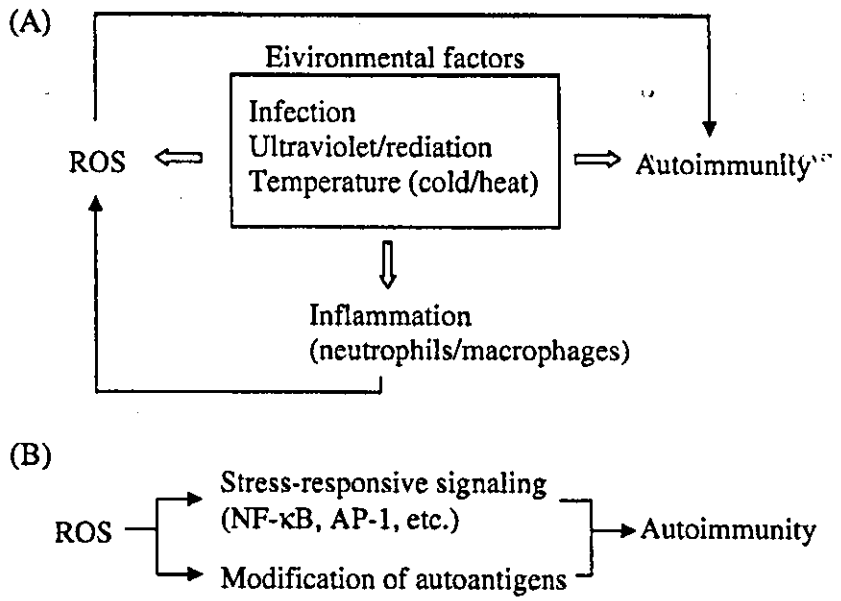

Figure 1 Involvement of ROS in autoimmunity and RA. (A) Reactive oxygen species production is either directly or indirectly induced by environmental factors during the inflammatory process. (B) Induction of autoimmune response by ROS through the stress-responsive signaling and oxidative modification of autoantigens.

defense system for the organism contains a potentially dangerous option conforming a positive feed-back loop which is considered responsible for further augmentation and expansion of the response if the feed-back regulatory system, antioxidant system, and negative-cytokine network do not work efficiently.

Rheumatoid arthritis is a common human autoimmune disease with a prevalence of about 1% (2). While there has been progress in defining its etiology and pathogenesis, these are still incompletely understood (3–5). Rheumatoid arthritis is characterized by a chronic inflammation of the synovial joints associated with proliferation of synovial cells and infiltration of activated immuno-inflammatory cells, including memory T cells, macrophages, and plasma cells (4–6), leading to progressive destruction of cartilage and bone. This process is considered to be mediated by a number of cytokines

such as TNFα, IL1, IL-6, IL-8, IL-12, IL-16, IL-18, and IFNγ (reviewed in Refs. 2–5).

II. PATHOPHYSIOLOGY OF RA

Proposed causes for RA include: (i) genetic preposition; (ii) pathogenetic immuno-inflammatory responses triggered by environmental agents, particularly microbes; (iii) autoimmunity directed against components of synovium and cartilage; (iv) dysregulated production of cytokines; (v) recruitment of immuno-inflammatory cells through induction of inflammatory cell adhesion molecules (e.g., E-selectin, ICAM-1, and VCAM-1); and (vi) transformation of synovial cells into autonomously proliferating cells with tissue-infiltrating nature [often referred to as "transformed-like" phenotype (7)]. We have recently clarified the transformed-like nature of rheumatoid synoviocytes by performing gene expression profile analyses of synoviocytes and elucidated cellular genes specifically activated in RA synoviocytes (8,9). When compared with control synoviocytes obtained from healthy individuals (upon injury) or osteoarthritis (OA) patients, we found that both PDGF receptor α and SDF-1, a chemokine, genes are activated in RA synoviocytes without any external stimulus (8). Interestingly, from the gene knockout studies, it was shown that these factors are required for the development of limb joints. Moreover, when synoviocytes were stimulated with physiological concentration of TNFα, a principal proinflammatory cytokine, cell fate-determining factors, including Notch 1, Notch 4, and Jagged-2, a ligand for Notch proteins were activated only in RA synoviocytes (9). These findings indicate that RA synoviocytes may have reacquired the "revertant" phenotype mimicking the primordial synoviocytes that exhibit hyperproliferation and invasion, at least in a part. It is possible that this peculiar feature is caused by the long-term inflammatory stimulation, through which constitutive activation of particular signaling and transcription pathways lead to the change in "histone code" proposed by Allis (see Ref. 10 for review) and eventually change the epigenetic behavior of cells.

III. INVOLVEMENT OF NF-κB IN RA AS A PRIMARY PATHOGENIC DETERMINANT

Among the various signaling and transcription regulation pathways, nuclear factor-κB (NF-κB) and activator protein-1 (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 (reviewed in Ref. 11). Particularly, various cytokines 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 feed-back loop in the logic of the inflammatory responses associated with RA (Fig. 2). F2

In addition, besides its action in upregulating inflammatory cytokines and cell adhesion molecules, NF-κB also

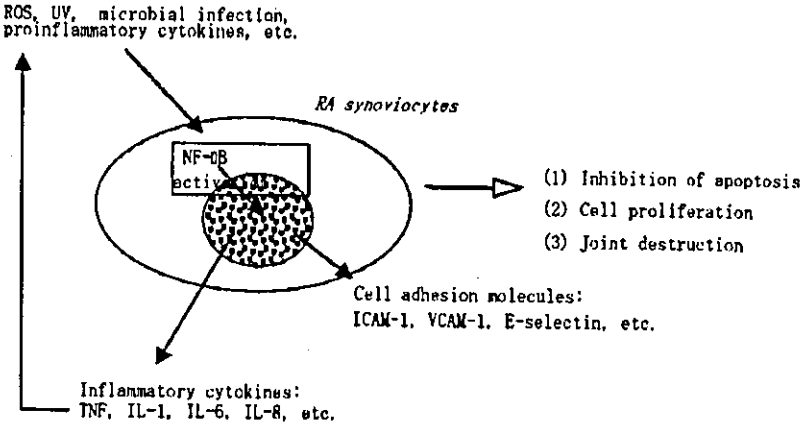

Figure 2 Involvement of NF-κB in the RA pathophysiology. Nuclear factor-κB induces gene expression of inflammatory mediators such as cytokines and cell adhesion molecules. As proinflammatory cytokines, TNF-α and IL-1β, stimulate the NF-κB activation cascade that induces expression of these cytokines, there will be a positive feed-back loop that perpetuates and expands the inflammatory responses even systemically. Nuclear factor-κB also stimulates synovial proliferation by inhibiting apoptosis. See also Fig. 9.

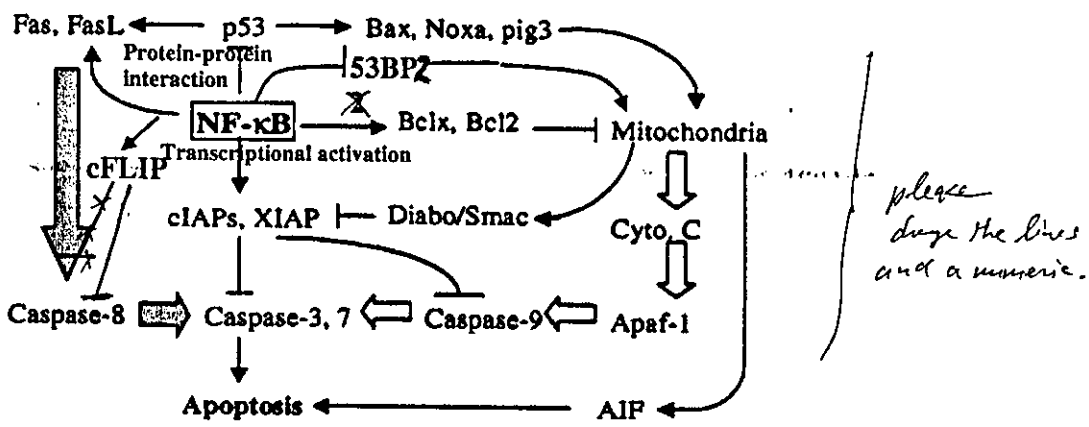

Figure 3 Antiapoptotic actions of NF-κB. Nuclear factor-κB inhibits apoptosis by: (i) transcriptional activation of antiapoptotic factors including cIAPs, XIAP, cFLIP, Bclx, and Bcl2, and (ii) direct inhibition of proapoptotic proteins such as p53 and 53BP2. Oxidation of NF-κB by gold cation. The active DNA binding form of NF-κB is hypothesized to contain zinc ions. When gold compound is added, Au(I) can take the electron from thiolate anions due to its higher oxidation potential compared with that of Zn^{2+}. Thus, Au(I) eventually oxidizes the thiolate anions of NF-κB into disulfide. The oxidation of NF-κB abolishes the DNA-binding activity.

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 (12,13) (Fig. 3). Moreover, it is shown that NF-κB blocks apoptosis in the absence of de novo protein synthesis (14) through protein–protein interaction with p53 and proapoptotic protein 53BP2 (15,16).

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 is formed to perpetuate and even expand the inflammatory responses (11). Thus, the intervention therapy against using anti-TNF antibody and IL-1β receptor antagonist has been developed (17,18). In addition, some of the drugs for RA have been shown to block

Table 1 List of RA Drugs that Inhibit NF-κB

Acetylsalicylic acid	Dexamethasone
Aurothioglucose	Ibuprofen
Aurothiomalate	Sodium salicylate
Auranofin	Sulfasalazine

NF-κB-activation cascade or its actions (Table 1) (19–22). However, there is no evidence to support the possibility that NF-κB or its signaling cascade is impaired in RA. Gene expression profile analysis using rheumatoid synoviocytes did not show any significant difference with regard to the responsiveness of NF-κB target genes (9). Thus, further studies are needed to elucidate a common mechanism by which NF-κB is activated in RA synovium.

IV. OXIDATIVE STRESS IN RA

It is well known that the synovial cavity of patients with RA is full of oxidative stress. First, Jayson and Dixon (23) found that the intra-articular pressure is much higher in RA joints as the result of a decreased compliance of the joint wall due to synovial membrane swelling and fibrosis of the capsule. Because of this elevated intra-articular pressure, the capillary flow rates of the inflamed joint tissues greatly fell, and reperfusion was delayed, thus associated with a decrease in the synovial O_2 tension (pO_2), an elevated pCO_2, an increase in the concentration of synovial fluid (SF) lactate, and a decrease in pH (24–26). Second, the synovial hypoxia was shown to cause accumulation of adenosine and its breakdown products including hypoxanthine and xanthine (27), which subsequently activates the xanthine oxidase system (28) leading to repeated episodes of oxidative injury in the rheumatoid joints. Third, ROS production was detected in the joints of RA patients including the direct measurement of superoxide anion by electron spin resonance (28), ROS-modified IgG (29), increase in lipid peroxidation product (26), depletion of ascorbate (29), and ROS-mediated fragmentation of glycosaminoglycans such as synovial hyaluronic acid (30).

As mentioned earlier, the degradation of hyaluronic acid is considered responsible for the decreased viscosity of joint fluid and the increase in intra-articular pressure, and the oxidatively damaged IgG accounts for the generation of reactive epitopes for the production of rheumatoid factor.

V. OXIDO-REDUCTION OF PROTEINS AS A SIGNAL: REDOX REGULATION

Reactive oxygen species are highly reactive with biological macromolecules to result in producing lipid peroxides (which are often radicals), inactivating proteins and mutating DNA (by producing 8-OH-dG or breaking nucleic acid chains). Therefore, cells must have acquired the multiplicated endogenous antioxidant system for the maintenance of a stable form of life under such harmful conditions. These defense mechanisms include reducing enzymes such as thioredoxin (Trx) and glutaredoxin (Grx) (31–33). Oxidized protein molecules by ROS are reversibly reduced by Trx or Grx. Importantly, this reversible oxidation and reduction involving Cys residues of a functional protein sometimes work as a regulatory modification that determines its biological/biochemical activities. This is analogous to the regulatory modification of proteins such as phosphorylation (involving Ser, Thr, and Tyr residues), acetylation and methylation (Lys), and ubiquitination (Lys) (Fig. 4). Thus, the term "redox regulation" has been proposed indicating the active role of oxido-reductive modifications of proteins in regulating their activities. In other words, oxidation and reduction of biomolecules can be regarded as "signals" through which the organism communicates with external environment. There are accumulating evidences indicating that such redox control system works for the maintenance of cellular homeostasis (11,33–35).

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VI. SIGNALING CASCADE FOR NF-κB ACTIVATION

The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, RelA (p65), RelB, NFkB1

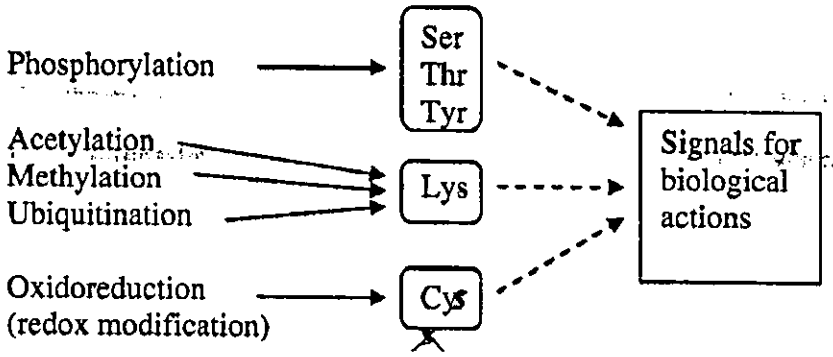

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Figure 4 Biochemical modifications of proteins. Oxido-reduction (redox regulation) involves Cys residues. These amino acid modifications will generate biological signals.

(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 homo-dimers with distinct specificities in various combinations (11,36–38). 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 (38,39). Upon stimulation of the cells by proinflammatory cytokines, IL-1β and TNF-α, IκBs are degraded and NF-κB is translocated to the nucleus and activates expression of target genes. In addition to these physiological stimuli, NF-κB activation cascade is also triggered by ionizing UV irradiation and oxidative reagents such as H_2O_2 (11,40–42). It is well established that two major signaling cascades are involved in NF-κB activation: kinase cascade and redox regulation.

VI.A. Kinase Cascades Involved in the NF-κB Activation Cascade

At least two distinct types of kinase pathways are known to be involved in NF-κB activation: IκB kinase and NF-κB kinase (Fig. 5). The IκB kinase complex, capable of specifically F5

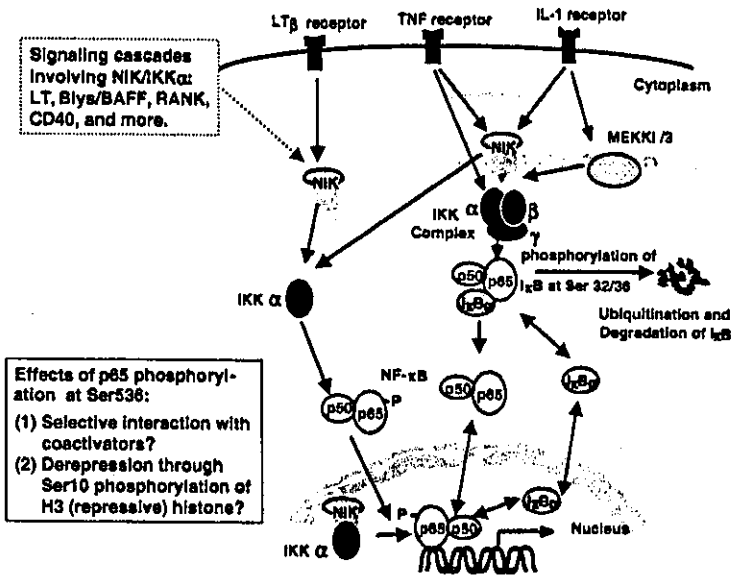

Figure 5 Nuclear factor-κB activation cascades. In addition to canonical pathway involving IκB phosphorylation and ubiquitination followed by its proteolytic degradation in 26S proteasome within the cytoplasm, there appears to be another cascade not involving IκB phosphorylation. Lymphotoxin β receptor 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 de-represses 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.

phosphorylating serines 32 and 36 of IκBα, was originally identified as ~700 kDa of high molecular complex (29,38,43). Subsequently, two catalytic subunits (IKKα and IKKβ) and a scaffold subunit of this complex (IKKγ/NEMO/IKKAP) were identified and cloned (37,44). The IκB kinase (IKK) complex, consisting of IKKα, β, and γ, can be activated by a variety of stimuli, including TNF-α, 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. The IKK complex is stimulated by upstream kinases that belong to mitogen-activated protein kinase kinase kinases (MAP3Ks), such as MEKK1, MEKK2, MEKK3, and to NIK, capable of phosphorylating these serines in vitro, and activating NF-κB (62,65). Phosphorylation on specific serine residues of IκBs leads to ubiquitination of IκBs and subsequent degradation by proteasome complex.

There are accumulating evidences suggesting the involvement of additional kinases that phosphorylate the p65 (RelA) subunit of NF-κB and regulate its transcriptional competence (45–47). We recently found that IKKα is responsible for the p65 phosphorylation at Ser536 upon the lymphotoxin (LT)β receptor signaling mediated by NIK and induces NF-κB activation independently of the IκB phosphorylation and its degradation (48–50). Interestingly, this NIK–IKKα cascade is also involved in Blys/BAFF and RANK, and most likely CD40, signaling (50–52). Because 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 TNF-α-dependent phosphorylation of serine 529 has also been demonstrated to increase the transcriptional activity of p65. For example, casein kinase II was implicated in the TNF-α-dependent phosphorylation of p65 on serine 529 (53). It was shown that serine residues 529 and 536 of p65 were required for the transcriptional activation of p65 by AKT and the IL-1β signaling (50,54). Although Ghosh and colleagues (47) have proposed a model in which the catalytic subunit of PKA (PKAc) is associated with the NF-κB/IκBα complex in the cytoplasm in an inactive form, and signal-induced degradation of IκBα allows PKAc to phosphorylate p65 on serine 276 for transcriptional activation, we and others (55–57) found that PKA activation did not stimulate NF-κB-dependent gene expression.

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 (38). 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 (58–60). Moreover, recent evidences have demonstrated that upon signaling IKKα translocates to the nucleus and phosphorylates Ser10 of the histone H3 component of nucleosome (61,62) (Fig. 5). Although the histone H3 with methylated Lys9 of H3 renders the local nucleosome to be "repressive," the adjacent Ser10-phosphorylation of H3 histone reverses this effect and de-represses the transcriptional activity of the genes located in the "de-repressed" nucleosome (10).

In addition, it was recently shown that ischemia/reperfusion injury and H_2O_2 induce Src family kinases that subsequently phosphorylate the Tyr42 of IκBα and induce NF-κB in the absence of ubiquitin-dependent degradation (63,64). It appears that Src family kinases act as redox sensors for NF-κB activation. Thus, IKK-independent pathway can function under specific redox-mediated stimuli to activate NF-κB. Although NF-κB activation may reduce tissue damage following the ischemia/reperfusion injury by blocking apoptosis, it may promote synovial cell proliferation in the affected joints of RA patients.

VI.B. Redox Regulation of NF-κB Activation

The induction of NF-κB following liver ischemia/reperfusion injury is regulated by acute redox-activated responses involving an NADPH oxidase Rac1 (65). Other evidences also indicate the involvement of Rac1 in the generation of ROS and activation of NF-κB (66,67). Divergent stimuli that activate NF-κB are considered to generate ROS on the basis of the facts that most such signaling cascades could be blocked by antioxidants, e.g., agents like *N*-acetyl-L-cysteine (NAC), PDTC, and α-lipoic acid were shown to block NF-κB activation in response to diverse stimuli (40,68–72). However, a recent study has clarified that NAC and PDTC block the NF-κB signaling by not necessarily blocking ROS but by lowering the affinity of TNF receptor to its ligand and inhibiting the ubiquitin ligase activity for IκB, respectively (73). No direct evidence of ROS production in response to various NF-κB

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stimulating agents, such as TNFα, IL-1β, and LPS, is yet to be obtained. Therefore, the general involvement of ROS in the NF-κB signaling is still elusive.

Intriguingly, there are accumulating evidences that support the positive effect of Trx in the NF-κB activation cascade (74–77). Thioredoxin is a cellular reducing catalyst and is known to participate in the 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. Interestingly, human Trx has been initially identified as a factor responsible for induction of the α subunit of the IL-2 receptor, which is known to be under the transcriptional control of NF-κB (78). We and others (74–76,79) have demonstrated in vitro that NF-κB cannot bind to the κB DNA sequence of the target genes until it is reduced.

Structural and biochemical approaches have provided evidences supporting the molecular model of the redox regulation of NF-κB by Trx (Fig. 6). Within the NF-κB DNA F6

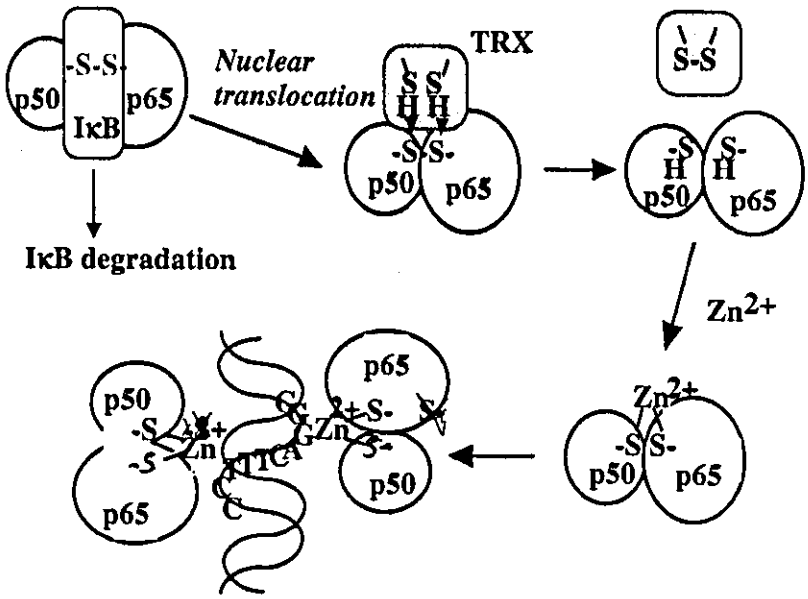

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Figure 6 Redox regulation of NF-κB by Trx. See the text for the details.

recognition domain, there is a redox-sensitive Cys (74,75) in the loop of the β-barrel structure that makes a direct contact with the DNA (80,81). Qin et al. (82) has solved the 3D NMR structure of Trx molecule that is associated with the DNA-binding loop of p50 subunit of NF-κB and showed that a redox-active Cys located in the depth of the boot-shaped hollow on Trx surface is in the close proximity with the redox-sensitive Cys of the DNA-binding loop of p50 and likely to reduce the oxidized cysteine on p50 by donating protons in a structure-dependent fashion. However, the inter-molecular disulfide bridge bet-ween Trx and NF-κB must be transient because the binding of Trx to the NF-κB DNA-binding loop prevents the recognition of target DNA. On the basis of biochemical reactions, we have postulated that zinc ion replaces the inter-molecular disulfide bridge and dissociates NF-κB from Trx (11,20,41). In favor of this model, we have demonstrated with cultured rheumatoid synoviocytes that NF-κB and Trx concomitantly migrated to the nucleus during the early phase of the NF-κB activation process induced by TNFα (83). Thioredoxin was relocated in the cytoplasm after 30 min of stimulation, whereas the NF-κB was predominantly present at the nucleus for several hours. Thus, it is possible that NF-κB associates with Trx immediately after dissociation from IκB, translocates to the nucleus together, and dissociates from Trx through displacement of the inter-molecular disulfide by zinc ions.

VII. ROLES OF THIOREDOXIN IN RA PATHOPHYSIOLOGY

In order to examine the roles of Trx in RA, we have measured the Trx level in the joint fluid and explored its effects on the NF-κB activation cascade. Others have elucidated additional roles of Trx in the hyperproliferative nature of rheumatoid synoviocytes.

VII.A. Elevated Trx in the RA Joint Fluid

We found that the serum Trx level was elevated in patients with RA when compared with healthy individuals and AQ4 OK

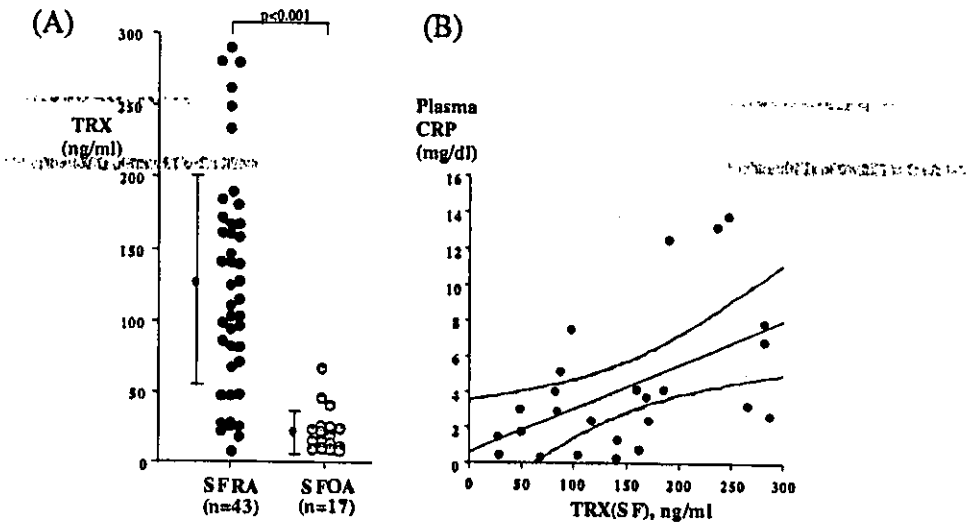

Figure 7 Elevated levels of Trx in RA patients. (A) The Trx concentration is elevated in the joint fluids of RA patients when compared with that of OA patients. (B) Positive correlation of Trx (SF) and serum CRP (its production is stimulated by IL-6 in the liver), indicating that Trx also plays a role in systemic inflammation.

patients with OA (Fig. 7) (77). Moreover, the Trx level in the F7
SF was much greatly elevated in RA patients than in OA patients. In fact, Taniguchi et al. (84) identified a cis-regulatory element in response to oxidative stress within a promoter region of human Trx gene. Thus, the increase of Trx level in SF could be ascribed to the production of ROI by activated macrophages and by hypoxic-reperfusion injuries in the inflamed rheumatoid joints as discussed earlier (23–30). Moreover, multiple regression analysis revealed that the serum C-reactive protein (CRP) level, a clinical laboratory parameter of inflammation, was better correlated with the linear combination of TNF-α (SF) and Trx (SF) levels than TNF-α (SF) alone, which suggested that Trx might play a subsidiary role in the rheumatoid inflammation (Fig. 7) (77).

When the effect of Trx on the TNF-α-induced IL-6 and IL-8 production using rheumatoid synovial fibroblast cultures was examined, we found that the extents of IL-6 and IL-8

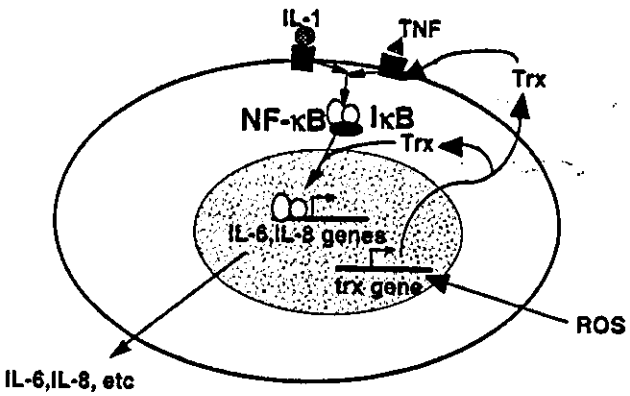

Figure 8 Effect of extracellular Trx on the NF-κB activation stimulated by proinflammatory cytokines. Reactive oxygen species stimulates Trx production in addition to NF-κB activation. See the text for the details.

production in response to TNF-α were greatly augmented by Trx when compared with TNF-α alone (Fig. 8). Furthermore, F8 we found that Trx accelerated the nuclear translocation of NF-κB and facilitated the IκBα phosphorylation and subsequent degradation in response to TNF-α. The elevated Trx level indicates the persistent presence of oxidative stress in the joints of RA patients. Thus, these findings show that Trx has an active role in RA by augmenting the proinflammatory response of TNFα.

VII.B. Regulation of ASK1 Activity by Trx

More intriguingly, another piece of evidence linking the cellular redox status to specific signaling pathways came from the fact that the apoptosis signal regulated kinase 1 (ASK1) interacts with Trx. ASK1 is a member of the MAP3K family that activates downstream kinases including JNK and p38 MAPK. Screening for ASK1-associated proteins using the yeast two-hybrid system led to the identification of Trx as an ASK1-interacting molecule (85). When Trx binds to ASK1, the activity of ASK1 is inhibited (Fig. 9). The rise in ROS F9 levels after TNF-α stimulation leads to activation of ASK1

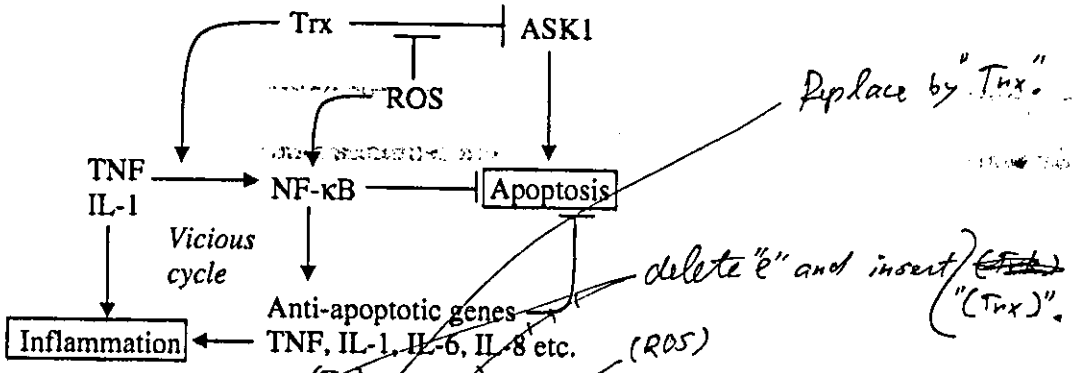

Figure 9 Thioredoxin as a redox sensor and biological switch to control apoptosis. Thioredoxin inhibits the apoptosis-inducing ASK1 kinase. Reactive oxygen species blocks the interaction between Trx and ASK1, thus stimulating apoptotic cascade. Nuclear factor-κB induces gene expression of various cytokines and antiapoptotic factors. In the presence of excessive ROS, both the anti- and proapoptotic processes are stimulated. Thus, the actual cell fate depends on the level of cellular antioxidant system. Signal transduction pathways for NF-κB activation. The first step involves kinase pathways such as IKKs and NF-κB kinases. The second step involves "redox regulation" by Trx. After the stimulation of the cells by TNF-α or IL-1β, for example, ROS are produced. ROS activate kinase cascades by direct or indirect mechanisms. Reactive oxygen species also induces production of Trx. TRAF molecules transduce signals from TNF receptor or IL-1 receptor and stimulate the downstream NF-κB activation pathway. Phosphorylation of NF-κB or IκBs will lead to dissociation of NF-κB from IκBs. The phosphorylated IκBs by IKK complex will be ubiquitinated and then degraded by proteasome. After liberated from IκBs,NF-κB must go through the Trx-mediated reduction of the "redox-sensitive" cysteine to recognize the target DNA sequence (κB site). The transactivation potential of the NF-κB p65 subunit is modified by interaction between with coactivators or corepressors, including CBP/p300 or TLE/AES.

by dissociation of Trx from ASK1 (85,86). It appears that cellular reducing enzymes, such as Trx and Grx, could also function as redox sensor molecules. If the cellular antioxidant system is sufficient, Trx will be stably associated with ASK1

and act as an antiapoptotic factor, thus supporting the synoviocyte proliferation. However, when excessive ROS is present, due to I/R stress, for example, synoviocytes undergo apoptosis in the presence of severe inflammatory responses. In fact, apoptotic synoviocytes were often observed within the hyperproliferative synovial tissues ~~(refs)~~. AQ6

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VIII. CONCLUSION

Rheumatoid arthritis is a complexed process of chronic and progressive inflammation involving numerous transcription factors and signaling molecules. Although majority of pathologic changes of RA appeared to be limited in the joints, similar pathophysiological considerations are applicable to a wide variety of chronic and acute/severe inflammatory diseases including inflammatory bowel diseases, surgical inflammatory response syndrome, multiple sclerosis, and atherosclerosis. In terms of NF-κB involvement, the RA pathophysiology shares with HIV infection and cancer. Thus, findings and therapeutic measures discovered in RA are readily applicable to the understanding and the treatment of these fatal diseases. In other words, when disease processes are broken down into actions of each molecule that governs critical step of many events that build up the entire process, there will be no restriction in applying the concept obtained from the behavior of each molecule to other disease by crossing the border of different scientific disciplines.

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