cells [90,93]. Interestingly, these activities could be inhibited by specific IKK inhibitor, suggesting that upstream molecule of IKK might be responsible for constitutive activation in a Tax-independent mechanism. In chronic myelogenous leukemia (CML) or ALL containing t(9;22) chromosomal translocation, *bcr-abl* expression causes the constitutive NF-κB activation through its tyrosine kinase activity [94]. High levels of NF-κB activity were also demonstrated in CLL B-cells [95]. Moreover, in mucosa-associated lymphoid tissue (MALT) lymphoma, NF-κB is activated through the overexpression of Bcl-10 protein [96,97].

In addition, constitutive activation of NF-κB is reported in many solid tumors such as breast cancer [21,76, 98], prostate cancer [99,100], pancreatic cancer [101], colorectal cancer [102,103], and hematocellular carcinoma [104]. In breast cancer, overexpression of *p100* and/or *bcl-3* lead to constitutive NF-κB activation followed by overexpression of *CyclinD1* [98,105]. These NF-κB activities are correlated with those of ErbB2 (HER2/neu). In colorectal cancer and hepatocellular cancer, chronic inflammation maintains the persistent activation of NF-κB and conceptually contributes to tumorigenesis.

There is a new twist in the role of NF-κB in tumorigenesis. Although both classical and alternative NF-κB activation pathways are involved in carcinogenesis and tumor development, previous studies suggest that the classical pathway mediated by IKKβ is mainly responsible for the carcinogenic function of NF-κB in inflammation-associated tumor "promotion", but not in tumor "initiation" [103,104,106]. For example, in a mouse model of colitis-associated colon cancer, the tumor promoting function of NF-κB in enterocytes was not associated with its ability to activate pro-inflammatory genes but rather with its ability to suppress the apoptosis of pre-neoplastic progenitors [103]. Ablation of IKKβ in enterocytes decreased the tumor incidence without affecting the tumor size or composition, indicating that the IKKβ-dependent NF-κB activation operates during early phase of tumor promotion in enterocytes. It is noted that, in these models, the ablation of IKKβ only in myeloid cells leads to decrease not only tumor number but also tumor size. In addition, it is reported that injection of neutralizing antibodies against IL-6 receptor decreased tumor number and size in the colitis-associated colon cancer model [107]. These findings suggest that myeloid cells infiltrated into the inflammation tissues promote tumor promotion by producing inflammatory cytokines such as IL-6 through classical NF-κB activation pathway.

Similarly, the promoting effect of IKKβ-NF-κB activation pathway in tumor promotion was demonstrated in the inflammation-associated hepatocellular carcinoma model [104]. In contrast to these two studies, Maeda et al recently reported that the deletion of IKKβ in hepatocytes with pre-malignant state, which is initiated by pro-carcinogen diethylnitrosamine (DEN), results in marked increase of tumor incidence and size [106]. Interestingly, the activation of IKKβ in Kupffer cells is essential for production of IL-6, and additional deletion of IKKβ in Kupffer cells resulted in a remarkable decrease in tumor load, supporting the essential role of NF-κB in hepatocellular carcinogenesis. It is postulated that loss of NF-κB activation could not rescue the hepatocytes from DEN-induced apoptotic or necrotic cell death, and that cellular constituents released from necrotic cells might activate Kupffer cells, which produce pro-inflammatory cytokines such as TNF or IL-6, resulting in the stimulation of compensatory hepatocyte proliferation [3]. These inflammation-associated carcinogenic models provide us additional roles of NF-κB in tumor development.

In contrast to these findings, the opposite roles of NF-κB in tumorigenesis are reported. For example, NF-κB inhibition in the epidermis promotes the development of spontaneous squamous-cell carcinoma [108]. In this model, inhibition of NF-κB causes sustained JNK activation through TNFα-mediated signaling, resulting in increasing the risk for skin carcinogenesis.

10. p53 tumor suppressor and NF-κB

Tumor suppressor protein p53 contains both DNA binding and trans-activation domains, and acts, like NF-κB, as a transcriptional activator. It is interesting that p53 and NF-κB subunits share the similar DNA-binding domain characterized by the presence of β-barrel structure although their biological actions are opposite: whereas NF-κB activates genes that are involved in cell cycle progression and apoptosis inhibition, p53 activates genes that are responsible for cell cycle arrest, such as *p21*, and induction of apoptosis, such as *Bax* and *Noxa* [51]. In addition, NF-κB and p53 are known to compete in binding to common transcriptional coactivators such as p300 and CBP. Thus, p53 and NF-κB

Apoptosis is a necessary step for an organism to eliminate the cells in which excessive mutations are accumulated. Thus the action of p53 in inducing various proapoptotic genes, including *Bax, Noxa*, and *Fas*, is considered to play a major role in

natural defense mechanism against cancer and leukemia. However, this action of p53 is known to be inhibited by NF-κB such as by inducing anti-apoptotic genes and also by directly interacting with a pro-apoptotic 53BP2 protein. Whereas Bax and Noxa induce apoptosis by directly acting on mitochondria and reducing its membrane potential, thus releasing cytochorome C, Apaf1 and Diablo/Smac, NF-κB inhibits this process by inducing anti-apoptotic Bcl-x_L and Bcl-2, acting to restore the mitochondrial membrane potential, and c-IAPs and XIAP, inhibitors of caspase 9 and Diablo/Smac. In addition, we recently found that 53BP2, identified as an interacting protein to p53 [109](Iwabuchi et al., 1994)and NF-κB p65 [110], induces apoptosis through the mitochondrial death pathway [111] and the NF-κB activation could inhibit the 53BP2-induced apoptosis [112]. It was shown by others that p53 appears to stabilize 53BP2 protein and induce apoptosis [113]. Thus, 53BP2 appears to play a central role in the regulation of apoptotic pathway by interacting with p53, Bcl-2 family proteins, and NF-κB (Fig.3). In contrast to regulation of apoptosis by NF-κB, cellular apoptotic process appears to regulate the action of NF-κB. For example, UV-activated caspase-3 sensitizes cells with apoptosis by cleaving the N-terminal domain (called "DRHDS") of IκBα, which then becomes resistant to the signal-induced phosphorylation and acts as a strong inhibitor of NF-κB, and by demolishing the transcriptional activity of NF-κB by cleaving the trans-activation domain of p65 [94,114].

On the other hand, both p53 and NF-κB are crossly involved in cell cycle checkpoint mechanisms in opposite fashion (Fig.4). Cell cycle checkpoints are operational at G1/S and G2/M stages involving CDK inhibitors at G1/S, p21, p16 and p27, and G2/M inhibitor for Cyclin B/CDC complex, GADD45, respectively. p21 is also involved in G2/M checkpoint through directly interacting with Cdc25C [115]. Whereas production of p21 is under the transcriptional control of p53, a G1/S cyclin Cyclin D1 is controlled by NF-κB. In murine fibroblasts and regenerating liver tissues, for example, the NF-κB activity is increased during G0 and G1 [116], and inhibition of NF-κB in HeLa cells resulted in the depressed production of Cyclin D1, reduction of Rb phosphorylation, and cell cycle arrest at G1 [117].

Thus, cellular apoptotic response is regulated by NF-κB and p53 in a competitive fashion at multiple levels. Since loss-of-function mutations in p53 gene often predispose cells to carcinogenesis, activation of NF-κB could also be regarded as tumor promoting step irrespective of the presence of p53 mutation. In fact, a number of factors

that are known to stimulate NF-κB, including phorbor esters, okadaic acid, UV irradiation and chronic inflammation, were identified as tumor promoters.

11. NF-κB as a feasible target in cancer treatment

Since 1960s, non-steroidal anti-inflammatory drugs (NSAIDs) such as sodium salicilate have been reported to prevent the tumor development, and recent prospective clinical trials concluded that NSAIDs can reduce the incidence of cancer [118]. Although one of the main targets of NSAIDs is *cox-2*, a specific *cox-2* inhibitor celecoxib failed as a chemopreventive drug in multicenter clinical trials with non-familial adenomatous polyposis [119]. On the other hand, Kopp and Ghosh showed that sodium salicylate or aspirin could inhibit the activation of NF-κB in 1994 [120], and many reports demonstrated that conventional NSAIDs including ibuprophen, acetoaminophen, indomethacin or sulindac, could inhibit NF-κB activation [100,121]. These results suggest that in addition to Cox-2, NF-κB could be a crucial target of NSAIDs in the cancer treatment.

Recently, a number of synthetic or natural compounds have been reported as NF-κB inhibitors. For example, several plant extracts or natural resourses including 1'-acetoxychavicol acetate (ACA) [122], (-)-epigallocatechin gallate [123], avicin [124], curcumin [125], genistein [126] or kaurane diterpene [127], have been shown to have inhibitory effect on NF-κB activation. Anti-oxidant compound such as isovitexin [128] or ascorbic acid [129] also inhibits the NF-κB activity. In experimental studies, MG-132 [130], pyrrolidine dithiocarbamate (PDTC) [131] or SN-50 [132] is frequently used as a NF-κB inhibitor. In addition, several clinical drugs including histamine blocker such as desloratadine [133], glucocorticoid [134] or statin [135] were shown to inhibit the NF-κB activity. We have previously reported that gold compound, a clinical therapeutic agent for rheumatoid arthritis, could inhibit the DNA-binding activity of NF-κB through oxidation of the cysteins associated with zinc [42,43]. These natural compounds or drugs have great advantages in safety and appliance, and could be used as chemopreventive agents. Regarding the clinical use of NF-κB inhibitors for cancer treatment, PS-341 (bortezomib, ®velcade), a proteasome inhibitor, is successfully used for multiple myeloma [132]. Phase II clinical trials of bortezomib for relapsed or refractory multiple myeloma showed approximately 30% response rate [136].

Thus, there are many NF-κB inhibitors with potential anti-cancer activity. However, most of these agents have other targets, in addition to NF-κB. In order to select the malingnancies, for which anti-NF-κB therapy could be beneficial, it is necessary to clarify the status of NF-κB and the roles of NF-κB in each neoplasm besides further development of more specific NF-κB inhibitors are needed.

There are some specific NF-κB inhibitors experimentally shown to be effective in the treatment of neoplasms such as Bay 11-7082 [137], dehydroxymethylepoxyquinomicin (DHMEQ) [138] and PS-1145 [132]. We have recently reported the therapeutic effecacy of a novel IKK inhibitor 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP) [139,140] on the tumor cell growth of multiple myeloma and adult T-cell leukemia (ATL) [92,93] (Fig.5). In these cells, NF-κB is constitutively activated as evidenced by the phosphorylation of IκBα and p65 subunit of NF-κB, activation of NF-κB DNA binding, and upregulation of various target genes including *bcl-x_L, bcl-2, XIAP, c-IAP1, survivin, cyclinD1, ICAM-1* and *VCAM-1*. ACHP exhibited a high selectivity for IKKβ and IKKα over other kinases (whereas the 50% of inhibitory concentration (IC_{50}) values for IKKβ and IKKα are 8.5 nM and 250 nM, respectively, those for IKKγ, Syk and MKK4 are over 20μM, measured by *in vitro* kinase assays) [139,140]. In fact, ACHP could inhibit the phosphorylation of IκBα and p65 and induce cell growth arrest and apoptosis in these cells. Moreover, ACHP increased the cytotoxic effect of conventional chemotherapeutic agents [92]. Thus, anti-NF-κB agents can be effectively used in a combination with conventional agents. Interestingly, p65 Ser536 phosphorylation was detected in both myeloma and ATL cells. Remarkably, in fresh ATL cells, p65 Ser536 phosphorylation in the absence of IκBα phosphorylation was detected. These findings suggest that IKKα might be involved in the constitutive NF-κB activation in these cells although currently there is no effective IKKα-specific inhibitor.

12. NF-κB p65 interacting proteins and novel molecular targets for NF-κB actions

As discussed above, NF-κB appears to play major roles in carcinogenesis and its progression by promoting cell proliferation though cell cycle progression, endowing cells with resistance to apoptosis and inducing cell adhesion molecules for distant metastasis of cancerous cells. We also found that NF-κB blocks apoptosis by direct

protein-protein interaction with a proapoptotic 53BP2 protein [110,112]. These actions of NF-κB are exhibited through interaction with promoters of target genes and with target proteins. Since these NF-κB actions do not require genetic mutations, thus are considered at reversible steps of carcinogenesis, prevention of carcinogenesis can be achieved by blocking the activation process of NF-κB or the direct actions of NF-κB, although the mechanism by which most cancers or leukemia achieve constitutive activation of NF-κB may greatly differ in individual cases. However, since normal cells do not exhibit constitutive NF-κB activation, these steps could be feasible targets for novel anti-cancer therapeutic strategies.

We thus initiated comprehensive genetic screening to identify interacting proteins for the transcription-competent subunit of NF-κB, p65 (RelA), by yeast two-hybrid screening utilizing Gal4-based transcription and SOS-Ras signaling. Fig. 6 describes 6 interacting proteins and p65 baits in these studies. These interacting proteins include 53BP2 [110], that we described earlier in this manuscript, RelA-associated inhibitor (RAI) [141,142], FUS/TLS coactivator [143], AES/TLE Groucho family of repressor [144], a novel transcriptional coactivator AO7 [145] and RNA helicase A (RHA) [146].

(1) RAI: This protein was identified with Gal4-based yeast two-hybrid screen using the central portion of p65 as a bait [141]. RAI is located in the nucleus and inhibits the DNA binding activity of NF-κB, thus considered to be a fail-safe mechanism for NF-κB activation. Interestingly, we found that RAI also inhibits the DNA binding of Sp1. It is therefore acts as a strong inhibitor of transcription of genes that are under the control of NF-κB and Sp1 such as TNFα and HIV [142]. Interestingly, others have reported that RAI also inhibits the DNA binding of p53 [147] although we and others could not confirm such activity. It is of note, however, that RAI and 53BP2 shares amino acid sequence homology in the C-terminal region [110] although their subcellular localizations and biological activities are distinct.

(2) AES/TLE: This protein belongs to a Groucho family corepressor which was initially identified as a corepressor protein for Dorsal, a Xenopus homologue of p65(RelA) [148]. We identified AES/TLE as the interacting protein of the C-terminal sub-region of p65 transactivation domain, devoid of the two transactivation (TA) domains, and found that AES/TLE inhibits the NF-κB transactivation by direct interaction with p65 subunit of NF-κB [144]. Although the detailed mechanism is not known, AES/TLE exhibits transcriptional silencing not involving HDAC and

presumably through blocking the chromatin remodeling [149].

(3) FUS/TLS: TLS was initially identified from the chromosomal translocated region in liposarcoma [150] and later found to be a homologue of TAFII68, one of TBP-associated factor (TAF) [151]. We identified FUS/TLS as an interacting protein of the same C-terminal sub-region of p65 transactivation domain utilized in the identification of AES/TLE, and found that it augments the NF-κB transactivation by direct interaction with p65 subunit of NF-κB [143]. Interestingly, FUS/TLS appears to counteract the transcriptional repression of NF-κB mediated by AES/TLE apparently by competing the binding to p65 subunit of NF-κB (our inpublished data). However, it is currently unknown in what conditions either FUS/TLS and AES/TLE selectively interacts with p65. It is possible that tumor cells containing chromosomal translocation involving FUS/TLS exhibit constitutive NF-κB activation.

(4) RNA helicase A (RHA): This protein was similarly identified as AES/TLE and FUS/TLS in the yeast two-hybrid screen [146]. It is considered as one of functional subunit of RNA pol II [152]. We found that RHA augments the transcriptional activity of NF-κB through direct interaction in a manner dependent on the ATPase activity of RHA. Since, both RHA and FUS/TLS contain RNA-binding domain, it is possible that these proteins may participate in the later phase of transcriptional activation presumably through interacting with nascent mRNA driven by NF-κB.

(5) AO7: This protein is characterized by its RING finger domain and was initially identified as an interacting protein for E2 ubiquitination conjugase. We have identified AO7 as an interacting protein to p65 by SOS-Ras yeast hybrid screen using the C-terminal region containing the tranactivation domain of p65 [145]. AO7 is constitutively located in the nucleus and acts as a cofactor of NF-κB-mediated transactivation. Interestingly, AO7 mutant, in which an essential Cys residue conforming the RING finger was mutated and ubiquitination action was abolished, acted as a strong inhibitor of NF-κB in a dominant negative fashion. Although the mechanism by which AO7 exerts transcriptional activation is not known, there are a number of precedental transcription factors such as VP16 and ERα where ubiquitination is required for their transcriptional activity. Therefore, it is considered that ubiquitination does not necessarily involve proteolysis for

transcription and that ubiquitination may rather act as a tag for the interaction with general transcription factor or RNA pol II.

Fig.7 depicts a putative model of actions of these p65-interacting proteins. It is postulated that NF-κB sequentially interacts with these proteins even after the binding to DNA, thus pushing the transcriptional steps from initiation through elongation. These findings support a possibility that there are a number of mechanisms involved in carcinogenesis by augmenting NF-κB actions and these proteins could serve as targets for such carcinogenic processes. Therefore, further elucidation of the actions of these proteins should provide novel therapeutic measures to block NF-κB actions in cancers and leukemia in which NF-κB is constitutively activated. Since each cancer cell might have acquired different strategy by which NF-κB is activated, elucidation of a mechanism of NF-κB activation in each cancer could be a basis for the tailor-made anti-cancer therapy with NF-κB as a final target.

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Figure Legends

Fig. 1. NF-κB inducers and its target genes. A, NF-κB is activated by various inducers including cytokines, mitogens, microorganism product and stress/carcinogens. B, The activated NF-κB induces a number of gene expressions involved in inflammation, immune response, viral replication, anti-apoptosis and proliferation. Interestingly, NF-κB also regulates the gene expressions of Rel/IκB family genesas a self-regulatory mechanism.

Fig. 2. Two NF-κB activation pathway. Canonical pathway is mediated by IKKβ, leading to the phosphorylation of IκBα and its degradation. Noncanonical pathway is mediated primarily by IKKα in an IKKβ-independent manner, leading to the phosphorylation and processing of p100 subunit. We found that the phosphorylation of p65 at Ser536 is mediated by NIK-IKKα pathway in lymphotoxin-β (LTβ) receptor signaling [36].

Fig. 3. Anti-apoptotic actions of NF-κB. NF-κB promotes the gene expressions of anti-apoptotic genes such as bcl-X_L, bcl-2, c-IAPs and XIAP and survivin. In addition, NF-κB directly inhibits the functions of p53 or p53BP2 by protein-protein interaction. It appears that NF-κB counteracts with p53.

Fig. 4. Promotion of cell cycle progression by NF-κB. NF-κB induces gene expression of cyclinD1 and inhibits the functions of p53 and GADD45. Thus, together with anti-apoptotic actions of NF-κB (Fig. 3), NF-κB activation is equivalent to p53 inactivation in the carcinogenic process.

Fig. 5. Redox regulation of NF-κB and involvement of thioredoxin (Trx). Because antioxidants effectively block the signal-induced IκB phosphorylation and its subsequent degradation by 26S proteasome, oxidative action of ROS is proposed to activate the IKK pathway. After dissociation of NF-κB from its inhibitor IκB, NF-κB goes through redox regulation mediated by Trx, a cellular reducing catalyst, which is involved in redox regulation of cellular proteins by reducing the redox-active cysteins through reversible oxidation of the active center dithiol of Trx molecule to a disulfide

[38,39]. Structural and biochemical approaches have provided evidences supporting the molecular model of the redox regulation of NF-κB by Trx. Trx associates with the redox-sensitive Cys within the DNA-binding loop of p50 subunit of NF-κB and reduces the oxidized cysteine on p50 by donating protons in a structure-dependent fashion. The inter-molecular disulfide bridge between Trx and NF-κB must be transient because the binding of Trx to the NF-κB DNA-binding loop prevents NF-κB to recognize the target DNA. Based on biochemical reactions we postulated that zinc ion replaces the inter-molecular disulfide bridge and dissociates NF-κB from Trx [42].

Fig. 6. A novel IKK inhibitor ACHP inhibits the cellular growth of adult T-cell leukemia (ATL) cells. A, The chemical structure of ACHP. B, Inhibitory effect of ACHP on phosphorylations of IκBα and p65 in ATL cell lines. ACHP inhibits the constitutive phosphorylations of IκBα at Ser32 and p65 at Ser536. MT-2 is a Tax-active HTLV-1 infected T-cell line. ED-40515 (-) is a Tax-inactive ATL cell line. C, Inhibitory effect of ACHP on NF-κB DNA binding. ACHP inhibits the DNA binding activity of NF-κB. The arrowhead shows the NF-κB-DNA binding complex. D, Inhibitory effect of ACHP on expression of genes under the control of NF-κB. ACHP downregulates the gene expressions of cyclinD1 and anti-apoptotic genes at mRNA level. The results of RT-PCR are shown. E, Growth inhibitory effect of ACHP on ATL cell lines. ACHP inhibited cellular growth of ATL cell lines. Open symbols show Tax-active cell lines, and closed symbol show Tax-inactive cell lines. The data were obtained from Sanda et al. (2006) [93] with the permission of authors.

Fig. 7. Interacting proteins of p65. We identified 6 proteins as interacting molecules with p65 by yeast two-hybrid screen. A novel protein RAI inhibits p65 in the nucleus (Yang et al., 1999a). 53BP2 acts as a proapoptotic factor [110-112]. AES/TLE inhibits the transcriptional activity of p65 as a corepressor [144]. RHA [146], AO7 [145] and FUS/TLS [143] promote the transcriptional activity of p65 as coactivators.

Fig. 8. The putative model of actions of p65-interacting proteins. It is postulated that NF-κB sequentially interacts with these proteins even after the binding to DNA, thus pushing the transcriptional steps from initiation through elongation. The biochemical reactions and protein-protein interactions exhibited by these proteins could serve as