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Human Papillomavirus E6 and E7 oncoproteins affect the cell microenvironment by classical secretion and extracellular vesicles delivery of inflammatory mediators

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ABSTRACT

The connection between chronic inflammation and risk of cancer has been supported by several studies. The development of cancer might be a process driven by the presence of a specific combination of inflammatory mediators, including cytokines, chemokines and enzymes, in the tumor microenvironment. Virus-induced tumors, like HPV-induced Squamous Cell Carcinomas, represent a paradigmatic example of the interplay between inflammation, as integral part of the innate antiviral response, and malignant transformation. Here, the role of inflammatory microenvironment in the HPV-induced carcinogenesis is addressed, with a specific focus on the involvement of the immune molecules as well as their delivery through the microvesicle cargo possibly correlated to the different HPV genotype.

The expression of the inflammatory mediators in HPV positive cells has been analyzed in primary human foreskin keratinocytes and keratinocytes transduced by E6 and E7 from mucosal HPV-16 or cutaneous HPV-38 genotypes. HPV E6 and E7 proteins can modulate the expression of immune mediators in HPV-infected cells and can affect the levels of immune molecules, mainly chemokines, in the extracellular milieu. HPV-16 E6 and E7 oncoproteins have been silenced to confirm the specificity of the modulation of the inflammatory microenvironment.

Our results suggest that the expression of HPV oncoproteins allows the modification of the tumor milieu through the synthesis and release of specific pro-inflammatory cytokines and chemokines, affecting the efficacy of the immune response. The microenvironment can also be conditioned by an altered mRNA cargo delivered by extracellular vesicles, thereby efficiently affecting the surrounding cells with possible implication for tumorigenesis and tumor diagnosis.

1. Introduction

The inflammatory microenvironment, established by inflammatory cells, cytokines, chemokines and enzymes, plays a fundamental role in the development of tumor [1]. Indeed, people inclined to chronic inflammatory diseases have a higher risk to develop tumors [2].

Cancer secretome represents the whole proteins released by transformed cells or tissues (cytokines, hormones, coagulation and growth factors) that possibly participate to several physiological and pathological processes. Moreover, the interplay among cells or cell-to-extracellular matrix is another fundamental process of carcinogenesis [3].

Some chemokines (CXCL1/GRO-α, CXCL5/ENA-78, CXCL8/IL-8, CXCL12/SDF-1) and pro-inflammatory cytokines as interleukin (IL)-1α and IL-6 have the potentiality to support tumor growth otherwise the treatment with non-steroidal anti-inflammatory drugs has effects on the reduction of cancer incidence and aggressiveness [4]. On the other hand, toll like receptors (TLRs) can induce both anti- and pro-tumorigenic pathways thus affecting the tumor microenvironment [5]. Various members of the Toll Like Receptor (TLR) family can be inhibited by

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some viruses like Epstein-Barr Virus (EBV), Hepatitis B virus (HBV) and mucosal Human Papillomavirus (HPV) type 16 that reduce TLR9 expression and function [6–8]. About 15% of the global cancer burden is attributable to infectious agents and a persistent inflammation characterizes these chronic infections; for example, hepatocellular, colorectal, cervical and gastric carcinomas share a chronic inflammatory response [1]. Oncogenic viruses, such as Hepatitis C virus (HCV), HPV and HBV, produce persistent infection in which the viral oncoproteins alter specific cellular pathways promoting tumorigenesis. A continuous and deregulated immune response produces serious systemic and tissue damage, impaired tumor surveillance and, finally, cancer characterized by metastatic phenotype and resistance to chemotherapy [9].

HPVs are a large family of small DNA viruses that can be classified by their tropism to skin or mucosa [10]. High risk (HR)-HPVs belong to mucosal genotypes and are the aetiological agent of cervical cancer whereas the involvement of specific cutaneous HPV types in human carcinogenesis is not completely demonstrated. High risk genotypes exert their dangerous functions mainly through E6 and E7 oncoproteins that are already highly expressed in precursor lesions where they interact with numerous cellular proteins finally fostering viral immune-evasion and tumor development. About 60% of all cervical cancers are caused by mucosal HPV-16, as well as some anogenital and head and neck cancers. The properties of most cutaneous HPV types are unknown but it has been reported that E6 and E7 proteins of some cutaneous HPVs, such as HPV-38, have transforming properties [11].

In contrast to the increasing knowledge of the intracellular activities of E6 and E7 oncogenes, the possible effects on the intercellular communication of HPV-positive cancer cells is not yet well known.

The production and release of extracellular vesicles (EVs) like microvesicle, which blebs directly from plasmamembrane and exosomes, small extracellular vesicles of endocytic origin that can contain proteins, RNA, microRNAs and DNA [12], is deregulated in cancer, indicating their important role in tumors [13–15]. The release of EVs from cancer cells can impair the microenvironment, affecting tumor development and chemoresistance [16,17]. Rising evidence suggests that cancer cells use EVs transmitted nucleic acids and proteins to evade an immune response [18].

Tumor viruses can lead to modifications of the content of EVs ultimately implying them into the intercellular communication of virally transformed cells. Novel diagnostic markers for virus-associated preneoplasia or neoplasia could be found from the characterization of new virus-induced molecular signatures, as, for example, EVs.

Here we report that the inflammatory microenvironment appears modified in the HPV-induced transformation. In particular HPV E6 and E7 proteins can modulate the expression of immune mediators in HPV-infected cells and can affect the levels of immune molecules, mainly chemokines, in the extracellular milieu. In addition, the inflammatory immune mediators delivery through the EVs is affected by the expression of the HPV oncoproteins.

2. Materials and Methods

2.1. Cell cultures and treatments

Primary Human Foreskin Keratinocytes (HFK) were transduced with pLXSN16E6E7 (K16) and pLXSN38E6E7 (K38) as already described [19] and were grown in serum free keratinocytes medium (KBM BulletKit, Lonza). HFK were utilized as control. SiHa cell line was grown in DMEM + 10% fetal calf serum. Cells were cultivated in a humidified air of 5.0% CO_2 at 37 °C.

2.2. Extracellular vesicles isolation

HFK, K16 and K38 cells were seeded at 3×10^6 cells/plate in 100 mm tissue culture plates. After 5 days supernatants were collected and centrifuged at 500*g* for 10 min, 2000*g* for 10 min and 100000*g* for 60 min. At the end of the last spin, supernatants were discarded and the pellet containing the EVs was stored at −80 °C for the subsequent analyses.

2.3. HPV-16 E6, HPV-16 E7 silencing

Small interfering RNAs (siRNAs) conjugated with phosphoramidite (FAM) and targeted to HPV-16 E6 and HPV-16 E7 were designed and approved by Qiagen and a nonsilencing siRNA (Qiagen) was used as control.

HPV-16 E6 siRNAs were:
sense GAGGUAUAUGACUUUGCUU;
antisense AAGCAAAGUCAUAUACCUC.
HPV-16 E7 siRNAs were:
sense AGGAGGAUGAAAUAGAUGG;
antisense CCAUCUAUUUCAUCCUCCU.
Control siRNAs were:
sense UUCUCCGAACGUGUCACGU;
antisense ACGUGACACGUUCGGAGAA.

Briefly, before transfection 2×10^5 cells were seeded in 35-mm dishes in 1 ml of supplemented culture medium. siRNA was diluted in 50 µl of culture medium up to 10 nM final concentration. Successively 3.5 µl of HiPerfect Transfection Reagent (Qiagen) was added to siRNA-medium solution and mixed by vortexing. After 10 min at room temperature, the transfection complex was included drop-wise onto the cells. Twenty-four hours after transfection, transfection efficiency was evaluated by cytometric analysis (FACS Aria II as instrument and FACS Diva 6.1.1 as software, both from Becton–Dickinson). At least 10^4 events were acquired and analyzed using Flowing software (version 2.5.1, Turku Centre for Biotechnology). The level of transfected cells typically was superior to 50%.

2.4. RNA purification and Real Time RT-PCR

Cells were seeded in six well plates, 2×10^5 cell/ml and, after 72 h, were washed twice in PBS. EVs were isolated as described above. Cellular or EVs-derived RNA was purified utilizing the Total RNA Purification Kit (Norgen Biotech Corp.). Five hundred ng to 1 µg of total RNA were retro-transcribed using the Tetro cDNA synthesis kit (Bioline) and cDNA were examined by real-time PCR using the Sensi-Mix SYBR Hi-ROX Kit (Bioline) and specific primers (Table 1).

2.5. Western blot

K16 cells were seeded at 3×10^6 cells/plate in 100 mm tissue culture plates and 2 h later cells were transfected with HPV-16 E6 and E7 siRNAs or control siRNA. Following 72 h cells were tripsinized and pellets were gathered. Cells were lysed in lysis buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 10 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and 30 µg of total proteins were resuspended in 5X SDS sample buffer (40% Glycerol, 240 mM Tris–HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol), resolved on SDS–PAGE and transferred onto nitrocellulose membrane (Whatman). The membranes were incubated with mouse anti-p53 (clone Bp53-12 Santa Cruz) and rabbit monoclonal anti-GAPDH (clone 14C10, Cell Signaling). Immune complexes were detected with horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit (Calbiochem).

2.6. Human cytokine Antibody Array – Membrane

K16 and K38 cells were seeded in six well plates (2×10^5 cells/ml). After 72 h supernatants were collected and centrifuged at 2000*g* for 10 min. Subsequently, Human Cytokine Antibody Array – Membrane encompassing 80 cytokines, chemokines, growth and angiogenic factors (Abcam) was performed according to the manufacturer's instructions.