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Original Article

The effects of HAART on the expression of MUC1 and P65 in a cervical cancer cell line, HCS-2

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ABSTRACT

Cervical cancer is the third most commonly diagnosed cancer globally and it is one of three AIDS defining malignancies. Highly active antiretroviral therapy (HAART) is a combination of three or more antiretroviral drugs and has been shown to play a significant role in reducing the incidence of some AIDS defining malignancies, although its effect on cervical cancer is still unclear. The aim of this study was to investigate the relationship between cervical cancer and HAART. This was achieved by studying the expression of two signalling molecules expressed in cervical cancer; MUC1 and P65. Following the 24-hour treatment of a cervical cancer cell line, HCS-2, with drugs, which are commonly used as part of HAART at their clinical plasma concentrations, real-time qPCR and immunofluorescence were used in order to study gene and protein expression. A one-way ANOVA followed by a Tukey-Kramer post-hoc test was conducted using JMP 11 software on both sets of data. The drug classified as a protease inhibitor (PI) (i.e. LPV/r) reduced *MUC1* and *P65* gene and protein expression more than the other drug tested. PIs are known to play a significant role in cell death; therefore, the cells were thought to be more susceptible to cell death following treatment with PIs. In conclusion, the drugs used, especially the PI showed some anticancer effects by facilitating cell death through decreased gene and protein expression of MUC1 and P65 and present promising agents for cancer treatment.

1. Introduction

Cervical cancer, non-Hodgkin's lymphoma and Kaposi's sarcoma are known to be AIDS defining malignancies because they have been observed to occur more frequently in HIV infected people [1,2]. The development of the acquired immunodeficiency syndrome (AIDS) defining malignancies is mainly through immuno-suppression; however, the risk of developing cervical cancer is further increased because both HIV and Human Papillomavirus (HPV) are sexually transmitted viruses and have common sexual behavioural risk factors [1,2]. The introduction of combination therapy known as highly active antiretroviral therapy (HAART), which consists of nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI), has generally led to major improvements to the clinical outcome and life expectancy of people infected with HIV/AIDS [3,4]. There has been no clear reduction in the burden and severity of cervical disease with the use of HAART, unlike other AIDS-related malignancies [4].

Cancer involves the disturbance of tissue growth [5]. Normal tissue growth is regulated by the balance between cell proliferation and cell death [5]. Alteration of the genes that control cell growth and cell death is required for the transformation from normal to cancer cells [5]. Proteins that play a role in both cell growth and cell death provide useful subjects to study drug effects on cancer cell lines.

Mucins have been shown to harbour complex relationships with many cellular pathways associated with cell growth, proliferation and apoptosis [5]. In addition, overexpression and downregulation of mucins is often used in order to alter the tumorigenicity and metastasis of various cancer cells *in vitro*, thus demonstrating their function in cancer pathogenesis [5].

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MUC1 is an oncogene and thus makes a suitable therapeutic target against cancer [6]. In many cancers, the overexpression of MUC1 signals reduced survival for patients because it promotes cancer, therefore reducing the expression of MUC1 is important for therapeutic intervention [6]. MUC1 expression is important for the normal function of organs and produces a protective mucous over epithelial surfaces [6], for example, in the uterus prior to the time of uterine receptivity [7]. The overexpression of MUC1 has been linked to metastasis in breast cancer [8]. The MUC1 protein undergoes post-translational proteolytic cleavage which results in the formation of two MUC1 subunits forming a heterodimer on the cell membrane [8]. The cytoplasmic domain of MUC1 has been shown to bind to the epidermal growth factor receptor (EGFR) family members, c-Src and β-catenin, suggesting that MUC1 plays an additional role in cell growth related pathways and signal transduction [8].

RelA (P65) is a transcription factor, which belongs to the family of nuclear factor kappa beta (NFkβ) transcription factors [9–11]. NFkβ family plays a role in regulating the expression of several genes that play a role in carcinogenesis and inflammation, for example, cyclin D1, c-myc, bcl-2, cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) [12,13]. The activation of NFkβ in cancer cells promotes cell proliferation, survival, angiogenesis, metastasis, epithelial to mesenchymal transition and inflammation [12,13]. Tumour promoters activate the NFkβ pathway and therefore enhance cell survival; when the NFkβ pathway is downregulated cells are susceptible to apoptosis [14].

The aim of this study was to examine the expression of MUC1 and P65 in cervical cancer cells (HCS-2) following treatment with different antiretroviral drugs which together comprise the HAART cocktail in order to better elucidate the role of HAART in cervical cancer. The outcome of this study will add new knowledge regarding the role of HAART in cervical cancer. In addition, it will identify a potential drug against cancer.

2. Materials and methods

2.1. Cell culture

Human squamous cell carcinoma from Uterine Cervix, HCS-2 cell line was purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank (National Institute of Health Sciences, Tokyo, Japan). The cells were routinely grown as a monolayer in Nunc tissue culture grade flasks of 25 cm^2 or 50 cm^2 (Thermo Scientific, Pittsburgh, PA, USA). The cells were maintained in a humidified environment of 5% CO_2 at a temperature of 37 °C in Eagle's Minimal Essential Medium (EMEM) (Lonza, Basel, Switzerland) supplemented with 15% foetal calf serum (GIBCO, Darmstadt, Germany). The cells were incubated for approximately 2 days or until they reached ~ 60–80% confluency and were then harvested with 0.1% trypsin and 0.02% EDTA and seeded onto coverslips, alternatively, the cells were frozen down in the culture medium supplemented with 5% DMSO (Sigma, St Louis, MO, USA).

2.2. Drug solutions and treatment

The mean steady-state peak plasma concentration (C_{max}) is the most physiologically relevant concentration for the ARVs because it represents naturally occurring concentrations of the drugs following their intake [15–18], hence, it was used to treat the cells for 24 h. C_{max} concentrations are as follows for the drugs: Emtricitabine (FTC) C_{max} = 1.8 μg/mL [16], Tenofovir Disoproxil Fumarate (TDF) C_{max} = 0.3 μg/mL [15], Efavirenz (EFV) C_{max} = 4.07 μg/mL [17], Ritonavir (RTV) C_{max} = 2.45 μg/mL [18], Lopinavir (LPV) C_{max} = 9.8 μg/mL [18], Cocktail 1 (ATP) 1.8 μg/mL FTC + 0.3 μg/mL TDF + 4.07 μg/mL EFV, Cocktail 2 (LPV/r) 2.45 μg/mL RTV + 9.8 μg/mL LPV (Table 1). The drugs were initially dissolved in 1 mL of primary diluent; 3600 μg/mL FTC and 600 μg/mL TDF were dissolved in distilled water whereas 6000 μg/mL RTV, 98,000 μg/ml LPV and 40,700 μg/mL EFV were dissolved in methanol (diluents were specified in the certificate of analysis obtained from Toronto Research Chemicals Inc. for each drug). The drugs were further diluted in EMEM in order to obtain the desired C_{max} concentrations as indicated above. The final concentration of methanol in working solution was 0.02%.

Table 1
The mean steady-state peak plasma concentrations (C_{max}) for the drugs used.

Drug	C_{max} (μg/mL)
FTC	1.8
TDF	0.3
EFV	4.07
Cocktail 1(ATP); FTC:TDF:EFV	1.8:0.3:4.07
Cocktail 2 (LPV/r); LPV:RTV	9.8:2.45

2.3. RNA extraction, cDNA synthesis, and real-time qPCR analysis

The Gene-JET RNA Purification Kit purchased from Thermo Scientific was used to isolate RNA from cells and the manufacturer's protocol was followed. RNA was isolated from a minimum of 1×10^6cells/mL. Following a 24-hour treatment with ATP and LPV/r, the growth medium (EMEM) containing the drugs was removed from the cells and the cells were rinsed once in PBS (pH 7.4) in order to remove residual medium. The cells were then harvested in trypsin EDTA and transferred to an RNAse-free microcentrifuge tube (Whitehead Scientific) following which the RNA extraction procedure was followed to isolate RNA from the cells. An aliquot of 5 μL was prepared in order to measure the RNA concentration and purity using a Nanodrop-1000 spectrophotometer. Genomic DNA was removed from the total RNA using the DNase I, RNase-free kit. The high capacity cDNA reverse transcriptase kit (purchased from Applied Biosystems) was used to synthesize cDNA and the manufacturer's protocol was followed. The final reaction volume was 20 μL and the Multiscribe Reverse Transcriptase was use to synthesize cDNA from 100 ng of RNA. The reverse transcription reaction was carried out in a GeneAmp PCR System 2400 Thermal Cycler for 10 min at 25 °C, 120 min at 37 °C and the enzyme was deactivated for 5 min at 85 °C. The cDNA was used in qPCR reactions for *MUC1* and *P65*, with large ribosomal protein PO (RPLO) and transferrin receptor (TFRC) used as reference genes. Primer sequences for *MUC1*, *P65*, *RPLPO*, and *TFRC* are indicated in Table 2. The Power SYBR® Green PCR Master Mix was purchased from Applied Biosystems (Carlsbad, CA, USA). The final reaction volume was 20 μL and the ABI 7500 real-time machine was used. The PCR reactions were amplified for 40 cycles under the following conditions; DNA polymerase was activated for

Table 2
Oligonucleotide sequences used for qPCR.

Gene	Sequences (5′–3′ direction)	Gene Bank accession number
MUC1	F: TGC CGC CGA AAG TAC G R: TGG GGT ACT CGC TCA TAG GAT	NM_001204294
P65	F: GTG GGG ACT ACG ACC TGA ATG R: GGG GCA CGA TTG TCA AAG ATG	NM_001145138
RPLPO	F: TGC AGC TGA TCA AGA CTG GAG ACA R: TCC AGG AAG CGA GAA TGC AGA GTT	NM_053275.3
TFRC	F: GGC ACC ATC AAG CTG CTG AAT GAA R: GTT GAT CAC GCC AGA CTT TGC TGA	NM_003234.2

MUC1: mucin 1, cell surface associated; *P65*: transcription factor P65 (RELA); *RPLPO*: large ribosomal protein PO; *TRFC*: transferrin receptor.