The influence of the signal dynamics of activated form of IKK on NF-κB and anti-apoptotic gene expressions: A systems biology approach

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Abstract NF-κB activation plays a crucial role in anti-apoptotic responses in response to the apoptotic signaling during tumor necrosis factor (TNF)-α stimulation. TNF-α induces apoptosis sensitive to the hepatitis B virus (HBV) infected cells, despite sustained NF-κB activation. Our results indicate that the HBV infection induces sustained NF-κB activation, in a manner similar to the TNF-α stimulation. However, these effects are not merely combined. Computational simulations show that the level of form of the IKK complex activated by phosphorylation (IKK-p) affects the dynamic pattern of NF-κB activation during TNF-α stimulation in the following ways: (i) the initial level of IKK-p determines the incremental change in IKK-p at the same level of TNF-α stimulation, (ii) the incremental change in IKK-p determines the amplitudes of active NF-κB oscillation, and (iii) the steady state level of IKK-p after the incremental change determines the period of active NF-κB oscillation. Based on experiments, we observed that the initial level of IKK-p was upregulated and the active NF-κB oscillation showed smaller amplitudes for a shorter period in HepG2.2.15 cells (HBV-producing cells) during TNF-α stimulation, as was indicated by the computational simulations. Furthermore, we found that during TNF-α stimulation, NF-κB-regulated anti-apoptotic genes were upregulated in HepG2 cells but were downregulated in HepG2.2.15 cells. Based on the previously mentioned results, we can conclude that the IKK-p level changes induced by HBV infection modulate the dynamic pattern of active NF-κB and thereby could affect NF-κB-regulated anti-apoptotic gene expressions. Finally, we postulate that the sensitive apoptotic response of HBV-infected cells to TNF-α stimulation is governed by the dynamic patterns of active NF-κB based on IKK-p level changes.

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1. Introduction

The hepatitis B virus (HBV), a member of the hepadnavirus family, has infected 350 million people worldwide [1]. In 5–10% of patients, the infection advances to lifelong chronic hepatitis B, which is frequently a precursor of cirrhosis and hepatocellular carcinoma [2]. Generally, in host cells, NF-κB is activated by pathogenic viruses [3]. HBV also activates NF-κB in the host cells; this activation is mediated by HBx, preS2, etc. [4–6]. NF-κB may be involved in oncogenesis. In a manner similar to HBV, many oncoproteins are able to activate NF-κB, and its activity is required for subsequent transformation [7,8]. Furthermore, the viral counterpart of NF-κB–v-Rel is highly oncogenic [9]. Some recent reviews have indicated the role of NF-κB in oncogenesis with respect to its function in promoting cell proliferation and transformation [7,8].

NF-κB is a collective term and refers to a class of dimeric transcription factors that belong to the Rel family, which includes RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52) [10]. Traditional NF-κB activation comprises the nuclear localization of RelA/NF-κB1 via inhibitor κB (IκB) degradation [11], NF-κB activation signals, such as stimulation of tumor necrosis factor (TNF)-α and lipopolysaccharide stimulation, result in IκB kinase (IKK) activation by phosphorylation [12]. This activated IKK complex (IKK-p) phosphorylates the IκBα that is bound to RelA/NF-κB1; this process is primarily mediated by IκKβ. The phosphorylated IκBα is subjected to ubiquitin-mediated degradation. IκBα degradation enables the nuclear translocation of the transcription factor RelA/NF-κB1, and this leads to various gene transcriptions, including IκBα transcription. This traditional pathway has a negative feedback loop system that comprises IκBα bound to RelA/NF-κB1, IκBβ, and RelA/NF-κB1. An increasing amount of attention is being focused on this negative feedback loop because it plays the significant role of regulating RelA/NF-κB1. In an alternative NF-κB activation pathway, the NF-κB-inducing kinase (NIK)-IKKα cascade is
activated, and the activated IKK-α then phosphorylates the precursor of NF-κB2 (p100) [11]. This phosphorylated protein is further processed to NF-κB2 (p52) via the degradation of its inhibitory region. Although NIK is not essential for IKK-β-dependent NF-κB activation, it participates in the overall activation of NF-κB, including the activation of NF-κB via IKK-α and IKK-β [13–15].

Recently, various computational models have been proposed and successfully applied in the context of systems biology to solve some interesting biological questions that are otherwise difficult to answer by using conventional biological approaches alone [16]. For instance, some subcellular systems have been investigated by using ordinary differential equations (ODEs), and their intrinsic dynamical properties such as adaptation [17,18], amplification [19], ultrasensitivity [19], multistability [20], and hysteresis [20] were revealed by using the systems biology approach. A recent study on computational modeling of the negative feedback loop system in the traditional NF-κB activation pathway showed that the temporal control of active NF-κB can differently regulate the corresponding gene expressions [21]. Following this study, the active NF-κB regulation system has been widely studied to date [22,23]; however, the computational model of its upstream signaling component has not yet been well studied. Recently, a few attempts have been made to propose a computational model of the entire TNF-α-mediated NF-κB signaling system based on ODEs [24]; however, the dynamic properties of the upstream signaling systems and their effects on NF-κB regulation still remain unclear. In this study, we have developed a computational model of the TNF-α-mediated NF-κB activation pathway by further extending Hoffmann et al.’s model [21]. We have investigated the dynamical properties of traditional NF-κB complex (RelA/NF-κB1) activation in the traditional pathway induced by TNF-α stimulation during HBV infection. Computational simulations demonstrate that not only the initial level of IKK-p but also the quantity that comprises this level changes due to external stimulation; these levels play a crucial role in producing the different dynamic patterns of NF-κB activation in the TNF-α-mediated NF-κB pathway. By using in vitro experiments, these results were then further investigated with HBV infection model systems since HBV induces IKK-p upregulation. The upregulation of IKK-p results in different dynamic patterns of TNF-α-mediated NF-κB activation compared to those at a basal level of IKK-p. These dynamic patterns may cause different regulations of the corresponding anti-apoptotic gene transcriptions; this could then explain the sensitive apoptotic responses of HBV-infected cells to TNF-α. Finally, we postulate that the state change of a system that is caused by an HBV infection changes the initial status of the system, which may then distinctly regulate the corresponding anti-apoptotic gene transcriptions even at the same level of TNF-α stimulation.

2. Materials and methods

2.1. Computational modeling and simulations

Based on the law of mass action, we were able to build a computational model of the NF-κB pathway by using a set of nonlinear ODEs. We employed power law functions, which, in turn, give rise to nonlinear models of the form

\[
\frac{d}{dt} m_i(t) = \sum_{j=1}^{n} k_{ij} \prod_{p_{ij}} m_{p_{ij}}(t) - \sum_{k=1}^{n} h_{ik} \prod_{q_{ik}} m_{q_{ik}}(t)
\]

for 1 ≤ i ≤ n, where \( m_i(t) \) describes the temporal concentration of the ith component, \( g_{ij} \) and \( h_{ik} \) are kinetic orders corresponding to the elementary processes contributing to the production and degradation of \( m_i \), additively, and \( k_{ij} \) and \( h_{ik} \) are the respective rate constants for these processes. In order to facilitate the design and analysis of the computational model, we employed the “modular approach” [25,26]; we decomposed the entire pathway system into three substructures based on their function and dynamical properties (see Supplementary material). Each of these substructures is characterized and modeled by using sequential binding reactions (TNF-α receptor system; stage 1), a series of enzymatic activations (kinase cascade; stage 2), and a complex reaction network forming a negative feedback loop (NF-κB regulation system; stage 3). Further, based on this computational model, we take into account the HepG2/HepG2.2.15 models by introducing additional reaction networks comprising constitutive IKK activation (denoted by the Factor X), HBV inducible NIK-IKK activation (denoted by the Factor Y), and NIK inhibition (denoted by the Factor I) by the TNF receptor-associate factor (TRAF) complex, including TRAFs-and-NIK-associated protein (TNAP). The kinetic parameters and initial concentrations of the participant molecules that were used for the computational simulations were sourced from either literature [17,18,21–27,29] or public databases (http://docs.ncbs.res.in) or estimated from our own experiments by using relevant assumptions (see appendix in Supplementary material). A complete set of 52 differential equations and 84 parameters are summarized in the appendix. Computational simulations have been performed using a 2.7 GHz Pentium 4 PC, and the ODE solver (ode15s function) Matlab R14 (MathWorks, Inc.) was used to solve the differential equations.

2.2. Cells and transfections

HepG2 cells were obtained from American Type Culture Collection, and HepG2.2.15 cells were obtained from Hyesec Cho (Ajou University, Suwon, Korea). The HepG2.2.15 cells are HBV-transfected variants of HepG2 cells that have the ability to replicate HBV [30]. The cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Life Technologies Inc.).

2.3. TUNEL assays

Either the HepG2 or HepG2.2.15 cells were seeded on poly-L-lysine-coated coverslips. At 24 h after the seeding, the cells were treated with TNF-α (40 ng/ml) for 12 h. After the treatment, the cells were fixed with 4% methanol-free formaldehyde solution in PBS (pH 7.4) for 25 min at 4°C. Next, the apoptotic cells were detected with the DeadEnd Fluorometric TUNEL System (Promega).

2.4. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts (5 μg) that were obtained from either the HepG2 cells or HepG2.2.15 cells were incubated for 15 min at 20°C in 10 μl reaction buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 1 μg of poly(dI–dC) with 1 μl [³²P] 5′-end-labeled dsDNA oligonucleotide (10³ cpm/reaction). The dsDNA oligonucleotide, which was used as either the probe or competitor, contained an NF-κB binding site corresponding to the sequence 5′-AGTTGAGGGGACTTTCC-3′ (Promega). The resolved DNA–protein complexes were analyzed by electrophoresis through 5% PAGE containing 50 mM Tris–HCl (pH 8.5), 200 mM glycerol, and 1 mM EDTA. The gels were dried and autoradiographed. When performing the assays with an unlabeled competitor, a 100-fold molar excess of unlabeled dsDNA NF-κB oligonucleotide was added simultaneously with the labeled probe. In the case of the antibody-blocking experiment, an unlabeled competitor, a 100-fold molar excess of unlabeled dsDNA NF-κB oligonucleotide was added simultaneously with the labeled probe.
turer’s instructions. The cDNA was produced by reverse transcription (RT). Following RT, the synthesized cDNA was amplified with 2.5 U Taq Polymerase (Qbio), a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primer set, and an appropriate primer set. The sequences of the primer set were as follows: BCL-xL-specific, Bcl-1-specific, FLICE inhibitor protein (FLIP)-specific, growth arrest- and DNA-damage-inducible proteins 45B (Gadd45B)-specific, X-chromosome-linked inhibitor of apoptosis (XIAP)-specific, and GAPDH-specific. A polymerase chain reaction (PCR) was performed by using a Gene Amp PCR system 2400 (Perkin–Elmer) with 5 min initial denaturation at 95 °C and 20–30 cycles of 30 s at 95 °C, 50 s at 60 °C, and 50 s at 72 °C; these cycles were followed by 7 min of extension at 72 °C. To separate the PCR fragment, 1% agarose gel was used.

3. Results

3.1. HepG2.2.15 cells have a more sensitive response to TNF-α with regard to apoptosis

A previous study demonstrated that TNF-α kills HBx-expressing cells with more sensitivity than it does control cells [31]. Moreover, the hepatocytes of an HBV-infected liver have a faster turnover rate than those of a healthy liver [32]. This could be due to the death of the HBV-infected hepatocytes as a result of immune responses [32]. In this study, the sensitivity of TNF-α-induced apoptosis was tested on HepG2 and HepG2.2.15 cells by using TUNEL assays that detect the DNA fragmentation that is induced by apoptotic events. The results of the analysis reveal that with regard to apoptosis, the HepG2.2.15 cells responded to TNF-α more sensitively than HepG2 cells (Fig. 1).

3.2. Computational model of the TNF-α-mediated NF-κB pathway during HBV infection

It has been reported that both HBV and TNF-α activate NF-κB, and this activation generally prevents apoptosis. On the other hand, it has also been observed that TNF-α induces the apoptosis of HepG2.2.15 cells more sensitively. Based on previous reports, we can infer that the TNF-α-mediated NF-κB activation may be affected by HBV infection. In order to investigate this mechanism in great detail in our study, we employed computational modeling and simulation as well as in vitro experiments. Prior to the development of the computational model, a schematic diagram (so-called, a “biological cartoon”) of the NF-κB pathway was drawn using data from a previously reported review article [11]; this diagram had a slight modification based on recently published data [15,33] (Fig. 2). Recent data revealed that NIK participates in the traditional and alternative NF-κB pathways via complex formations with different adaptor proteins [15]. Moreover, it was discovered that TNAP inhibits NIK in the TNF-α-mediated NF-κB activation [33]. Further, previous reports [4,34] indicate that HBV activates NF-κB via NIK-dependent pathways. In this model, we did not consider the alternative NF-κB activation pathway since it is not activated by TNF-α.

3.3. Dynamic patterns of NF-κB activation depend on the initial level of IKK-p and the amount of its level change by external stimulation

The computational simulations demonstrated that the same level of IKK-p can lead to different NF-κB activation dynamics. Moreover, even a smaller level of IKK-p often results in a higher level of NF-κB activation. These observations motivated us to further investigate the underlying causal relationship in greater detail. In order to investigate whether different initial levels of IKK-p affect the dynamic patterns of NF-κB activation by upstream signals, the dynamic patterns of NF-κB activation were analyzed by using computational simulations with various levels of upstream signal input and various initial levels of IKK-p. The computational simulation of TRAFs complex-IKKK-IKK cascade indicates that in response to the same level of activated TRAFs complex, ΔIKK-p (the amount of IKK-p level change) is larger for a lower IKK-p(I) (initial level of IKK-p) as compared to that for a higher IKK-p(I) (Fig. 3B). In our computational model, ΔIKK-p is restricted by the total amount of IKK. In the computational simulations of NF-κB (Fig. 3D, E, and F), signals are introduced in the form of upregulating the IKK-p level after the initialization for 4000 min. Based on the computational simulations, we discovered that the amplitude of active NF-κB (Aκ) after the introduction of the signal has a positive correlation with ΔIKK-p. As seen in Fig. 3E, the amplitude 2Δκ is almost the same as 1Δκ since the ΔIKK-p of line 1 is equal to that of line 2. On the other hand, Fig. 3D shows that

![Fig. 1. TNF-α induces apoptosis more preferentially in HepG2.2.15 cells as compared to HepG2 cells. Cells were treated with TNF-α (40 ng/ml) or 1x PBS as the control. DNA fragmentations were then analyzed by using TUNEL assay, as described in Section 2. In this experiment, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).](image)
2A1 is larger than 1A1 or 3A1 as ΔIKK-p of line 2 is larger than that of line 1 or line 3. We noted that 2A1 is larger (or not smaller) than 1A1 even though IKK-p(I) and IKK-p(E) (equilibrium level of IKK-p) of line 1 are larger than those of line 2, as seen in Fig. 3D and E. These observations suggest that after the signal onset, ΔIKK-p is more important than IKK-p(E) in determining the amplitude of active NF-κB. Similarly, we know that IKK-p(E) determines the period (Pn) of active NF-κB oscillation since 1P1 is almost identical to 2P1 when lines 1 and 2 have the same IKK-p(E) (Fig. 3F), and on comparing 1P1 with 2P1 (Fig. 3D and E), NF-κB activity was observed to exhibit a shorter period corresponding to higher IKK-p(E). We therefore postulate that the dynamic patterns of NF-κB activation depend not only on IKK-p(E) but primarily on IKK-p(I) and ΔIKK-p.

3.4. Levels of activated NF-κB and IKK-p in HepG2.2.15 cells are higher than those in HepG2 cells before TNF-α signaling

In this study, HepG2.2.15 cells (HBV producing cell line) were used to investigate the NF-κB activation induced by HBV, while HepG2 cells were used as a control. The level of IKK-p in HepG2.2.15 cells is higher than that in HepG2 cells, as seen in Fig. 4A. This may result in constitutive activation of NF-κB in the HBV-infected cells. In order to estimate the NF-κB activity in HepG2.2.15 and HepG2 cells, EMSA was performed with their nuclear extracts. Fig. 4B, C, and D shows that the NF-κB complexes were activated in both – HepG2 and HepG2.2.15 cells, but the activation in HepG2.2.15 cells was higher than that in the HepG2 cells; this could have been caused by HBV. The shifted bands were efficiently decreased by p50 antibody; this represents that the band is the NF-κB/NF-κB probe complex (Fig. 4C and D).

3.5. IKK-p governs the dynamic patterns of NF-κB activation in computational simulations of the NF-κB pathway for HBV infection in response to TNF-α

In order to build a computational model of the NF-κB pathway during HBV infection, HepG2 and HepG2.2.15 cells are studied. From these cell lines, the initial concentrations of some proteins (TRAF2, NIK, IKKα, and IKKβ) in the NF-κB pathway were estimated by immunoblot analysis with standard proteins. The results revealed that the HepG2.2.15 cells had a more active (approximately 2.5-fold on an average) form of NF-κB than the HepG2 cells, as can be seen in Fig. 4. In addition, this 2.5-fold ratio was not severely changed by the sampling time if the time was at least 12 h after the seeding. This implies that the difference level of active NF-κB at equilibrium is constant.

Based on the above experimental observations of HepG2 and HepG2.2.15 cells, a computational model is built by extending the previous NF-κB signaling pathway model. The extended computational model includes Factor X, Factor Y, and three stages of the NF-κB pathway, as described in Section 2. To reflect sustained NF-κB activation in HepG2 cells, which is primarily independent of NIK [4,34] (see Supplementary material), Factor X was introduced in the form of an IKK activating factor. In addition, Factor Y was introduced in the form of an NIK activating factor to address the NF-κB activity in HepG2 cells after TNF-α treatment induced by HBV, which primarily depends on NIK (see Supplementary material).

Based on a step change of TNF-α stimulation after initialization from 0 to 0.1 μM, the computational simulation shows that ΔIKK-p in HepG2 cells is higher than that in HepG2.2.15 cells (Fig. 5). This is due to the level of IKK-p(I) in HepG2 being different as compared with that in HepG2.2.15. On the other hand, the IKK-p(E) in HepG2 is smaller than that in HepG2.2.15 (Fig. 5). These differences (i.e., ΔIKK-p and IKK-p(E)) are responsible for the oscillatory pattern of active NF-κB with prominently higher amplitudes; further, a longer period of activity is observed in HepG2 cells after TNF-α treatment as compared with that in HepG2.2.15 cells.

3.6. Dynamic patterns of the active NF-κB complex in HepG2 cells differ from those in HepG2.2.15 cells during TNF-α stimulation

To analyze the NF-κB activation pattern during TNF-α signaling, the NF-κB activities were estimated by EMSA. EMSA was performed by using NF-κB probes with nuclear extracts obtained from HepG2 and HepG2.2.15 cells at the indicated time immediately after TNF-α treatment, as shown in Fig. 6. On analysis, we found that in response to TNF-α treatment, the active NF-κB complex in HepG2.2.15 cells exhibits oscillation for a shorter period and of smaller amplitudes as compared to the damped oscillatory pattern of HepG2 cells. This result is presumably due to the differences between the HepG2 and HepG2.2.15 cells with regard to the levels of ΔIKK-p and IKK-p(E). Moreover, the differences between the HepG2 and HepG2.2.15 cells with regard to the levels of IKK-p(I) may affect the ΔIKK-p and IKK-p(E), as shown in the Fig. 3B. However, we observed a discrepancy in the experimental data.
Fig. 3. ΔIKK-p and IKK-p(E) after input signal determine the amplitude (\(A_n\)) and period (\(P_n\)) of active NF-κB oscillation. (A) Definitions of notations are indicated. IKK-p(I), initial level of IKK-p before a stimulation; ΔIKK-p, level of decrement or increment of IKK-p after a stimulation; IKK-p(E), equilibrium level of IKK-p after a stimulation. (C) A schematic diagram of the NF-κB activation pathway model used for computational simulations. A computational model of the two-step kinase cascade was constructed, and ΔIKK-p was monitored after a uniform level of input signal at 10 different levels of IKK-p(I). Based on stage 3 of the computational model, the effects of ΔIKK-p and IKK-p(E) on \(A_n\) and \(P_n\) were analyzed. Left panel, IKK-p(I), ΔIKK-p, and IKK-p(E); right panel, dynamic patterns of NF-κB activation. (D) ΔIKK-p varies at the same level of IKK-p(I). (E) IKK-p(I) varies at the same level of ΔIKK-p. (F) ΔIKK-p is adjusted to the same level of IKK-p(E). In figures (D, E, and F), line colors in IKKp correspond to line colors in NF-κBn, active NF-κB complex in the nucleus.
3.7. The undershoot phenomenon in HepG2.2.15 cells during TNF-α treatment may arise due to the inhibition of NF-κB activation induced by HBV.

To explicate the undershoot phenomenon observed throughout the computational simulations, the computational model of the NF-κB pathway was further revised based on the fact that TNAP inhibits NIK-mediated NF-κB activation during TNF-α signaling [33]. Based on this revised model of HepG2.2.15 cells, a new computational simulation demonstrates that the level of IKK-p is immediately reduced after the introduction of a TNF-α signal (Fig. 7). As a result, the observed undershoot of active NF-κB that is specific to HepG2.2.15 cells is revealed in the computational simulation for HepG2.2.15, while the simulation result for HepG2 is unchanged (Fig. 7). The computational simulation further shows that the decrement in IKK-p (i.e., the downward D IKK-p) affects the amplitude of NF-κB activation, even if the effect is not as dominant as compared to the effect of upward ΔIKK-p (see Supplementary material). As observed in the simulation in Fig. 7, ΔIKK-p in HepG2.2.15 cells is smaller than that in HepG2 cells, while IKK-p(E) in HepG2.2.15 cells is higher.

Fig. 4. Levels of IKK-p and activated NF-κB in HepG2.2.15 cells were increased as compared to their levels in HepG2 cells. (A) Total extracts from HepG2 and HepG2.2.15 cells were prepared with RIPA lysis buffer, and levels of IKK-p were estimated by immunoblot analysis with a IKK-p-specific antibody. In this experiment, β-actins were used for the internal control. (B, C, and D) Nuclear extracts were prepared as described in Section 2, and the activated forms of NF-κB were estimated by EMSA with NF-κB labeled with 32P. Shifted NF-κB/NF-κB probe complexes were confirmed by antibody-mediated blocking experiments using anti-p50 antibodies (Ab), as described in Section 2.

Fig. 5. Computational simulations of TNF-α-mediated NF-κB activation with/without HBV infection. (A) A schematic diagram of the NF-κB activation pathway model used for computational simulations. (B) Dynamic patterns of active IKK and active NF-κB have been indicated. The computational simulations were initialized for 4000–6000 min and TNF-α signal was introduced at the zero time point. NF-κBn, active NF-κB complex in the nucleus and IKK-p, active IKK.
than that in HepG2 cells. We note that most of the oscillatory patterns that are observed in Fig. 5 are still preserved, except for the undershoot.

3.8. Analysis of anti-apoptotic gene expressions in HepG2 and HepG2.2.15 cells reveals that the gene expressions differ in response to TNF-α stimulation

Liver regeneration followed by apoptosis is an important mediator of chronic liver disease caused by HBV infection [32]. Previous reports also indicate that an overexpression of the HBx protein sensitizes the cell to apoptosis [31]. Our experiment also showed that with regard to apoptosis, HepG2.2.15 cells were more sensitive to TNF-α stimulation than HepG2 cells (see Supplementary material). TNF-α activates two opposite pathways: the anti-apoptotic pathway and the apoptotic pathway [35]. It is well known that the NF-κB activation inhibits apoptosis via the upregulation of anti-apoptotic proteins such as BCL-xL (preserves mitochondrial integrity), Bfl-1 (inhibits the release of cytochrome c and caspase-3 activation), FLIP (interferes with death receptor signaling), Gadd45β.

Fig. 6. The dynamic pattern of active NF-κB complexes in HepG2 cells treated with TNF-α differs from that in HepG2.2.15 cells. Nuclear extracts were prepared as described in Section 2 at each instance of sampling HepG2 (A and C) and HepG2.2.15 cells (B and D). EMSA was performed with a NF-κB probe labeled with 32P. The intensities were analyzed with the 1D Image Analysis software (Kodak Digital Science), and the relative intensities of the active NF-κB complexes with respect to those of HepG2 cells were calculated (C and D). NF-κB, active NF-κB complex in the nucleus.

Fig. 7. Computational simulation of a model that is updated with regard to the undershoot phenomenon. The inhibition of NIK by the TRAFs complex was introduced such that it competed with downstream molecules such as IKK. (A) A schematic diagram of the revised model used for computational simulations. (B) Dynamic patterns of active IKK and active NF-κB have been indicated. The computational simulations were initialized for 4000–6000 min, and TNF-α signal was introduced at the zero time point. NF-κB, active NF-κB complex in the nucleus and IKK-p, active IKK.
was extracted from these cells. With the extracted RNA, RT-PCRs were performed with primers specific to BCL-xL, Bfl-1, FLIP, Gadd45β, and XIAP. In this analysis, GAPDH was coamplified as the internal control. Amplified products were separated on 1.2% agarose gels, and the band intensities were analyzed by using the 1D Image Analysis software (Kodak Digital Science); the relative intensities in HepG2.2.15 cells with respect to each zero time point were calculated. Note that the number of amplification cycles was adjusted to avoid saturation.

![Image](82x470 to 510x735)

Fig. 8. Analysis of anti-apoptotic gene expressions in HepG2 and HepG2.2.15 cells after TNF-α treatment. After TNF-α treatment, the total RNA was extracted from these cells. With the extracted RNA, RT-PCRs were performed with primers specific to BCL-xL, Bfl-1, FLIP, Gadd45β, and XIAP. In this analysis, GAPDH was coamplified as the internal control. Amplified products were separated on 1.2% agarose gels, and the band intensities were analyzed by using the 1D Image Analysis software (Kodak Digital Science); the relative intensities in HepG2.2.15 cells with respect to each zero time point were calculated. Note that the number of amplification cycles was adjusted to avoid saturation.

... (downregulates pro-apoptotic JNK signaling), and XIAP (inhibits caspase-3, -7, and -9) [35,36]. In order to explore the relationship between different oscillatory patterns of NF-κB activation and anti-apoptotic gene expressions, we compared the mRNA expression levels in HepG2 and HepG2.2.15 cells after TNF-α treatment. Fig. 8 shows that TNF-α activated most of the anti-apoptotic genes in HepG2 cells, while it did not activate most of the anti-apoptotic genes in HepG2.2.15 cells. This indicates that with the same amount of TNF-α treatment, the overall transcriptions of the anti-apoptotic genes chosen in this experiment were mostly upregulated in HepG2 cells but mostly downregulated in HepG2.2.15 cells.

4. Discussion

The dynamical properties of molecular biological systems are difficult to uncover by means of conventional molecular biology methods alone. A systems biology approach, on the other hand, enables us to generate/verify hypotheses concerning the hidden dynamical properties via interdisciplinary studies [37]. From this point of view, a systems biology approach has been introduced in this paper in order to investigate the dynamical property underlying the sensitive apoptosis of HBV-infected cells on TNF-α stimulation. It has been known that TNF-α induces both – an anti-apoptotic response via NF-κB activation as well as an apoptotic response via caspase activation. These two counteractive responses with respect to the TNF-α stimulation are normally balanced but can be prominently inclined to an apoptotic response during HBV infection. Also it has been known that HBV also induces both anti-apoptotic and apoptotic responses [38]. Under HBV infection, NF-κB activation resulting in upregulations of anti-apoptotic genes may compensate the apoptotic responses. The present study has shown that the HBV infection induces sustained NF-κB activation in a manner similar to the TNF-α stimulation, but these effects are not merely added up. Throughout the computational simulations, we postulated that the previously mentioned balance can be disrupted due to different dynamics of NF-κB activation that are caused by not only the initial level of IKK-α but also the quantity that comprises its level change. The dynamics of TNF-α-mediated NF-κB activation under HBV infection may result in the reinforcement of the apoptotic responses. It was reported that the mathematical model for CD95-mediated apoptosis pathway [39] and this model can be applied to the TNF-α-mediated apoptosis under HBV infection in our future study.

Using the HBV infection model system, these results have been further verified by experimental data on the dynamic patterns of NF-κB activation and on the expressions of corresponding anti-apoptotic genes. Recently, it was reported that temporal controls of IKK activity by different stimulations are important for NF-κB activation pattern [40]. The report focused on responses for different stimulation. In this report, however, we focused on responses for the same stimuli at different initial states.

In our data, oscillatory pattern of NF-κB obtained from computational simulation nearly matched the pattern obtained from experimental data. Although this is successful data, points for improving our model are present. In our present model, the frequencies of the oscillations in computational simulation seem to be different from the frequency in the experimental results. In computational simulation, generally, the dependencies of all results on the parameter values are a critical point. In addition, many factors, which need to be considered in this model, still remain because biological systems are more complicated than the model presented in our report. It can be inferred that increasing biological data help to make a more precise computational model.

In this report, we found that the initial state of IKK is very important for NF-κB activation dynamics. These results may explain the TNF-α-sensitive apoptosis of HBV-infected cells and demonstrate the benefits of the systems biological approach in unraveling the hidden dynamics.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.01.004.

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