REGULATION OF HUMAN GENE OF INFLUENZA A VIRUS-ASSOCIATED NS1-BINDING PROTEIN BY 2,3,7,8-TCDD: MECHANISTIC DATA AND EPIDEMIOLOGICAL FINDINGS

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Abstract
Agonist-induced recognition of a cognate DNA enhancer dioxin responsive element (DRE) does epitomize wide range of mammalian genes expression mediated via the Ah receptor pathway. The same was postulated also for viral DRE-containing genes expression caused by 2,3,7,8-TCDD in infected human cells. In this study, such mechanistic concept applied to type A influenza virus nonstructural protein 1 binding protein (NS1BP) induction in humans and chicken. The data are presented at genetic, cellular, and population levels. Primers for mutation analysis were constructed for two DRE identified within enhancer region of the *IVNS1ABP* gene. Treatment of HeLa cell line with 0.1 nM of TCDD resulted in substantial increase of NS1BP protein level. This might add to influenza virus A non-structural protein 1 (NS1) inhibitory effect on cellular interferons, which determines antiviral resistance of emerging H5N1 virus. Recent H5N1 outbreaks among poultry in China and Vietnam might partially relate to chicken NS1BP, as outbreaks occurred in areas highly contaminated by dioxin-like compounds. Minimal dose of TCDD upregulating human *IVNS1ABP* gene was estimated moderately above current TCDD blood level in general population. So, in human subgroups exposed to TCDD, its body burden might facilitate spreading of H5N1 if avian flu pandemic were to occur.

Introduction
Mechanistic studies on 2,3,7,8-TCDD (TCDD) numerous toxic effects in mammals have revealed mediatory role of the Ah receptor (AhR). TCDD-activated AhR was proven to upregulate the expression of sensitive genes through direct gene transcriptional activation. That resulted from the nuclear AhR-Arnt heterodimer complex binding to DRE in the 5' flanking region of target gene.[1,2] Earlier we discovered that TCDD might also activate *HIV-1* gene expression in infected human cells,[3] and later the AhR-mediated transcriptional pathway was demonstrated for activation by TCDD of *CMV* gene.[4] Mechanistic comprehension of gene activation by TCDD eventuated from DRE (core nucleotide sequence 3' A-CGCAC 5') identified in regulatory region of mammalian and viral genes.[1,2,5,6] In this study, the above mechanistic concept was applied to the *IVNS1ABP* gene encoding influenza virus A NS1 binding protein (NS1BP) in humans and chicken. Alongside an important role this protein plays in the virus life cycle, two DRE sequences were computationally detected in the *IVNS1ABP* 5'-flanking region, at positions - 7942 and - 687.[7]

The NS1 protein of influenza type A viruses (IAV) is known to prevent transcriptional induction of antiviral interferons (IFNs).[8] This viral protein also modulates host cell gene expression and inhibits double-stranded RNA (dsRNA)-mediated antiviral responses. The data suggest that the inhibition of splicing by the NS1 may be mediated by binding to NS1BP of the target cells.[9] In this study, we treated human HeLa cells with TCDD and analyzed whether DRE sites detected in enhancer region of *IVNS1ABP* gene are functionally active, in terms of NS1BP induction. The data obtained were hypothetically extrapolated to recent H5N1 strain outbreaks among poultry and humans reported in geographic regions of China and Vietnam,[10,11] exactly known as areas contaminated with dioxin-like compounds.[12,13]

Materials and Methods
Immunoblotting of the NS1BP. Confluent monolayers of HeLa cells, pretreated with 0.1 nM TCDD for 36 h, were lysed in RIA-precipitation assay buffer. Soluble proteins from equivalent volumes of extract corresponding to 10^4 cells were separated by SDS gel electrophoresis, transferred to a nitrocellulose membrane, and probed with affinity-purified NS1BP-specific antibodies. The antibodies were raised against recombinant human NS1BP, a polypeptide of 69.8 kDa.

Mutation analysis. Fragments containing the putative DRE sites were obtained by cleaving the ~500-bp fragment with PvuII. These were subcloned directly into the Nru I site of the pSEAP2-Promoter. Concatamers consisting of wild type or mutant copies of more proximal DRE site (-687) were prepared by ligation of ds oligonucleotides corresponding to its sequence and inserted into the Nru site of pSEAP2-Promoter. The set used in this study contained two copies of the site. To facilitate site-directed mutational analysis, the segment was subcloned into pGL3 vectors (Promega), which contain the reporter gene luciferase. Mutagenesis, performed using the QC site-directed mutagenesis kit (Stratagene) and oligonucleotides mutated in the core binding sequence of DRE, was confirmed by sequencing.

Quantitative analysis. Determination of TCDD and dioxin-like compounds in tissues of humans and chicken were performed according to earlier described protocols.[14]

Results and Discussion

The IAV replicate and transcribe their RNA genome in the nucleus of the infected host cell. All RNA synthesizing activities associated with influenza virus are performed by the virally encoded RNA-dependent RNA polymerase (RdRp). However, viral transcription is critically dependent on on-going cellular transcription, in particular, on activities associated with the cellular DNA-dependent RNA polymerase II (Pol II). Some viral transcripts require splicing and since influenza virus does not encode splicing machinery, it is dependent on host splicing, an activity also related to Pol II transcription. For purpose of multiplication, the virus not only relies on cellular factors to support its own RNA synthesis, but utilized interaction of the viral NS1 protein with factors involved in cellular pre-mRNA processing.[15]

NS1 is abundantly expressed in infected cells, and characterized by pleiotropic effects, which provide NS1 the key role in determining the host range and virulence of IAV strains. Thus HS1 influences host gene expression, including pre-mRNA splicing, nucleocytoplasmic transport of poly(A) RNA, and translation. NS1 can block the activation of the double-stranded RNA-activated protein kinase (PKR), presumably due to its double-stranded RNA binding activity. The activation of PKR results in a downregulation of translation and is part of the cellular IFNs antiviral defense mechanism.[8] Moreover, NS1 of highly virulent avian H5N1 virus, circulating in poultry and waterfowl and transmitted to humans in Southeast Asia in 1997, is shown associated with resistance of lethal H5N1 to antiviral effects of INFs and TNF-alpha.[16]

Little has been known about the cellular factors recognized by the NS1 protein, until in 1998 a novel human ~70-kDa protein, termed NS1BP, was identified and characterized.[9] Because the wild-type NS1BP promoted pre-mRNA splicing, it was suggested that the inhibition of pre-mRNA splicing by the viral NS1 protein may be mediated by its binding to NS1BP. That protein-protein physical interaction occurs when NS1BP is relocalizing throughout the nucleus of infected cell, presumably via the binding to the viral NS1.[9]

Genetic fragment of this study deals with 13 DRE-containing sequences previously identified in the *IVNS1ABP* gene.[7] Three separate probes were analyzed, in each of those two DREs located in the gene 5'-flanking region, at positions: (-687, -7942,), (-687, -7942), and (-645, -7900). Since determining DRE positions, primer for mutational analysis of DRE at position –687, was designed. Eventually, we showed that mutation of the core binding sequence at –687 (-645) suppressed TCDD-induced reporter gene activity by app. 55%. The results indicate that this DRE site within *IVNS1ABP* promoter is supposedly functional. Functionality of another DRE site in the human *IVNS1ABP* gene identified much further 5' (-7942) remains to be determined. Also, because DRE sequences localized in the positions downstream of TSS were suggested to manifest enhancing effect,[17] that can be addressed to DRE positions identified in the probes of *IVNS1ABP*, namely (+351, +2041), (+351, +2041), and (+695, +811, +1669, +6347, +7810).[7]

In this study, an ability of sub-nanomolar TCDD to induce NS1BP was demonstrated. Detail information on this subject (figures and tables) is presented on accompanied poster/slides. The fact of the matter is that treatment of human HeLa cells with only 0.1 nM TCDD led to a 3- to 4-fold increase in level a NS1BP polypeptide of 69.8 kDa, determined by Western blot. The data obtained might be interpreted in terms of cytosolic Ah receptor activation in HeLa cells by TCDD, followed by AhR/Arnt heterodimer recognition and binding to DRE(s) located in regulatory region of the *IVNS1ABP* gene.

DREs are established acting as enhancers for genes regulated by dioxin-like compounds (DLC). Since the enhancer sites are usually situated far upstream of the gene promoter, gene activation by TCDD probably involves nucleosomal disruption and interaction with transcriptional co-activators/co-corepressors.[1] This coheres our data on TCDD-caused expression of *IVNS1ABP* gene and induction of NS1BP with information on mediatory role of NS1BP in inhibition of pre-mRNA splicing by viral NS1 protein, and physical interaction between NS1 protein and NS1BP when the latter relocates throughout the nucleoplasm of IAV-infected cell.[9] In addition, it was demonstrated that concentration of functional AhR, and its signaling in response to agonists like TCDD are regulated through physical interaction of AhR with NS1BP (also named Ah receptor associated protein 3).[18]

There were several reasons why we began molecular epidemiological study of H5N1outbreaks in South East Asia. First, resistance to antiviral defense of the Asian virulent H5N1strain was demonstrated to be directly associated with NS1.[10,16,19] Second, alongside with known physical and regulatory interactions between NS1BP and viral NS1,[9] and between NS1BP and AhR,[18] we demonstrated in this study that TCDD, at concentration as low as 0.1 nM, was able to activate the AhR transcriptional pathway thus targeting newly DRE-possessing gene, human *IVNS1ABP*.

Third, the *IVNS1ABP* gene was also identified in chicken (*Gallus gallus*). Moreover, a significant similarity was found between human and chicken *IVNS1ABP* genomic alignments. Thus according to GeneCard, HomoloGene, euGenes, and MGD data for chicken protein-coding *IVNS1ABP* (GC01M183532), percent similarity of chicken *IVNS1ABP* to the human orthologous gene is 82.89 (for nucleic acid based comparisons) or 90.64 (for amino acid based comparisons).

Specifically, the Asian H5N1 virus was first detected in Guangdong Province, China, in 1996, but it received little attention until it spread through live-poultry markets in Hong Kong to humans in 1997, killing 6 of 18 infected persons,[20] and human bird flu infection was confirmed by the Chinese Ministry of Health.

At the same time, it was demonstrated that mothers born in Guangdong province, have had a significantly higher CALUX-TEQ (a luciferase expression bioassay for maternal exposure to TCDD/DLC body load). Higher seafood consumption was associated with higher maternal CALUX-TEQ level.[21]

As regards Vietnam, WHO was informed in late 2004 of suspicious chicken deaths in southern Vietnam, in provinces Dong Thap, Tien Giang, and Ben Tre. Earlier in 2005, Vietnam informed WHO that chicken in these southern provinces were infected with H5N1. In a week, Vietnam advised WHO that humans had been admitted to a hospital with a severe respiratory infection. It was announced that tests proved positive for the H5N1 virus. The southern cases had a case fatality rate approaching 100%, while the fatality rate in northern Vietnam fell to 10-20%.[22]

A study was conducted by the TropCenter on the content of TCDD in humans and chicken (Wild Red Juglefowl *Gallus gallus*) from Northern and Southern regions of Vietnam.[14] Thus, in samples from northern provinces Ha Noi, Thang Hoa, and Nghe An, the following levels of TCDD (TCDD-based TEQ, ppt) were determined: human blood - 1.2 ± 0.19 (n=168), chicken fat tissues – 2.02 ± 0.22, chicken eggs – 0.015 ± 0.002. That differs from TCDD levels determined in samples from southern provinces Tay Ninh, Binh Duong, and Can Tho. Thus, TCDD-based (ppt) concentration in human blood (n = 1210) was 3.53 ± 1.04, in chicken fat tissues – 3.13 ± 0.43, and in chicken eggs – 0.35 ± 0.04. There data collaborate with the data of others summarized earlier.[23]

To this end, suggested here induction of chicken NS1BP by body burden TCDD, and its functional interaction with both viral NS1[9] and target cell AhR,[18] more attribute to TCDD role in spreading of IAV among birds than TCCD-caused immunotoxicity is commonly viewed to be, because AhR-mediated immunotoxicity was only demonstrated in avian B-lymphocytes treated with 100 nM TCDD.[24]

So, it was suggested that the data obtained in this study might explain why reported provinces with poultry dying caused by H5N1 strain of AIV in China and Vietnam are that very provinces where environment and birds were found to be contaminated with DLC.

The human *IVNS1ABP* gene possesses two promoter DREs, which is twice as much as in *HIV-1* promoter, and five times less than in *CMV* promoter. Correspondingly, a minimal concentration of TCDD effectively upregulating *IVNS1ABP* gene human cells (0.1 nM), is lower than earlier shown for *HIV-1* gene (1 nM),[3,6] and higher than shown for *CMV* gene (0.3 pM).[4] On the other hand, the concentration of TCDD causing *IVNS1ABP* induction (32 ng/kg) is inside the range of lowest observed adverse (health) effect concentrations determined by WHO (from 10 to 73 ng/kg),[25] which all are within a factor of 10 of the average TCDD body burden in the human population.

Thus in human subgroups slightly exposed to TCDD, its body level might be considered a factor, which could facilitate spreading of H5N1 if avian flu pandemic were to occur.

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