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Modulation of telomerase activity, bTERT and c-Myc induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin during Bovine Herpesvirus 1 infection in MDBK cells

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) influences infection of kidney cells (MDBK) with Bovine Herpesvirus 1 (BHV-1) through an increase in virus replication and an acceleration of BHV-1-induced apoptosis. Previously our group demonstrated that BHV-1, in the early stages of infection, significantly up-regulates telomerase activity in MDBK cells, while, in the late phases of infection, when BHV-1-induced apoptosis occurred, a down-regulation of telomerase activity was detected. Hence, herein, for the first time, we described the influences of TCDD on telomerase activity during virus infection. In kidney cells (MDBK) infected with BHV-1 and exposed to different doses of TCDD we explored telomerase activity by TRAP assay. Concomitantly, we examined protein levels of both bTERT and c-Myc by Western blot analysis. In all groups, TCDD induced an acceleration in down-regulation of telomerase activity. Particularly, TCDD drastically and significantly decreased telomerase activity when virus-induced apoptosis took place. This result was accompanied from an accelerated down-regulation of bTERT and c-Myc. Finally, in the presence of TCDD, we evidenced a dose-dependent overexpression of aryl hydrocarbon receptor. Hence, our data suggest that TCDD, through a significant acceleration in down-regulation of telomerase activity, bTERT and c-Myc, may contribute to accelerated BHV-1-induced apoptosis.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), commonly known as dioxin, is a highly toxic and persistent environmental contaminant. Several studies suggest that the exposure to TCDD suppresses immune responses and decreased host resistance to infectious disease (Mandal, 2005). In particular, dioxin enhances mortality in mice infected with influenza virus (Burleson et al., 1996), increases gene expression of human immunodeficiency virus type-1 (HIV-1) in chronically infected promonocytic U1 cells (Gollapudi et al., 1996) or activates replication of cytomegalovirus in human fibroblasts (Murayama et al., 2002). Moreover, TCDD activates HIV-1 replication in OM 10.1 cells, promyelocytic cell line latently infected with HIV-1 (Ohata et al., 2003), and increases Bovine Herpesvirus 1 (BHV-1) replication in kidney cells (Fiorito et al., 2008a). Recently, high levels of TCDD have been detected in dairy products from some areas of Campania Region (Italy) (Diletti et al., 2003; Santelli et al., 2006; Esposito et al., 2009, 2010) where BHV-1 is widespread (2004/558/CE; Ackermann and Engels, 2006).

BHV-1, a member of the alpha-herpesvirinae subfamily, is an important pathogen that, in cattle, can provoke infectious bovine rhinotracheitis (IBR), conjunctivitis, abortions and shipping fever, which is a complicated infection of the upper respiratory tract. BHV-1 initiates the disorder through immunosuppression that could render the animals more susceptible to secondary bacterial infections, leading to pneumonia and occasionally to death (Tikoo et al., 1995; Jones, 2003). BHV-1 establishes latency in sensory neurons of the infected host. Reactivation from latency is stimulated by dexamethasone treatment or increases in natural corticosteroids resulting in virus shedding and spread to susceptible hosts (Jones, 2003). The genes of BHV-1 are expressed in three temporally distinct phases identified as immediate-early (IE), early (E), and late (L) and it is generally approved that tissue-specific

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; MDBK cells, Madin-Darby bovine kidney cells; AhR, aryl hydrocarbon receptor; bTERT, bovine telomerase reverse transcriptase.

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factors mediate latency and/or pathogenesis by influencing viral gene expression (Jones, 2003).

In Madin-Darby bovine kidney (MDBK) cells BHV-1 induces apoptosis which occurred only during the late stages of infection (Devireddy and Jones, 1999; Fiorito et al., 2008b) while, TCDD was able to accelerate BHV-1-induced apoptosis by accelerating the activation of initiator caspases 8 and 9, as well as of executioner caspase 3, through modulation of Bcl-2 family members (Fiorito et al., 2008b).

Telomerase, a multi-component ribonucleoprotein complex, is the enzyme responsible for telomere elongation that catalyzes the synthesis and extension of telomeric DNA repeats (Greider and Blackburn, 1989). Telomerase activity is specifically expressed in immortal cells, cancer and germ cells, where it compensates for telomere shortening during DNA replication and thus stabilizes telomere length, while it is absent in most somatic cells (Shay and Bacchetti, 1997). The absence of telomerase activity often leads to progressive telomere shortening, known to initiate cellular senescence and irreversible cell cycle arrest. Several viruses have been shown to modulate telomerase activity. Kaposi's sarcoma herpesvirus (Verma et al., 2004), Marek's disease virus (Djeraba-AitLounis et al., 2004) and herpes simplex virus type-1 (HSV-1) (Yang et al., 2003), all up-regulate telomerase activity, while some viruses can also negatively regulate telomerase activity such as human immunodeficiency virus (Franzese et al., 2004) and Hepatitis B virus (Fan et al., 2000). Previously, our group demonstrated that BHV-1, in the early stages of infection, significantly up-regulates telomerase activity in MDBK cells and that the effect was mediated by an immediate-early viral gene (Pagnini et al., 2006) whereas, in the late phases of infection, when BHV-1-induced apoptosis occurred (Devireddy and Jones, 1999; Fiorito et al., 2008b), a down-regulation of telomerase activity was detected (Fiorito et al., 2012). Structurally, telomerase is a ribonucleoprotein enzyme complex which contains a catalytic protein subunit and an essential RNA component. The catalytic protein subunit contains the telomerase reverse transcriptase activity. In humans, the telomerase reverse transcriptase (hTERT) catalytic subunit is a protein which is actively up-regulated by c-Myc (Wu et al., 1999).

The mechanism of action by which TCDD exerts the biochemical effects on vertebrate species is through activation of the aryl hydrocarbon receptor (AhR), a ligand-activated basic helix-loop-helix transcription factor, and a member of the PER-ARNT-SIM (PAS) superfamily of transcription factors. Binding of AhR/ARNT leads to changes including induction of the cytochrome P-450 1A1 (Mandal, 2005). It has been demonstrated that, in human choriocarcinoma cells (BeWo), TCDD induces telomerase activity mediated through AhR signaling with an increase in hTERT copy number as well as in c-Myc protein levels (Sarkar et al., 2006). Hence, the aim of current study was elucidate the influence of dioxin on telomerase activity, bovine TERT (bTERT) and c-Myc in kidney cells infected with BHV-1. Moreover, to explore the mechanism of action of TCDD, we investigated the involvement of aryl hydrocarbon receptor in this cell model line.

2. Materials and methods

2.1. Cell cultures, virus infection and TCDD exposure

MDBK cells (American Type Culture Collection, CCL22) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 2% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin, 0.2% sodium pyruvate and 0.1% tylosin, a macrolide-class antibiotic. Cells were maintained in an incubator at 37 °C (in 5% CO_2/95% air). This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus by real time PCR analysis (Cordero Camacho et al., 2011). The BHV-1 Cooper strain was used throughout the study. Virus stocks were routinely grown on MDBK cells and were also used for determination of virus titers (De Martino et al., 2003). We used 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 10 μg/ml in toluene (Supelco, 48599). TCDD was initially diluted to give a 10,000 pg/ml stock solution by mixing with DMEM. This stock solution was then diluted to give working solutions of 0.01, 1 and 100 pg/ml in DMEM (from 3.1×10^{-14} to 3.1×10^{-10} M), which were added to cultures, according to Fiorito et al. (2008a,b,2010,2011), Santamaria et al. (2011). All other chemicals were of the highest commercially available purity.

MDBK cells, at confluency, were washed with DMEM and then infected or not with BHV-1, at multiplicity of infection (MOI) of 5, at the same time, in the presence or not of different concentrations of TCDD (0.01, 1 and 100 pg/ml). After 1 h of adsorption at 37 °C, the cells were incubated for 4, 8, 12, 24, 36 and 48 h post infection (p.i.) and then processed.

2.2. Cell viability

Cell viability was evaluated by MTT test, as we previously described (Fiorito et al., 2008b, 2011). Data are calculated as a percentage of the control, and results are the mean ± SEM of four independent experiments performed in duplicate.

2.3. Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were carried out as we previously described (Fiorito et al., 2008b, 2010, 2011). The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: anti-caspase-3 PAb (dilution 1:1000) (Cell Signaling, 9661), anti-caspase-9 PAb (dilution 1:2000) (Stressgen, AAP149C), anti-c-Myc MAb (Santa Cruz Biotechnology, sc-70463) (1:2000), anti-hTERT PAb (Rockland, 600-401-252) (1:1000) (Fiorito et al., 2011), anti-AhR (Sigma, AV32243) (1:250), and anti-ß-actin MAb (Sigma, A5316) (1:7500).

2.4. Detection of telomerase activity

Telomerase activity was evaluated by Telomeric Repeat Amplification Protocol (TRAP) assay using the TeloTAGG Telomerase PCR ELISA^{PLUS} (Roche Applied Science, 12013789001) which is an extension of the original method described by Kim et al. (1994), as our group previously described (Pagnini et al., 2006; Fiorito et al., 2011).

2.5. Statistical analysis

Data are presented as mean ± S.E.M. One-way ANOVA with Tukey's post-test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). P value < 0.05 was considered statistically significant.

3. Results

In order to evaluate the effect of TCDD on cell death during BHV-1 infection, MDBK cells were infected with BHV-1 alone or in the presence of different concentrations of TCDD and underwent, at different hours post infection, to MTT assay, as described in Section 2. In agreement with previous reports (Devireddy and Jones, 1999; Fiorito et al., 2008a,b), in the present study we confirmed that TCDD induces a significant time and dose dependent viability decrease of BHV-1 infected cells (Fig. 1). In particular, after 8 h p.i., we observed a significant ($p < 0.05$) reduction of cell viability only in the presence of the highest dose of dioxin

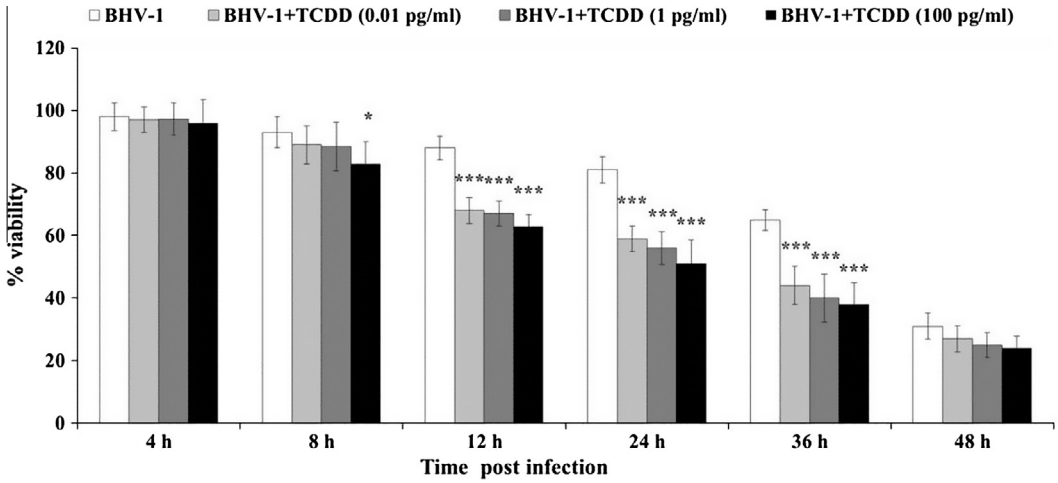

Fig. 1. Dose response curve of MDBK cells treated with different concentrations of TCDD and observed at different times on cell viability. Viable, adherent cells were stained with MTT at different times of incubation and the absorbance assayed as described in Materials and Methods section. Data are presented as mean ± S.D. of four independent experiments performed in duplicate. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability *p*. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

(100 pg/ml). From 12 h to the end of infection, TCDD drastically and significantly ($p < 0.001$) increased cell death of infected groups in a dose-dependent manner.

To understand the role of TCDD in the apoptotic mechanism induced by BHV-1 in MDBK cells, the effects of different concentrations of TCDD (0.01, 1 or 100 pg/ml) on the caspases 3 (initiator caspase) and 9 activation (executioner caspase) were examined. Analysis of BHV-1 infected cells exhibited activation of caspases 3 and 9 already at 4 h after infection in all TCDD exposed groups, whereas only at 12 h p.i. in unexposed groups (Fig. 2A and B). In particular, the activation of caspases 3 and 9, in all treated groups, was regulated in a dose-dependent manner, as shown in Fig. 2A and B.

To understand the effects of TCDD on telomerase activity, BHV-1 infected cells were exposed or not to TCDD (0.01, 1 or 100 pg/ml) and analyzed, at different hours post exposure, by TRAP assay. Following infection in MDBK cells, in BHV-1 infected groups, we detected high levels of telomerase activity, which significantly decreased at the end of infection (Fig. 3A). While, in infected groups, starting to 4 until 8 h p.i., TCDD induced a down-regulation of telomerase activity ($p < 0.05$ and $p < 0.01$), which drastically and significantly ($p < 0.001$) decreased from 12 h to 36 h p.i. (Fig. 3A). No significant differences between unexposed and exposed infected groups were detected at the end of infection (Fig. 3A). Analysis of bTERT protein expression by Western blot showed a significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$) decrease in the levels of bTERT in all TCDD treated groups when compared to untreated infected cells (Fig. 3B and C). Then, we carried out Western blot analysis for c-Myc. As we displayed in Fig. 3B and D, from 8 to 48 h, dioxin significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$) down-regulated c-Myc protein levels, at all doses tested. Our data suggest that TCDD may influence BHV-1 infection, through an acceleration in down-regulation of telomerase activity, bTERT and c-Myc.

Finally, to explore the mechanism of action of TCDD, we investigated the involvement of AhR in kidney cells by Western blot analysis. Results from this analysis showed: (i) the presence of AhR in MDBK cells (4A); (ii) the expression of AhR was not influenced by BHV-1 infection (4A,B); (iii) following BHV-1 infection, TCDD induced a significant overexpression of AhR, in a dose-dependent manner (Fig. 4A and B).

4. Discussion

This study suggests that TCDD, through a dose-dependent overexpression of AhR in MDBK cells, is a potent inhibitor of telomerase activity in BHV-1 infection. Concomitantly, reduced levels of bTERT and of c-Myc were found in BHV-1 infected cells in the presence of dioxin. In particular, following infection in MDBK cells, in BHV-1 infected groups, we detected high levels of telomerase activity, which significantly decreased at the end of infection (Fig. 3A), as we previously showed (Pagnini et al., 2006; Fiorito et al., 2012), while, in all infected groups, TCDD induced a down-regulation of telomerase activity, which drastically and significantly decreased starting from 12 h p.i. (Fig. 3A) when, in the same experimental conditions, accelerated virus-induced apoptosis occurred (Fiorito et al., 2008b and here), to the end of infection. As reported by Esposito et al. (2010), an extraordinary plan of official control was carried out in 2008 in Campania Region (Italy) with the aim to monitor polychlorinated dibenzo-p-dioxins (PCDDs) levels in 460 samples of buffalo milk collected from 304 farms in the province of Caserta (Italy). The range of WHO-TEQ values for the PCDD/Fs in milk was 0.17 pg TEQ/g fat and 87.0 pg TEQ/g fat with a mean value 3.63 pg TEQ/g fat and medium value 2.25 pg TEQ/g fat. The geo-referencing analysis allowed to individuate a restricted area of the region where was located the majority of the non-compliant farms. The study of the congeners distribution suggested that the likely cause of contamination is to be attributed to the illegal burning of waste. As showed by Devireddy and Jones (1999), in the same experimental conditions, following infection of BHV-1 in MDBK cells, inhibition of caspase activity increased the overall virus yield, indicating that caspases enhance virus release. Furthermore, BHV-1 can infect CD4(+) T cells in cattle, leading to apoptosis and suppression of cell-mediated immunity, during acute infection of cattle (Winkler et al., 1999). Finally, in our recent study, we performed an epidemiological analysis on diffusion of IBR in some areas where high levels of TCDD have been detected in dairy products. We collected serum and milk samples to detect antibodies for IBR from cattle raised on farms in contaminated areas in Campania Region. Then, by using both IBR-gB and IBR-gE E.L.I.S.A. kit (IDEXX), which represents the test procedure of choice in many European IBR programs, we revealed a significant diffusion of IBR on samples collected from farms in contaminated areas, compared to samples collected in uncontaminated areas (data not shown).

It is known that immediate-early BHV-1 infected cell protein 0 (bICP0), the bovine homologue of HSV-1 ICP0, regulates all three the phases of virus replication by acting as a strong activator or as a repressor of specific viral promoters (Wirth et al., 1991; Jones et al., 2006). Recently, following BHV-1 infection in kidney cells, in the presence of TCDD, we detected an increase of both bICP0 gene

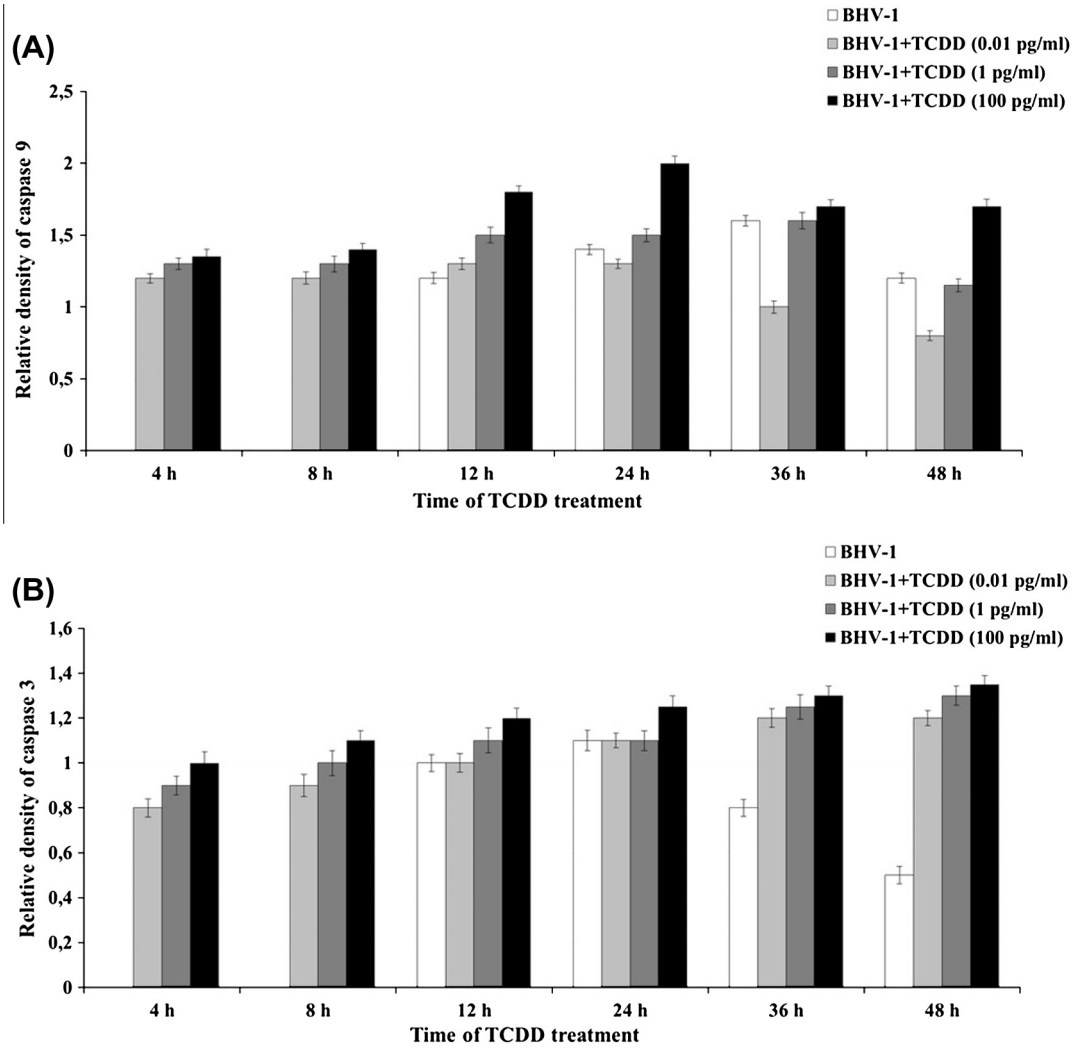

Fig. 2. TCDD anticipates of activation of caspases 9 and 3 during BHV-1 infection. Whole-cell lysate was prepared from untreated cells, infected cells, or infected and exposed to different concentrations of TCDD (0.01 pg/ml), (TCDD 1 pg/ml) or (TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h p.i., Western blot analysis was performed with an antibody which specifically recognized caspase 9, caspase 3 or β-actin. β-actin was used as an internal loading control. (A) Densitometry analysis of caspase 9; (B) densitometry analysis of caspase 3. Results are the mean ± S.D. of three separate experiments.

and protein expression levels (Fiorito et al., 2011). Hence, we hypothesize that the accelerated down-regulation of telomerase activity, herein showed, may be the result of a relationship between TCDD and bICP0. We supported this idea because Pagnini et al. (2006) also showed that the modulation of telomerase activity induced by BHV-1 was mediated by an immediate-early viral gene. Future studies will be crucial to elucidate the mechanism by which TCDD acts during BHV-1 infection in kidney cells or *in vivo*.

Among the various components associated with telomerase complex, the telomerase catalytic subunit TERT has been demonstrated to be the rate-limiting determinant of telomerase activity (Nakamura et al., 1997). Hence, bTERT protein levels were examined to establish if telomerase inhibition by TCDD was indeed mediated through modulation of the catalytic subunit bTERT. Herein, the levels of bTERT protein significant decreased following TCDD treatment when compared to untreated infected cells. By comparing telomerase activity and bTERT protein level measurements, we observed a good correlation, showing a similar trend. These data were in agreement with previous studies carried out on MDBK cells (Fiorito et al., 2011) and on esophageal epithelial cells, describing an evident correlation between telomerase activity and the amount of cellular hTERT mRNA (McGruder et al., 2006).

To determine if the regulation of c-Myc was also involved in telomerase down-regulation by TCDD, we carried out Western blot analysis for c-Myc, and we showed that dioxin significantly down-regulated c-Myc protein levels. As reported above, hTERT is actively regulated by c-Myc (Wu et al., 1999). hTERT proximal core promoter contains two binding sites for c-Myc, which acts as a regulatory factor for hTERT transcription (Wu et al., 1999; Kyo et al., 2000). Results from our analysis showed that during BHV-1 infection in MDBK cells there was a significant repression of c-Myc protein levels by TCDD. Inhibition of c-Myc expression has been closely associated with cell death and hTERT activity, which were consistent with its down-regulation induced by dioxin and the resulting decrease in telomerase activity. Furthermore, in TCDD exposed groups, both bTERT and c-Myc exhibited significant differences until the end of infection while no differences in cell viability as well as in telomerase activity were observed at 48 h between unexposed and exposed groups.

To date there are very few studies describing how TCDD impacts on telomerase activity. In particular, TCDD did not affect telomerase activity either in normal human epidermal cells or

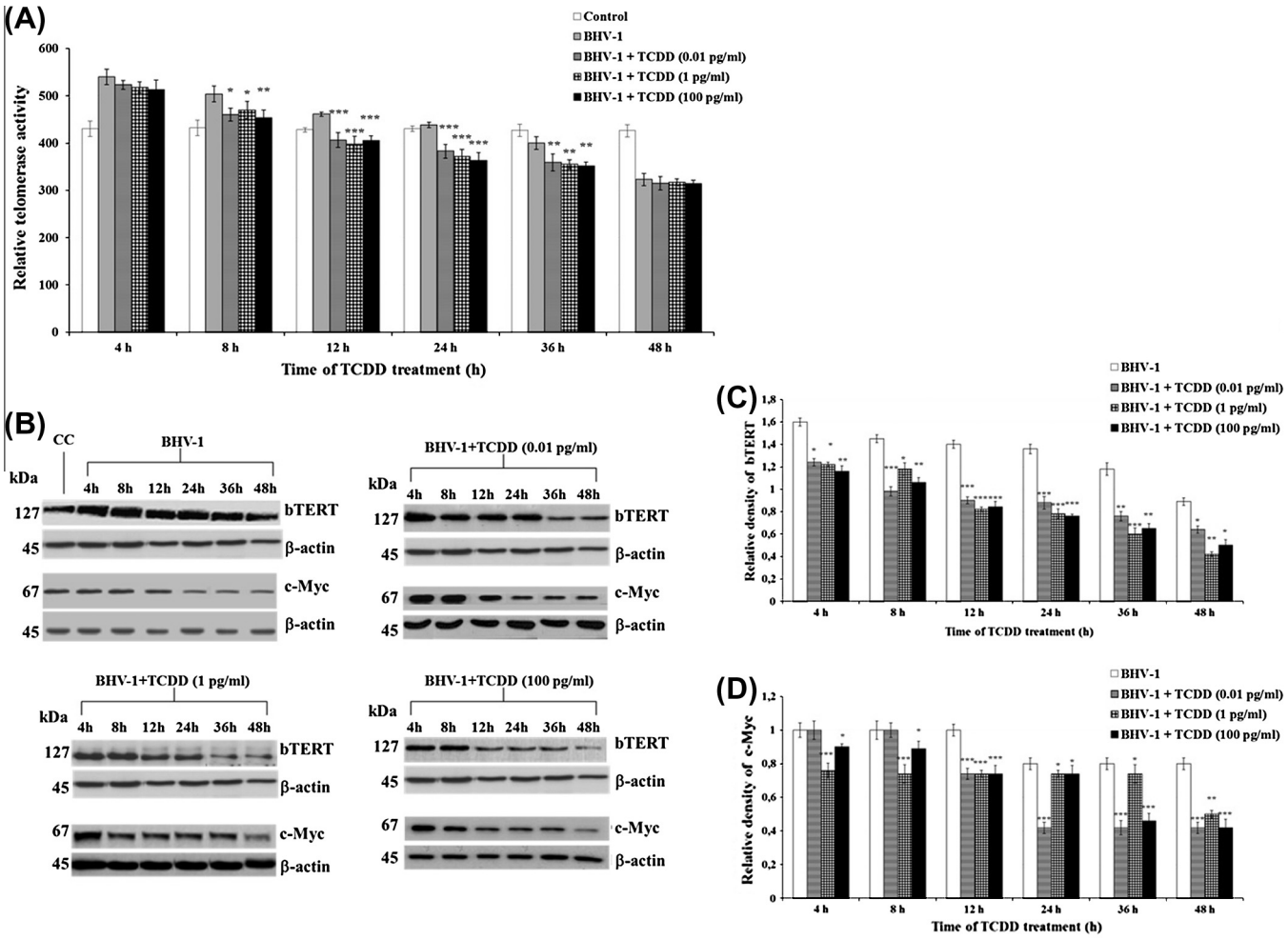

Fig. 3. TCDD accelerates down-regulation of telomerase activity, bTERT and c-Myc protein levels during BHV-1 infection in MDBK cells. (A) Telomerase activity in MDBK cells infected with BHV-1, exposed or not to TCDD, after 4, 8, 12, 24, 36, or 48 h of telomerase activity was measured by the TRAP assay and results are expressed as relative telomerase activity (RTA). Results are the mean ± SD of three separate experiments performed in duplicate exposure. (B) Whole-cell lysates were prepared from untreated cells (lane CC), infected unexposed (lane BHV-1) or infected and exposed to different concentrations of TCDD (lane BHV-1 + TCDD 0.01 pg/ml), (lane BHV-1 + TCDD 1 pg/ml) or (lane BHV-1 + TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h, Western blot analysis was performed with antibodies which specifically recognized bTERT, c-Myc or β-actin, as described in Section 2. β-actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (C) Densitometry analysis of bTERT blots shown in B. (D) Densitometry analysis of c-Myc blots shown in B. Results are the mean ± S.D. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

immortalized cells or malignant keratinocytes (Rea et al., 1998). Nevertheless, as above reported, BeWo cells treated with two concentrations of TCDD, showed a dose-dependent increase in telomerase activity, mediated through AhR signaling, with an increase both in hTERT copy number and in c-Myc protein levels (Sarkar et al., 2006). Conversely, analysis of telomerase activity showed that TCDD down-regulated telomerase activity in MDBK cells, with a significant decrease in bTERT protein levels (Fiorito et al., 2011). As above reported, viruses has been demonstrated to be able to increase telomerase activity, as well as, to down-regulate telomerase activity. Generally, viruses may up-regulate telomerase activity of infected cells and inhibit apoptosis to prevent pre-mature cell death, but some viruses, such as BHV-1, may increase telomerase activity in the first time of infection (Pagnini et al., 2006) and may down-regulate telomerase activity (Fiorito et al., 2012 and here) to induce apoptosis in the late phase of infection to increase virus yield (Devireddy and Jones, 1999; Fiorito et al., 2008b). Herein, for the first time, we described the effect of dioxin on telomerase activity during virus infection. Moreover, to our knowledge, this is the first report showing the presence of AhR in MDBK cells, as well as the induction of AhR due to dioxin. Our data suggest that TCDD may influence BHV-1-induced apoptosis, by accelerating down-regulation of telomerase activity, bTERT and c-Myc. Such observations correlate with the finding that TCDD exposure in kidney cells infected with BHV-1 causes a dose dependent increase in cytopathy, an increased viral titer (Fiorito et al., 2008a) and an acceleration of BHV-1-induced apoptosis (Fiorito et al., 2008b). Taken together, the results suggested that TCDD may act as an additional risk factor for disease progression.

Conflict of Interest

None.

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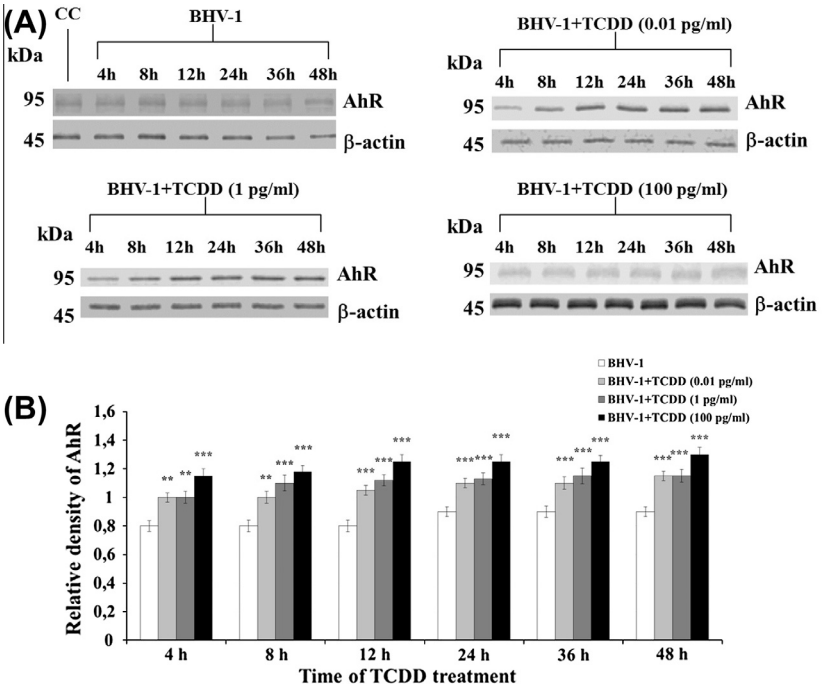

Fig. 4. TCDD induces overexpression of AhR protein levels during BHV-1 infection in MDBK cells. (A) Whole-cell lysates were prepared from untreated cells (lane CC), infected unexposed (lane BHV-1) or infected and exposed to different concentrations of TCDD (lane BHV-1 + TCDD 0.01 pg/ml), (lane BHV-1 + TCDD 1 pg/ml) or (lane BHV-1 + TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h, Western blot analysis was performed with antibodies which specifically recognized AhR or β-actin, as described in Section 2. β-actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (B) Densitometry analysis of AhR blots shown in A. Results are the mean ± S.D. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

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