the effect of autophagy inhibition by using 3-methyladenine, an inhibitor of autophagic/lysosomal protein degradation and knocking down by Beclin1 siRNA. Inhibition of autophagy increased the PIs-mediated cell death induced by sorafenib. However, sorafenib inhibited protein ubiquitination and UPR induction in an autophagy-independent manner.

We also demonstrated that sorafenib inhibited phosphorylation of K8 and K18 at the major phosphorylation sites, including K8 Ser73, K18 Ser33 and K18 Ser52, in hepatoma cells [29]. In addition, sorafenib inhibited PIs-induced MDB-like inclusion body formation. As described above, keratin phosphorylation is very important for cytoprotection from various stresses. Therefore, inhibition of keratin phosphorylation was considered to be one of the cytotoxic effects of sorafenib, in particular when co-treating with other drugs including PIs.

Of note, sorafenib-induced inhibition of cellular defense mechanisms, UPR and keratin phosphorylation, and enhancement of PIs-mediated cell death were also shown in hepatocyte derived cells. Therefore, sorafenib is useful in the treatment for HCCs, although we must be careful of severe liver injury as an important adverse effect of sorafenib, especially in combination with other agents.

**Fig. 1. The scheme of the effect of sorafenib and PIs on cellular defense mechanisms.** PIs induce accumulation of abnormal proteins and endoplasmic reticulum (ER) stress, and subsequently induce apoptosis. Sorafenib inhibits both unfolded protein response and keratin phosphorylation and enhances the proteasome inhibitors (PIs)-mediated cell death. PI: proteasome inhibitor, ER: endoplasmic reticulum, K8: keratin 8, K18: keratin 18, MDB: Mallory-Denk body.
Conclusions

The UPR and keratin phosphorylation are very important mechanisms in cellular defense against ER stress. Our study revealed that sorafenib inhibits these cellular defense mechanisms and enhances cytotoxicity in combination with PIs in hepatoma cells. Because the prognosis of advanced HCC is still very poor, our data shows the potential to substantially increase the clinical activity of these agents and offers a new therapeutic strategy. Remarkably, sorafenib can also inhibit cellular defense mechanisms in hepatocyte derived cells, although we must be careful of severe liver injury as severe side effect.

Conflict of Interest

There is no conflict of interest (COI) status to disclose.

References

分子標的薬によるストレス防御機構の阻害を介した肝細胞癌に対する新たな治療戦略

本間 雄一, 原田 大
産業医科大学 医学部 第3内科学講座

要 旨: 非切除進行肝細胞癌の予後は依然として不良であるが、分子標的薬であるソラフェニブの承認により生命予後の改善を認めている。しかし、ソラフェニブの肝癌細胞への直接的な分子作用など、その詳細な機序については不明である。ソラフェニブの臨床効果は十分とは言えず、重篤な副作用も報告されていることから、その詳細な分子機序の解明が重要である。小胞体ストレスは肝細胞癌を含めた様々な肝疾患の病態に関与する。Unfolded protein responseとケラチンのリン酸化は、小胞体ストレスに対する細胞防御機構として重要である。ソラフェニブは小胞体ストレスに対する細胞防御機構として重要なUnfolded protein responseやケラチンのリン酸化を抑制し、プロテアソーム阻害薬との併用で抗腫瘍効果を増強させることが示された。本稿では進行肝細胞癌に対する新たな治療戦略として、小胞体ストレスに対する細胞防御機構と分子標的薬の作用について概説する。

キーワード: ソラフェニブ、小胞体ストレス、小胞体ストレス応答、ケラチン、オートファジー。