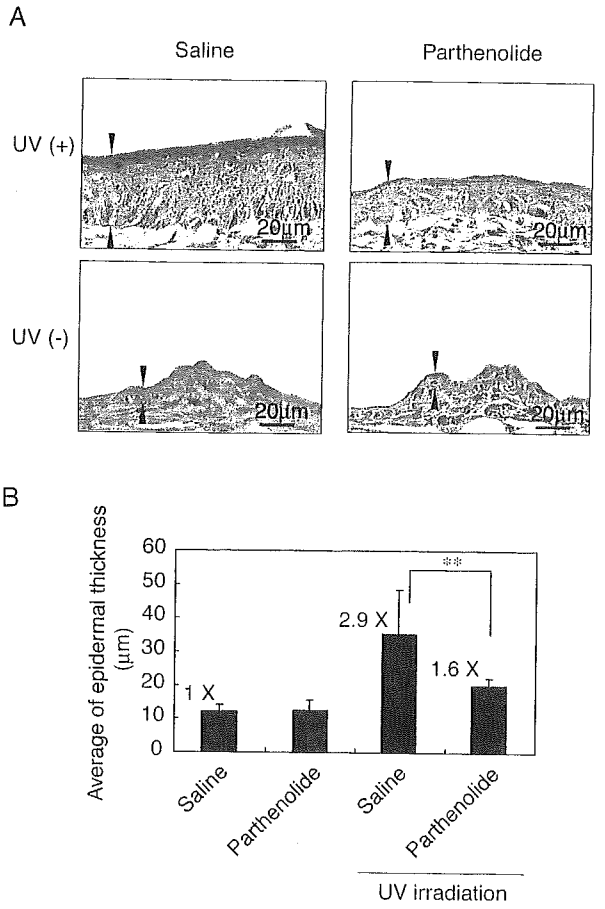

Fig. 4. Epidermal hyperproliferation by UV and inhibition by parthenolide. A, epidermal hyperproliferation by UVB. Parthenolide (250 μg/kg) or physiological saline as control was administered to DBA/2 mice (n = 10 for each group) by repeated i.p. injections every day for 12 days. Half of the mice in each group were exposed to UVB irradiation at 180 mJ/cm^2 every other day. Ear samples were prepared from the anesthetized mice, paraffin-embedded, and cut with a sliding microtome to 5-μm thickness. Tissue sections were subjected to histological examination (H&E staining). Representative pictures are shown. Arrowheads indicate the width of epidermis. B, measurement of the epidermal hyperproliferation induced by UVB and effect of parthenolide. All specimens (n = 5 per group) were examined microscopically for the measurement of epidermal thickness. Data shown are the mean ± S.D. (n = 5) of the average epidermal thickness measured using software for image analysis (Win ROOF). -Fold increase in the epidermal thickness compared with the control is indicated. **, $p < 0.01$; scale bar = 20 μm.

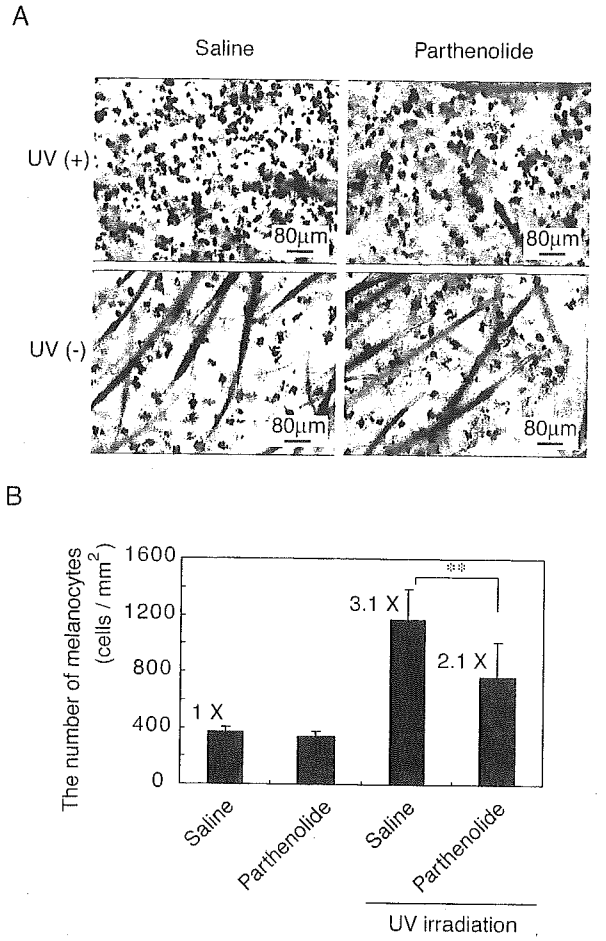

Fig. 5. Induction of melanocyte proliferation by UVB and its inhibition by parthenolide. A, L-DOPA staining of skin melanocytes. DBA/2 mice were treated with parthenolide (250 μg/kg) and exposed to UVB (180 mJ/cm^2) as in Fig. 4. Parthenolide or physiological saline was repeatedly administered to DBA/2 mice by i.p. injection once a day for 12 days. Half of the mice in each group were exposed to UVB irradiation of 180 mJ/cm^2 every other day. To stain melanocyte, the ears obtained from each mouse were soaked in 2 N NaBr solution for exfoliating epidermis and immersed in 0.14% L-DOPA solution for 3 h at room temperature. B, melanocyte counting. All specimens (n = 5 per group) were examined microscopically for counting the DOPA-positive melanocytes in the epidermis. Data shown are the mean ± S.D. of the number of melanocytes per millimeter squared. -Fold increase in the melanocyte numbers compared with the control is indicated. **, $p < 0.01$; scale bar = 80 μm.

Promotion of the Melanocyte Growth by Keratinocytes and Involvement of bFGF. In Fig. 6, we examined if bFGF stimulated the growth of melanocyte and if keratinocytes, upon NF-κB activation, produced bFGF. After 48 h of the bFGF treatment, a dose-dependent stimulation of melanocyte growth was observed (Fig. 6A). Since keratinocytes are known to produce bFGF (Bielenberg et al., 1998), which acts as a growth factor for melanocytes (Krasagakis et al., 1995), and NF-κB has been shown to activate production of bFGF (Wakisaka et al., 2002), we examined the effect of the culture supernatant of keratinocytes in which NF-κB was activated on the melanocyte growth. In Fig. 6B, p65 was overexpressed in HaCaT keratinocytes (HaCaT-p65), and the culture supernatant was examined for the growth-promoting effect on melanocytes. As shown here, a significant increase in the melanocyte growth was observed after incubation with the culture supernatant of HaCaT-p65 (Fig. 6B). In fact, bFGF was detected in the HaCaT-p65 culture supernatant (14.8 ± 1.53 ng/ml), whereas the level of bFGF in parental HaCaT cells was less than 6 ng/ml. When the HaCaT-p65 supernatant was preincubated with the neutralizing antibody to bFGF, the melanocyte growth-promoting effect was completely abolished (Fig. 6B), whereas the anti-EGF antibody did not show such effect. From these observations, it was suggested that keratinocytes were responsible for the melanocyte proliferation and that this effect was mediated by bFGF.

Discussion

UV is one of the most harmful environmental factors for skin (Kligman, 1989; Ulrich et al., 2004; Wulf et al., 2004) and is responsible for the skin aging. Most characteristic changes of the skin induced by UVB have been ascribed to the production of proinflammatory cytokines, including IL-1 and TNFα (Wlaschek et al., 1994; Fisher et al., 1996; Corsini

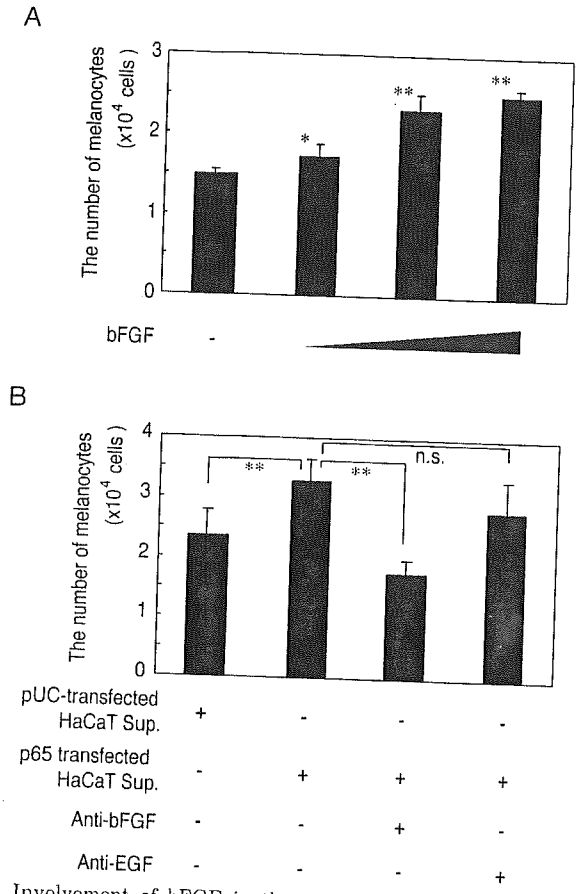

Fig. 6. Involvement of bFGF in the melanocyte proliferation and its production from keratinocytes. A, induction of melanocyte proliferation by bFGF. Primary human melanocytes were cultured in melanocyte basic medium, and the effects of bFGF of 1, 10, and 100 ng/ml were examined. The numbers of melanocytes were determined by WST method after 48 h of culture. B, involvement of bFGF in the keratinocyte-mediated melanocyte proliferation. HaCaT cells were transfected with pCMV-p65 as in Fig. 3, and the culture supernatant was collected after 48 h of transfection. As a control, pUC19 plasmid was transfected into HaCaT. The anti-bFGF or anti-EGF antibody was incubated with the supernatants of these transfected HaCaT cells, and bFGF or EGF was removed. These supernatant samples were then added to the melanocyte and incubated for an additional 72 h. The numbers of melanocyte were counted by WST method. Data shown are the mean ± S.D. (n = 5) of the number of melanocytes. *, $p < 0.05$; **, $p < 0.01$.

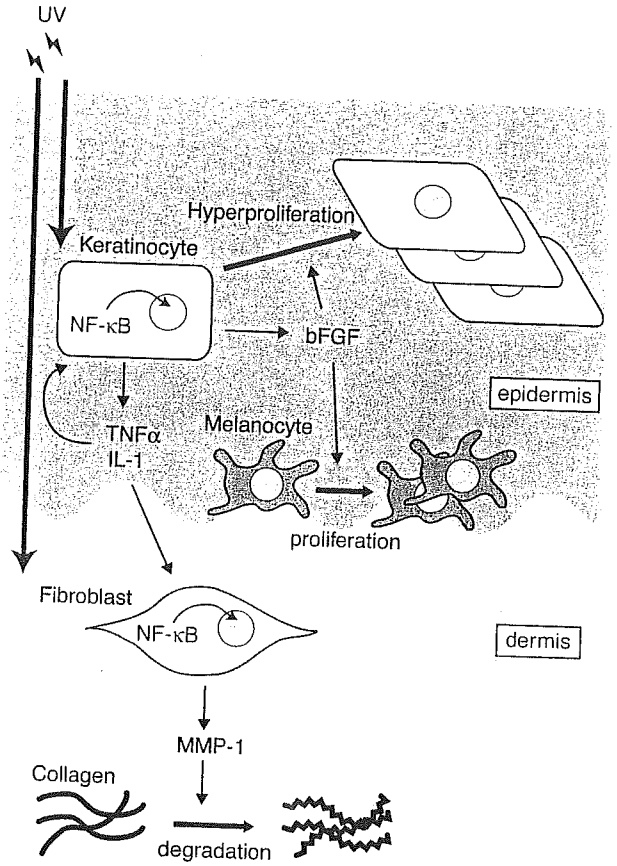

Fig. 7. Diagrammatic representation of the action of NF-κB in the process of UVB-mediated cutaneous alterations (photoaging). Environmental stimuli, such as UV irradiation and inflammatory signaling, induce the NF-κB activation that leads to the production of MMP-1 and bFGF in epidermal keratinocytes. The up-regulation of bFGF promotes proliferation of keratinocytes and melanocytes as a protection mechanism to these environmental insults. In dermis, skin fibroblasts are stimulated by UV and proinflammatory cytokines, such as IL-1 and TNFα, produced by keratinocytes, and then NF-κB is activated, leading to the MMP-1 production. Thus, NF-κB inhibitors are considered to be effective in preventing the UVB-mediated cutaneous alterations and eventually the skin photoaging process.

et al., 1997; Yarosh et al., 2000) and effector molecules, such as MMP-1 and bFGF (Wlaschek et al., 1994; Barchowsky et al., 2000). It is well established that expressions of IL-1, TNFα, MMP-1, and bFGF are controlled by NF-κB, which is activated by UV irradiation (Barchowsky et al., 2000; Wakisaka et al., 2002). NF-κB is a crucial factor for the immunoinflammatory responses and is also implicated in various skin diseases including allergic dermatitis, psoriasis vulgaris, and skin cancer (Bell et al., 2003). Hence, although NF-κB is involved in maintaining the skin homeostasis (Pasparakis et al., 2002; Takao et al., 2003), excessive activation is pathogenic. Thus, inhibition of NF-κB is considered to prevent the pathogenetic changes induced by UVB. In this study, we have examined the causal association of NF-κB with the UVB-induced changes in the skin and the efficacy of one such inhibitor, parthenolide.

Although parthenolide is known to have inhibitory action on NF-κB (Heinrich et al., 1998; Hehner et al., 1999), such as inhibitions of NF-κB DNA binding (Garcia-Pineres et al., 2001) and IκB kinase (Hehner et al., 1999), its specificity has not been clearly demonstrated. We found that parthenolide specifically inhibited the NF-κB-dependent gene expression and did not affect the actions of other transcription factors such as AP-1 and CREB. Although Won et al. (2004) recently reported the inhibitory action of parthenolide on AP-1 and claimed its chemopreventive activity against UVB-induced skin cancer, we did not observe such inhibitory effect on AP1, which might be due to the differences of cell lineages used or the experimental procedures applied.

Thus, the effect of parthenolide seems to be specific. Since parthenolide was effective in blocking the induction of MMP-1 and bFGF even in cells overexpressing p65, bypassing the activation signaling of NF-κB, it is possible that parthenolide can inhibit NF-κB DNA binding or transactivation process following the DNA binding such as the recruitment of basal transcription factors or coactivators (Uranishi et al., 2001; Jiang et al., 2003). However, further analysis of parthenolide action is needed to clarify its mode of action.

The possible involvement of NF-κB in the UV-mediated cutaneous alteration, or skin photoaging, through induction of

MMP-1 and bFGF has been implicated (Bond et al., 1999; Abeyama et al., 2000; Barchowsky et al., 2000; Wakisaka et al., 2002). We have confirmed these findings in this study using cultured cells and short-term irradiation experiments using mice. In addition, we found that NF-κB activation is involved in the melanocyte proliferation, which was consistent with the previous observations by others such as the involvement of bFGF in melanocyte proliferation (Pittelkow and Shipley, 1989). Interestingly, impaired bFGF production is reported in the vitiliginous patient skin where melanin deposition in the skin is partially affected (Moretti et al., 2002). In addition, kojic acid and ascorbic acid, identified as active whitening ingredients for the skin, have been shown to inhibit the NF-κB-mediated gene expression (Ahn et al., 2003).

These findings support an idea that NF-κB is involved in various steps in the UVB-mediated skin change both directly and indirectly. Thus, inhibition of NF-κB activation should be effective in preventing the process of UVB-mediated cutaneous alterations and eventually photoaging (schematically demonstrated in Fig. 7). Although we did not observe any side effect in experimental parthenolide therapy of mice as long as we observed (12 days), longer treatment might cause unexpected side effects such as immunosuppression and deteriorated host defense considering NF-κB being a major determinant for immunological and inflammatory responses. Further studies are needed regarding the chronic effects and the possible toxicity of parthenolide.

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REVIEW ARTICLE

Takashi Okamoto

The epigenetic alteration of synovial cell gene expression in rheumatoid arthritis and the roles of nuclear factor κB and Notch signaling pathways

Abstract Rheumatoid arthritis (RA) is a complex process of chronic and progressive inflammation associated with activation of numerous signaling molecules and transcription factors and hyperproliferation of synoviocytes of the affected joints, although the greater part of its pathophysiological process is explained by activation of nuclear factor κB (NF-κB). For example, the self-perpetuating nature of the rheumatoid inflammation is ascribable to overexpression of the proinflammatory cytokines tumor necrosis factor α and interleukin-1β, known to elicit the activation cascade for NF-κB and activator protein-1 that are responsible for transcriptional induction of these cytokines among other target genes, which conform a positive feedback loop for continuation and expansion of the inflammatory responses. In addition, comparative gene expression profile analyses have revealed activation of a number of genes that explain the "transformed-like" phenotype of synoviocytes. Among the genes expressed in rheumatoid synoviocytes upon inflammatory stimuli, induction of gene expression of Notch proteins and its ligand have been found. Possible roles of Notch signaling in RA synoviocytes are discussed.

Key words Notch · Nuclear factor κB (NF-κB) · Rheumatoid arthritis (RA) · Signal transduction · Synoviocyte

Introduction

Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of about 1%.[1] While there has been progress in defining its etiology and pathogenesis, these are still incompletely understood.[1–3] Proposed causes for RA include (i) genetic predisposition, (ii) pathogenetic immunoinflammatory responses triggered by environmental agents, particularly microbes, (iii) autoimmunity directed against components of synovium and cartilage, (iv) dysregulated production of cytokines (usually upregulation of proinflammatory and inflammatory cytokines and chemokines), (v) recruitment of immunoinflammatory cells through induction of inflammatory cell adhesion molecules (such as E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1), and, last but not least, (vi) transformation of synovial cells into autonomously proliferating cells with highly invasive nature (often referred to as "transformed-like" phenotype[4–6]).

Rheumatoid arthritis is characterized by a chronic inflammation of the synovial joints associated with proliferation of synovial cells and infiltration of activated immunoinflammatory cells including memory T cells, macrophages, and plasma cells,[1,2,7] which eventually leads to progressive destruction of cartilage and bone. This process is considered to be mediated by a number of cytokines including tumor necrosis factor α(TNFα), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-16, IL-18, and interferon γ(IFNγ) (reviewed in Refs. 1–3). Most of these pathophysiological features of RA can be explained by activation of limited number of transcription factor and its activation signals such as nuclear factor κB (NF-κB) and activator protein (AP)-1.[3,8] In fact, some effective anti-RA drugs are now known to inhibit NF-κB and its activation cascade (reviewed in Ref. 8). However, the mechanism by which rheumatoid synoviocytes exhibit the tumor-like nature has been yet to be clarified.

Involvement of NF-κB in RA as a primary pathogenic determinant

Among the various signaling and transcription regulation pathways, NF-κB and AP-1 are known to be the target of inflammatory responses. In fact, most of the factors involved in RA pathophysiology are under the control of these transcription factors.[3,8] Particularly, various cytokines

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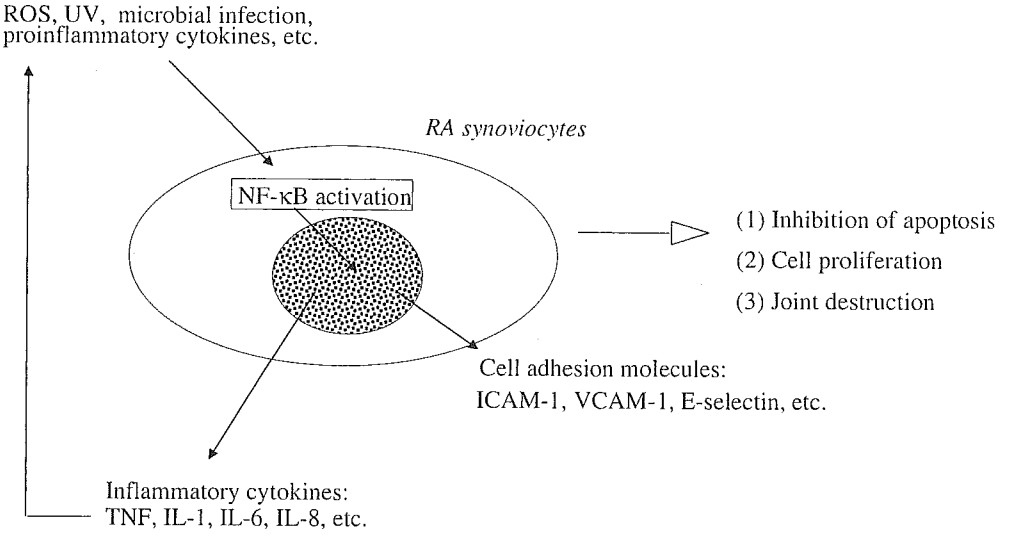

Fig. 1. Involvement of nuclear factor κB (*NF-κB*) in rheumatoid arthritis (*RA*) pathophysiology. NF-κB induces gene expression of inflammatory mediators such as cytokines and cell adhesion molecules. Since proinflammatory cytokines, tumor necrosis factor α (*TNF*), and interleukin (*IL*)-1β stimulate the NF-κB activation cascade that induces expression of these cytokines, there will be a positive feedback loop that perpetuates and expands the inflammatory responses even systemically. NF-κB also stimulates synovial proliferation by inhibiting apoptosis. See also Fig. 2. *ROS*, reactive oxygen species; *ICAM*, intracellular adhesion molecule; *VCAM*, vascular cellular adhesion molecule

and cell adhesion molecules activated in the rheumatoid joints are under the transcriptional control of NF-κB. The self-perpetuating nature of rheumatoid inflammation is ascribable to TNFα and IL-1β, known to elicit the activation cascade for NF-κB and AP-1, as they constitute another positive feedback loop in the logic of the inflammatory responses associated with RA (Fig. 1).

In addition, besides its action in upregulating inflammatory cytokines and cell adhesion molecules, NF-κB also induces gene expression of cell growth-promoting factors such as cyclin D1 and c-Myc, and physiological inhibitors of apoptosis such as cIAPs, Bcl-X_L, and cFLIP.[9,10] (Fig. 2). Moreover, it has been shown that NF-κB blocks apoptosis in the absence of de novo protein synthesis[11] through protein–protein interaction with p53 and proapoptotic protein 53BP2.[12,13] These actions of NF-κB explain not only the inflammatory responses but also the hyperproliferation of synovial tissues in RA, indicating that NF-κB acts as a major determinant for RA pathophysiology. Nuclear factor κB induces TNFα and IL-1β gene expression, and both TNFα and IL-1β stimulate NF-κB signaling, a vicious cycle formed to perpetuate and even expand the inflammatory responses.[8] The intervention therapy using anti-TNF antibody and IL-1β receptor antagonist has been thus developed.[14,15] In addition, some of the drugs for RA have been shown to block NF-κB-activation cascade or its actions (Table 1).[16–18]

The signal transduction cascade for NF-κB activation

The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, Rel A (p65), Rel B, NFkB1 (p50/105), and NFkB2 (p52/p100). These proteins share a conserved 300-amino-acid region known as the Rel homology domain, which is responsible for DNA binding, dimerization, and nuclear translocation of NF-κB. In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations.[8,19,20] A common feature of the regulation of NF-κB family is their sequestration in the cytoplasm as inactive complexes with a class of inhibitory molecules known as IκBs.[20,21] Upon stimulation of the cells such as by proinflammatory cytokines, IL-1β and TNFα, IκBs are degraded, and NF-κB is translocated to the nucleus and activates expression of target genes (Fig. 2).

The IκB kinase complex capable of specifically phosphorylating Ser32 and Ser36 of IκBα was originally identified as a ~700kDa of high molecular complex.[21,22] Subsequently, two catalytic subunits (IKKα and IKKβ) and a scaffold subunit of this complex (IKKγ/NEMO/IKKAP) were identified and cloned (for review see Refs. 20–22). The IKK complex, consisting of IKKα, β, and γ, can be activated by a variety of stimuli, including TNFα, IL-1β, and LPS. Activation of the complex involves the phosphorylation of two serine residues located in the "activation loop" within the kinase domain of IKKα and IKKβ. IKK complex is stimulated by upstream kinases that belong to MAP kinase kinase kinases (MAP3Ks), including MEKK1, MEKK2, MEKK3, and NIK, capable of phosphorylating these serines in vitro, and activating NF-κB.[23,24] Phosphorylation on specific serine residues of IκBs leads to ubiquitination of IκBs and subsequent degradation by the proteasome complex.

There is accumulating evidence suggesting the involvement of additional kinases that phosphorylate the p65 (RelA) subunit of NF-κB and regulate its transcriptional

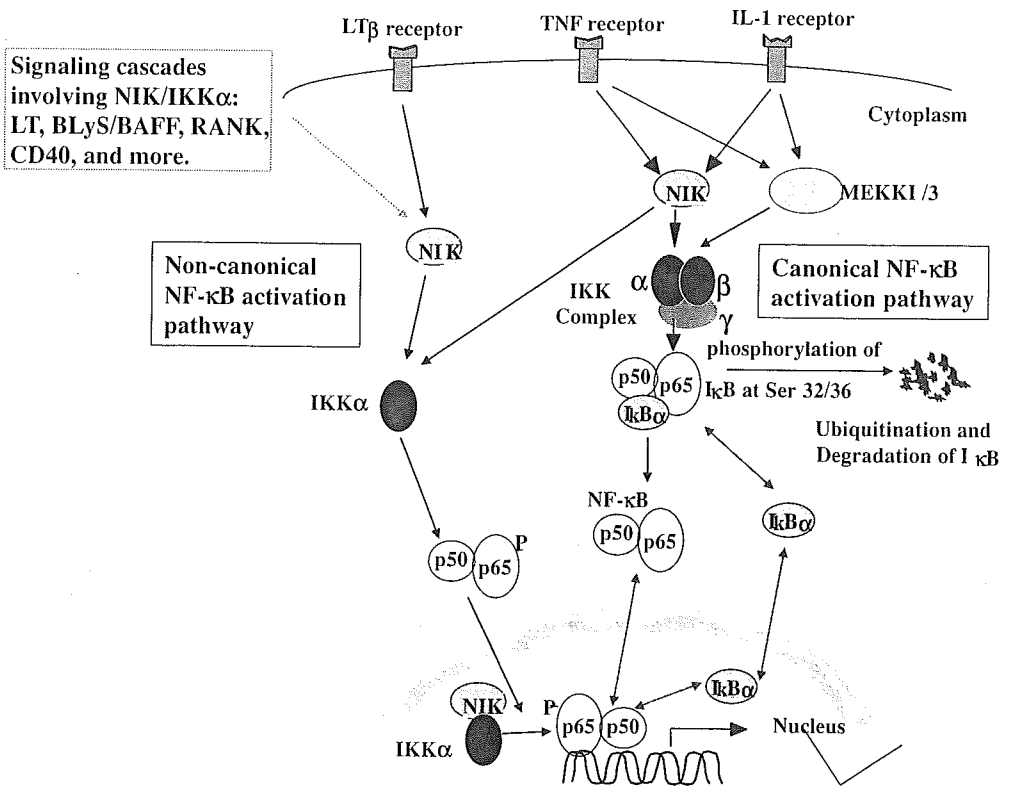

Fig. 2. NF-κB activation cascades. In addition to the canonical pathway involving IκB phosphorylation and ubiquitination followed by its proteolytic degradation in 26S proteasome within the cytoplasm, there appears to be another cascade not involving IκB phosphorylation. Lymphotoxin (*LT*) β-receptor signaling, CD40, RANK, and BLyS/BAFF stimulate the NIK–IKKα cascade that leads to p100/p52 processing and p65 phosphorylation at its C-terminal transactivation (Ser536). IKKα also phosphorylates histone H3 in the nucleus and derepresses the otherwise silent nucleosome, thus reactivating the dormant genes. The effect of p65 (Ser536) phosphorylation is considered to activate the transcriptional competence of NF-κB

Table 1. List of rheumatoid arthritis drugs that inhibit nuclear factor κB

Acetylsalicylic acid
Aurothioglucose
Aurothiomalate
Auranofin
Dexamethasone
Ibuprofen
Sodium salicylate
Sulfasalazine

competence.[25–27] We recently found that IKKα is responsible for the p65 phosphorylation at Ser536 upon the lymphotoxin β receptor signaling mediated by NIK, and induces NF-κB activation independently of the IκB phosphorylation and its degradation.[28–30] Interestingly, this NIK–IKKα cascade is also involved in BLyS/BAFF, RANK, and most likely CD40 signaling.[31,32] In contrast to the classical (or canonical) pathway involving IKKβ and the phosphorylation of IκB, this cascade ("non-canonical pathway") does not necessarily involve IKKβ and IκB phosphorylation but involves p100 (NFkB2) processing and p65 phospho-rylation (Fig. 2). Since BLyS/BAFF and CD40 signaling cascades induce B-cell activation and RANK signaling is involved in osteoclast differentiation, the NIK–IKKα cascade is considered to play important roles in disease progression of RA. The TNFα-dependent phosphorylation of Ser529 has also been demonstrated to increase the transcriptional activity of p65. For example, casein kinase II was implicated in the TNFα-dependent phosphorylation of p65 on Ser529.[33] It was shown that Ser529 and Ser536 of p65 were required for transcriptional activation of p65 by AKT and the IL-1β signaling.[30,34]

Inducible phosphorylation of p65 appears to function at many different levels, including conformational changes in the transcriptional activation domain and promoting association with coactivator proteins CBP/p300.[20] It is possible that the phosphorylation of p65 may lead to dissociation from corepressor proteins such as histone deacetylases and Groucho proteins (TLE/AES) and selective interaction with FUS/TLS coactivator protein.[35–37] Regarding the cross-talk with the camp–PKA cascade, although my group and others found that it downregulates the NF-κB-dependent gene expression presumably mediated by C/EBPβ,[38–40] it has also been reported that the catalytic subunit of PKA (PKAc), associated with the NF-κB/IκBα complex, upregulates the NF-κB-dependent gene expression by directly phosphorylating p65 on serine 276,[27] thus pending the physiological relevance.

Cytological characteristics of rheumatoid synoviocytes

Although it appears that NF-κB plays a major role in the pathophysiology of RA, there is no evidence to support the possibility that NF-κB or its signaling cascade is impaired in RA. To clarify the transformed-like nature of rheumatoid synoviocytes, my group have performed gene expression

profile analyses of synoviocytes.[41] When compared with control synoviocytes obtained from healthy individuals (upon injury) or osteoarthritis patients, we found that both platelet-derived growth factor (PDGF) receptor α and a chemokine, SDF-1, genes are activated in RA synoviocytes without any external stimulus. Gene knockout studies showed that PDGF receptor α is required for the development of limb joints. During the early developmental stages, PDGF and SDF-1 are known to act as chemotactic factors for fibroblasts[42] and macrophages,[43,44] respectively. It is possible that synovial fibroblasts (type B synoviocytes) and synovial-lining macrophage-like cells (type A) communicate with each other by producing SDF1A and PDGF, respectively, in order to form the primordial joint tissue during the early embryonic development (reviewed in Ref. 41). Thus, it is likely that rheumatoid synoviocytes may have reacquired the "revertant" phenotype of the primordial synoviocytes, like cancer cells, although the underlining mechanism is yet to be clarified.

As mentioned above, rheumatoid synovial tissues are usually under inflammatory stimuli as synovial fluid contains high concentrations of TNFα, IL-1, and oxidants (Ref. 46 and references therein). Thus, we extended the gene expression profile analysis with synoviocytes in the presence of physiological concentration (200pg/ml) of TNFα and compared the genes induced by TNFα in RA and control synoviocytes. Although well-known genes under the control of NF-κB were similarly stimulated by TNFα, we found that the genes encoding Notch-1, Notch-4, and Jagged-2, a ligand for Notch proteins, were activated only in RA synoviocytes.[49] (Fig. 3). We also found that genes encoding MMP-11 and -17, and Wee1 and cyclin B1, were induced by TNFα only in rheumatoid synoviocytes. These findings indicate that one of the effects of phenotypic reversion of rheumatoid synoviocytes, as described above, could be attributable to the induction of Notch signaling and that the activation of Notch signaling, known to be involved in cell-fate determination, may directly or indirectly cause induction of genes responsible for cell proliferation (such as induction of Wee1 and cyclin B1 genes) and tissue invasion (such as induction of MMP-11 and -17). These findings support an idea that RA synoviocytes may have reacquired the "revertant" phenotype mimicking the primordial synoviocytes, by presumably involving Notch signaling, and exhibit the hyperproliferative and invasive nature of cells.

Activation of Notch signal in RA

As TNFα induced Notch-1 and its ligand Jagged-2 in RSF, we examined if the Notch signaling is elicited by the TNFα stimulation. Rheumatoid synovial fibroblasts (RSF) and normal synovial fibroblasts (NSF) were stimulated with TNFα and the intracellular localization of Notch intracellular domain (NICD) of Notch-1 was examined by immunostaining. We found the nuclear translocation of Notch-1 NICD, a hallmark of the Notch signaling,[48,49] only in TNFα-stimulated RSF[47] (Fig. 4). These results suggested that in response to TNFα stimulation RSF expressed both Notch-1 and Jagged-2 proteins, which then interacted with each other between adjacent cells and elicited the signaling. In RA tissues we found that hyperproliferative synovial tissues were clearly stained by Notch-1, Notch-4, and Jagged-2 antibodies, and that some of the RA synovial cells showed the nuclear staining of Notch-1 and Notch-4.

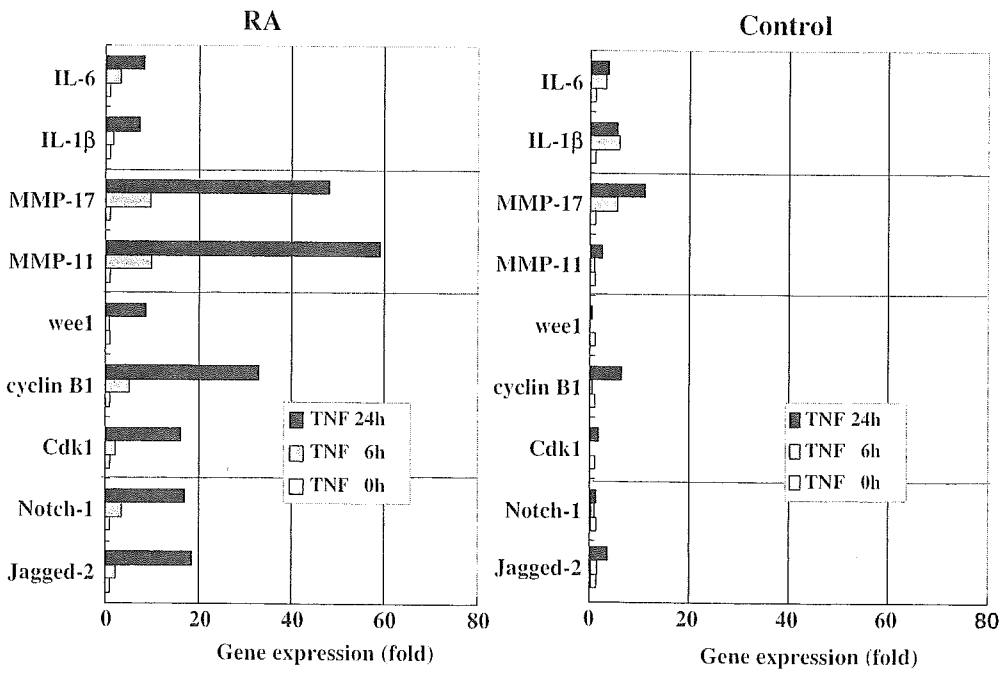

Fig. 3. Comparative gene expression profile analysis of rheumatoid and control synoviocytes upon stimulation with tumor necrosis factor α (*TNF*). Synoviocytes were cultured with or without TNF (200pg/ml). The mRNA was purified from each cell culture harvested at 0, 6, and 24h after TNF stimulation, cDNA probe was synthesized, then hybridized with a cDNA array membrane. The quantitation of gene expression level was performed and standardized based on the average levels of housekeeping genes. Based on the observation by Ando et al.[47] Reproduced with permission[47]

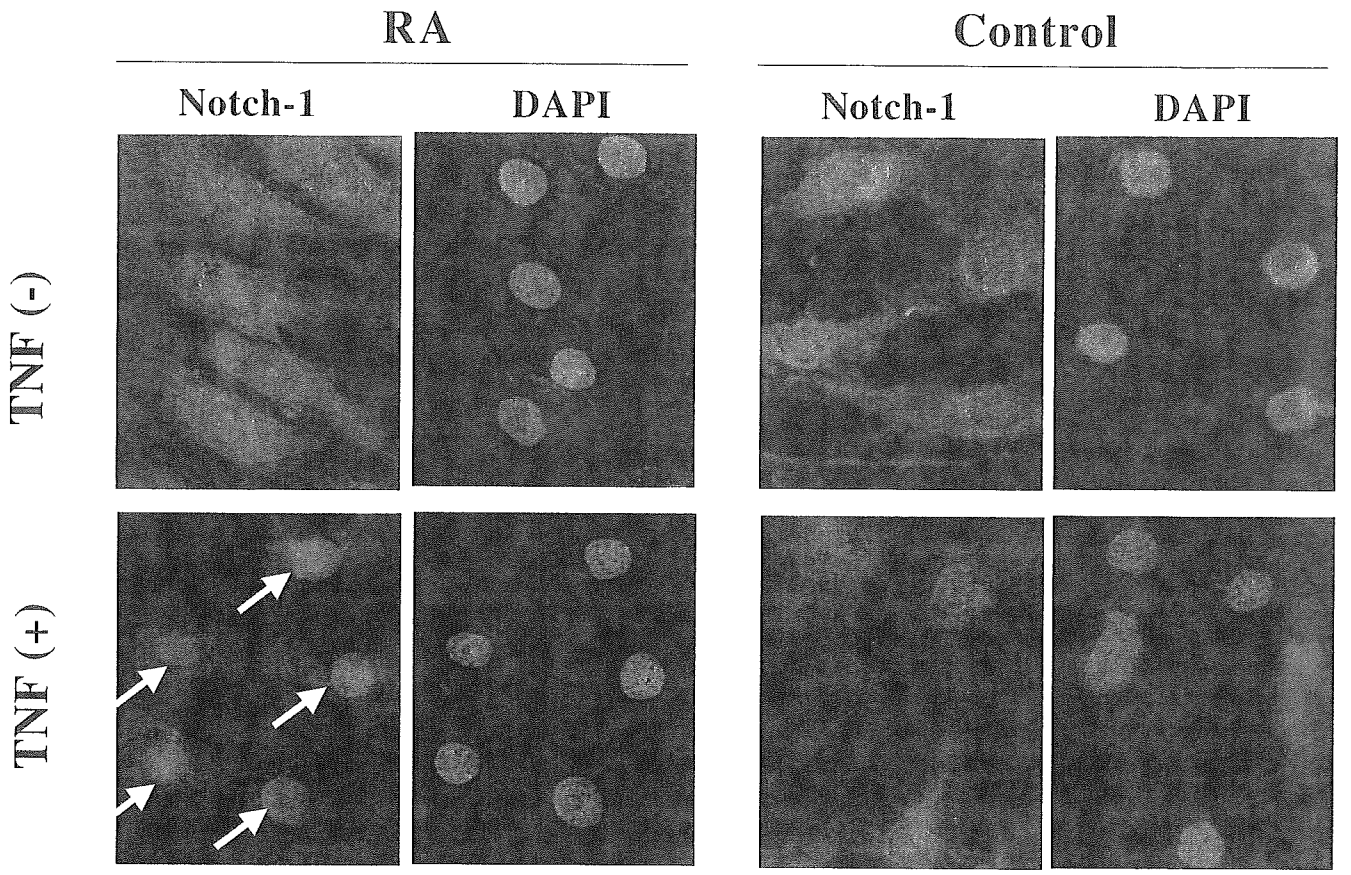

Fig. 4. Nuclear translocation of Notch-1 intracellular domain (NICD) in rheumatoid synoviocytes after tumor necrosis factor α (*TNF*) stimulation. Cells were immunostained with anti-Notch-1 C-terminus polyclonal antibody (C-20) before and after 12 h of TNF stimulation and examined by fluorescent microscopy. *Green*, Notch-1 intracellular domain (detected by fluorescein isothiocyanate-conjugated rabbit antigoat IgG as secondary antibody); *Blue*, nuclear staining with 4′,6′-diamidino-2-phenylindole hydrochloride (*DAPI*). Based on the observation by Ando et al.[47] Reproduced with permission[47]

We also detected expression of these proteins in the developing synovial and cartilage tissues of embryonic mice.[47] In more developed joints of newborn mice, expression of these proteins was restricted in the synovium, raising a possibility that the Notch signaling pathway might control the differentiation and development of joints.

Biological implications of Notch signal activation in RA

Notch signaling is involved in three different biological processes including (i) lateral specification, in which adjacent equipotent precursor cells coordinate each other's developmental fate, (ii) inductive signaling, in which one cell type determines the differentiation of another cell type, and (iii) cell-autonomous effects, in which a developing precursor (stem) cell regulates its own fate and maintains its status (reviewed in Refs. 50 and 51). Notch genes encode single-pass transmembrane receptors that transduce the extracellular signals responsible for cell fate determination during crucial steps of metazoan development.[52,53] The large transmembrane receptors encoded by Notch genes interact with membrane-bound ligands encoded by the Delta and Jagged (Serrate) genes at the extracellular surface of cells. The signal induced by this ligand binding leads to proteolysis of Notch, generation and nuclear translocation of NICD, and regulation of target gene expression (Fig. 5). Genes homologous to members of the Notch signaling pathway have been cloned from numerous vertebrate organisms and many have been shown to be essential for normal embryonic development. In humans, the importance of Notch signaling for growth and development is supported by the findings that T-lymphoblastic leukemia[54] and some inherited diseases involving affected organogenesis[55,56] can be ascribed to the mutations in Notch/Jagged (Delta) genes. The Notch signaling pathway is evolutionarily conserved, and mutations in its components disrupt cell fate specification and embryonic development in diverse organisms.[48,50]

Interestingly, a targeted mutation that removes a domain of the Jagged-2 protein required for the interaction with Notch-1 caused perinatal death associated with defects in craniofacial morphogenesis and syndactyly (digit fusions) of the fore- and hindlimbs, implicating that Jagged-2/Notch signaling is indispensable for the development of the joint.[57]

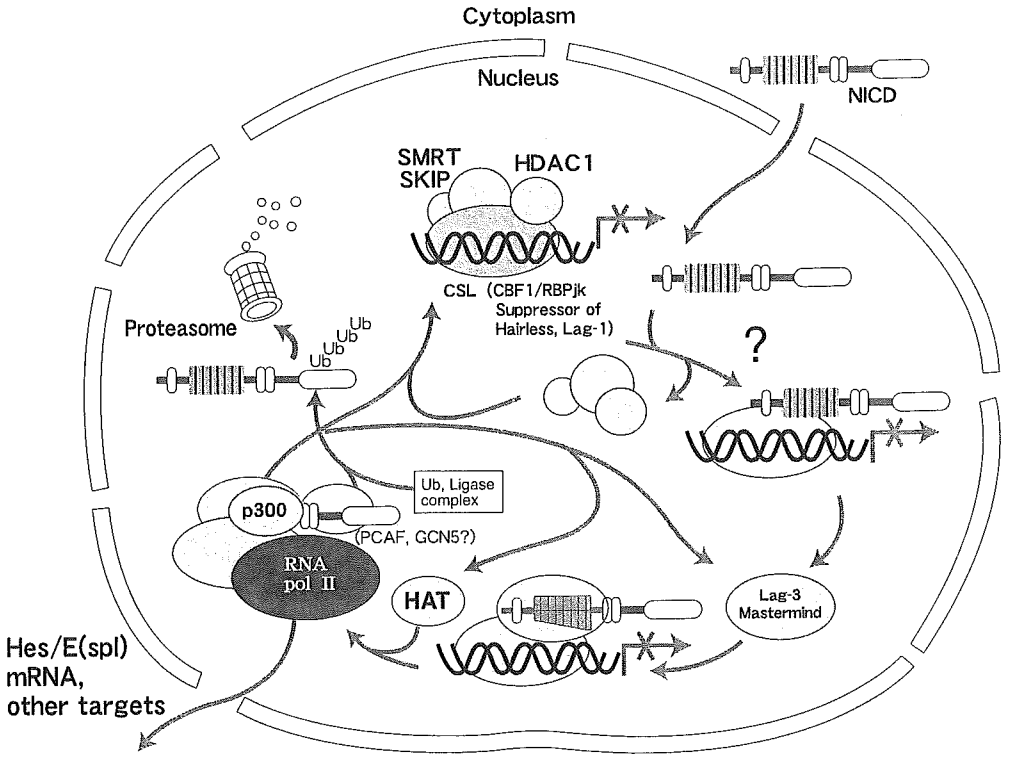

Fig. 5. Notch signaling and the transcriptional control by the Notch intracellular domain (*NICD*). Upon ligand binding, Notch protein is proteolytically cleaved by furin and presenilin to generate NICD, which is translocated to the nucleus. Once in the nucleus, NICD displaces the corepressor proteins such as *SKIP*, *SMRT*, and *HDAC-1* from the specific DNA-binding proteins *CSL* (CBP or RBP-Jκ in vertebrates, Su(H) in *Drosophila*, and Lag-1 in *Caenorhabditis elegans*; thus collectively called "CSL") and associates with the coactivator complex containing *Mastermind* (MAM) and *p300*;[62] MAM is considered to bridge the NICD/RBP–Jκ complex and p300.[63] The Notch intracellular domain is subjected to ubiquitination followed by proteasome-mediated degradation, thus terminating the transcriptional activation

A similar phenotype has been observed in mice lacking the IKKα subunit of IκB kinase complex.[58] In embryonic day 16 (E16) mutant embryos, forelimbs (but not hindlimbs) were visible but were considerably shorter than those of normal ($Ikk\alpha^{+/+}$ and $Ikk\alpha^{+/-}$) littermates and lacked separated digits. At an earlier stage, E14.5, the fore- and hindlimbs of mutant embryos were not much shorter than those of normal counterparts, but were devoid of distinct digits. Therefore, it appears that the TNF-mediated NF-κB activation through IKKα is involved in expression of Jagged-2 in the developing joints. Thus, activation of the Notch signaling found in rheumatoid synoviocytes not only confirms the phenotypic reversion of synoviocytes but also indicates its active role in pathophysiological processes of RA, which presumably involve NF-κB cascade.

Cross-talk between NF-κB activation cascade and Notch signaling

In mammals, all four known Notch family members can physically interact with recombinant signal binding protein Jκ (RBP-Jκ), a DNA-binding repressor protein, and inhibit the activity of RBP-Jκ.[59,60] Oswald et al.[61] reported that NICD overcame the RBP-Jκ-mediated repression and strongly activated NF-κB2. In the absence of Notch signaling, RBP-Jκ interacts with SKIP and SMRT that recruit transcriptional corepressor complex[62] (Fig. 5). However, upon Notch signaling NICD induces changes in the DNA-bound protein assembly containing RBP-Jκ in the nucleus, thus displacing the corepressor complex and converting it to a transcriptionally active complex. It has been shown that a non-DNA-binding transcriptional coactivator Mastermind (MAM) is essential for the Notch/RBP-Jκ complex to recruit p300 coactivator to DNA.[63] Thus, activation of Notch signaling observed in rheumatoid synoviocytes appears to stimulate the noncanonical NF-κB pathway (Fig. 5).

It is conceivable that this noncanonical NF-κB activation pathway may be responsible for the altered response to the inflammatory environment involving IKKα. It is known that IKKα is translocated, together with NF-κB, to the nuclear chromatin compartments where target genes are present, and phosphorylates Ser10 of the histone H3 component of nucleosome[64,65] (Fig. 5). Although the histone H3 with methylated lysine 9 of H3 renders the local nucleosome to be "repressive," the adjacent serine 10-phosphorylation of H3 histone reverses this effect and derepresses the transcriptional activity of the genes located in the "derepressed" nucleosome.[66] Thus, chronic and persistent NF-κB stimulation in synoviocytes of RA patients could also lead to the change in "histone code"[66] and eventually transform synoviocytes.

Conclusion

Rheumatoid arthritis is a complex process of chronic and progressive inflammation involving numerous transcription factors and signaling molecules. Based on the unexpected transcriptomic characteristics of rheumatoid synoviocytes, suggesting the phenotypic reversion, I have explored the mechanism by which chronic inflammatory stimuli could

endow normal synoviocytes with "transformed-like" phenotype and could ascribe activation of the Notch signaling to this altered cellular status. This may explain the progressive and self-perpetuating nature of the rheumatoid inflammation, at least in part. Based on these considerations, future therapeutic strategy of RA should be developed based on the action of Notch signaling on its pathophysiology, which includes the action of the noncanonical NF-κB activation pathway, its therapeutic intervention, and elucidation of the Notch target genes, particularly in synoviocytes.

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Current Pharmaceutical Design
Review

Title:
NF-κB signaling and carcinogenesis

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Key words:

1. Summary

NF-κB is an inducible transcription factor that is controlled by the signal activation cascades. NF-κB controls a number of genes involved in immuno-inflammatory responses, cell cycle progression, inhibition of apoptosis and cell adhesion, thus promoting carcinogenesis and cancer progression. Interestingly, some proteins encoded by oncogenes and oncogenic viruses have been shown to be involved in NF-κB activation pathway. In fact, NF-κB is constitutively activated in some cancer and leukemia cells. These findings have substantiated the old concept of the link between chronic inflammation and carcinogenesis. In this review, we have attempted to overview the possible involvement of NF-κB in cancer and discuss the feasibility of anti-cancer strategy with NF-κB and its signaling cascade as novel molecular targets.

2. Inflammation and carcinogenesis

In 1863, Rudolf Virchow pointed out the lymphoreticular infiltration in cancer tissues and implied the involvement of chronic inflammation in carcinogenesis. Recent studies have supported this notion and highlighted the positive correlation between inflammation and cancer [1-3]. In fact, about 15% of the neoplasms have been considered to be associated with chronic inflammation caused by infectious, chemical or physical agents [4].

In general, inflammatory responses are elicited to repair the damaged tissues by promoting cell cycle progression and inhibiting apoptosis. On the other hand, reactive oxygen species (ROS) and nitric oxide, forming highly reactive peroxynitrite, generated by local inflammatory responses are now known to cause DNA oxidization, resulting in genetic mutations [5,6]. If such DNA damages are too severe to be repaired, apoptotic cell death is induced though the activation of p53 [7]. However, in most precancerous cells p53 protein is often mutated or functionally inactivated, thus resulting in the restoration of genetic mutations. In addition, migration inhibitory factor (MIF), produced by inflammatory responses, was shown to overcome the activity of p53 by blocking its transcriptional activity [8]. Moreover, activation of NF-κB similarly offset the function of p53 as discussed later. These inflammatory microenvironments are associated with production of pro-inflammatory cytokines, growth factors, pro-angiogenic factors and matrix metalloproteases in a paracrine manner, allowing the

malignant cells to proliferate, invade and metastasize [1, 9].

3. The role of NF-κB in virus-associated carcinogenesis

Viral infection often triggers initiation of carcinogenic steps in some neoplasms. For example, hepatocellular carcinoma is closely related to chronic hepatitis caused by infection of hepatitis B or C virus (HBV or HCV). Nasopharengeal carcinoma or some types of malignant lymphomas are associated with chronic Epstein-Barr virus (EBV) infection [10,11]. In adult T-cell leukemia (ATL), infection of human T-cell leukemia virus type-I (HTLV-1) triggers the initial step of its tumorigenesis [12]. Cervical cancer is associated with the infection of human papillomavirus [13].

In these virus-associated neoplasms, although viral gene products play important roles in carcinogenesis, however, that viral proteins such as latent membrane protein-1 (LMP-1) or gp350, encoded by EBV, do not have transforming activity by themselves [10], suggesting that EBV infection alone cannot attribute to carcinogenesis. Similarly, core-protein encoded by HCV also does not have an oncogenic activity [14]. Interestingly, it is reported that local production of cytokines or growth factors is responsible for the development of wound-induced tumorigenesis in chicken models infected with Raus-sarcoma virus, expressing the *ras* oncogene [15]. These findings indicate that inflammatory responses followed by expression of viral proteins are crucial for virus-associated tumorigenesis. Chronic inflammatory responses wherein virus-encoded LMP-1, gp350 and HCV core-protein are produced have been reported to activate NF-κB, supporting an idea that NF-κB is the major player in virus-associated carcinogensis [16].

4. NF-κB as a central regulator for the inflammatory and immune response and its target genes

Baltimore and his coworkers discovered NF-κB in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin [17]. It has since been shown to be expressed ubiquitously in the cytoplasm of all cell types, form Drosophila to man. Once NF-κB is activated, it translocates to the nucleus, where it regulates the expression of over 200 genes, the majority of which participate in the host immune response. NF-κB is thought to be as a master switch for inflammation and immune response.

NF-κB activates a variety of gene's expression including genes encoding cytokines, cell adhesion molecules, cell cycle regulators and apoptosis inhibitors (Fig. 1). Genes regulated by NF-κB activation include those encoding *TNF-α, IL-6, E-selectin, ICAM-1, Cyclin D1* and *Bcl-X_L*. NF-κB involves several biological events such as inflammation and immune response, viral infection and tumorigenesis through transcriptional regulation of its target genes. Interestingly, NF-κB regulates the expression of NF-κB family themselves and its inhibitor protein, IκB family.

NF-κB is a hetero- or homo- dimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100. Phosphorylation-dependent cleavage of p100 produces p52, whereas p105 is cleaved to form p50. NF-κB mainly exists the heterodimer consisting of p65 and p50 in the cell, and normally present in the cytoplasm in association with its inhibitor IκB. IκB also consists a family of ankylin-domain containing proteins including IκBα, IκBβ, IκBγ, IκBε, p100, p105 and bcl-3. After stimulation with pro-inflammatory cytokines such as TNF-α and IL-1, IκB is degraded and NF-κB translocates to the nucleus, leading to stimulate transcription of genes containing the consensus κB sequence 5'-GGGPuNNPyPyCC-3' (Pu:purine, Py:pirimizine.). The specificity with structural variants of the κB DNA binding site may be determined by the forming combination of a hetero- or homo- dimer consisting of Rel family proteins [18].

5. Two NF-κB regulation pathways; canonical and non-canonical pathway

NF-κB is activated by a variety of stimuli, including inflammatory cytokines such as TNF-α and IL-1, CD40 ligand [19], B-cell activating factor (BAFF) [20] and lymphotoxin-β (LT- β) [21] (Fig. 2, left panel). These stimuli utilize distinct NF-κB-activation pathways that involve different kinase cascades. The "canonical pathway" involves IκB kinase (IKK) β that is primarily responsible for the NF-κB activation through IκB degradation. When cells are stimulated with TNF-α, for example, adaptor proteins such as TRADD, RIP and TRAF2 bind to the TNF receptor and recruit IκB kinase (IKK) complex [22,23]. The IKK complex is a large molecular weight complex and contains two kinase catalytic subunits, IKKα and IKKβ [24] and the associated regulatory subunit IKKγ [25]. Some reports demonstrated that Hsp90 and Cdc37 also are included in the IKK complex and involve the shuttling form the cytoplasm to the nucleus [26]. Moreover, ELKS is responsible for the binding between IKK complex

and IκB [27].

Activated IKK complex phosphorylates IκBα on two specific serine residues, Ser32 and Ser36, and this phosphorylation leads to polyubiquitination of IκB, its subsequent degradation by 26S proteasome and NF-κB nuclear translocation. Similarly, IκBβ is phosphorylated on Ser19 and Ser23 by IKK, leading to its polyubiquitination and subsequent degradation. IκBα is responsible for NF-κB activation in the early step, whereas IκBβ is involved in the constitutive activation of NF-κB. There is a possibility that distinct IκB family proteins may involve NF-κB activation in the different step by depending on the inflammation stage. Interestingly, but not surprisingly, NF-κB and IκBα, not IκBβ, shuttle continuously between the nucleus and the cytoplasm without NF-κB stimulation, suggesting that a sole inhibition of IκB phosphorylation or ubiquitination is not sufficient to block NF-κB activation and that a unique downstream regulation exists in the nucleus.

Since the identification of IKK complex, much attention has been focused on the upstream kinases in different signal transduction pathways and the mechanism by which these pathways are converged on the IKK complex. Upstream kinases reported to activate IKKα and IKKβ include NF-κB-inducing kinase (NIK), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1), PKC, Akt (or PKB), and mixed-lineage kinase 3 (MLK3). For example, TNF-α activates PI3K-Akt cascade and Akt activates IKKα, leading to the phosphorylation of p65 transcriptional activation domain (TAD) on Ser536 and enhances the transcriptional activity of NF-κB. Moreover, Akt also activates p38MAPK, which activates IKKβ, thus promoting the phosphorylation and degradation of IκB. Interestingly, a tumor suppressor protein PTEN phosphatase, which is frequently mutated in a large number of cancers including glioblastoma, melanoma and lung cancer and negatively regulates Akt/PKB signaling pathway though its phosphatase activity, prevents NF-κB activation by inhibiting phosphorylation of p65 TAD upon TNF-α stimulation.

The second pathway involved in NF-κB activation is called "non-canonical pathway (or alternative pathway)", in which activation is triggered by LT-β [21], BAFF [20] and CD40 signaling [19]. This pathway depends on the NIK and IKKα, but not IKKβ or IKKγ, activities. For example, when CD40 signaling is activated by its ligand (CD154), TRAF2, TRAF3 and TRAF6 are recruited to CD40 and form a complex, leading to the activation of NIK and IKKα homodimer [28] (Fig. 2, right panel).

IKKα phospholylates p100, which contains the inhibitory domain consisting of ankyrin repeats on the C-terminal, and subsequently it is ubiquitinated and cleaved to generate p52 subunit of NF-κB [28]. p52 binds to RelB to form a NF-κB heterodimer and translocates to the nucleus, where it induces expression of target genes.

There is another pathway, independent of IKK, that is triggered by DNA damage such as UV [29] and doxorubicin [30]. UV radiation activates casein kinase 2 (CK2), though p38 MAPK activation, which in turn phospholylates IκBα, leading to its degradation by proteasome.

Among signal molecules in the NF-κB activation pathway, IKKα and p38 MAPK are important for the epigenetic modification of NF-κB target genes as a molecular linkage between inflammation and tumorigenesis since those kinases can phosphorylate histone H3, thus converting the repressive chromatin to the transcriptionally active form [31,32]. Therefore, precancerous status of cells can be achieved without involving genetic mutations.

6. Modification of NF-κB transcription activity by p65 phosphorylation

Besides the phosphorylation and subsequent degradation of IκB, phosphorylation of p65 is also required for optimal NF-κB activation. For example, protein kinase A catalytic subninit (PKAc), which is maintained in an inactive form by binding with IκBα and NF-κB, phosphorylates p65 on Ser276 [33]. This phosphorylation of p65 enhances NF-κB transcription activity by recruiting histone acetyltransferase such as cAMP response element-binging (CREB)-binding protein (CBP) and p300. Ser276 of p65 is also phosphorylated by mitogen- and stress-activated protein kinase-1 (MSK1) upon TNF-α stimulation and id dhoen to be required for NF-κB activation [33]. In addition, Ser536 of p65 is phosphorylated upon stimulation with TNF-α [34] or by activated IKK complex through the action of human T-cell lymphotropic virus type 1 Tax protein [35]. IKKα is also shown to phosphorylate Ser536 upon stimulation with LT-β through the non-canonical pathway involving the NIK-IKKα cascade [36].

Thus, in order to investigate the activation status of NF-κB on tumor cells and tissues, it is necessary to examine not only the expression level and phosphorylation status of IκB but also the p65 phosphorylation status. Recently, it is revealed that the activation of S6-kinase upon DNA-damaging agents such as doxorubicin and etoposide involves p65 Ser536 phosphorylation and is dependent on p53 [37].

7. Redox regulation in the NF-κB activation pathway

It has been well established that reversible oxidation-and-reduction ("redox") reaction is involved in the NF-κB activation pathway and well-known therapeutic effects of antioxidants can be understood by their anti-NF-κB actions. There are at least two steps where redox regulation is involved: (1) involvement of radical oxygen species (ROS) in activation of Syk kinase that phosphorylates IκBα at Tyr42 that leads to ubiquitination of IκBα and subsequent degradation by proteasome, (2) involvement of thioredoxin (Trx), a physiological reducing catalyst, that reduces the redox-sensitive Cys on NF-κB leading to the activation of DNA its binding activity [38,39]. Interestingly, Trx itself is induced by oxidative stress [40], thus induction of Trx and activation of NF-κB is considered as a feed-back mechanism of cells in self-defense response to harmful oxidation. Thus, actions of antioxidants are exerted by blocking the actions of Syk and Trx induction, both acting as NF-κB activators. Although polyphenols and flavonoids contained in plants, and a physiological antioxidant α-lipoic acid inhibit NF-κB by this mechanism, the effective concentrations are too high to be easily used in the treatment. In addition, we found that one of anti-rheumatic compounds, monovalent gold ion compounds, such as aurothiomalate and aurothioglucose, could effectively block the DNA binding activity of NF-κB at concentrations that are clinically attainable [41]. We found that monovalent gold salts appeared to block NF-κB DNA binding of the reduced form of NF-κB that associates with Zn ion by extracting electron and making the disulfide ("oxidized") cysteins at the DNA-binding loop by liberating Zn^{2+} through conversion of Au^{+} to metal Au because of the enormous difference of their ionic tendencies [39, 42]. In fact, we reported the actions of gold salt compounds in inhibiting HIV replication [43], cytokine production [44] and cancer cell metastasis by blocking NF-κB activity [45].

8. The role of NF-κB in tumorigenesis

As discussed above, NF-κB is implicated in the control of cellular growth and apoptosis in neoplasia. The viral protein *v-rel*, a homolog of cellular *c-rel*, was originally identified as an oncogene encoded by an avian reticuloendotheliosis virus strain T (REV-T) [46]. Human NF-κB have been shown to be associated with tumorigenesis in mammalian cells through regulation of a number of oncogenic genes

[47]. However, *v-rel* is the only member that is actually oncogenic in animal model systems [48, 49]. The target genes of NF-κB include anti-apoptotic genes such as *bcl-xL, bcl-2, IAPs, XIAP* and *surviving* [50-52], cell cycle regulator such as *cyclinD1* [53], and adhesion molecules such as *ICAM-1, VCAM-1* and *E-selectin* [54,55] (Fig.1). *VEGF, iNOS, MMP-9* and urokinase type of plasminogen activator (uPA), which are involved in angiongenesis or invasion, are also under the control of NF-κB [56,57,58]. For tumor-associated macrophage, exposure to IL-1 leads to NF-κB-dependent upregulation of *COX-2* expression, which is responsible for the induction of hypoxia-inducible factor 1α that controls VEGF gene expression [59]. Interestingly, NF-κB also induces cellular transformation by regulation of *c-myc* [60]. Furthermore, NF-κB contributes the drug-resistance through multi-drug resistance-1 expression in cancer cells [61], thus making cancer cells resistant to anti-cancer chemotherapy.

Recent studies reveal constitutive activation of NF-κB in many neoplasms as a result of genetic or chromosomal alterations. Unlike *p53*, these alterations are relatively frequent in lymphoid malignancies, but not so common in solid tumors. Chromosomal aberrations involving the location of *c-rel, relA, p100* or *p105* are found both in hematological malignancies and solid tumors [62] (Table 1). For example, gene amplification of *c-rel* on chromosome 2p14-15 was observed in 23% of diffuse large B-cell lymphoma (DLBCL) [62,63,64](Brownell et al., 1988; Mathew et al., 1993; Rayet & Gelinas, 1999), and is associated with extranodal progression [65,66]. Similarly, 2p duplication is correlated with poor prognosis in DLBCL [67]. Since the genetic mutations which affect conformation of c-rel protein has not been found, it is considered that direct amplification of *c-rel* could increase the tumorigenesity by a gene-dose effect. Overexpression of *c-rel,* not invoving genetic amplification, is reported in non-small cell lung cancer (NSCLC) [68]. Chromosomal alterations containing *relA* on 11q13 are observed in only a few cases of lymphoid malignancies and several solid tumors such as breast cancer, squamous head/neck cancer and NSCLC [65,69]. On the other hand, chromosomal aberrations containing *p100* gene locus on 10q24 are found in several lymphoid malignancies such as cutaneous T-cell lymphomas (CTCL), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL) and multiple myeloma (MM) [48,70-73]. Importantly, truncated p100 protein caused by chromosomal alteration lacks a part of c-terminal ankyrin repeat, resulting constitutive nuclear translocation and DNA binding of p100 protein without processing to p52 [71-

74](Chang et al., 1995; Migliazza et al., 1994; Neri et al., 1991; Thakur et al., 1994). It is reported that truncated p100 protein appears to act as a constitutive activator of gene expression [74](Chang et al., 1995). Overexpression of p100 protein is also reported in breast cancer and colon cancer [75,76]. Unlike p100, although *p105* alterations are rare in hematological malignancies, there are some cases of *p105* gene rearrangement in acute lymphoblastic leukemias (ALL) [77]. Interestingly, p105 can associate with LYL1, which is involved in a translocation in T-cell ALL [78]. *p105* overexpression is also observed in solid tumors such as NSCLC [68,79].

In addition to NF-κB proteins, functional alterations of IκB proteins containing IκBα, bcl-3 and IκBε are reported. Chromosomal alteration of *Bcl-3*, which was originally cloned from CLL due to its location at a breakpoint in 19q13.1, is rarely observed in CLL and associated with poor prognosis [80,81,82]. Translocation containing *bcl-3* locus attend the overexpression of intact bcl-3 protein. Bcl-3 facilitates NF-κB-mediated transcription by removing inactive p50 homodimers from DNA and acts as a potent coactivator for homodimers of p52. It is reported that Emu-*bcl-3* transgenic mouse develop a lymphoproliferative disorder [83], and Franzoso et al showed that *bcl-3* deficient mouse displayed a loss of B-cell [84], suggesting that overexpression of *bcl-3* leads to leukemogenesis. In Hodgkin disease, inactivating mutations of IκBα and IκBε lacking various portions of ankylin repeats and c-terminal region have been observed [85,86]. These mutations produce nonfunctional IκBα proteins, resulting in constitutive NF-κB activation [87].

9. Constitutive NF-κB activation in neoplasms

Constitutive activation of NF-κB has been reported in many hematological malignancies (Table 2). In Hodgkin disease, constitutive NF-κB activation is found in Reed-Sternberg cell [85-88], and inhibition of NF-κB induces apoptosis in these cells. Similarly, in childhood acute lymphoblastic leukemia (ALL), persistent activation of NF-κB is highly observed (more than 90% cases) [89]. These constitutive NF-κB activations are caused by hyperactivtion of IKK or IκB alteration. In addition, we and others reported the constitutive NF-κB activation in multiple myeloma cells and adult T-cell leukemia (ATL) cells [90-93]. In ATL, Tax protein encoded by a human T-cell leukemia virus type-I (HTLV-1) leads to NF-κB activation through interaction with IKK and NF-κB. However, constitutive activation is also observed in Tax-inactive ATL