Chapter from the book *Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities*

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Molecular Mechanisms of Rheumatoid Arthritis Revealed by Categorizing Subtypes of Fibroblast-Like Synoviocytes

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1. Introduction

The immune system is a highly organized defense system, which recognizes invading microorganisms and aims to exclude them. In order to do this effectively and safely, the immune system must distinguish between self- and non-self-antigens, and be tolerant of self-antigens. Autoimmune diseases develop through the breakdown of self-tolerance, as a result of immune deregulation. This is caused by the combined influence of genetic and environmental factors, including infectious microorganisms. Rheumatoid arthritis (RA) is a systemic autoimmune disease, characterized by synovial hyperplasia leading to the destruction of bones and joints. This severely impairs the life of patients. RA is a relatively common autoimmune disease, occurring in approximately 1% of the population. However, its etiology and pathophysiology are not completely understood. The incidence of RA is correlated with certain human leukocyte antigen (HLA)-DR haplotypes, and the production of autoantibodies such as rheumatoid factor and anticitrullinated protein autoantibody. Thus, the involvement of the deregulated immune system is strongly implicated. Various molecules, including type II collagen, gp39, citrullinated peptides, and glucose-6-phosphoisomerase, have been reported as potential pathogenic autoantigens. However, their involvement explains only a proportion of RA cases. Autoantigens are abundant in the body and, theoretically, the immune response to them continues indefinitely. Thus, systemic autoimmune diseases exhibit the characteristics of chronic inflammation.

In the pathological condition of RA, the joints are infiltrated with T cells, B cells, macrophages, and plasma cells, all of which are characteristic chronic inflammation cells driven by the immune system. Recently, Th17, a novel helper T-cell subset producing interleukin (IL)-17, has been recognized as a pivotal player in the local inflammation driven by acquired immunity. In addition to immune-competent cells, there is accumulating evidence for abnormalities in non-hematopoietic cells, especially fibroblast-like synoviocytes (FLSs) (Bartok & Firestein, 2010; Firestein, 2009; Mor et al., 2005; Pap & Gay, 2009). The cartilage and bone are destroyed by the invasion of pannus, which is formed from proliferating FLSs and multi-nucleated osteoclasts. Osteoclasts are specialized to resolve bone, and play a major role in bone destruction in RA. However, there is strong evidence that FLSs themselves are aggressive enough to destroy bone. When cultured FLSs derived from RA or osteoarthritis (OA) were co-implanted with human cartilage under the renal
capsule of a severe combined immunodeficiency (SCID) mouse, the FLSs derived from RA, but not from OA, destroyed the cartilage (Muller-Ladner et al., 1996; Pierer et al., 2003). In RA, cytokines produced by surrounding cells in the inflamed joints, such as basic fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, tumor necrotizing factor (TNF)-α, and IL-1β, are thought to be responsible for the hyperplasia of FLSs. On the other hand, activated FLSs produce TNF-α, IL-1β, IL-6, chemokines, and matrix metalloproteinases (MMPs), thereby establishing the chronic and destructive inflammatory circuit driven by cellular interaction. Thus, it appears that some passively activated FLSs may be changed to be in a distinctly activated state, autonomously destroying bone and joints.

The critical roles of inflammatory cytokines are evidenced by the effectiveness of cytokine-blockade therapies for RA, using anti-TNF-α or anti-IL-6 receptor antibodies (Brennan & McInnes, 2008; Nishimoto & Kishimoto, 2006). In spite of the promising effects shown by these anti-cytokine therapies, several problems remain, such as suppression of the normal immunity and substantial numbers of resistant cases (Firestein, 2007). To overcome these difficulties, increased knowledge of the molecular mechanisms involved in the complex and multi-factorial pathophysiology of RA is required. In this context, our research on the disease-associated genes of RA is based on the theory that FLSs are heterogeneous in physiological, and also in pathological situations. In this chapter, we briefly overview the current understanding of FLSs in RA, and introduce the pathophysiological natures of FLSs as revealed by our subtyping studies.

2. Fibroblast-like synoviocytes

2.1 Fibroblast-like synoviocytes in the normal synovium

The synovium is a membranous structure that extends from the margins of articular cartilage and lines the capsule of diarthrodial joints. The synovium supports the joint structure, provides nutrition to the cartilage and lubricates the surface. The synovial membrane has 2 compartments: the intimal lining layer and the sublining layer. The intimal lining layer is the superficial layer that faces the intra-articular cavity, and produces synovial fluid as lubricant. This lining layer is normally 2 to 3 cells thick and consists of 2 types of synovial cells: macrophage-like synoviocytes (Type A synoviocytes), and fibroblast-like synoviocytes (Type B synoviocytes). Type A synoviocytes are hematopoietic in origin, bone marrow-derived, and terminally differentiated, as are other tissue-resident macrophages. Type B synoviocytes are mesenchymal cells with vimentin in the cytoskeleton, and Thy-1 (CD90) on their surface. Type B synoviocytes display many characteristics of fibroblasts, such as the production of extracellular matrix, and collagen type IV and V. Specific characteristics for FLSs in the intimal lining layer include expression of cadherin-11 for homotypic aggregation (Lee et al., 2007) and uridine diphosphoglucose dehydrogenase for synthesis of hyaluronic acid, an essential joint lubricant. Expression of decay accelerating factor, CD55, and adhesion molecules (VCAM-1 and ICAM-1) is also characteristic.

2.2 Fibroblast-like synoviocytes in the synovium of RA

In the synovium of RA, the histopathological characteristics are hyperplasia of FLSs, and infiltration with inflammatory cells. The pathophysiological reactions are joint destruction and perpetuation of inflammation.
2.2.1 Hyperplasia

Hyperplasia of FLSs exhibits features of stable activation—the so-called tumor-like transformation. Features of tumor-like transformation include anchorage-independent growth, adhesion to the extracellular matrix of cartilage, resistance to apoptotic signaling, and invasiveness to cartilage and bone. Tumor-like transformation may be cell-autonomous or non-cell-autonomous. The non-cell-autonomous pathway is indirectly driven by factors produced by autoimmune-competent cells in the microenvironment. These include cytokines, growth factors, lipid mediators, and reactive oxygen species. By contrast, the cell-autonomous pathway results from the cell-intrinsic changes of FLSs themselves. Reflecting cell-intrinsic changes, FLSs in RA have a characteristic morphology, i.e., an abundant cytoplasm; a dense, rough endoplasmic reticulum; and large, pale nuclei with several prominent nucleoli (Pap & Gay, 2009). One of the important molecular characteristics of FLSs in RA is the expression of proto-oncogenes (Bartok & Firestein, 2009), including c-fos (Aikawa et al., 2008), ras, raf, sis, myb, and myc (Roivainen et al., 1999). Interestingly, proto-oncogenes are predominantly expressed by FLSs attached to cartilage and bone (Muller-Ladner et al., 2000). Furthermore, some of these proto-oncogenes regulate gene expression of MMPs or cathepsin L. Thus, in the SCID mouse, inhibition of c-Raf-1 or c-Myc significantly reduced the expression of MMP-1 and MMP-3, resulting in decreased invasiveness of FLSs to the cartilage (Pap et al., 2004).

Among the various cells in the inflamed synovium, macrophages and T cells are thought to be most responsible for producing various stimuli for stable activation of FLSs. Various combinations of PDGF, TGF-β, TNF-α, IL-1, and the arachidonic acid metabolites induce the proliferation of FLSs (Konttinen et al., 1999). On the other hand, FLSs in RA have been shown to exhibit defective apoptosis, rather than enhanced proliferation (Jacob et al., 1995; Korb et al., 2009). Apoptosis was rapidly induced in RA-derived FLSs by retroviral transduction of a combination of dominant-negative c-Raf-1 and dominant-negative c-Myc (Pap et al., 2004), indicating that some proto-oncogenes are involved. Death receptor Fas is expressed and is functional in FLSs in vitro. However, apoptosis induced by anti-Fas antibody was prevented by TNF-α, IL-1β, and IL-6, suggesting that FLSs in the inflamed joints are resistant to apoptosis (Ohshima et al., 2000; Wakisaka et al., 1998). The anti-apoptotic function of nuclear factor (NF)-κB activated by TNF-α signaling, and the induction of the anti-apoptotic molecule Bcl-xL by IL-1β are involved (Jeong et al., 2004). In addition to the effects of cytokines, the adhesion molecule VLA-5 (integrin α5β1), upon ligation with fibronectin, is involved in this resistance to Fas-mediated apoptosis (Kitagawa et al., 2006). Under conditions of genotoxic stress, the tumor-suppressor p53 induces cell-cycle arrest, followed by either DNA repair or apoptosis, depending on the degree of DNA damage (Gudkov & Komarova, 2010). A main effector of p53-dependent apoptosis, PUMA (p53 up-regulated modulator of apoptosis) is present in very low concentrations in the synovium. Adenovirus-mediated transfer of the p53 gene into FLSs induced production of the p53 protein, leading to p21 expression; however, PUMA expression was not enhanced and apoptosis was not induced (Cha et al., 2006). This suggests that, under conditions of genotoxic stress, the FLSs in RA tend to undergo cell-cycle arrest rather than apoptosis.

2.2.2 Infiltration with inflammatory cells

Infiltration with inflammatory cells mainly involves chemokines, cytokines, lipids of chemical mediators, and adhesion molecules. It comprises the mutual activation of interacting cells of distinct lineages, leading to the perpetuation of inflammation.
Inflammatory mediators produced by FLSs include IL-15 (Miranda-Carus et al., 2004), IL-16 (Pritchard et al., 2004), IL-18 (Gracie et al., 1999), TNF-α, TGF-β (Pohlers et al., 2007), NO, and prostaglandin E2 (Kojima et al., 2003). Various chemokines are reported to be produced by FLSs in RA (Iwamoto et al., 2008). The production of IL-8/CXCL8 and GROα/CXCL1, which recruit neutrophils, is induced by stimulation of FLSs with IL-1α, IL-1β, TNF-α, or IL-17 (Hosaka et al., 1994; Kehlen et al., 2002; Koch et al., 1991, 1995). Neutrophils are abundant in the synovial fluid of RA, but rare in the synovial tissue. The levels of lymphotactin/XCL1 are elevated in the synovial fluid and tissues of RA. Moreover, infiltrating mononuclear cells and FLSs in the tissues of RA express XCR1, a receptor for lymphotactin/XCL1 (Wang et al., 2004). The levels of macrophage inflammatory protein (MIP)-1α/CCL3 (a ligand of CCR1 and CCR5) are higher in the synovial fluid of RA. Furthermore, upon stimulation with lipopolysaccharide and TNF-α, isolated FLSs produce MIP-1α/CCL3 (Koch et al., 1994). The migration of CD4+ memory T cells to the synovium of RA, and the inhibition of activation-induced apoptosis of T cells, are induced by stromal cell-derived factor (SDF)-1/CXCL12. Thus, the accumulation of CD4+ memory T cells in the synovium plays an important role in RA (Nanki et al., 2000). The production of RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted)/CCL5 is histologically detected in the synovial lining and sublining layers of affected rheumatoid joints (Robinson et al., 1995). All these suggest that, in microenvironments rich with TNF-α and IL-1β, FLSs themselves recruit monocytes, neutrophils, Th1 cells, eosinophils, and basophils (Rathanaswami et al., 1993).

In addition to the regulation of migration, stimulation with MCP-1/CCL2, SDF-1/CXCL12, IP-10/CXCL10, Mig/CXCL9, and MCP-4/CCL13 enhances the proliferation of FLSs, leading to synovial hyperplasia (Garcia-Vicuna et al., 2004; Iwamoto et al., 2007). Furthermore, continuous infusion of human IL-8/CXCL8 into the knee joints of rabbits for 14 days led to severe arthritis, characterized by erythema, joint pain, infiltration of leucocytes and mononuclear cells in the synovial tissue, and hypervascularization in the synovial lining layer (Endo et al., 1994). Thus, the angiogenic properties of chemokines, such as IL-8/CXCL8, GROα/CXCL1, MCP-1/CCL2, SDF-1/CXCL12, and fractalkine/CX3CL1 (Koch et al., 1992; Salcedo et al., 1999, 2000; Volin et al., 2001) may play an important role in the development of RA. Angiogenic factors, including FGF (Thomas et al., 2000), vascular endothelial growth factor (VEGF) (Cho et al., 2002), IL-18, and angiopoietin (Scott et al., 2002), are also produced by FLSs. This suggests that FLSs are involved in neovascularization, and may cause critical pathological changes to sustain pannus formation in RA (Szekanecz & Koch, 2007).

### 2.2.3 Joint destruction

Proteinases, such as MMPs and cathepsins, are produced by FLSs attached to cartilage and bone, and play an important role in joint destruction. The expression of MMP-1/interstitial collagenase and MMP-3/stromelysin correlates with the invasive growth of FLSs in RA (Tolboom et al., 2002). MMP-1 is found in the synovial membranes of all RA patients. Moreover, the levels of MMP-1 in the synovial fluid, but not in the sera, correlate with the degree of synovial inflammation (Konttinen et al., 1999; Maeda et al., 1995; Sorsa et al., 1992). MMP-3 plays a key role in joint destruction, not only by degrading matrix molecules, but also by activating other pro-MMPs into their active forms (Okada, 2009). The major source of MMP-3 is FLSs in the lining layer (Tetlow et al., 1993). High concentrations of MMP-3 have been detected in the synovial fluid and sera of RA patients (Beekman et al., 1997; Taylor et al., 1994). Moreover,
elevated serum levels of MMP-3 are correlated with systemic inflammation at the clinical and also the serologic level (Manicourt et al., 1995; Yoshihara et al., 1995). Although expression of MMP-13/collagenase-3 correlates with elevated levels of systemic inflammatory markers, this is not specific to RA (Lindy et al., 1997; Westhoff et al., 1999). MT1-MMP/MMP-14 degrades the extracellular matrix, and activates MMP-2/gelatinase A and MMP-13 (Pap et al., 2000a). The expression of MMPs in synovial cells is regulated by several extracellular signals, including inflammatory cytokines, growth factors, and molecules of the extracellular matrix, such as collagen and fibronectin (Pap & Gay, 2009). Among these, IL-1 is the most potent inducer of MMPs, including MMP-1, MMP-3, MMP-8, MMP-13, and MMP-14. FGF and PDGF also act as potent inducers for MMPs, by enhancing the effects of IL-1. TNF-α and TGF-β induce MMP-1 and MMP-13, respectively, while IL-17 induces MMP-1 and MMP-9.

Another group of proteinases involved in joint destruction is the cathepsins, which cleave cartilage types II, IX, and XI, and proteoglycan. The expression of the cysteine proteases, cathepsins B and L, was increased in the synovium of RA, especially at the sites of cartilage invasion (Keyszer et al., 1995, 1998). Similarly to MMPs, the production of cathepsins is induced by proto-oncogene, IL-1, and TNF-α (Joseph et al., 1987; Huet et al., 1993; Lemaire et al., 1997). Cathepsin K, which plays an important role in bone resorption by osteoclasts, is also expressed by FLSs and macrophages at the site of synovial invasion into the articular bone (Min et al., 2004).

### 2.2.4 Perpetuation of inflammation

When cells producing soluble factors or expressing ligands on their surfaces are located close to cells that receive signals through the specific receptor, a circuit of chronic inflammation may be generated through an exchange of cell roles. For example, activated Th1 cells produce IFNγ, which activates macrophages. The activated macrophages produce IL-1 and TNF-α, which in turn activate T cells. In cases of RA, IL-1 and TNF-α from macrophages can activate FLSs, creating another circuit with a distinct cellular combination. Although one can easily imagine the operation of such a circuit at a certain time point of autoimmune diseases, it is difficult to demonstrate the mechanism by a suitable model system. Recently, Ogura et al. (2008) proposed that IL-17 secreted from Th17 cells induces fibroblasts to produce more IL-6, in a manner dependent on the transcription factor NF-κB, and the signal transducer and activator of transcription (STAT) 3. The mechanism, designated as “IL-17A-triggered positive-feedback loop of IL-6 signaling”, is thought to amplify the inflammatory responses mediated by interactive cytokines. Enhancement of this loop was shown to be involved in the development of RA-like arthritis or experimental autoimmune encephalomyelitis in knock-in mice gp130F759, which are defective in the negative regulation of signaling through a common receptor subunit of IL-6 family cytokines, gp130. The identification of such a powerful circuit, specific to each autoimmune disease, will facilitate the development of a critical target point for effective therapy.

### 3. Progress in the study of RA by the molecular genetic approach

#### 3.1 Genome-wide screening for disease-related genes

The risk of developing RA and the severity of the disease are significantly affected by genetics. It has long been recognized that certain HLA alleles, especially HLA-DR4, are
associated with increased risk of onset and severity of RA (Weyand et al., 1992). A shared epitope on certain HLA haplotypes is thought to affect the binding of peptides derived from self-antigens, leading to autoimmune responses by T cells (Wordsworth et al., 1989). To identify non-HLA genes that regulate the development and severity of RA, human genome-wide studies have been performed. Some of these studies have used a combined approach, with factors such as microsatellites (Tamiya et al., 2005), or disease subsets; serum autoantibody alone (Stahl et al., 2010) or combined with a shared epitope (Sugino et al., 2010); race, or nation (Freudenberg et al., 2011; Martin et al., 2010); correlation with other autoimmune diseases (Cui et al., 2009; Zhernakova et al., 2011); or responsiveness to therapies targeting specific cytokines (Liu et al., 2008; Plant et al., 2011). Single nucleotide polymorphisms that may be involved in the development of RA include protein tyrosine phosphatase, nonreceptor-type 22 (PTPN22), cytotoxic T-lymphocyte antigen 4 (CTLA4), STAT4, and peptidylarginine deiminase type 4 (PADI4). Among these, PADI4 has been identified by genome-wide screening (Suzuki et al., 2003) as being able to modify self-antigens by citrullination. Moreover, the presence of anti-cyclic citrullinated antibody in the serum is highly specific to RA and has a high diagnostic value. The role of PADI4 in the pathogenesis of RA, especially with respect to “autoimmunity” to modified self-antigens, will be intriguing to clarify.

Large-scale, genome-wide association studies, based firmly on statistics, have provided valuable information on the candidate genes for RA. Nevertheless, to understand the complex pathophysiology of RA, data from studies on additional aspects must be integrated. Such studies should include molecular and cell-biological analyses of clinical materials from individual RA cases, and functional analyses of candidate genes in vitro and in vivo, including experimental system using engineered mutant mice.

3.2 Transcription profiling reveals disease-specific genes and heterogeneity in RA tissues
Gene expression profiling of FLSs, comparing RA and OA, has revealed disease-specific genes. The genes highly and exclusively expressed in RA were HOXD10, HOXD11, HOXD13, CCL8, and LIM homeobox 2. Further analysis of the relationships between gene expression on RA-FLSs and clinical disease parameters revealed specific and unique correlations as follows; HLA-DQA2 with Health Assessment Questionnaire (HAQ) score; Clec12A with rheumatoid factor; MAB21L2, SIAT7E, HAPLN1, and BAIAP2L1 with C-reactive protein level; and RGMB and OSAP with erythrocyte sedimentation rate (Galligan et al., 2007). The data indicated the heterogeneity of gene expression in patients with the same disease. These RA-specific or clinical state-related genes differ from those identified by genome-wide screening, indicating that the complete pathophysiology of RA, as a multifactorial disease, involves genomic and also epi-genomic regulation of genes. The functional roles of these genes remain to be determined.

Evidence for the heterogeneity of gene expression in synovial tissues from erosive RA cases has been demonstrated by large-scale profiling studies. Systemic characterization of the differentially expressed genes highlighted the existence of at least 2 molecularly distinct forms of RA tissues (van der Pouw Kraan et al., 2003). The first is RA tissue with high-grade inflammation (RA$_{high}$), which exhibits abundant expression of gene clusters indicative of adaptive immune responses, such as genes expressed by T cells, B cell, and antigen-presenting cell (APC). The second form of RA tissue is a low-grade inflammatory gene
expression signature (RA^{low}), common to the tissues from patients with OA, and characterized by increased expression of genes involved with tissue remodeling activity. Importantly, the cluster of RA^{high} showed an increased expression of STAT1-pathway related genes; STAT1-inducing receptors (IL-2Rγ, CCR5), and STAT1 target genes (MMP-1, MMP-3, caspase-1, TAP-1, and IRF-1), suggesting a prominent role for this pathway. Furthermore, patients with the high-grade inflammation tissue type had higher Disease Activity Scores in 28 joints, higher C-reactive protein levels, higher erythrocyte sedimentation rates, increased numbers of platelets, and shorter disease durations (van Baarsen et al., 2010). Several trials have profiled gene expression in the synovial tissues of RA patients undergoing molecular-targeting therapy (Lindberg et al., 2006; Wijbrandts et al., 2008). Further analysis is expected to yield valuable data on cytokine activity in the human body, facilitating the development of effective therapy with a clear target point.

4. Mouse models for RA

The generation of RA-like joint diseases in engineered mutant mice appears to reflect the heterogeneous and complicated mechanisms of human arthritis, diagnosed simply as rheumatoid arthritis. In contrast to previous years, when animal models for human diseases rarely emerged by point mutations in nature, current research on autoimmune diseases such as RA benefits from the existence of various engineered mutant mouse models. For example, mechanisms for RA-like disease revealed by murine models include, the abnormal T-cell receptor (TCR) signaling by a natural mutant ZAP70 in SKG mouse (Sakaguchi et al., 2003), an autoantibody to glucose-6-phosphoisoferase in K/BxN TCR transgenic mouse (Korganow et al., 1999), defective autoantigen clearance in DNaseII/− mouse (Kawane et al., 1991), and excessive amounts or activity of cytokines. RA-like disease developed in TNF-α transgenic mice (Keffler et al., 1991) and IL-1α transgenic mice (Niki et al., 2001) with overproduction of inflammatory cytokines and in TNF AU-rich elements-deficient (ΔARE) mice (Kontoyiannis et al., 1999) with increased stability of cytokine messenger RNA. Excessive activities of arthritogenic cytokines were evoked in IL-1 receptor antagonist knock-out mouse (Horai et al., 2000) lacking a physiological negative feedback molecule, and in gp130F759 with a defective, intracellular negative-regulatory signaling pathway (Atsumi et al., 2002; Ohtani et al., 2000).

These wide variety of murine arthritis models with a defined genetic defect will be useful for analyzing the mechanisms for the synergistic action of genetic and environmental factors in RA development (Ishihara et al., 2004), and also the mechanisms for initiation or perpetuation of joint inflammation (Murakami et al., 2011; Ogura et al., 2008). Furthermore, bone marrow transplantation experiment revealed a unique feature of gp130F759 that non-hematopoietic cells with a point mutation Y759F in gp130 are sufficient to induce passive but arthritogenic activation of wild type CD4+T cells (Sawa et al., 2006). In human TNF-α transgenic mouse, arthritogenic FLSs showed increased expression of MMP-1 and MMP-9, and also diminished adhesion to extracellular matrix components. These changes could induce increased proliferation and migration, which are critical for the spread of hyperplasia in the joints (Aidinis et al., 2003). Dispensable roles for RAG in arthritis have been observed in TNF^{ΔARE} mouse (Kontoyiannis et al., 1999) and DNaseII/− mouse (Kawane et al., 2010), indicating that synovial hyperplasia may develop independently of acquired immunity.
5. Fibroblast-like synoviocytes and mesenchymal stem cells

FLSs are characterized mainly by in vitro analyses. The synovial membranes are easily obtained by joint surgery. The cells liberated from the synovial tissues by treatment with collagenase can be cultured under the appropriate conditions. Although primary FLSs are useful, experiments must be carefully designed, because the composition of the cells in the culture changes after 4 passages, when contaminated hematopoietic cells disappear (Zimmermann et al., 2001).

The source of pathogenic FLSs proliferating in the synovium of RA is an intriguing issue, and several possibilities can be considered. Growth of FLSs can be stimulated by adjacent hematopoietic cells in the microenvironment, or initiated by the acquisition of cell-intrinsic properties for unregulated growth. Alternatively, growing FLSs can be derived from resident FLSs in the normal synovial membrane, or migrated from other organs. The latter possibility was demonstrated by experiments to inject FLSs from human TNF-α transgenic mice into the knee joint, and to transplant human synovial fibroblasts into SCID mice (Aidinis et al., 2003; Lefevre et al., 2009).

Fibroblast-like cells that initiate growth during the very early stages of RA can originate from mature FLSs or from other mesenchymal cells at the primitive stage, such as mesenchymal stem cells (MSCs). The presence of MSCs in the synovium has been reported by several researchers. MSCs with the potential to differentiate into 3 lineages, osteogenic, adipogenic, and chondrogenic cells, were obtained from the synovial membrane following digestion with collagenase and more than 3 passages (De Bari et al., 2001). Fibroblast-like MSCs expressing fibroblast marker D7-FIB, but not CD45, were detected in the synovial fluid (Jones et al., 2004). The number of these MSCs was lower in the synovial fluid of RA than in that of OA. In addition to the synovium, MSCs have been derived from blood (Zvaifler et al., 2000), adipose tissue (Zuk et al., 2002), and the periosteal region (De Bari et al., 2006). Although not genuine MSCs, circulating CD14+ monocytes may contain progenitors with the potential to differentiate into mesenchymal cells (Kuwana et al., 2003).

In terms of the underlying mechanism for the transformation-like phenotype of FLSs, and the involvement of MSCs, Li & Makarov (2006) reported intriguing data from animal models of RA. Arthritic FLSs contained a substantial fraction of bone marrow-derived precursors, with the ability to differentiate in vitro into various mesenchymal cell types. However, inflammation prevented multilineage differentiation. The transcription factor NF-kB played a key role in repressing osteogenic and adipogenic differentiation of arthritic FLSs. On the other hand, specific activation of NF-kB profoundly enhanced proliferation, motility, and matrix-degrading activity of FLSs by the induction of MMPs. These data suggest an intriguing mechanism, namely, that arthritic FLSs are bone marrow-derived MSCs, which are arrested during the early stages of differentiation, by the activation of NF-kB induced by inflammatory cytokines (Li & Makarov, 2006).

6. Search for RA-related genes through the classification of fibroblast-like synoviocytes

6.1 Subtypes of fibroblast-like synoviocytes in RA

Kasperkovitz et al. (2005) reported that subtypes of FLSs in RA differ in their gene expression. Complementary DNA microarrays of the synovial tissues and cultured FLSs obtained from RA patients revealed that the gene expression profiles of high- and low-grade
inflammation synovial tissues were characterized by high and low expression of genes of immune-competent cells (T cells, B cells, and APCs), respectively. Furthermore, hierarchical clustering identified 2 groups of FLSs, characterized by distinctive gene expression profiles and correlation with the inflammatory profiles of the synovial tissues. The first group correlated with the high-grade inflammation tissue, and exhibited increased expression of a TGF-β/activin A-inducible gene profile, which is characteristic of myofibroblasts, a cell type involved in wound healing. The second group correlated with the low-grade inflammation tissue, and showed increased expression of the genes involved in autocrine growth regulation, cell transformation, complement activation, and oxidative stress. Reflecting the gene expression profile, an increased proportion of myofibroblast-like cells in the heterogeneous population of FLSs were immunohistochemically detected in the high-grade inflammation tissue. These data suggest that the inflammatory state of the synovium is determined by the composition of heterogeneous FLSs.

6.2 Transformed fibroblast-like synoviocyte lines reveal heterogeneity irrespective of arthritis types

The data of Kasperkovitz et al. (2005), Galligan et al. (2007) and others indicate that combining gene expression profiling with other parameters, such as clinical data or characteristics of FLS lines, constitutes a powerful tool for identifying novel disease-related genes. To identify the cell-intrinsic abnormalities of RA-FLSs, we established transformed cell lines from the synovium of RA or OA cases, by immortalization with SV40 large T Ag (unpublished data of Ishihara et al.). Characterization of FLSs from 2 types of arthritis revealed no significant differences in surface molecules, growth rates, patterns of tyrosine-phosphorylated proteins, or expression of the genes related to inflammation (IL-1β, IL-6, MMP-1, MMP-3, etc.). Since the expression levels of these genes vary (ranges exceeding 1,000-fold) among FLS lines from each type of arthritis, we tentatively categorized them into 2 subtypes reflecting resting (r) and active (a) stages, based on the expression levels of IL-1β and MMP-1. Next, we performed a micro DNA array to obtain the gene expression profiles for 4 representative cell lines, r-OA-FLS, a-OA-FLS, r-RA-FLS, and a-RA-FLS, and obtained 10 gene clusters. Although no disease-specific clusters were obtained, 2 reciprocal, stage-specific clusters were detected, suggesting the validity of our hypothesis for the presence of subtypes in FLSs. Using these data we are presently searching for 2 types of candidate genes; master genes that determine the states of FLSs, and genes that could play a role in the pathophysiology of RA by inference based on our current understanding of FLSs. In the following sections, we will review the potential roles of activation-induced cytidine deaminase (AID) (Igarashi et al., 2010) and the A20/ABIN family.

6.3 Ectopic expression of AID and acquisition of a tumor-like phenotype by fibroblast-like synoviocytes

6.3.1 P53 mutation in fibroblast-like synoviocytes of RA and AID expression in inflammation

In addition to the properties described above, the expression of the tumor-suppressor gene p53 with somatic mutations (Firestein et al., 1997; Inazuka et al., 2000; Kullmann et al., 1999; Reme et al., 1998; Yamanishi et al., 2002), and the down-regulation of the tumor suppressor PTEN, a protein phosphatase gene, have been demonstrated in RA-FLSs (Pap et al., 2000b).
In particular, the somatic mutation of the $p53$ gene appears consistent, not only in terms of increased resistance to apoptosis, but also with respect to pro-inflammatory responses such as production of IL-6 and MMP-1 (Han et al., 1999; Sun et al., 2004; Yamanishi et al., 2005). However, little is known about the mechanism by which the somatic mutations are introduced into the $p53$ gene in RA-FLSs.

AID is a member of the APOBEC family, which is a cellular cytidine deaminase involved in protection from retroviral infection or regulation of cholesterol metabolism (Goila-Gaur & Strebel, 2008). AID was originally identified as an indispensable molecule for somatic hypermutation at the immunoglobulin variable region, and also for class-switch recombination in germinal center B lymphocytes (Di Noia & Neuberger, 2007; Honjo et al., 2004). Recently, several investigators have demonstrated up-regulation of AID in non-lymphoid tumor cells such as breast cancer, cholangiocarcinoma, hepatoma, and colorectal cancer cells (Babbage et al., 2006; Chan-On et al., 2009; Endo et al., 2007, 2008; Komori et al., 2008; Kou et al., 2007; Morisawa et al., 2008). During the process of oncogenesis, NF-κB activation in inflammation is thought to be important for aberrant expression of AID. For example, the infection of gastric mucosal cells with *Helicobacter pylori*, or of hepatocytes with hepatitis C virus, activates NF-κB and successfully induces local production of pro-inflammatory cytokines such as TNF-α and IL-1β. Together, these secreted cytokines also activate NF-κB, and lead to the induction of AID. In fact, stimulation with TNF-α or IL-1β induces AID expression even in non-tumor hepatocyte or colon epithelial cells. Moreover, the somatic mutations of $p53$ found in these cancer cells appeared to be a direct target of AID (Endo et al., 2008; Kou et al., 2007; Takai et al., 2009).

RA is characterized by an environment rich in pro-inflammatory cytokines and the existence of mutations in the $p53$ gene. Thus, under chronic inflammatory circumstances, it is possible that aberrant expression of AID could introduce mutations into the $p53$ gene of FLSs.

### 6.3.2 Aberrant expression of AID in RA-FLSs

First, we assessed the expression of the AID gene in the transformed FLS cell lines described in 6.2, by real-time reverse transcription polymerase chain reaction (RT-PCR). AID was transcribed in more than half of the RA-FLS cell lines (5 out of 9) and in none of the OA-FLS cell lines. Quantitative assay by RT-PCR showed 7- to 18-fold higher AID transcription in the RA-FLS lines compared to the OA-FLS lines that expressed a low but detectable level of AID transcription. The possibility of contaminated signals from AID-expressing B cells was excluded by the absence of pan B cell marker transcription. The translation of AID was further confirmed by the detection of protein in the cell lysate from RA-FLSs, with western blot analysis.

Patients who provided AID-expressing FLSs showed a tendency toward higher levels (approximately 2.7 times) of CRP in the serum. Regarding gender, the number of female patients with AID⁺ FLSs was approximately 1.9 times higher than the number of male patients. Although our data are not statistically significant because of the small sample numbers used, it appears that AID expression in FLSs is facilitated under conditions of inflammation in female patients. Indeed, we observed that estrogen, a representative female hormone, or TNF-α, a representative pro-inflammatory cytokine, augmented the transcription of AID in AID⁺ RA-FLSs to more than 20-fold higher levels compared with the basal levels in OA-FLSs. These results are similar to those previously reported for other cells.
(Endo et al., 2007, 2008; Pauklin et al., 2009). The transcription levels of TNF-α, or the pro-inflammatory cytokines IL-6 and IL-1β, did not correlate with that of AID, suggesting that AID transcription is not induced by autocrine cytokines. No clear relationship was observed between aberrant expression of AID and other clinical parameters, such as age, serum MMP-3 levels, or medication.

6.3.3 Accumulation of p53 gene mutations in AID-expressing RA-FLSs

The mutations of the p53 tumor-suppressor gene frequently found in RA-FLSs could contribute to the tumor-like, and also the pro-inflammatory properties of RA-FLSs, such as aggressive growth, invasion, and destruction of cartilage and bone (Firestein et al., 1997; Inazuka et al., 2000; Kullmann et al., 1999; Reme et al., 1998; Sun et al., 2004; Yamanishi et al., 2002). Although genotoxic and oxidative stresses have been speculated to be causative candidates for the somatic mutation in the p53 gene in RA-FLSs, the molecular mechanism has not yet been elucidated. As mentioned in 6.3.1, a clear relationship between AID expression and the frequency of p53 somatic mutations has been demonstrated in some non-B lymphocytes, such as hepatocytes and colon epithelial cells (Chan-On et al., 2009; Endo et al., 2008; Komori et al., 2008; Kou et al., 2007; Morisawa et al., 2008). Thus, we speculated that aberrant expression of AID might be involved in the introduction of the p53 gene mutation. We amplified the coding region of p53 from 3 AID+ RA-FLS cell lines with high-fidelity polymerase. We then determined the nucleotide sequence corresponding to that region and compared it with the intact p53 gene sequence. AID+ RA-FLSs harbored approximately 2- to 3.5-fold more mutations than the control RA-FLS subsets, which expressed AID at a lower level. In addition, the frequency of non-silent mutations was 3 times more than that of silent mutations. Notably, the base substitution pattern in p53 was biased toward the transition type, which is typical for AID-mediated mutations at the variable region of the immunoglobulin gene (Di Noia & Neuberger, 2007). The mutations were distributed intensively at the DNA-binding domain of the p53 gene, where the hotspot of somatic mutations is found in some malignant tumors. The Arg248 mutation, one of the cancer hotspot mutations (Ko & Prives, 1996), was found in p53 from our AID+ RA-FLSs. In addition, among the amino acid mutations that we identified, 17% were identical to those previously reported. A further 33% were distinct amino acid mutations; however, the positions of base change were located in the same codons as previously reported. The apparent correlation between ectopic expression of AID and increased frequency of somatic mutations of p53 strongly suggests that AID may be involved in the introduction of mutations to p53. Such mutations could lead to reductions or increases in the function of p53, which in turn may result in the tumor-like or anti-apoptotic phenotypes of FLSs in RA.

6.3.4 AID is produced by non-transformed RA-FLSs and in the RA synovium outside the B-cell follicles

The aberrant expression of AID in some RA-FLS transformed cell lines is not caused by the effects of transformation with SV40 large T Ag. Indeed, 3 to 8 times higher transcription levels of AID were observed, even in non-transformed primary FLS cell lines (4 out of 11 RA-FLSs, but none of the 6 OA-FLSs). In addition, cyto-staining with anti-AID antibody revealed a positive signal in AID-expressing primary RA-FLSs. Furthermore, dual-color immunohistostaining of the synovial sections from AID+ RA patients clearly demonstrated
the production of AID by FLSs in the RA synovial tissues (Figure 1), providing definitive evidence for the occurrence of ectopic and aberrant expression of AID in RA.

![Immunofluorescence staining of AID on synovial tissue sections from a representative RA patient. Sections were stained simultaneously with rat mAb for AID and anti-CD20 (B-cell marker) mAb. AID was visualized with alexa 488 fluoro-dye conjugated anti-rat secondary Ab (green); CD20 was visualized with alexa 594 fluoro-dye conjugated anti-mouse secondary Ab (red). The nucleus was stained with 4',6-diamino-2-phenylindole (blue). Scale bar is 100 µm.](image)

We concluded that AID is selectively expressed by a proportion of RA-FLSs and that its expression is associated with an increased frequency of somatic mutations in p53 (Igarashi et al., 2010). Thus, it is possible that the aberrant expression of AID within certain RA-FLSs induces somatic mutations in p53, leading to the acquisition of pro-inflammatory or tumour-like phenotypes.

6.4 Heterogeneous responsiveness of fibroblast-like synoviocytes to TNF-α

6.4.1 RA-FLS cell lines differentially respond to TNF-α

The chronic inflammation circuit in the joints of RA is initiated by the production of inflammatory cytokines by FLSs, following stimulation with TNF-α secreted from the surrounding inflammatory cells. In this context, the TNF-α/NF-κB pathway plays an essential role in the transcription of pro-inflammatory cytokines. However, the regulation of NF-κB activity downstream of TNF-α in FLSs is not fully understood. To investigate the heterogeneous responsiveness of RA-FLS cell lines to TNF-α stimulation, we examined the panels of primary RA-FLS cell lines for their induction levels of pro-inflammatory cytokines following TNF-α stimulation. Interestingly, RA-FLS cell lines can be clearly categorized into 2 types based on the responsiveness to TNF-α, namely, whether the transcription levels of pro-inflammatory cytokine gene are high (designated as the high-responder group) or not (designated as the low-responder group). This facilitated production of pro-inflammatory cytokines can be explained by the significant elevation of NF-κB activity in the high-responder FLS lines compared with that in the low-responder lines.
6.4.2 Possible positive effect of A20/ABINs on pro-inflammatory cytokine induction
A20, also termed TNFAIP3 (TNFα-induced protein 3), was originally identified as an inducible zinc finger protein in human umbilical vein endothelial cell lines following stimulation with TNF-α. A20 has dual enzymatic activities, namely, ubiquitination and deubiquitination (Dixit et al., 1990). The induction of A20 upon stimulation with TNF-α is NF-κB dependent; moreover, induced A20 reversely suppresses the activation of NF-κB through the regulation of ubiquitin-mediated degradation of NF-κB activator (Vereecke et al., 2009). This negative feedback loop is thought to be necessary to terminate inflammation and protect tissues from unnecessary damage. Recently, it was reported that the expression level of A20 in RA-FLSs was lower than that in OA-FLSs (Elsby et al., 2010). Although the difference was not significant, this finding could provide in vitro evidence of altered A20 transcription by 6q23 intergenic SNPs associated with RA (Dieguez-Gonzalez et al., 2009; Orozco et al., 2009). Thus, we speculated that the down-regulation of NF-κB inhibitors might be a possible mechanism for enhanced activation of NF-κB in high-responder FLSs. Contrary to our speculation, the high-responder group with abundant mRNA levels of pro-inflammatory cytokines also exhibited marked induction of A20 following stimulation with TNF-α. Furthermore, the transcription of the NF-κB inhibitory molecules ABIN (A20-binding inhibitor of NF-κB activation, also called TNIP, TNFAIP3 interacting protein)-1 and ABIN-3, but not of ABIN-2, was increased (Igarashi et al., in press).

These observations indicate that there is heterogeneity of RA-FLSs in the responsiveness to TNF-α stimulation and suggest that these “inhibitors” might not play negative regulatory roles in RA-FLS. The precise mechanism, cell-lineage specificity, disease specificity, and significance in cell biology of this unexpected possible positive role for A20/ABINs are currently under investigation.

7. Conclusion
Anti-cytokine therapy for RA is a prominent achievement in the field of autoimmune diseases. Accumulated evidence from clinical and basic medical research indicates pivotal roles for FLS in the pathogenesis and pathophysiology of RA. Data from genome-wide screening, transcriptional profiling, and animal models indicate that RA consists with heterogeneous disease subsets. Together with several other researchers, we have presented evidence for heterogeneity in FLS. Based on this finding, we have successfully searched for disease-related genes by subtyping FLS. We have identified 2 groups of genes, AID and A20/ABINs. AID is involved in the irreversible transformation of FLS, whereas A20/ABINs participate in the reversible, but potentially harmful, responsiveness of them. Both groups of genes are constituent elements for distinct levels of heterogeneity in FLS, which may be involved in resistance to anti-cytokine therapies. Subtyping of FLS based on expression of AID did not coincide with that based on responsiveness to signal-utilizing NF-κB, which is reasonable because RA is a multi-factorial disease. We believe that our approach to categorizing subsets of FLS based on differential gene expression, or on responsiveness to inflammatory stimuli, will facilitate a comprehensive understanding of the pathogenesis and pathophysiology of RA.

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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 16 chapters, with contributions from numerous countries (e.g. UK, USA, Japan, Sweden, Spain, Ireland, Poland, Norway), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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