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Review Article

Interactions between Hsp90 and oncogenic viruses: implications for viral cancer therapeutics

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Abstract: Oncogenic viruses are the etiologic agents for a significant proportion of human cancers, but effective therapies and preventative strategies are lacking for the majority of virus-associated cancers. Targeting of virus-induced signal transduction or virus-host protein interactions may offer novel therapeutic strategies for viral cancers. Heat shock protein 90 (Hsp90) is a well-characterized, multifunctional molecular chaperone involved in regulation of signal transduction, transcriptional activation, oncogenic protein stabilization, and neovascularization—pathogenic elements relevant to viral cancer pathogenesis. This review will summarize mechanistic concepts involving regulation of viral oncogenesis by both intracellular and extracellular Hsp90, as well as current therapeutic implications of these data.

Keywords: KSHV, heat shock protein, signal transduction, cancer, viruses

Introduction

Molecular chaperones were initially discovered as mediators of the cellular heat shock response [1], and subsequent studies have demonstrated promiscuous functions for these proteins, including those related to both cancer and virus-associated pathogenesis [2-4]. Heat shock protein 90 (Hsp90) is an evolutionarily conserved chaperone that has been linked to the maturation of over 200 proteins [5, www.picard.ch/downloads/Hsp90interactors.pdf]. Hsp90 functions in concert with heat shock protein 70 (Hsp70) and other co-chaperones to prevent misfolding, thereby ensuring proper conformation and function of its client proteins [6,7]. Studies have also demonstrated an important role for Hsp90 in the protection of oncoproteins from the cellular editing machinery [8], and Hsp90 is a pivotal co-factor for cell signaling [9], neovascularization [10], chromatin remodeling and transcriptional regulation [9,11,12].

Hsp90 association with the cell surface and Hsp90 secretion, which we refer to collectively as extracellular Hsp90, have also been recently characterized [13]. The precise mechanisms by which Hsp90 arrives in the extracellular compartment are poorly understood, although exosomal secretion is likely involved [14,15]. Hsp90 secretion is also initiated by cell stress and various growth factors [14,16] and may be regulated by post-translational modification of Hsp90 [17]. Published data indicate that Hsp90 association with the cell surface is pivotal for cell migration in wound healing [14] and cancer cell metastasis [13,18].

It is estimated that approximately 20% of cancers worldwide are caused by viruses [19] including the Hepatitis B virus (HBV), Hepatitis C virus (HCV), Epstein-Barr virus (EBV), Human T-cell Leukemia virus (HTLV), Kaposi's sarcoma-associated herpesvirus (KSHV), and Human papillomavirus (HPV). Viral cancer pathogenesis is dependent upon expression of viral oncogenes and virus-induced inflammation that promotes cell transformation, migration, and angiogenesis in the microenvironment [20,21]. Other than the demonstrated efficacy for HPV and HBV vaccines in the prevention of infection by

oncogenic virus, effective treatments and preventative strategies are lacking for the majority of cancers caused by viruses [21]. Therefore, a better understanding of virus-induced cancer pathogenesis is needed to facilitate development of targeted therapies for viral tumors.

Hsp90 regulation of the cellular stress response, signal transduction, oncogene transcription and expression, stabilization of oncogenic protein structure, and neovascularization has clear relevance for virus-host interactions and viral oncogenesis. This review attempts to summarize the relevant literature addressing the role of Hsp90 in viral oncogenesis and the potential utility of Hsp90 inhibitors for the prevention and treatment of virus-associated malignancies.

Hsp90 regulation of gene expression and replication of oncogenic hepatitis viruses

Increasingly, data support a role for Hsp90 in facilitating gene expression and replication for HBV and HCV [22,23]. HBV, a DNA virus and member of the hepadenavirus family, infects over 300 million people worldwide and remains a common cause of cirrhosis and hepatocellular carcinoma (HCC) [24,25]. Unique from other DNA viruses, HBV utilizes reverse transcription during its replication cycle. To accomplish this, HBV encodes a polymerase with intrinsic reverse transcriptase, DNA polymerase, and RNAaseH activity (the term "polymerase" is often used synonymously with "viral reverse transcriptase" in the literature when referring to this protein for HBV; in this review, we will refer to the viral reverse transcriptase, or vRT, when referencing literature related to this protein) [22]. Initiation of reverse transcription requires the binding of the vRT to a pregenomic RNA template [22], and Hsp90, along with its co-chaperones Hsp40, Hop, p23 and Bag-1 (collectively referred to as the "Hsp90 machinery," including Hsp70), facilitates this process [22,26]. Importantly, proteomics and immunohistochemical analyses reveal that expression of Hsp90, Hsp70, and Hop in HBV-associated HCC lesions correlates directly with tumor progression [27,28].

Initial studies using a duck hepatitis B virus (DHBV) model system suggested that Hsp90, in cooperation with p23, plays key a role in facilitating the binding of the vRT to the ε RNA sequence to initiate the "protein priming reaction" which refers to the formation of the Ribonucleoprotein complex (RNP)—a necessary step for initiation of reverse transcription [22,29]. The interaction of Hsp90 with the vRT is also important for RNA packaging, suggesting a prolonged interaction between Hsp90 and the vRT whereby Hsp90 maintains the association of the vRT with template RNA [29]. Supporting this concept, Hsp90 is found in extracellular DHBV virion fractions [29]. Subsequent studies focusing on interactions between Hsp90 and the vRT have identified additional proteins involved in this process. The Hsp90 machinery allows the vRT to maintain a conformation capable of binding the ε RNA sequence and initiating the protein priming reaction. Some reports indicate that Hsp70 and Hsp40 are indispensable for this role, while Hsp90, Hop, p23 and Bag-1 appear to serve more ancillary roles in accelerating kinetics of the protein priming reaction [30-33]. However, controversy exists regarding the relative contributions of Hsp90, Hsp70 and Hsp40 for efficiently stimulating binding of the vRT to the ε RNA and subsequent protein priming compared with other chaperones [31-34]. Consequently, the interplay of cellular chaperones in this process is still not well defined. The Hsp90 machinery also plays a role in vRT folding by helping to overcome auto-inhibitory sequences within the protein [35]. One proposed model, therefore, is that the Hsp90 machinery complexes with the vRT early in the replication cycle to ensure correct folding of the protein as it matures, after which Hsp90 facilitates binding of the vRT to the ε RNA sequence by maintaining the polymerase in the correct binding conformation.

The development of an efficient *in vitro* model system for HBV has exposed some critical differences between HBV and DHBV virology, especially with regard to the role of Hsp90. Analogous to DHBV, early characterization of Hsp90 and HBV interactions suggest that both the amino- and carboxy-terminus of Hsp90 interact independently with specific domains of the HBV vRT, and these interactions appear necessary for the generation of infectious virions [36-38]. Despite one contradictory report [39], these interactions appear to be important for vRT binding to ε RNA for HBV as well [40], and Hsp70 and Hsp40 may be involved in this process [38,40]. However, using similar methods employed in DHBV-based experiments [35], one

report suggests that elements of the Hsp90 machinery facilitate HBV vRT binding to ε RNA, but not protein priming [40], implicating other cellular factors in protein priming and replication for HBV. Supporting the latter, proteomics analysis of a cell line utilized for generating infectious HBV identified additional Hsp90-associated factors [38]. Hsp90 also appears critical for the assembly of the HBV core protein where it may form a complex with the HBV vRT and core protein before their incorporation into the viral nucleocapsid [41]. In summary, while similar to the role of Hsp90 in DHBV replication, the role of Hsp90 in the HBV replication cycle is a more complicated process that may require other cellular factors besides chaperones. Regardless, these data underscore the importance of the Hsp90 machinery for vRT interactions with ε RNA and protein priming necessary for replication, RNA packaging, and nucleocapsid formation. Interestingly, HBV up-regulates Hsp90 expression via c-Myc activation [42], and Hsp90 influences c-Myc induced cell transformation [43], leading to speculation that Hsp90 upregulation may be another means though which HBV induces oncogenesis and tumor progression.

HCV, an RNA virus and member of the Flaviviridae family, is also a common cause of cirrhosis and HCC worldwide [44-46]. Following viral entry, HCV RNA is translated by cellular ribosomes to form a polyprotein that is cleaved by both cellular and viral proteases to create functional viral proteins, after which the virus generates the RNA template for replication [47-49]. Hsp90 plays a critical role in the cleavage and activation of at least two HCV proteins, NS2 and NS3 [50], although the precise mechanisms through which Hsp90 coordinates this process is unknown. One potential mechanism involves direct interaction between Hsp90 and NS3, thereby protecting NS3 from proteasomal degradation [51]. The prevention of proteasomal degradation of viral proteins by Hsp90 is at least partially mediated through circumvention of the "unfolded protein response"— the process by which cells degrade unfolded or misfolded proteins [51,23]. Hsp90 also associates with HCV-encoded NS5A, a key step for initiation of viral replication, through a joint interaction with several host proteins [52,53]. As with HBV, Hsp90 requires other cellular proteins to facilitate regulation of HCV replication, with at least one of these proteins functioning as a p23 analog or co-chaperone [54,23].

Although HBV and HCV exhibit considerably different replication cycles, these studies reveal conserved roles for Hsp90 in hepatitis virus biology, including interaction with viral proteins to ensure their correct processing and folding, and participation in the replication complex. During the evolution of hepatitis viruses, the hijacking of cellular machinery for replication has, therefore, led to a dependence on properly functioning host chaperone proteins for viral propagation—a finding that could potentially be exploited for therapeutic benefit.

Hsp90 regulates signal transduction induced by oncogenic viruses

Oncogenic viruses hijack cell signaling pathways, in part through expression of unique oncogenes which either mimic endogenous cellular oncogenes or inactivate tumor suppressor genes [21]. Hsp90 regulates transcription of cellular genes in non-viral malignancies [9,11,12], and existing data support a similar role for Hsp90 in transcriptional regulation of viral genes that regulate cell signaling. The gamma-herpesvirus EBV is linked to a variety of cancers including NK/T-cell lymphoma (NKTL), Burkitt's lymphoma, nasopharyngeal carcinoma, and diffuse large B-cell lymphoma [20,21]. Like other herpesviruses, EBV establishes latent infection wherein expression of several viral genes, such as latent membrane protein-1 (LMP-1) and Epstein-Barr nuclear antigen (EBNA), is critical for the maintenance of viral latency and cell transformation [55,56]. EBNA is critical for EBV replication and propagation given its roles in tethering the viral particle to the host chromosome, mediating viral episome replication and packaging during cell division, and inhibiting apoptosis [57-60,55]. LMP-1 function as a constitutively active CD40 mimicker, resulting in stimulation of cell proliferation following NF-κB activation and the inhibition of apoptosis through overexpression of BCL-2, MCL-1, and BCL-2 related proteins [61,21].

Hsp90 plays a multifactorial role in EBV-associated cancers, as it modulates cell signaling and viral gene expression [62,63]. During initial EBV infection and transformation of B-cells, Hsp90 expression is induced and transformation is Hsp90-dependent [64]. In addition, Hsp90 is necessary for suppression of apop-

tosis for EBV-infected NK/T lymphoma cells through promotion of LMP-1-induced Akt phosphorylation [62]. Recently published studies also indicate that Hsp90 plays a novel role in the modulation of EBNA1 expression for a variety of EBV-positive cell lines [63]. Of note, Hsp90 mediates the translation of EBNA1 through modification of a specific amino acid motif within the protein. Exactly how this occurs is unknown, as direct association of Hsp90 with EBNA1 is not necessary for this function [63].

Kaposi's sarcoma-associated herpesvirus (KSHV), another human gamma-herpesvirus most closely related to EBV, is the causative agent for three clinical entities: Kaposi's sarcoma (KS) [65], Primary effusion lymphoma (PEL) [66], and Multicentric Castleman's disease (MCD) [67]. Central to KSHV pathogenesis is its manipulation of cell signaling following viral gene expression. Two genes important for KSHV oncogenesis are the viral FLICE inhibitory protein (VFLIP) and K1 [68,69]. vFLIP regulates proliferation and apoptosis of cells through its ability to inhibit procaspase-8 cleavage following Fas-Fas ligand interactions [70]. In addition, vFLIP modulates constitutive NF-κB activation [71,72] to promote survival of KSHV infected PEL cells [73] and latent viral gene expression [74]. Hsp90 complexes with vFLIP and the IKK complex, and in this capacity serves as a co-factor for KSHV activation of NF-κB [75]. KSHV-encoded K1 is a transmembrane glycoprotein which activates the PI3K/Akt pathway and inhibits CD95-mediated apoptosis [76,77]. Underscoring the designation of K1 as an oncogene, ectopic K1 expression induces cell transformation in isolation [78]. Hsp90 directly associates with K1 and facilitates its expression [79]. Furthermore, Hsp90, in association with Hsp40, is critical for K1 modulation of cellular anti-apoptotic functions [79].

HTLV is an oncogenic retrovirus whose infection of T-cells may result in their malignant transformation [21]. Similar to KSHV-encoded vFLIP, the HTLV-encoded protein Tax plays a major role in the ability of the virus to induce growth and transformation of T-cells via chronic stimulation of NF-κB activation through activation of IKK [80-82]. Tax requires the presence of Hsp90 in the IKK complex for prolonged activation of NF-κB and long-term survival of HTLV transformed cells [83-85]. Hsp90 may also regulate HTLV activation of AP-1- and Akt-dependent signaling pathways important for cell proliferation [85].

Extracellular Hsp90 regulates signal transduction induced by oncogenic viruses.

When used in published literature, the phrase "extracellular Hsp90" (eHsp90) refers to either cell surface-associated Hsp90 (csHsp90) or Hsp90 secreted into the extracellular space. eHsp90 was only recently identified with the advent of experimental tools for isolating its role in cancer pathogenesis from its intracellular counterpart. Studies have implicated eHsp90 in cell migration [14,16,86] and cancer metastasis [13]. Expression of eHsp90 also correlates with melanoma metastasis [87] and breast cancer invasiveness [88], possibly through matrix metalloproteinase (MMP)-dependent interactions [13]. Furthermore, eHsp90 is critical for Her-2 signaling by maintaining the receptor in an activated state, suggesting that it may regulate signal transduction initiated by extracellular signals [88].

Burgeoning data suggest a role for eHsp90 in viral cancer pathogenesis, especially for the two human oncogenic gamma-herpesviruses—KSHV and EBV. We demonstrated that csHsp90 is critical for KSHV activation of mitogen-activated protein kinase (MAPK) signaling following viral entry and establishment of latent viral gene expression [89]. As mentioned previously, NF-κB plays a critical role in KSHV pathogenesis, including secretion of pro-angiogenic factors and invasiveness for KSHV-infected cells [74]. We recently found that csHsp90 regulates KSHV-induced, NF-κB-mediated endothelial cell invasiveness and secretion of pro-angiogenic factors [90]. These results support those of previous studies suggesting a role for eHsp90 in NF-κB-dependent invasiveness for other tumor cell types [91]. Another recent report indicates that eHsp90-CD91 interactions are important for regulation of Ephrin receptor A2 (EphA2) signaling with downstream effects on cell invasiveness [92]. Therefore, we hypothesize that EphA2 may also be important in csHsp90 regulation of invasion following *de novo* KSHV infection.

eHsp90 may also play an important role in the immune response to virus-infected cells and virus-associated tumors. Existing data support a role for eHsp90, via interactions with CD91 and other cellular receptors, in both adaptive and

innate immune responses [93-95]. Furthermore, one study has demonstrated the importance of eHsp90-CD91 interactions for cross presentation of viral antigens by dendritic cells during the immune response to KSHV [96]. EBV infection of B-cells promotes expansion of gamma-delta T-cells via upregulation of Hsp90 [97], and gamma-delta T-cells are important in the immune response to KSHV infection [98]. It follows that augmentation of Hsp90 secretion, or csHsp90 interactions with protein clients at the cell surface, may enhance specific immune responses to KSHV-infected tumor cells.

Targeting Hsp90 for suppression of viral oncogenesis

There are currently thirteen Hsp90 inhibitors in clinical trials for a variety of cancers [3]. Hsp90 inhibitors have also shown activity against a wide range of viruses [99,100]. Interestingly, viral resistance to Hsp90 inhibitors is uncommon [101]. These data rationalize additional studies to determine the utility of Hsp90 inhibitors for suppressing viral cancer pathogenesis.

Inhibition of Hsp90 prevents release of HBV particles *in vitro*, likely through the prevention of pregenomic RNA packaging and inhibition of core protein assembly [40,41]. Newer generation Hsp90 inhibitors that demonstrate more favorable toxicity profiles than the first generation inhibitor, geldanamycin (GA), also demonstrate antiviral activity for HBV [100].

A better understanding of HCV biology and the development of treatment strategies were initially hindered by difficulties in developing an efficient cell culture model [102]. The development of HCV replicon systems (both genomic and subgenomic) provided useful insight into HCV biology [103,104], including validation of Hsp90 as a viable drug target for HCV. Initial reports demonstrated inhibition of HCV replication by GA and its derivatives, possibly through disruption of an FKBP8/NS5A/Hbind1/Hsp90 complex [52,54,23]. In addition, 17-AAG, a second-generation GA derivative, showed marked inhibition of HCV replication in two different HCV replicon models wherein a significant reduction in NS3 protein levels (but not NS5A levels) were noted [51]. This suggests that the inhibitory effects of Hsp90 targeting were likely due to effects on NS3 stability in this model [51]. These results have been validated using GA derivatives and RNA interference targeting Hsp90 [105]. However, in studies using immunodeficient mice transplanted with humanized livers, Hsp90 inhibitors show effectiveness only when combined with interferon-alpha therapy [105]. When combined with proteasome inhibition, Hsp90 inhibitors also suppress intracellular HCV RNA replication at much lower concentrations (for both agents) than when tested alone [51]. Recent reports using newly synthesized GA derivatives indicate that these conpounds may retain anti-HCV activity while exhibiting reduced toxicity [106]. In one report, inhibition of Hsp90 also blocked HCV replication in treatment-resistant replicon cells, suggesting that Hsp90 may be a useful target for drug-resistant HCV strains [107]. These results support Hsp90 inhibition as a viable strategy for inhibiting HCV replication and HCC pathogenesis, although it is important to once again note that the majority of these studies were done using replicon systems that are unable to support HCV virion assembly and release. Additional *in vivo* data are, therefore, needed to determine whether Hsp90 inhibition, alone or in combination with existing agents, is effective in the prevention of HCV replication and pathogenesis.

In vitro studies have demonstrated initial promise for Hsp90 inhibition in the abrogation of EBV pathogenesis for a variety of EBV-associated cancer cell lines [62,63,108]. Initial studies using GA demonstrated that Hsp90 inhibition induces apoptosis for EBV-associated NK/T-cell lymphomas, possibly through inhibition of virally mediated Akt activation [62]. Validating these results, treatment of EBV-infected cell lines with 17-AAG, especially when combined with rapamycin, induces cell death [108]. Furthermore, the GA derivative 17-DMAG induces apoptosis in EBV-immortalized lymphoblastoid cell lines, and 17-AAG significantly suppresses EBV-induced lymphoproliferative disease in SCID mice [63,109]. Of clinical relevance, immunohistochemistry and proteomics analyses of tumors from patients with EBV-associated post transplant lymphoproliferative disorder (PTLD) demonstrate a marked upregulation of Hsp90 relative to control tissue [108].

As mentioned above, Hsp90 serves as a cofactor promoting expression of KSHV genes relevant for cell cycle progression and anti-apoptotic signaling [75,79]. Hsp90 inhibition reduces proliferation of KSHV-infected PEL cell lines *in vitro,* possibly through inhibition of viral

gene expression [75,79]. The involvement of Hsp90 in promoting constitutive signaling through several pathways in PEL cells would suggest, however, that multiple mechanisms responsible for PEL proliferation are suppressed with Hsp90 inhibition. As with KSHV-associated tumors, Hsp90 inhibition also prevents proliferation and induces apoptosis in HTLV-infected cell lines and freshly isolated adult T-cell lymphoma (ATL) cells through inhibition of several cell signaling pathways [84,85]. Additional *in vivo* studies are needed to establish the putative clinical utility of Hsp90 inhibitors for the treatment of both KSHV- and HTLV-associated diseases.

Inhibition of csHsp90 may provide a more desirable targeted approach than inhibition of intracellular Hsp90 for the treatment of viral cancers because of its reduced toxicity profile [89], perhaps related to csHsp90 regulation of a more restricted subset of signaling pathways relative to intracellular Hsp90 [13]. KSHV is the only oncogenic virus to date for which inhibition of csHsp90 has been evaluated as a potential therapeutic approach [89,90]. These studies indicate that csHsp90 inhibition suppresses KSHV-induced gene expression and cell invasiveness following *de novo* infection, supporting csHsp90 inhibition as a therapeutic approach for either KS or PEL.

Conclusions

Many human cancers are caused by viruses, and effective treatment and prevention strategies are lacking for most of these cancers. The essential role that Hsp90 plays in oncogenic viral gene expression, replication and downstream pathogenesis suggests that oncogenic virus evolution in the human host has created a dependency on cell chaperones for the survival of cells infected by these pathogens. Whether this concept can be exploited for therapeutic benefit requires additional characterization of the effects of Hsp90 inhibitors using *in vivo* models of viral cancer pathogenesis.

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