Proteases and HPV-Induced Carcinogenesis

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Contributor: Gabriel Viliod Vieira , Fernanda Somera dos Santos , Ana Paula Lepique , Carol Kobori da Fonseca , Lara Maria Alencar Ramos Innocentini , Paulo Henrique Braz-Silva , Silvana Maria Quintana , Katiuchia Uzzun Sales

Human papillomavirus (HPV) infection is a sexually transmitted disease with high prevalence worldwide. Although most HPV infections do not lead to cancer, some HPV types are correlated with the majority of cervical cancers and with some anogenital and oropharyngeal cancers. Moreover, enzymes known as proteases play an essential role in the pathogenic process in HPV-induced carcinogenesis.

HPV carcinogenesis proteases cervical cancer

1. Introduction

Viral infection in humans leads to a wide variety of diseases, such as smallpox, polio, and measles [1]. Some have caused recent significant pandemics, such as H1N1 influenza in 2009 and the SARS-CoV-2 coronavirus in 2020 [2][3]. Some viruses can increase the host cell's lifespan and deregulate critical signaling pathways through the activation of oncogenes and/or the suppression of tumor suppressor genes [4]. Such viruses are classified as carcinogenic to humans (group 1) by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) [5][6]. At least seven viruses are related to cancer development in humans: Epstein–Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), human T-cell lymphotropic virus (HTLV-1), Kaposi's sarcoma-associated herpesvirus (KSHV), and Merkel cell polyomavirus (MCV or MCPyV); they contribute to 10–15% of cancers worldwide [5][7][8]. These viruses, known as tumor viruses, induce changes in cellular functions that ultimately lead to cancer development [4][9].

The transformation of a healthy cell into a tumor cell is a complex, multi-step process [4]. During the carcinogenic process, the malignant cell suffers genetic and epigenetic modifications that are selected and expressed as capabilities known as the hallmarks of cancer: genome instability and mutation, resistance to cell death, the deregulation of cellular energetics, sustained proliferative signaling, the evasion of growth suppressors, the avoidance of immune destruction, the enabling of replicative immortality, tumor-promoting inflammation, and the activation of invasion and metastasis, inducing angiogenesis [9][10][11][12].

Although oncoviruses can participate in oncogenesis, they are not sufficient for the development of cancer, and inflammation, host immune response and environmental conditions are also involved in this process [4][5][10][13].

Papillomaviruses are epitheliotropic, small, double-stranded DNA viruses that infect the mucosa or the skin of many animals' species mucosa [14][15]. Although more than 200 genotypes can infect humans, only 12 HPV genotypes with carcinogenic properties—classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC)—are known [6][16][17]. Mucosal transmission occurs mainly by sexual contact [17][18]. However, other transmission routes are also known [18]. Studies have demonstrated the vertical transmission of HPV from mother to fetus, as well as the presence of HPV viral DNA in breast milk, amniotic fluid, the umbilical cord, and the placenta [19][20][21]. Newborns can also become infected through skin-to-skin contact with other relatives, as well as oral lesions related to HPV infection, such as oral squamous papilloma, condyloma acuminatum, verruca vulgaris, and multifocal epithelial hyperplasia; genital HPV infection in children is a warning sign for sexual abuse [22][23][24][25].

HPV viruses are divided into five genera according to the sequence of their genotype, known as α, β, γ, μ, and ν [26]. The HPV alpha and gamma groups infect skin and mucosal tissue, whereas the beta-, nu- and mu-subtypes infect cutaneous sites, even without clinical manifestations [27][28]. All of the 12 HPV genotypes that are classified as group 1 carcinogens belong to the alpha genus: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 [29]. Indeed, alpha-HPVs are transmitted through sexual contact, and can be considered the leading group of causative agents of sexually transmitted infections globally [28]. Moreover, their incidence rises sharply after the first sexual intercourse [30]. As the natural immune response to this virus is weak and variable, one person may acquire different types of HPV infections [14]. Low-risk alpha HPV types causing benign genital warts or condylomata acuminata, as well as common and plantar warts, are also found [29].

2. HPV Carcinogenesis

HPV infection has been linked to several malignancies, such as cervical carcinoma, female and male anogenital carcinomas (vulvar, vaginal, anal, and penile), and head and neck squamous cell carcinomas (HNSCCs) [14]. In these anatomical sites, however, the behavior of HPV is less understood, as the prevalence is lower than that of cervical carcinomas [14].

According to the type of lesion they generate, they are subdivided into low-risk HPVs and high-risk HPVs [28]. Low-risk HPVs are associated with the development of warts and benign lesions, while high-risk HPVs are associated with precancerous and cancerous lesions [31]. HPV16 and HPV18 stand out for their greater capacity to lead to cancer development; they account for approximately 60% and 15% of cases of invasive cervical cancer worldwide, respectively [14].

The HPV genome can be divided into two types of genes: (i) early genes (from E1 to E7), which are responsible for viral genome gene expression and replication, and which also modulate host cell proliferation and differentiation [32][33], and (ii) late genes (L1 and L2) which are responsible for the formation of viral capsid [34][35][36]. During infection, the viral genome may integrate into the host cell genome. When found in cancer cells, which occurs in most cases, the viral genome is disrupted in the E1/E2 region. E2 is a transcription factor that binds to the HPV LCR (Long Control Region) and maintains the weak transcriptional activity of the promoter. If E2 expression is lost, other

transcription factors bind to the LCR and increase the expression of E6 and E7, which are two bona fide oncogenes present in the HPV genome. This event is important for cellular immortalization and transformation by HPV [37][38][39]. Persistent infection is a major risk factor that increase HPV genome integration.

Molecular events caused by infection and, in some cases, cellular transformation will cause lesions classified histopathologically as cervical intraepithelial neoplasia (CIN) and further to cancer, which is then sub-classified as CIN 1—mild dysplasia, CIN 2—moderate dysplasia, or CIN 3—severe dysplasia to carcinoma in situ [40][41].

The main orchestrators of cellular transformation by HPV are the oncoproteins E6 and E7. They inactivate p53 and retinoblastoma protein (pRB), respectively, leading to the cell's inability to control the cell cycle checkpoints correctly, and thus exacerbating cell proliferation [38]. Significantly, the E7-dependent inhibition of pRB leads to the cell cycle S phase transition, promoting cell proliferation and viral transcription [42][43]. Another critical aspect of E7 is that it binds to p21 and p27, which are proteins belonging to the cyclin-dependent kinase (CDK) interacting protein/kinase inhibitory protein (CIP/KIP), and are involved in regulating the cell cycle, which increases cyclin-dependent kinase 2 (CDK2) activity, collaborating with the cell entering the G1 to S phase [44][45]. In a normal physiological condition, p53 would counteract the effects of exacerbated cell proliferation while activating the cellular DNA damage response (DDR) during viral DNA integration into the host genome, leading to the inhibition of cell growth and apoptosis [46][47]. However, HPV E6 inactivates p53 by targeting its proteasomal degradation and forming a complex with the E3 ubiquitin-protein ligase E6-associated protein, E6AP [48][49]. Moreover, it is also important to highlight the fact that high-risk E6 has been reported to bind the hTERT protein as well as the repeating DNA sequence of telomeric DNA, in addition to controlling telomerase activity [50]. High-risk E6′s role in hTERT, telomerase, and telomeric DNA is thus multilayered, emphasizing its crucial and overlapping role in immortalization [51].

Cells infected with HPV are able to stop infection by activating signaling pathways that result in the induction of anti-viral status and IFN type I secretion. However, both E6 and E7 display mechanisms of suppressing this response. Among other effects, E6 binds to IRF-3 (Interferon Response Factor-3), inhibiting its activity. E7 binds to IRF-1 and recruits HDAC (histone deacetylase) to the promoters that would be activated by IRF-1 but are suppressed due to E7 activity [52]. Moreover, E6 and E7 can inhibit the STAT-1 and protein kinase R (PKR) pathways in infected cells [53].

3. Proteases and HPV Carcinogenesis

Proteases, also known as peptide hydrolases, are found in all organisms (from viruses to vertebrates), and are classified as enzymes that can cleave peptide bonds [54][55][56][57]. There are more than 400 proteases described in humans, and more than 14% have the potential to serve as drug targets for a variety of diseases [58][59].

A critical aspect of the proteases is substrate specificity. Some proteases, such as trypsin, display broad specificity and are capable of cleaving many different substrates [60][61][62][63]. Other proteases, such as the urokinase-type plasminogen activator (uPA), are selective and cleave a limited number of substrates [57][63].

Proteases can be subdivided into two major groups: exopeptidases and endopeptidases [64]. Exopeptidases are known to hydrolyze the substrate chain's amino or carboxy terminal, and the conferred specificity is determined by the fragment size [65][66]. Endopeptidases are proteases that can cleave the amino acids that are non-terminal, and the classification relies on the chemical group present in the catalytic domain. Six classes of endopeptidases have been described: (i) cysteine proteases, (ii) aspartic acid proteases, (iii) threonine proteases, (iv) glutamic acid proteases, (v) serine proteases, and (vi) metalloproteases [67].

Proteases are known to participate in different physiological processes, from the degradation of proteins for recycling, to apoptosis, the cell cycle, skin desquamation, semen liquefaction, epithelial differentiation, the regulation of blood pressure, and homeostasis [68][69][70][71]. It is essential to highlight the facts that proteases are synthesized as inactive zymogens, and that they have to be cleaved to be activated, which can occur irreversibly through post-translational modifications, co-factors ligation, and changes in pH, among others [71]. Therefore, the activation of proteases is a very regulated process that prevents the uncontrolled activation of the enzymes in the cell [67][72]. The dysregulation of some proteases' expression can lead to the development of diseases, such as cancer. These enzymes are also involved in the degradation of the extracellular matrix and the activation of growth factors and pro-inflammatory mediators, which participate in malignant transformation and tumor progression [73].

Serine proteases are a family of proteases characterized by the amino acids responsible for the catalytic activity in proteolysis, which are serine, aspartate, and histidine [74]. This family of proteases is involved in different biological processes, such as epithelial barrier formation, skin desquamation, fertilization, embryonal development, cell signaling, and tissue morphogenesis [75].

As part of this family, there is a subgroup composed of membrane-anchored serine proteases, which is divided into (i) GPI—serine proteases that are anchored in the plasma membrane by a glycosylphosphatidylinositol anchor, (ii) Type I—serine proteases that have a single pass domain in the plasma membrane located close to the C-terminus end, and (iii) Type II—serine proteases that are anchored in the plasma membrane and have an anchor sign located close to the N-terminus end [71][76].

Matriptase, a type II transmembrane serine protease, is expressed in different epithelial tissues, such as the skin, gastrointestinal tract, lungs, kidneys, prostate, and mammary glands [77][78]. This protease is responsible for activating uPA (urokinase plasminogen activator), which is related to cell adhesion and migration regulation, and the activation of growth factors and metalloproteases zymogens [79][80]. Different studies have shown that when matriptase is less expressed, there is also less activation of uPA in the cells of ovary and prostate cancer [81][82]. Matriptase is also responsible for activating the PI3K-Akt-mTOR pathway after the proteolytic activation of the hepatocyte growth factor precursor (pro-HGF), which can promote cell proliferation and decrease apoptosis [76]. PAR-2 (protease-activated receptor 2), a receptor expressed in different cell types, is related to cell adhesion, the maintenance of the skin barrier, and inflammatory responses [83][84]. One study has shown that the absence of PAR-2 inhibited the appearance of premalignant lesions and spontaneous or induced carcinogenesis in mice that overexpress matriptase in the basal layer of the epithelia, which highlights the importance of PAR-2 activation by

matriptase in oncogenesis [84]. It has already been described that matriptase is dysregulated in different types of epithelial cancers and, more specifically, carcinomas [78][84][85][86][87][88][89].

Furthermore, matriptase is inhibited by the hepatocyte growth factor activator inhibitor-1 and -2 (HAI-1 and HAI-2), which are serine proteases inhibitors [90][91]. HAI-1 is a type I transmembrane serine protease inhibitor encoded by the SPINT1 gene. One study has shown that HAI-1 is a potent inhibitor of hepsin, matriptase, and prostasin in HPV-positive cells (SiHa and HeLa). In cervical tissue analysis, HAI-1 expression was correlated with higher rates of tumor growth, the stage of the disease, stromal invasion, vaginal invasion, and lymph node metastasis [92]. Moreover, patients exhibiting higher levels of HAI-1 exhibited decreased disease-free and overall survival [92]. Similarly, analyzing cervical cancer specimens and the biological functions of HPV-positive cell lines, findings have indicated that a lower expression of HAI-2 in cervical cancer may be correlated with poor prognosis as well [93].

Other subgroups of serine proteases are the ones that are secreted to the extracellular space, such as the kallikreins. Kallikreins are serine proteases that can be divided into (i) plasma kallikrein, with one member, KLKB1; and (ii) tissue kallikreins, with fifteen members, which have either tryptic or chymotryptic specificity [94]. KLK proteases are found in almost every tissue, with different physiological functions, such as skin desquamation and seminal clot liquefaction, related with various cancers, Parkinson's and Alzheimer's diseases [94]. The relationship between KLKs' activation and different types of viruses, such as influenza and HPV, has been described [95][96]. After HPV infection, the virus has to remove the capsid to expose the viral genome. The HPV16 virus can bind to heparan sulfate proteoglycans located in the host cell surface or the ECM [97][98][99]. After the binding, the late gene 1 (L1) undergoes a conformational change, which leads the protein to be cleaved by Kallikrein 8 (KLK8) [96]. Furthermore, the knockdown of KLK8 in HeLa and HaCaT cell lines has shown an inhibitory effect of HPV16 infection, while the irreversible serine protease inhibitor AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride] also had the same effect [100]. The conformational change caused after L1 cleavage by KLK8 facilitates access to late gene 2 (L2) protein, which is found in the capsid lumen and facilitates the uncoating of the virus [100]. In another study, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in cervical tissues, the researchers found that 95 proteins were dysregulated in the samples. Among those, the expression of ECM2 and the serine proteases KLK6 and MASP1 were increased in a stage-dependent manner. In particular, KLK6 was considered a highly significant prognostic marker, as it demonstrated a decrease in the overall survival (OS) and disease-free survival rates, showing that this protease may be considered a potential biomarker for the diagnosis and prognosis of cervical cancer [101].

Metalloproteases (MMPs) are critical enzymes which are responsible for the degradation of the extracellular matrix (ECM) [102]. These proteases participate in various physiological processes, especially in connective tissue remodeling, such as the postpartum involution of the uterus, ovulation, and wound healing, and in pathological processes such as joint destruction in rheumatoid diseases [103][104]. It is known that metalloproteases are secreted as zymogens, and that they are activated proteolytically. Metalloproteases are divided into their respective subfamilies: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-17), matrilysins (MMP-7 and MMP-26), membrane types (MMP-14, MMP-15, MMP-

16, MMP-24, and MMP-25), and other types (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-28, and MMP29) [66][105].

Studies of cervical tissues with cervical intraepithelial neoplasia and invasive squamous cell carcinomas (tested for HPV expression) have shown that the expression of MMP-2 in preinvasive lesions and MMP-1 and MMP-2 in invasive cancer suggests a gradual increase in the potential of cancer invasion [102]. Furthermore, analyses of the expression of MMP-1 in cell lines (transformed with HPV18) and in clinical samples of cervical squamous cell carcinomas (SCC) have shown that MMP-1 is more expressed in SCC samples when compared with normal tissues, and that this protein can serve as a marker of the invasiveness of SCC [106]. The production of MMP-9 was also up-regulated in cervical intraepithelial neoplasia (CIN 2 and 3) and in invasive carcinomas, which suggests a possible marker for early tumor progression [107].

The E6 HPV viral oncoprotein can interact with PDZ domains, which are 80–110 residue-containing domains that are part of the signaling proteins' C-terminal [108][109]. The serine protease HTRA-1, which contains a PDZ domain in its C-terminal region, is expressed in various tissues, and is also associated with different pathologies, such as some types of cancer [110][111][112][113]. One study has shown that the overexpression of the serine protease HTRA-1 is responsible for the prevention of cell proliferation in cervical HPV-negative cell lines and increasing cell proliferation in cervical HPV-positive cells, inferring that HTRA-1/E6 interaction is the underlying mechanism for the bypassing of growth arrest in HPV-positive cervical cancer cell lines [114].

Another important group of proteases that are related with HPV carcinogenesis are the Ubiquitin proteases. USP46 is recruited to deubiquitinate and stabilize Cdt2/DTL by the E6 of high-risk HPV but not low-risk HPV [115]. Cdt2—a component of the CRL4Cdt2 E3 ubiquitin ligase—is stabilized, which restricts the amount of Set8, an epigenetic writer, and promotes cell proliferation [115]. USP46 is required for HPV-transformed cells to proliferate, but not for non-HPV cells to proliferate [115]. Human cervical malignancies have a high level of Cdt2, and knocking down USP46 in xenografts stops HPV-transformed tumor growth [115]. Oncogenic E6 recruits a cellular deubiquitinase to stabilize critical cellular proteins, and because the E6-USP46-Cdt2-Set8 pathway is important in HPV-induced malignancies, USP46 is a target for cancer therapy [115]. Another study has shown that USP13 is essential for HPV-positive cervical cancer cells to proliferate, at least in part by deubiquitinating and stabilizing the prosurvival protein Mcl-1 [116]. Importantly, the pharmacological inhibition of USP13 sensitizes HPV-positive cervical cancer cells to BH3 mimetic inhibitors, implying that targeting USP13 could be beneficial in the treatment of these tumors [116]. Furthermore, another study discovered ubiquitin-specific protease 15 (USP15) as an HPV16 E6-interacting protein using the yeast two-hybrid technique [117]. HPV16 E6 polyubiquitin chains and/or ubiquitin precursors are cleaved by USP15, and could boost HPV16 E6 levels by preventing E6 degradation [117]. The degradation of HPV16 E6 was reduced by USP15 in a dose-dependent manner; these findings imply that USP15, as a deubiquitinating enzyme, can stabilize E6 and, as an oncoprotein, can influence biological activities in infected human cells [117]. Besides this, an important study highlighted that long-term hypoxia activates NF-κB, which is mediated via an effect of the HPV-encoded E6 protein on polyubiquitination and the subsequent degradation of the CYLD K63 deubiquitinase in HPV-positive cancer cells [118].

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