17 β -estradiol and progesterone effect on human papillomavirus 16 positive cells grown as spheroid co-cultures

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Abstract Human papillomavirus (HPV) is the key epidemiologic factor of cervical cancer, but additional cofactors are mandatory. Estrogen has been considered as one of those. Here, the aim was to study the effects of steroid hormones on HPV16 E6–E7, estradiol receptors ER α and ER β , and progesterone receptor (PR) in HPV16-positive cervical carcinoma cell lines SiHa and CaSki grown as epithelial and fibroblast spheroid co-cultures. The spheroid co-cultures were exposed to 17 β -estradiol or progesterone from day 7 onwards. mRNA levels of HPV16 E6–E7, ER α , ER β and PR normalized against GAPDH were

analyzed with quantitative reverse transcription–qPCR (RT–qPCR). 17 β -estradiol and progesterone decreased HPV16 E6–E7 mRNA expression in CaSki and increased in SiHa co-cultures. In CaSki co-cultures, ER β expression was blocked after 17 β -estradiol exposure while in SiHa cells it slightly increased ER β expression. PR expression was seen only in CaSki spheroids and it vanished after exposure to steroid hormones. Fibroblasts expressed all three hormone receptors as monolayers but ER β expression decreased and ER α and PR vanished after co-culturing. Cell culturing platform changes both oncogene and hormone receptor expression in HPV16 positive cervical cancer cell lines. This needs to be considered when in vitro results are extrapolated to in vivo situations.

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Introduction

Infection with high risk human papillomavirus (HR-HPV) is the main etiological agent for cervical and anal cancer and a subgroup of head and neck carcinomas (IARC 2012). HPV infection as such is not sufficient to induce cancer and additional risk factors are needed. It is known that long-term use of oral contraceptives and/or multiple pregnancies

increases the risk for cervical cancer (Gierisch et al. 2013). Recent studies on HPV16 E6 and E7 expressing transgenic mice (K14E6/K14E7) have shown that mice on estrogen will develop cervical cancer but not head and neck cancer (Chung et al. 2010; Elson et al. 2000). Thus, site specific additional risk factors are needed in HPV induced carcinogenesis. The transgenic mouse model (K14E7) confirmed that estrogen receptor α (ER α) is necessary for estrogen to cooperate with HPV in malignant transformation and growth of cervical cancer (Son et al. 2014; Chung et al. 2008; Chung and Lambert 2009).

ER α is the major ER expressed in the cervix. Increasing levels of estrogen together with ER α expression during normal menstrual cycle will result in hyperplastic cervical epithelium without pathological changes. However, continuous estrogen stimulation could lead to neoplasia. There is evidence that the removal of exogenous estrogen and/or inhibition of ER α function might lead to elimination and prevention of cervical neoplastic disease, providing further evidence for the role of estrogen and its receptor in cervical cancer (Chun et al. 2013).

In vitro studies have shown that estrogen leads to a more malignant phenotype of HPV immortalized cells partly by increasing expression of E6 and E7 oncogenes, which are the major driving force for cervical cancer (Yuan et al. 1999; Newfield et al. 1998). However, the mechanism by which estrogen increases HPV gene expression remains unclear. Our earlier studies indicate that estrogen provides a growth advantage to HPV16 positive cervical cell lines and protects them from apoptosis (Ruutu et al. 2006).

Tumor-stroma interactions are emerging as an increasingly active field of cancer research. The stroma is an active modulator of carcinogenesis, especially carcinoma-associated fibroblasts (Gascard and Tlsty 2016). Accordingly, temporal deletion of stromal ER α induced complete regression of cervical dysplasia in K14E7 mice which supports the hypothesis that stromal ER α is necessary for HPV-induced cervical carcinogenesis and implicates paracrine mechanisms involving ER α signaling in the development of estrogen-dependent cervical cancers (Chung et al. 2013).

Recently, several three-dimensional *in vitro* models have been developed to mimic the structural and functional characteristics of normal epithelia in a manner that closely approximates *in vivo* conditions

(Chow 2015). Spheroid co-cultures are well established in cancer studies (Kunz-Schughart et al. 2001; Seidl et al. 2002; Kilani et al. 2003; Weiswald et al. 2015; Nath and Devi 2016) to monitor multicellular interactions between stromal fibroblasts and epithelia, which cannot be studied with monolayers. Fibroblasts are important in assisting and maybe even driving the tumor cell growth (Chuang and Liao 2000; Zeisberg and Kalluri 2006; Gascard and Tlsty 2016). We have also found that the phenotype of the carcinoma cells in raft cultures could be modulated from benign to malignant phenotype only by the fibroblasts and/or their connections to epithelial cells (Atula et al. 1997). Moreover, in the field of HPV research, HPV needs epithelial differentiation for its lifecycle. Monolayer cultures do not provide this differentiation providing only basal like epithelial cells thus are not ideal for HPV studies (Doorbar 2016).

Spheroid co-cultures, where fibroblasts produce the matrix and epithelium differentiates and stratifies mimicking the *in vivo* condition, offer a valuable approach to studying HPV interaction with the host. So far, all tissue cultures for HPV studies have been done as raft cultures where epithelial layer is lifted and kept in air-medium interface to differentiate (Atula et al. 1997; Chow and Broker 1997; Hietanen et al. 1998). The main problem with the raft cultures is that differentiation occurs only if the epithelium is barely above the medium level. Thus, it is practically impossible to guarantee that all parts of the epithelium in raft cultures are always above the air-liquid interface. In addition, epithelium is fed through the underlying stroma, which makes the optimization of tissue cultures difficult. Furthermore, in raft cultures, the stroma is artificially made of fibroblasts seeded into the matrix gel consisting mostly of rat tail collagen. We have shown that the matrix as such modulates the phenotype of epithelium as well as its expression of certain proteins (Merne and Syrjänen 2003).

The present study was designed to evaluate the effects of estrogen and progesterone on mRNA expressions of HPV16 E6–E7, ER and progesterone receptors (PR) in two different HPV16 positive CaSki and SiHa cell lines grown as spheroid co-cultures with fibroblasts. We have previously shown that in monolayer culture estrogen and progesterone did not change the transcription levels of HPV16 E6/E7 or ER or PR levels and the hormone receptor antagonists had no

effect on transcription (Ruutu et al. 2006). The second aim was to compare the results achieved from the spheroid cultures with those from monolayer cultures.

Materials and methods

Cell lines

CaSki (ATCC[®] CRL-1550TM) (LGC Standards, Wesel, Germany) and SiHa (ATCC[®] HTB-35TM) (LGC Standards, Wesel, Germany) epithelial cell lines and fibroblasts established from healthy human gingiva (Willberg 2007) were used for the experiments. SiHa and CaSki cells have been established from squamous cell carcinomas of cervix uteri containing approximately 1–2 and 600 copies of HPV16, respectively.

Monolayer culture

CaSki and SiHa cells were cultured as monolayers as previously described (Ruutu et al. 2006). At 60% confluence the cells were exposed to either 17 β -estradiol (Sigma, St Louis, Mo, USA) or progesterone (Sigma, St Louis, Mo, USA) at the final concentration of 20 ng/ml for 2 days. 17 β -estradiol and progesterone were diluted in 100% ethanol and the final concentration of ethanol in the culture medium was 0.01%.

Spheroid co-culture

Spheroid co-culturing was done as described earlier (Kunz-Schughart et al. 2001). Shortly, 96-well plates were coated with 1.5% agar (Agarose M, Pharmacia Biotechnology, Uppsala, Sweden), boiled for 20 min. After 24 h, CaSki and SiHa cells were seeded separately on the agar coated microtiter plates, at an initial density of 2000/200 μ l of D-MEM/well. An initial cell density of approximately 4000/200 μ l was used for fibroblast spheroids. After 4 days of culturing, the medium was changed for the first time, and the spheroids were then exposed to either 17 β -estradiol or progesterone at the final concentration of 20 ng/ml for 2 days. At day 9, fibroblast spheroids were individually added with a Pasteur pipette to the wells harboring either SiHa or CaSki spheroids, to allow the formation of spheroid co-cultures individually between one

fibroblast and one epithelial spheroid. Thereafter, co-cultures were maintained in D-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 μ g/ml streptomycin (Gibco, Grand Island, USA), 100 IU/ml penicillin (Gibco) and 10% fetal calf serum (Gibco) and exposure with 20 ng/ml estradiol or progesterone for additional 14 days. The culture medium was changed every second day. The medium was changed by replacing always 100 μ l of the 200 μ l total volume. At the end of the culturing, the spheroids were collected individually into an Eppendorf tube. The spheroids were allowed to sediment for 3–5 min, after which the medium was removed by pipetting and 1 ml of Trizol (Gibco, Paisley, UK) was added. The samples were preserved at -70°C . A part of spheroids were formalin fixed and paraffin-embedded for basic morphology (Fig. 1). HaCaT cells (CLS Cell Line Service GmbH, Eppelheim, Germany) were aimed to use as control cells but they did not grow as spheroids, as also noticed by other researchers (Chandrasekaran et al. 2011).

Scanning electron microscopy (SEM)

For the SEM, harvested spheroids were fixed in 2% glutaraldehyde and 2% formaldehyde in phosphate buffered solution at pH 7.4 and dehydrated in ascending series of ethanol. Spheroids were collected into specimen holders and sputter coated with approximately 10 nm of Au (SCD 050, Bal-Tec AG, Balzers, Liechtenstein). Examinations were performed using SEM (JEOL JSM-5500, Tokyo, Japan) with additional software for image acquisition (Spirit, Princeton Gamma-Tech, Princeton, NJ, USA). SEM was used to estimate the size and morphology of the spheroids.

Quantitative reverse transcription–qPCR (RT-qPCR)

Spheroids were sonicated twice for 30 s to homogenize the tissue cultures followed by total RNA extraction from the samples with Trizol according to the manufacturer's protocol. First-strand cDNA was synthesized using 100 ng of total RNA (DNase-treated) with First-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). RT-qPCR reactions were performed in a 25 μ l mixture

Fig. 1 **a** The basic structure of a cross-section of spheroid. **b** Cytokeratin staining showing the fibroblast surrounding epithelium. **c** Vimentin staining showing fibroblasts

containing 15 ng sample with Universal MasterMix and TaqMan® Assays-on-Demand™ Gene Expression kits for PR, ER α and ER β (Applied Biosystems, Foster City, CA, USA) as previously described by us (Peitsaro et al. 2002). HPV16 E6–E7 mRNA expression was assayed with RT-qPCR as described earlier (Ruutu et al. 2006). All analyses were made in triplicates with ABI PRISM 7700 Sequence Detector. The results were normalized against GAPDH mRNA levels.

Immunohistochemical staining

Immunohistochemical stainings were performed with ER α antibody (M7047, Dako, Glostrup, Denmark) 1:50, PanCytokeratin antibody (M821, Dako, Glostrup, Denmark) 1:50 and Vimentin antibody (M7020, Dako) 1:150 with REAL Detection System Peroxidase/DAB-, Rabbit/mouse (Dako, K5001), TechMate 500 plus automatic immunostainer (Dako).

Statistical analysis

Statistical analyses were done using the SPSS for Windows (version 22.0.0.2) software. Two independent samples *t* test was used to compare the differences in expression of PR, ER α , ER β and HPV16 E6–E7 mRNA between the exposed and unexposed cultures. Levene's test was used to control for the validity of the equal variance assumption. In all calculations, a value of $p < 0.05$ was considered statistically significant.

Results

The spheroid morphology

A light microscopy image of basic spheroid structure is shown as Fig. 1. The size of the SiHa spheroids was always smaller than that of CaSki spheroids. Their morphology did not change after 17 β -estradiol or progesterone exposure (Fig. 2). The mean size of the CaSki spheroids was largest after progesterone exposure. In case of spheroid culture, the cells are always viable if the structure of spheroid is maintained.

Expression of HPV16 E6–E7

Compared to the level of CaSki cells grown as monolayers, 17 β -estradiol and progesterone exposure significantly decreased the HPV16 E6–E7 mRNA expression in CaSki spheroids ($p < 0.0001$) (Fig. 3). Contradictory to that HPV16 E6–E7 mRNA expression was slightly increased after steroid exposure in SiHa spheroids compared to SiHa monolayer cultures but the changes were not statistically significant. No HPV16 E6–E7 mRNA expression was found in the fibroblasts as expected.

Estrogen and progesterone receptors

ER α expression was not found in CaSki cells cultured either as monolayers or as spheroids (Figs. 4, 5). SiHa cells expressed tiny amounts of ER α when cultured as monolayers but not as spheroids. On the other hand,

Fig. 2 Spheroid co-cultures of SiHa control (**1A**, **1B**) and cells with estrogen supplementation (**2A**, **2B**), SiHa cells with progesterone supplementation (**3A**, **3B**), CaSki cells with estrogen supplementation (**4A**, **4B**) and CaSki cells with progesterone supplementation (**5A**, **5B**). Scale bars in the left column 200 μ m, right column 20 μ m

Fig. 3 Effects of 17 β -estradiol and progesterone on HPV16 E6–E7 mRNA expression presented as percentages of the expression levels. 17 β -estradiol and progesterone exposure significantly decreased the HPV16 E6–E7 mRNA expression in CaSki spheroids ($p < 0.0001$). The mRNA expression was

slightly increased after steroid exposure in SiHa spheroids compared to SiHa monolayer cultures but the changes were not statistically significant. No HPV16 E6–E7 mRNA expression was found in the fibroblasts as expected

ER β was expressed in all untreated cultures, whether monolayers or spheroids. The highest ER β levels were found in CaSki monolayers after exposure to progesterone followed by CaSki monolayers after exposure to estrogen and lastly, SiHa monolayers after estrogen exposure. ER β expression levels were similar in unexposed CaSki monolayers, CaSki spheroids and SiHa monolayers but were nearly twice as low in the SiHa spheroids. 17 β -estradiol exposure resulted in total vanishing of the ER β receptor expression in CaSki spheroids ($p > 0.002$). On the contrary, 17 β -estradiol exposure increased the ER β expression in SiHa spheroids ($p > 0.02$). PR expression was seen only in CaSki spheroids at very low levels, but vanished totally after exposure to steroid hormones.

ER α , ER β and progesterone receptors (PR) were expressed in fibroblasts grown in monolayers. Expression of PR was induced with progesterone exposure ($p < 0.01$) while 17 β -estradiol exposure ($p < 0.01$) caused a complete loss of PR expression.

Discussion

In the present study we have analyzed the effects of 17 β -estradiol and progesterone on mRNA expressions of HPV16 E6–E7 and their specific receptors (two forms of estradiol receptor, ER α and ER β , and PR) in two HPV16 positive cervical cancer cell lines grown as monolayers or spheroid co-cultures. In the present study, we made the following three observations of interest: (1) spheroid co-cultures provide totally different results than monolayers, (2) the two HPV16 positive cervical cell lines behave also differently in cell culturing. This aspect might be explained by the fact that the HPV16 copy numbers are 300–600 times higher in CaSki cells than in SiHa cells. (3) ER α and ER β , and PR exposure decreased HPV16 E6–E7 transcription in HPV16 copy number rich CaSki spheroids and slightly increased in HPV16 copy number poor SiHa spheroids. We have previously shown in monolayer culture that estrogen and

Fig. 4 Effects of 17β -estradiol and progesterone on ER α , ER β and PR mRNA expression in monolayers and spheroid co-cultures of CaSki and SiHa cell lines. Scale bar 50 μ m

Fig. 5 ER α expression was negative in CaSki spheroids, both in **a** control as well as after **b** 17β -estradiol and **c** progesterone exposure. **d** Breast carcinoma as a positive control. Spheroids sections cut show mostly the covering epithelial cells

progesterone did not change the E6–E7 levels and that the hormone receptor antagonists had no effect on transcription (Ruutu et al. 2006).

In addition to different results between monolayer culture and spheroid culture, SiHa and CaSki spheroids were morphologically different. SiHa spheroid grew more in clusters and the cross-sections of the spheroids were smaller than of the CaSki spheroids. This is likely a results from the fact that these epithelial cell lines are different per se, from

different persons and have in addition a different HPV content, e.g. different cellular contacts.

Our results on the absence of ER α expression in the cell lines of cervical uteri are in accordance with previous studies. López-Romero et al. (2013) found nuclear expression of ER α mainly in the first third of normal cervical epithelium but it was decreased or totally absent in invasive cervical carcinoma as detected with immunohistochemistry. However, they were able to demonstrate only slight ER α expression

with RT-qPCR. Also, the loss of ER α has been detected at protein expression level in these cell lines (Mittrani-Rosenbaum et al. 1989; Hwang et al. 1992; Kim et al. 2000). There is no definitive explanation why ER α expression is vanishing during the malignant progression. One explanation for the loss of ER α could be the dedifferentiation of the epithelial cells as discussed earlier by Smith et al. (2003). In our study, we had the possibility to assess the role of epithelial differentiation on ER α expression as the epithelium is stratifying in spheroids. However, neither the monolayers nor the differentiated CaSki cells in spheroids had any ER α expression. On the contrary, in SiHa cultures tiny amounts of ER α expression were found, which cannot be explained by the ER α expressed by fibroblasts as Caski spheroids were totally negative for ER α . Comparisons to normal epithelial cells would be preferable but the problem is that those cells do not grow well as spheroids (data not shown).

The role of ER β is less known. It has been shown that when ER α expression is lost as in cervical carcinomas, ER β could remain as the receptor for estrogenic effect in cervical carcinoma (López-Romero et al. 2013). We detected higher levels of ER β in both cell lines when grown in monolayers. 17 β -estradiol treatment induced ER β expression levels similarly in both cell lines but treatment with progesterone resulted in opposite effects in both the CaSki and SiHa cell lines. ER- β expression increased eightfold in Caski but declined in SiHa cells although in both cell lines no progesterone receptor expression was found. In spheroids the effects of hormone treatments were less obvious. It has been claimed that the biological effect of estradiol is dependent on PRs. However, this was not evident in our results. Only in fibroblasts we could show that progesterone treatment induced the expression of PR but with 17 β -estradiol treatment it vanished completely.

The absence of ER α or PR expression in carcinoma of cervix uteri associated with high-risk HPVs does not exclude the influence of estrogen and progesterone on HPV transcription. In recent years, it has been shown that activated ERs can have an effect on DNA, not only through the ERs, but also through transcription factor AP-1 (activating protein-1) and specificity protein 1 (SP1) (Jia et al. 2015; Berger et al. 2013; Jacquin et al. 2013; Björnström and Sjöberg 2004). Both ER α and ER β interact with the AP-1, which plays a crucial role in proliferation and differentiation of epithelial cells.

Importantly, long control region (LCR) of HPV16 genome has also binding sites for AP-1 and SP1, which regions can participate in the control of E6 and E7 expression (Jacquin et al. 2013; Nin et al. 2015). However, the influence of these individual types of receptors is dissimilar. Binding of receptor α with 17 β -estradiol activates transcription, while ER β deactivates transcription. In the present study we could not confirm this effect as HPV16 E6–E7 expression increased in CaSki monolayers over 600- and nearly 500-fold after 17 β -estradiol and progesterone treatment, respectively. However, the mechanism might be different when the expression of ER α is lost.

The expression of the ER and PR receptors have been shown to be high in stromal tissue of cervical cancers irrespective of the presence of HPV DNA and histopathological type of cancer whether squamous cell carcinoma or adenocarcinoma of the cervix (Kwasniewska et al. 2011). This highlights the importance of stromal factors in carcinogenesis (Chung et al. 2013; Hemmings 2013).

To summarize, spheroid co-cultures offer valuable approach to HPV studies because as an organotypic multicellular culture where fibroblasts produce the matrix, and epithelia differentiate and stratify mimicking the *in vivo* situation. Importantly, the effects seem to be cell line related which needs to be considered when *in vitro* results are extrapolated to *in vivo* situations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have conflict of interest.

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