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Research Paper

The Human Cytomegalovirus Strain DB Activates Oncogenic Pathways in Mammary Epithelial Cells

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ABSTRACT

Background: Human cytomegalovirus (HCMV) establishes a persistent life-long infection and increasing evidence indicates HCMV infection can modulate signaling pathways associated with oncogenesis. Breast milk is an important route of HCMV transmission in humans and we hypothesized that mammary epithelial cells could be one of the main cellular targets of HCMV infection.

Methods: The infectivity of primary human mammary epithelial cells (HMECs) was assessed following infection with the HCMV-DB strain, a clinical isolate with a marked macrophage-tropism. The impact of HCMV-DB infection on expression of p53 and retinoblastoma proteins, telomerase activity and oncogenic pathways (c-Myc, Akt, Ras, STAT3) was studied. Finally the transformation of HCMV-DB infected HMECs was evaluated using soft agar assay. CTH cells (CMV Transformed HMECs) were detected in prolonged cultures of infected HMECs. Tumor formation was observed in NOD/SCID Gamma (NSG) mice injected with CTH cells. Detection of long non coding RNA4.9 (lncRNA4.9) gene was assessed in CTH cells, tumors isolated from xenografted NSG mice and biopsies of patients with breast cancer using qualitative and quantitative PCR.

Results: We found that HCMV, especially a clinical strain named HCMV-DB, infects HMECs *in vitro*. The clinical strain HCMV-DB replicates productively in HMECs as evidenced by detection of early and late viral transcripts and proteins. Following infection of HMECs with HCMV-DB, we observed the inactivation of retinoblastoma and p53 proteins, the activation of telomerase activity, the activation of the proto-oncogenes c-Myc and Ras, the activation of Akt and STAT3, and the upregulation of cyclin D1 and Ki67 antigen. Colony formation was observed in soft agar seeded with HCMV-DB-infected HMECs. Prolonged culture of infected HMECs resulted in the development of clusters of spheroid cells that we called CTH cells (CMV Transformed HMECs). CTH cells when injected in NOD/SCID Gamma (NSG) mice resulted in the development of tumors. We detected in CTH cells the presence of a HCMV signature corresponding to a sequence of the long noncoding RNA4.9 (lncRNA4.9) gene. We also found the presence of the HCMV lncRNA4.9 sequence in tumors isolated from xenografted NSG mice injected with CTH cells and in biopsies of patients with breast cancer using qualitative and quantitative PCR.

Conclusions: Our data indicate that key molecular pathways involved in oncogenesis are activated in HCMV-DB-infected HMECs that ultimately results in the transformation of HMECs *in vitro* with the appearance of CMV-transformed HMECs (CTH cells) in culture. CTH cells display a HCMV signature corresponding to a lncRNA4.9

Abbreviations: ChIP, chromatin immunoprecipitation; HMECs, human mammary epithelial cells; HCMV, human cytomegalovirus; MOI, multiplicity of infection; UV, ultraviolet rays; HI, heat inactivated; Rb, retinoblastoma; hTERT, human telomerase reverse transcriptase; IE, immediate early; LA, late antigen.

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genomic sequence and give rise to fast growing triple-negative tumors in NSG mice. A similar lncRNA4.9 genomic sequence was detected in tumor biopsies of patients with breast cancer.

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1. Introduction

Worldwide breast cancer is the most common cancer diagnosed among women (Warner, 2011). Notably, majority of the breast cancers classified as carcinomas have been found to be originated from the mammary epithelial cells lining the duct responsible for converting most precursors into milk constituents and transporting them to the mammary lumen (Dimri et al., 2005). Breast cancer exhibits heterogeneous molecular characteristics and utilizing gene expression patterns several types of breast cancer have been identified including a normal breast epithelial-like group, a luminal epithelial cell type A, a luminal epithelial cell type B, an ErbB2-overexpressing group, a basal-like group and a claudin low group (Lehmann et al., 2011). Etiological factors involved in breast cancer include genetic and environmental risk factors (Hüsing et al., 2012), and among these latter viruses could be involved with close to one-fifth of all cancers in the world caused by infectious agents (Zur Hausen, 2009).

The human cytomegalovirus (HCMV), a highly host specific pathogen, is a member of *Betaherpesviridae* family. HCMV generally causes asymptomatic to mild infection in immunocompetent host. However, its infection in immunocompromised host may result in serious complications (Coquette et al., 2004). HCMV infects a broad range of cells including monocytes, macrophages, fibroblasts, endothelial cells, epithelial cells, stromal cells, hepatocytes, smooth muscle cells, and neural stem/progenitor cells (Belzile et al., 2014; Khan et al., 2009; Lepiller et al., 2013; Wang and Shenk, 2005). Although HCMV clinical isolates display a broad cellular tropism infecting among others fibroblasts and epithelial cells, the growth of laboratory HCMV strains is restricted to fibroblasts (Wang and Shenk, 2005). In infected patients, the blood monocytes and tissue macrophages are regarded as an important HCMV cellular reservoir responsible for the dissemination of virus and may also act as a site for the establishment of latency (Hargett and Shenk, 2010; Khan et al., 2009; Smith et al., 2004). Noteworthy, HCMV has the ability to induce a distinct inflammatory (M1) and immunosuppressive (M2) macrophages polarization (Chan et al., 2009). In addition, macrophage polarization into M1/M2 phenotype is associated with the secretion of cytokines that could play a pivotal role in viral replication and fitness, and favor breast cancer promotion (Grivnenkov et al., 2010; McKinney et al., 2014; Teng et al., 2012).

Role of HCMV in inflammatory diseases and cancer has been well speculated (Cobbs et al., 2002; Lepiller et al., 2011; Söderberg-Nauclér, 2006). Earlier studies demonstrated that HCMV was able to induce the *in vitro* transformation of human embryo lung fibroblasts (Clanton et al., 1983; Geder et al., 1976). More recently, HCMV DNA or antigen has been found in tumor tissues from brain (glioblastoma, medulloblastoma), colon, prostate, liver and breast cancer (Banerjee et al., 2015; Baryawno et al., 2011; Bhattacharjee et al., 2012; Harkins et al., 2010; Samanta et al., 2003; Taher et al., 2013). Besides a direct role of HCMV in cellular transformation, HCMV could infect the tumor tissue and acts as a cofactor in amplifying mechanisms of oncogenesis, a paradigm called as oncomodulation (Michaelis et al., 2009).

Previously, we isolated a clinical HCMV strain from a 30-year-old pregnant woman named as HCMV-DB (KT959235), which is highly macrophage-tropic, triggers a M2 phenotype and upregulates the proto-oncogene Bcl-3 (Khan et al., 2009). HCMV-DB strain is close from other primary clinical isolates which also infect macrophages such as PH and TR strains (Suppl. Fig. S1) (Michaelis et al., 2009). There is scarcity of direct evidence suggesting the involvement of HCMV in transformation of human mammary epithelial cells (HMECs) (Herbein and Kumar, 2014). Here, we assessed the potential direct

oncogenic role of HCMV in primary human mammary epithelial cells (HMECs) *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Reagents

Anti-p53, anti-Rb, anti-Ras, anti-pAktThr308, anti-pAktSer473, anti-Akt and anti-cyclinD1 antibodies were purchased from Cell signaling (Danvers, MA, USA). Anti-pSTAT3, anti-STAT3 and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-pp65, anti-pp85 (pUL25), anti-pp71 (pUL82), anti-IE1 and anti-IE2 antibodies were purchased from Santa Cruz Biotechnology. Recombinant Raf1-GST was purchased from Millipore (Molsheim, France).

2.2. Cell Cultures

Human primary mammary epithelial cells (HMECs) were obtained from Life Technologies (Carlsbad, CA, USA). MDA-MB-231 and MCF-7 cells were provided by Institut Hiscia (Arlesheim, Switzerland). HMECs were cultivated in HMEC medium (Life Technologies, Carlsbad, CA, USA) supplemented with HMEC supplement and bovine pituitary extract (Life Technologies, USA). Cell viability assay was performed as previously described (Khan et al., 2009). Cultures were free of mycoplasma.

2.3. HCMV Infection of HMECs

AD169 is a highly passaged laboratory strain of HCMV originally isolated from the adenoids of a child (Murphy et al., 2003). The clinical isolate HCMV-DB was isolated in our laboratory from a cervical swab specimen from a 30-year-old pregnant woman (Khan et al., 2009). Cell-free virus stocks were prepared by propagating AD169 in MRC5 human fibroblasts, meanwhile HCMV-DB were grown in macrophages, as described previously (Khan et al., 2009). The TB40/E strain was isolated from a throat wash of a bone marrow transplant recipient (Tomasec et al., 2005). MRC5 human fibroblasts were cultured as described previously (Coquette et al., 2004; Khan et al., 2009). HMECs (1×10^6) and MRC5 cells (1×10^6) were infected at a multiplicity of infection (MOI) of 1 or 10 for 2 h at 37 °C, washed thoroughly (three times with $1 \times$ PBS), and covered with fresh medium. Where specified, HCMV heat-inactivated at 95 °C for 10 min or treated with UV ($1200 \mu\text{J}/\text{cm}^2$ for 15 min) were used as controls. Supernatants were clarified by centrifugation and stored at -80°C until use. Virus titers were determined by plaque-forming assay in MRC5 human fibroblasts as described previously (Khan et al., 2009). The purity of our HCMV stocks was confirmed by the absence of detection of other viruses (HSV-1, HSV-2, varicella-zoster virus, Epstein-Barr virus, adenovirus, BK virus) using PCR screening (data not shown). Following HCMV infection of HMECs, viral replication was assessed by the appearance of a cytopathic effect (CPE) in the cultures and by detection of IE1, IE2, pp65 and pp85 by western blotting and IE1 antigen (clone E13, Argene-Biosoft, Varihes, France) using immunofluorescence microscopy (Nikon Eclipse E400, Kanagawa, Japan). For the detection of HCMV mRNA using RT-PCR assay, total RNA was extracted from uninfected, UV-treated and HCMV-DB infected HMECs with RNeasy mini kit (Qiagen). Total 2 μg of RNA was reverse transcribed into cDNA with Superscript III RT (Life Technologies) using oligo (dT) primers. The 5 μl of reverse transcription reaction product was amplified using primers against IE1, US28 and UL82 primers as

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