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Adenosine causes read-through into the late region of the HPV16 genome in a guanosine-dependent manner

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ABSTRACT

Adenosine plays an important role in cell death and differentiation as well as in tumorigenesis and the intra- and extra-cellular levels range from nanomolar to millimolar levels under various physiological or pathophysiological conditions. Here we report that adenosine can activate HPV16 late gene expression in a dose- and time-dependent manner, but only in the presence of guanosine. This activation occurred within hours after addition of the nucleosides and was primarily dependent on the ENT1 nucleoside transporter protein. Induction of HPV16 late gene expression was mainly the result of increased read-through at the early HPV16 polyadenylation signal into the late region of the HPV16 genome, thereby producing HPV16 late L2 mRNAs. The effect of guanosine and adenosine on HPV16 late gene expression was mediated by the increased binding to HPV16 mRNAs and nuclear export of the cellular HuR protein. Our results demonstrate that nucleosides can affect HPV16 gene expression.

1. Introduction

The papillomavirus family encompasses approximately 200 different human papillomaviruses (HPV), of which at least 12 have been termed high-risk types (Biological agents: a review of human carcinogens, I.A.R.C, 2012), defined as HPVs that are cancer-associated (zur Hausen, 2002). The most common high-risk HPV type is HPV16 (Schiffman et al., 2016; Walboomers et al., 1999). Long-term persistent infections with high-risk HPV types such as HPV16 may give rise to premalignant lesions on the uterine cervix in infected women. If such lesions are deemed to be of high-grade type, they represent true risk factors for cancer progression and should be treated (Chow et al., 2010). In contrast, HPV infections that are cleared within a year or two constitute no risk for cancer development. During the long-term persistence, HPV16 continuously expresses the HPV E6 and E7 genes. The E6 and E7 proteins drive cell proliferation, prevent apoptosis and cause genetic instability (Roman and Munger, 2013; Vande Pol and Klingelhutz, 2013). These effects of E6 and E7 increase the risk of mutations in the cellular genome that may cooperate with the HPV infection to cause cancer (Mighty and Laimins, 2014). In general, all HPV16 driven cancers are dependent on the continuous production of the HPV16 E6 and E7 proteins. At the same time, the highly immunogenic late viral structural proteins L1 and L2 are invariably suppressed in cancer cells to escape the immune surveillance of the host (Doorbar et al., 2012). The HPV16 life cycle is strictly linked to cell differentiation and entry into the late stage of the viral life cycle requires terminal cell differentiation (Doorbar et al., 2012; Hong and Laimins, 2013; Mighty and Laimins, 2014). Activation of HPV16 late gene expression is strictly regulated and includes activation of the HPV16 late promoter, inhibition of the HPV16 early polyadenylation signal and activation of HPV16 late splice sites (Graham and Faizo, 2017; Jia and Zheng, 2009; Johansson and Schwartz, 2013). A large number of alternatively spliced HPV16 mRNAs is generated (Johansson and Schwartz, 2013) and the presence of RNA regulatory elements on these mRNAs (Wu et al., 2017) and the interactions of these elements with cellular and viral regulatory proteins determine the relative levels of each mRNA (Kajitani and Schwartz, 2015). Thus, an orderly HPV16 gene expression depends on a strict control of HPV16 RNA processing.

Adenosine plays a key role in the control of various physiological processes and consequently has also been implicated in pathophysiological conditions including cancer (Antonioli et al., 2013; Burnstock and Di Virgilio, 2013). Human cells have an intracellular concentration of 1–10 mM of ATP whereas the adenosine concentration in the extracellular space normally varies between 20 and 300 nM. However, the concentration of adenosine inside and outside cells vary widely and may change quickly in response to physiological or pathophysiological conditions. For example, the millimolar levels of ATP inside cells may be dephosphorylated to adenosine. Millimolar levels of ATP may also be released into the extracellular space under conditions of tissue stress or trauma, or as a result of regulated release. The millimolar levels of ATP

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that enter the extracellular space are quickly converted to adenosine by cell-surface ectonucleotidases CD39 and CD73 creating high levels of adenosime in the extracellular space as well as inside cells as the newly generated adenosine molecules enter the cells via the nucleoside transporters. Thus, the levels of adenosine may quickly rise to fairly high local concentrations.

Due to the many effects of adenosine on the cell, it is of paramount importance to react rapidly to the altered levels of adenosine outside as well as inside the cells. Changes in extracellular levels of adenosine are sensed by at least four adenosine receptors (A1, A2A, A2B and A3) that activate various signal pathways depending on the type of adenosine receptor(s) on the cell surface (Antonioli et al., 2013; Burnstock and Di Virgilio, 2013), and altered levels of adenosine either outside or inside the cells, are rapidly equalized by the nucleoside transporters ENT1 and 2 and/or CNT1–3 (Young et al., 2013). If the intracellular milieu is challenged by a large influx of adenosine, adenosine is immediately metabolized by either adenosine kinase (ADK) (Boison, 2013; Park and Gupta, 2008), that has a low Km and handles minor changes in adenosine levels very well, or adenosine deaminase (ADA) (Whitmore and Gaspar, 2016), that has a larger Km and can metabolize higher levels of adenosine. ADK generates AMP from adenosine, that may be further phosphorylated to produce ADP and ATP. The inosine generated by ADA on the other hand, may either be further catabolized to hypoxanthine, xanthine and finally uric acid that is secreted by the cell, or alternatively to IMP and AMP.

The many effects of adenosine on cells and the rigorous control of the adenosine levels also suggest that adenosine is involved in many pathophysiological conditions (Fredholm, 2014). For example, adenosine has a potent anti-inflammatory function, presumably of importance to protect cells and tissues from detrimental effects of the immune system (Antonioli et al., 2013). On the other hand, tumour cells may produce high levels of adenosine as a means to escape immune responses (Burnstock and Di Virgilio, 2013). The cell surface ectonucleotidases CD39 and CD73 that convert ATP to AMP and AMP to adenosine, respectively, are often overexpressed on cancer cells and are believed to generate high levels of adenosine in the local environment of the tumour. Although the anti-inflammatory effect of adenosine may contribute to immune escape of tumour cells, adenosine may also affect the tumour cells directly (Chen et al., 2013). Depending on the type of tumour and the cell surface receptors it carries, the cell may either respond to adenosine by increased survival, proliferation and motility, or by apoptosis. Thus, adenosine may exert various effects on cancer cells.

Purinergic receptors for adenosine triphosphate (ATP) are expressed in cervical epithelium and it has been suggested that these receptors are involved in the differentiation and apoptosis of these cells (Greig et al., 2006). These receptors have also been shown to be overexpressed in human skin warts that contain HPV and on the HPV31 positive cell line CIN-612 (Greig et al., 2006), which suggested that HPV-infected cells could potentially be sensitive to ATP. Indeed, it has been reported that extracellular levels of ATP are converted to adenosine by cell surface ectonucleotidases on cervical cancer cells, and that the resulting adenosine causes apoptosis in these cells after uptake by adenosine transporter proteins (Mello Pde et al., 2014). The CD73 ectonucleotidase is overexpressed on various cancer cells, suggesting that generation of adenosine promotes carcinogenesis, perhaps through its anti-inflammatory function, or by more direct effects (Gao et al., 2014). It has also been reported that the tumour-promoting effect of CD73 is independent of its adenosine generating activity, and that adenosine exerts an antiproliferative effect on cervical cancer cells (Gao et al., 2017). However, the microenvironment of tumours is often rich in adenosine and ATP and other investigators have observed that transgenic mice expressing both HPV16 E7 and adenosine receptor 2A were characterized by the rapid occurrence and dissemination of malignant lesions (Coppee et al., 1996). Considering the important role of HPV16 in initiating and maintaining cervical cancer, as well as other anogenital cancers and head and neck cancers and the different effects of adenosine on cancer cells, we have investigated if nucleosides including adenosine, can affect HPV16 gene expression.

2. Results

2.1. Guanosine and inosine can activate HPV16 late gene expression

Intracellular concentrations of adenosine deoxy triphosphate (ATP) range from 1 to 10 mM. ATP may be released to the extracellular space by tissue stress or trauma, where it is quickly converted to adenosine by cell surface ectonucleotidases. The high levels of adenosine generated in the extracellular space is quickly normalized by transport of the nucleosides into healthy cells in the vicinity. This occurs via nucleoside transporter proteins located in the cellular membranes of most cells. Thus, adenosine levels can change very quickly, both outside and inside of cells, and human cells can be exposed to local concentrations of adenosine that lie in the millimolar range. Adenosine has been reported to have tumour-promoting as well as apoptosis-inducing effects on cancer cells (Coppee et al., 1996; Mello Pde et al., 2014). Less is known about inosine and guanosine and the pyrimidines uracil and cytidine. Here we have investigated if nucleosides affect HPV16 late gene expression. We used the C33A2 reporter cell line for HPV16 late gene expression that we had established previously by stable transfection of the subgenomic HPV16 reporter plasmid pBELsLuc into HPV-negative cervical cancer cell line C33A (Fig. 1**A**) (Johansson et al., 2015; Kajitani et al., 2017; Li et al., 2013a, b). Upon induction of HPV16 late gene expression, the late mRNAs encoding the secreted luciferase (sLuc) reporter gene are produced and sLuc levels increase. sLuc activity in the cell culture medium is therefore a measure of the level of HPV16 late gene expression. This cell line was treated with various concentrations of adenosine, guanosine or inosine, and sLuc activity in the cell culture medium was monitored. As can be seen, adenosine had no detectable effect on HPV16 late gene expression (Fig. 1**B**), whereas guanosine activated HPV16 late gene expression between 2 and 3-fold at concentrations of 900uM and above (Fig. 1**B**), and inosine at concentrations of 1.5 mM and above (Fig. 1**B and C**). In contrast, the modified guanosine 6-thioguanosine had no effect on HPV16 late gene expression (Fig. 1**B**). Guanosine increased the levels of HPV16 E2, E4 and late L2 mRNAs (Fig. 1**D**). We concluded that the purines guanosine and inosine modestly increased HPV16 late gene expression while adenosine did not.

2.2. Adenosine activates HPV16 late gene expression in a guanosine-dependent manner

It has previously been reported that guanosine can augment the ability of adenosine to regulate cell proliferation (Jackson et al., 2013; Jackson and Gillespie, 2013). This “guanosine-adenosine” effect, could potentially be active on the C33A2 cells used here. We therefore investigated if adenosine could activate HPV16 late gene expression in the presence of guanosine. As can be seen in Fig. 2**A**, adenosine could induce HPV16 late gene expression up to 9-fold and in a concentration-dependent manner, but only in the presence of guanosine (Fig. 2**A**). In contrast, the pyrimidines uracil and cytidine did not have a measurable effect on HPV16 late gene expression, neither in the absence, nor in the presence of guanosine (Fig. 2**B**). Adenosine also induced HPV16 late gene expression in the presence of inosine (Fig. 2**C**). A combination of the three purines adenosine, guanosine and inosine activated HPV16 late gene expression up to 15-fold, and adenosine and guanosine up to 9-fold, in a time-dependent manner (Fig. 2**D**). The effects of guanosine and adenosine or guanosine, adenosine and inosine was readily detected after 6 h (Fig. 2**D**). Adenosine and inosine or guanosine or inosine alone, caused a lower induction (Fig. 2**D**). We concluded that adenosine could activate HPV16 late gene expression in the presence of inosine, but preferably with guanosine.