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CD40/CD40 LIGAND INTERACTIONS AND TNFα TREATMENT REDUCE ACTIVITY OF P105 PROMOTER OF THE HUMAN PAPILLOMA VIRUS-18 *IN VITRO*

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***Background*: Cervical carcinoma cells including those infected with the oncogenic human papilloma virus (HPV) and several cervical carcinoma cell lines show a strong expression of the CD40 receptor, unlike benign cervical epithelial cells infected with HPV. The functional relevance of this up-regulated expression in the tumor is not fully understood. Nevertheless, it might offer a unique possibility to target those malignant cells due to the antiviral and antitumoral effects of the CD40/CD40 ligand (CD40L) interactions. *Aim*: *In vitro* assessment of the effect of CD40L on HPV 18-P105 promoter activity and the subsequent release of IL-6 by the promoter transfected $HeLa_{CD40}$ cells, which express CD40 constitutively. *Material and Methods*: Transfection of $HeLa_{CD40}$ cells was achieved by electroporation after optimizing the parameters by the pCMV-β-Gal vector and β-Gal stain. Transfected $HeLa_{CD40}$ cells were challenged with BHK_{CD40L} and TNFα, in addition to BHK_{wt} and medium alone as controls. HPV18-P105 promoter activity was demonstrated by luciferase reporter gene assay while IL-6 was assessed by ELISA. *Results*: CD40/CD40L interactions and TNFα treatment significantly reduced HPV18-P105 promoter activity ($56.0 \pm 10.2\%$ and $64.1 \pm 9.1\%$ *vs*. control, respectively; $p < 0.001$). Likewise, IL-6, which is a sensitive cytokine of CD40 activation, was significantly increased in $HeLa_{CD40}$ cells in the same experiments (2.7 fold after stimulation with BHK_{CD40L} and 5.2 fold after stimulation with TNFα *vs*. control; $p < 0.01$ and $p < 0.001$, respectively). *Conclusion*: It is likely that the CD40/CD40L interactions and TNFα are effective against cervical carcinomas by repressing transcriptional activity of HPV promoter. This can result in new adjuvant treatments.**

***Key Words*: human papilloma virus, CD40, CD40 ligand, promoter, repression.**

INTRODUCTION

CD40 is a member of the TNFR family. It is expressed on B cells, monocytes, dendritic cells, and a variety of nonhemopoietic cells, including normal keratinocytes, tumor cells, and many *in vitro* transformed and carcinoma-derived cell lines. CD40L, a member of the TNF superfamily, is expressed primarily by activated T cells, activated B cells and platelets and under inflammatory conditions on monocytic cells, natural killer cells and mast cells. CD40/CD40L engagement is required for B-cell differentiation, isotype switching, maturation of dendritic cells and subsequent stimulation of antigen-specific T cells [1].

The CD40/CD40L interactions in tumor cells seem to be pleiotropic and context dependent as it may not only inhibit tumor growth via immunologic mechanisms and induction of apoptosis but also may stimulate tumor growth via various cytokines and growth factors including IL-6 and VEGF, among others [2]. Likewise, TNFα exerts selective direct tumor cytotoxicity but also promotes tumor development and metastases via NO release and angiogenesis [3]. *In vivo* and *in vitro* studies revealed that oncogenic human papilloma viruses (HPV) positive cervical carcinoma and HPV positive cervical carcinoma cell lines expressed CD40 at the high levels, in contrast to normal cervical epithelium and non-malignant keratinocyte cell lines transfected with HPV-DNA [4, 5]. This discrepancy may provide an explanation for persistence of HPV infection by evading the immune system as shown by increased production of IL-18 binding protein that antagonizes the inflammatory cytokine IL-18 and attenuates the response to CD40 ligation and the capacity to attract peripheral blood mononuclear cells [6, 7]. This immune escape may be the rational for the poor prognosis of cervical carcinoma in the presence of HPV-18 genotype [8]. The selective effect of TNFα on HPV infection is not known but previous studies have shown that both CD40/CD40L interactions in the presence of a protein inhibitor and TNFα exerted a direct $HeLa_{CD40}$ cytotoxicity, however it is unclear whether this effect also affects $HeLa_{CD40}$ cells' HPV infection [9].

The aim of the present work is to examine the functional consequences of CD40/CD40L interactions and TNFα treatment on the HPV-18-p105 promoter activity of transfected $HeLa_{CD40}$ cells *in vitro*.

MATERIALS AND METHODS

Cell lines and formaldehyde fixation. The cervical carcinoma cell line $HeLa_{CD40}$ (for cloning of CD40-cDNA and transfection see [10], CCL 2; American

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Abbreviations used: BHK – baby hamster kidney; CD40L – CD40 ligand; HPV – human papilloma virus; IL-6 – interleukin-6; IL-18 – interleukin-18; LCR – long control region; RLU – relative light units; SN – supernatant; STAT3 – signal transducer and activator of transcription 3; TNF – tumor necrosis factor; VEGF – vascular endothelial growth factor.

Type Culture Collection, Kockville, MD) and the baby hamster kidney (BHK_{CD40L}) cells, transfected with CD40L cDNA [11] (ACC 61; German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany) were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-alanyl-L-glutamine and antibiotics (all from Life Technologies, Eggenstein, Germany). Formaldehyde-fixed BHK_{CD40L} cells and the wild type variant BHK_{wt} were prepared as described previously [9]. Formaldehyde fixation of BHK cells prevents cellular adhesion to culture plates and release of autologous cytokines in an appreciable amount.

Transient transfection and assays. *1. Optimization of transfection efficiency by electroporation*. The pCMV-β-Gal vectors and β-Gal stain method were used for adjustment and optimization of the electroporation variable parameters as previously described (Fig. 1, *a*) [12]. The voltage was varied at a range of 220 to 350 V with stepwise increments of 10 V, while the capacitance was tested at a range of 900 to 1400 μF with stepwise increments of 150 μF. The optimal parameters were found to be 300 V/1050 μF as demonstrated by the count and the density of the deep blue color of the transfected cells (Fig. 1, *b*, *c*). *2. Reporter gene assays* (Luciferase assay). The luciferase expression test represents a reporter system for analysis of regulatory DNA sequence in which luciferase catalyzes the D-luciferin to emit a green luminescence that can be quantitatively measured. Electroporation cuvettes, each containing 0.8 ml of $HeLa_{CD40}$ cell suspension (adjusted at ca. $8–9 \cdot 10^6$ cells/ml), to which 10 μg 4321-luc DNA (the entire HPV18 long control region (LCR) and the p105 promoter are conjugated on the luciferase gene) (Dr. Steger, Cologne, Germany) or 10 μg pCMV-β-Gal vector that served as a control were added. Mixtures were left at room temperature for 10 min and followed by application of the optimum electric pulses (300 V, 1050 μF). Cells of 10 cuvettes were collected, gently mixed and seeded at equal number in 20 culture plates for 24 h followed by addition of formaldehyde-fixed BHK_{CD40L} (6 plates, $2 \cdot 10^6$ cells/plate), formaldehyde-fixed BHK_{wt} (6 plates, $2 \cdot 10^6$ cells/plate) and TNFα (4 plates, each contains 1000 U/ml) while the remaining 4 plates contained medium only and served as controls. Culture plates were incubated at 37 °C for 43 h followed by a gentle wash with warm medium to remove the BHK cells and TNFα. The transfected cells were washed with cold PBS and retrieved by a rubber scraper in 1.8 ml PBS. After centrifugation (250 g, 4 °C, 7 min) cells were resuspended in 50 μl extraction buffer, lysed by 4 freeze-thaw cycles (each for 2 min in liquid nitrogen at −196 °C and 2 min in water bath at 37 °C), and followed by ultracentrifugation (1400 g, 5 min at 4 °C). The extract was retrieved and kept on ice. The luciferase activity was measured by mixing 15 μl of the extract with 100 μl of the luciferase assay buffer (containing rATP). After injection of 300 μl solvent (0.356 mM D-luciferin dissolved in luciferase buffer) the relative light units (RLU) were immediately measured in a LB 9501 luminometer (Berthold Technologies, Bad Wildbad, Germany). On the other hand, the control reaction consisted of 470 μl β-galactosidase-buffer, 100 μl ONPG solution (4 mg/ml O-Nitrophenol-β-D-galactosidase in 0.1 M Na_2HPO_4-buffer, pH 7) and 30 μl cell extract which were incubated till they attain yellow color. Wells with β-Gal buffer and ONPG only served as controls. After stopping the reaction with 250 μl $NaCO_2$, the fluorescence was measured at a wave length of 420 nm (Fig. 1, *a*).

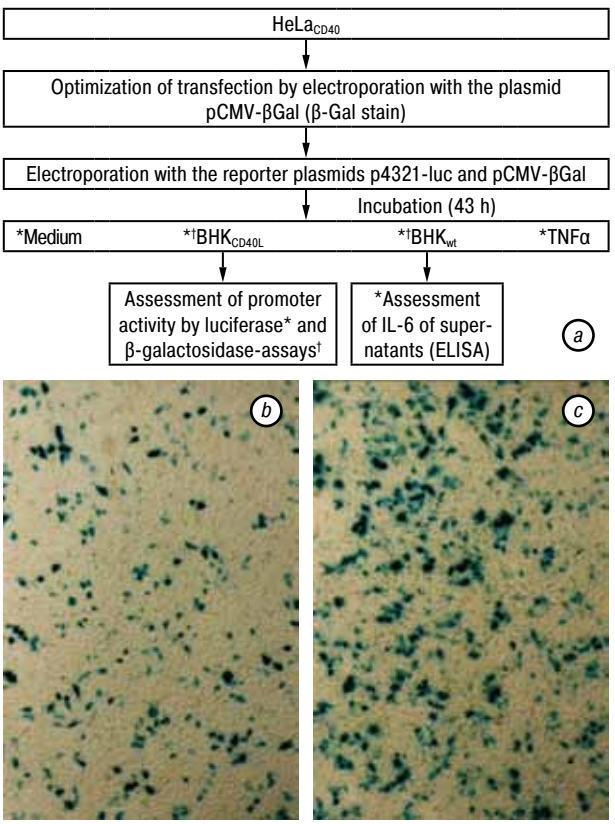

Fig. 1. Schematic representation of the experimental design, steps and methods (*a*). Detection of β-galactosidase activity after electrotransfection of $HeLa_{CD40}$ cells (with pCMV-β-Gal; original magnification, × 40; *b* — electroporation parameters 240 V and 1050 μF; *c* — electroporation parameters 300 V and 1050 μF; the latter represents the optimal parameters as shown by the count and density of the blue dye)

Assessment of IL-6 by ELISA. Cells were seeded in 24-well plates at a density of $1.5 \cdot 10^5$ cells/well and were challenged after 24 h with BHK_{CD40L} cells, BHK_{wt} cells (each with $2 \cdot 10^6$ BHK/well), TNFα (1000 U/ml) or left with medium alone that served as a control. After 16 h, supernatants (SNs) were collected, cellular debris was removed by centrifugation and the IL-6 contents were assessed by ELISA as previously described [11]. Briefly, Maxisorp plates (Nunc, Wiesbaden, Germany) were coated with 1 mg/ml anti-IL-6 overnight. After blocking of the plates for 1 h with PBS containing 0.5% BSA, 0.05% Tween 20 (Serva, Heidelberg, Germany), and 0.02% NaN_3, SNs and serial dilutions of the recombinant human IL-6 (Tebu, Frankfurt, Germany) as standards were added for 6 h. Plates were then incubated with polyclonal anti-

IL-6 overnight (Tebu, Frankfurt, Germany) followed by a 2-hour incubation with peroxidase-labeled goat anti-rabbit F(ab) after which the substrate was applied and the absorbance was measured with an ELISA reader (SLT Lab Instruments, Hannover) at 405 nm (Fig. 1, *a*).

Statistical analysis. The mean and standard deviation of values obtained from each assay were calculated and the percentage decrease of luciferase assays was estimated. Unpaired Student *t* test was used to evaluate the difference between means and SD of reporter gene assay and IL-6.

RESULTS

CD40/CD40L interactions and TNFα treatment reduced the HPV18-P105-promoter activity in HeLa cells that expressed CD40 constitutively. To examine the possible regulatory effect of CD40L on the HPV18 control region, the $HeLa_{CD40}$ cells transfected with reporter gene were stimulated for 43 h with formaldehyde-fixed BHK_{CD40L} cells and also with TNFα. Formaldehyde-fixed BHK_{wt} and medium alone served as controls. The activity of HPV18-P105-promoter stimulated by formaldehyde-fixed BHK_{CD40L} (RLU 2902.3 ± 291.49 *vs*. 4488 ± 459.62; $p = 0.017$) (in the four experiments) was repressed by a mean of 56 ± 10.2%, in comparison with BHK_{wt} (RLU 5114 ± 917.32 *vs*. 4488 ± 459.62; $p = 0.45$). Similarly, they were significantly stimulated by 1000 U/ml TNFα (RLU 1765 ± 321.02 *vs*. 4488 ± 459.62; $p = 0.02$). On the other hand, formaldehyde-fixed BHK_{wt} induced no significant effect in comparison with control medium (RLU 5114 ± 917.32 *vs*. 4488 ± 459.62; $p = 0.45$) (Fig. 2, *a*). It has been shown earlier that TNFα negatively regulates the HPV16 gene expression [13]. In the present work, activity of the HPV18-P105-promoter was repressed by TNFα by a mean of 64.9 ± 9.1% in comparison with medium. The standard deviations from several probes within each experiment were between 1.0 and 10.1%. In contrast, the control pCMV-β-Gal vector-transfected $HeLa_{CD40}$ cells incubated with formaldehyde-fixed BHK_{CD40L} or BHK_{wt} cells or TNFα or medium alone showed similar and non-significant CMV promoter activity in all probes ($p > 0.05$).

CD40/CD40L interactions and TNFα treatment induce IL-6 release by the $HeLa_{CD40}$ cells transfected with HPV18-P105. As IL-6 represents a very sensitive parameter for activation of the CD40 and TNFα receptors, its concentration was determined upon stimulation. It was found that levels of IL-6 produced by $HeLa_{CD40}$ cells increased significantly when challenged by both formaldehyde-fixed BHK_{CD40L} and TNFα but not by formaldehyde-fixed BHK_{wt}. The increase of IL-6 was up to 2.7 fold with BHK_{CD40L} ($p < 0.01$) and 5.2 fold with TNFα ($p < 0.001$) after 16 h incubation with $HeLa_{CD40}$ transfected with the HPV18–105 (Fig. 2, *b*). This illustrates the efficient stimulation with the functionally active CD40L and TNFα and also confirms the survival of the transfected $HeLa_{CD40}$ cells and their sustained efficiency in releasing IL-6 upon stimulation.

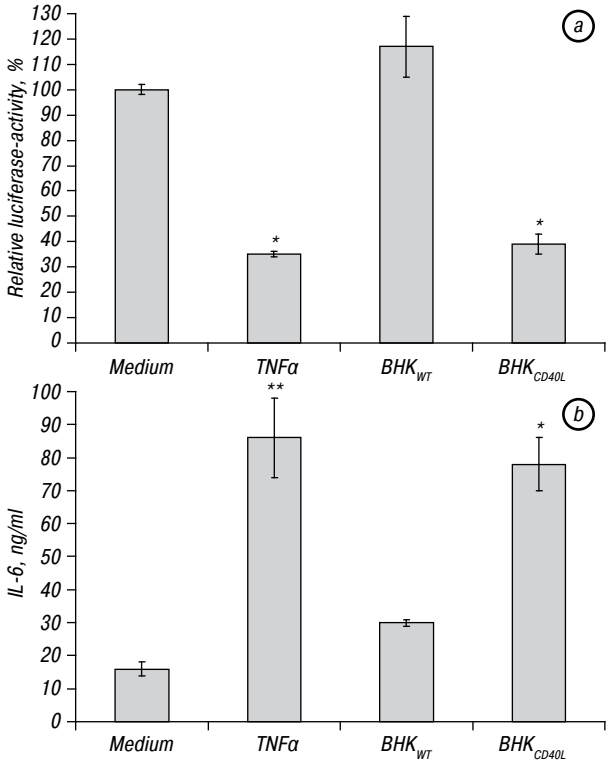

Fig. 2. Effect of CD40L and TNFα on transcription activity of HPV-LCR: *a* — transfected $HeLa_{CD40}$ cells stimulated by TNFα and formaldehyde-fixed BHK cells for 43 h showed repression by BHK_{CD40L} and TNFα but not BHK_{wt} (*$p < 0.01$ *vs*. BHK_{wt} and medium) as assessed by the luciferase activity; *b* — IL-6 was significantly elevated in the SNs of the corresponding experiment after 16 h incubation as assessed by ELISA (*$p < 0.01$ *vs.* BHK_{wt} and **$p < 0.001$ *vs*. medium)

DISCUSSION

The functional relevance of the CD40/CD40L interactions in malignant tumors is variable and likely context dependent. It has been shown that CD40/CD40L interactions or up-regulation of CD40 correlated with poor prognosis in pancreatic ductal carcinoma [14] and non-small lung cancer [15], whereas CD40 ligation exerts antiproliferative effects and apoptosis induction in malignant ovarian tumors [16] and melanoma cell lines [17]. Previous studies also showed that not only TNFα but also the CD40/CD40L interactions exert a direct cytotoxicity of $HeLa_{CD40}$ cells in the presence of a protein inhibitor [9].

In vivo studies revealed that CD40 expression correlated with HPV positivity and VEGF expression as well as microvessel density, which provides explanation for its high levels in cervical carcinoma and its absence in cervicitis and normal cervix [18]. Persistent HPV infection of the cervical epithelium may lead to progression from cervical dysplasia to cancer over years or decades. Resolution of epithelial HPV infection depends on complex interactions between the HPV-infected keratinocytes and both host cell-mediated and humoral responses [19, 20]. Repression of HPV oncogenes in HeLa cells resulted in the orderly restoration of dormant tumor suppressor pathways via mobilization of the p53 and Rb pathways to deliver growth inhibitory signals to the cells [21]. Interestingly, we could show that CD40/CD40L interactions and TNFα treatment resulted

in repression of the HPV18-p105-promoter in transfected $HeLa_{CD40}$.This mechanism could be effective in early stages of HPV18-infection and could also contribute to the regression of HPV18-associated high-grade squamous intraepithelial or tumor lesions expressing the HPV oncogene products. Accordingly, HPV-induced cancer therapeutic vaccines targeting the E6 and E7 oncoproteins are combined with adjuvant and costimulatory anti-CD40 antibodies. The CD40/CD40L interactions and TNFα treatment exert several antitumor effects, directly by cytotoxicity and indirectly *via* repression of oncogenes and eliciting antigen-specific T cell responses [22].

Various cytokines have been implicated in the pathogenesis of cervical cancer, among which IL-6 has received particular attention. Similarly to CD40/CD40L interactions and TNFα treatment, IL-6 shows a context-dependent pleiotropic effect as it promotes growth of certain tumors, such as cervical cancer, whereas it inhibits the growth of others. Recent studies showed that IL-6 favors proliferation and metastatic spread and its high expression in cervical malignant cells corresponds to bad prognosis. It has also been shown that IL-6 promotes cervical tumorigenesis mainly by activating VEGF-mediated angiogenesis via a STAT3 pathway [23]. In the present study, decreased activity of HPV18–105 promoter upon BHK_{CD40L} and TNFα treatment was associated with the significant increase of IL-6 levels. Although, the released IL-6 may provide neovascularization, promoting tumor growth and spread; this may also result in efficient recruitment of immune cells and better delivery of therapeutic cytotoxic drugs. Furthermore, certain cytotoxic drugs, such as zerumbone, not only induce cell death and apoptosis but also inhibit IL-6 release [24].

To conclude, the present study further confirms the antitumor properties of CD40/CD40L interactions and TNFα treatment in HPV-positive cervical carcinoma. Boosting of CD40 expression and release of TNFα might be considered as adjuvant therapies.

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