very similar capacity to interact and support the maturations of some population of MNCs were reported for FSCs of human skin (Iwagami S et al, 1994). Such cells we found in FSCs of bone marrow (12,14) and synovial tissue (13,14) of RA patients, and analysed the nurse like cell (NLC) functions contributing to the pathophysiology of RA (14).

Wekerle H, Ketelsen UP. 1980. Nature. 283: 402
Wekerle H, et al. 1980. J Exp Med. 925
Iwagami S, et al, 1994. J Immunol. 153: 2927

Fig 19. RA NLC induce pseudoemperipolesis of B and T lymphoma cells and bind peripheral B cells. The morphologic appearance of RA. NLC34syn is shown by phase-contrast microscopy when cultured alone (a), with normal peripheral B cells for 4 d (b), or with the T cell lymphoma line MOLT-17 (c), or the B cell lymphoma line , MC/car 8d). (x200)

V. Nurse- like cell functions observed by co-culture with MNCs

1) Pseudoemperipolesis observed in RA- NLC

To assess the pseudoemperipolesis, we cultured NLCs for 2 days and added MNCs for study (**Fig 20**). After coculture for 6 hrs, we counted the FSCs showing pseudoemperipolesis (**Fig 20b**), and assessed as the % to total FSCs in the field.

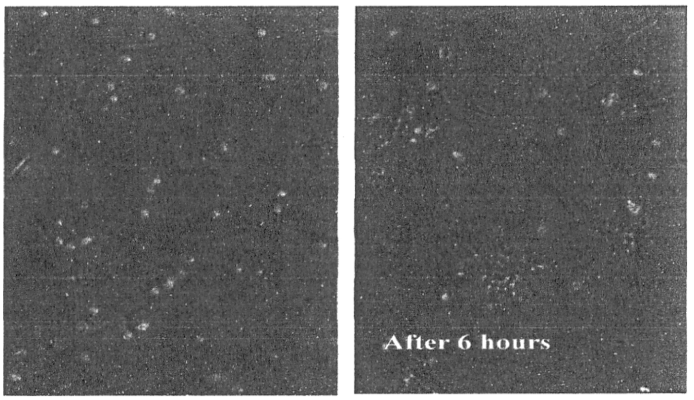


Fig.20a Fig20b

Figure 20. NLCs just added (left) and after 6 hrs (right) of coculturewith B cells

As shown in Table 3, pseudoemperipolesis was observed after 6 hrs in about half of FSCs co-cultured with MC/car and about a quarter of FSCs co-cultured with MOLT17. Such functions were observed in not only NLCs derived from the bone marrow, but also of those derived from the synovial tissue, although the ratios in OA were much less than RA.

Table 3. Pseudoemperipolesis observed in FSCs from synovial tissue and bone marrow cocultured with MC/car or MOLT17.

	Pseudoemperipolesis (%)	
Cell line	MC/car	MOLT17
IT-79MTNC3	50	26
RA		
SC1	51	28
SC2	48	22
BMC1	35	25
BMC2	40	21
OA		
SC1	2	2
SC2	3	0
SC5	14	8
HD		
SC1	4	0
SC2	2	1
BMC1	3	1
BMC2	6	2

From bone marrow (36) and synovial tissue (35) of RA patients, we established rheumatoid arthritis NLC (RA-NLC) clones having ability to promote pseudoemperipolesis. Those RA-NLC lines were determined to be of mesenchymal origin, given that they expressed vimentin but not cytokeratin. They did not exhibit desmosomes or classical junctional complexes, both of which were characteristic features of epithelial cells. Elongated and branching mitochondria were present in the cytoplasm of clones, and caveolae, which were unique to cells of mesenchymal origin,

were present on the surface (12,13).

NLCs were subsequently reported in patients with chronic lymphocytic leukemia (CLL) as well as RA patients. A subset of peripheral blood MNCs from patients with CLL were reported (Tsukada N et al. 2002) to differentiate into NLCs, which failed to differentiate from blood MNCs depleted of CD14(+) cells. They strongly suggested NLCs differentiated from CD14(+) cells. Various studies have been done to differentiate RA-NLC from CD14+ cells (23, 28, 38) or CD34+ cells (39, 44, 47, 50, 54), but not yet succeeded.

Burger JA et al, Blood 2000; 96: 2655
Burger JA et al. JCI 2001; 107: 305
Tsukada N et al, Blood 2002; 99:1030

2) Surface phenotypes of RA-NLC

Phenotypes of RA-NLC clones from bone marrow and synovial tissue were analyzed and identified (33,35,36) to express CD29, CD44, CD49c, CD54, CD106, and HLA-A, B, C (class I MHC) constitutively, but did not express CD1a, CD18 (LFA-1), CD35, CD40,, CD40L, and CD56. These surface phenotypes of RA- NLCs were similar to those of stromal cell lines derived from synovial cells and bone marrow cells from non-RA controls. Those of OA patients and human skin nurse cells, respectively were similar to those of RA-NLCs. RA-NLC constitutively expressed both CD106 and CD157 (BST-1), which were markedly enhanced by IFN γ . These seemed to be characteristic appearance of RA NLC lines, permitting them distinguished from fibroblasts (33).

3) B cell reactions co-cultured with RA-NLCs

i) Inhibition of spontaneous apoptosis of lymphocytes by RA-NLC

To examine the role of RA-NLC in promoting lymphocyte viability, co-culture experiments were carried out (Fig 20). Although peripheral blood B cells cultured in medium alone rapidly died, culture of B cells with RA-NLC markedly increased their viability. The loss of viability of B cells cultured alone related to the induction of apoptosis, whereas co-culture of B cells with RA-NLC substantially blocked their apoptosis. The mechanism of the prevention of apoptosis of B cells involved the contact dependent up-regulation of Bcl-x_L by RA NLCs (40).

Fig 21

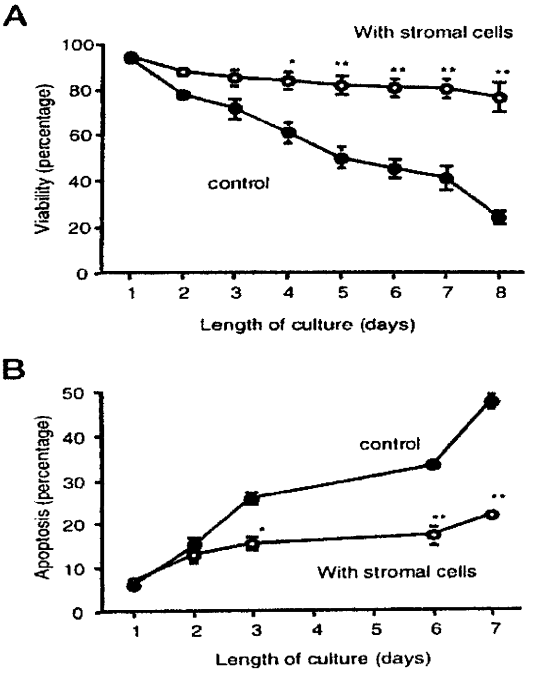


Figure 21. Peripheral B cells ($1x10^5$) were cultured with or without RA-NLC (Sy77; 4x103) in 96 well microtiter plates. After various length of culture, the ratio (%) of viable cells (A) and apoptotic cells (B) were determined. assessed (40).

ii) Cytokine production of NLC and the effect of adhesion molecules

Cytokine production by RA-NLC was studied (33,35,36). As shown in **Table 4**, RA-NLCs themselves from both bone marrow and synovial tissue produced detectable levels of IL-6, IL-8, and GM-CSF, and the production of IL-6 and IL-8 was quite large. After coculture with B cells, the levels of IL-6, IL-8, G-CSF, GM-CSF, and the levels of IgM were increased, and IL-1 β and TNF-α were detected. Direct contact with the B cell clone was required for RA-NLCs to produce IL-1 β and TNF-α and higher levels of the other cytokines.

Table 4

Cytokine production from RA-SNCs and Ig production from B cells in coculture.

Condition	Cytokines in cell culture supernatant, pg/ml †								IgM μg/ml †	
	IL-1α	IL-1β	IL-6	IL-8	G-CSF	GM-CSF	TNFα	TNFβ	Exp.2	Exp.3
RA-SNC	<5.0	<10.0	2,200	4,300	460	40	<5.0	<5.0	<1.5	<1.5
B cell	<5.0	<10.0	<10.0	<10.0	<10.0	<2.5	<5.0	<5.0	1.8	2.7
B cell + RA-SNC(separated) ‡	<5.0	<10.0	1,800	3,900	510	30	<5.0	<5.0	<1.5	<1.5
B cell + RA-SNC	<5.0	153	15,900	34,500	2,400	740	690	<5.0	5.6	8.6

* Bcell clones (1×10^5) and RA-SNC3 (5×10^4) were cultured under the indicated conditions for 3 days in 24-well plates.

† The amount of each cytokine and IgM in the culture supernatant was measured with an enzyme-linked immunosorbent assay kit.

‡ B cell clones were cultured in a Millicell culture insert.

The effects of various inhibitory factors on RA-NLC relating to pseudoemperipolesis (adhesion and transmigration) were examined using MC/car cells and a cloned RA-NLC line (RA-SNC77) (30). Treatment with anti-CD29 (integrin β1 chain) or anti- CD49d (integrin α4 chain) reduced adhesion by MC/car cells at approximately 50%. This result indicated that integrin α4β1 (VLA-4) on MC/car cells was involved, at least in part, in their ability to participate in pseudoemperipolesis with RA- SNL77 cells. Pretreatment of MC/car cells with the Rho-specific inhibitor C3 transferase significantly inhibited the transmigration of MC/car cells underneath RA-SNC77 cells in a dose-dependent manner, whereas the same treatment did not inhibit the adhesion of the MC/car cells at all. RA-SNC77 cells produced comparable levels of IL-6 and IL-8 when co-cultured with either untreated MC/car cells or C3 treated transmigration-defective MC/car cells. This suggested that adhesion and transmigration were thought to be independent reactions (46).

Other substances modifying the functions of FSCs in RA were thought to have enough possibilities for anti-RA drugs. Polyarthritis in CIA mice was reduced by gene trasfer of adiponectin (61), whose serum concentrations correlated with severity of RA (62). Interactions of FSCs with B cells in RA were specifically modified by osteopontin (63).

iii) NLC function relating to autoantibody production

We established RA-NLC- dependent B cell lines and clones from B cells cocultured for months with RA-NLC. Clones predominantly expressed

members of VH3 family with no representation of the VH2 or VH4 family. The homology of VH regions of B cell clones ranged from 89.0 to 96.6%. B cell clones predominantly produced IgG, while a minority produced either IgA or IgM (53). About IgM RF production from B cells, CD14+ cell function was shown to enhance the autoantibody production (18).

To investigate the RA-specific autoantigen recognized by CD4+ T cells in RA joints, we established CD4+CD8-CD45RO+ T cell clones from synovia and synovial fluid of RA patients. These clones responded to HLA-DR-matched allogeneic RA synovial nurse cells. It was suggested that the common (auto)antigen recognized by CD4+ T cells was presented on the HLA-DR molecule on synovial NLCs. Reactivity of the RA T cell clones was observed in the solubilized antigen extracted from RA-NLCs. In non-RA controls, the reactivity was observed only in the presence of HLA-DR- matched monocytes (37).

VI. CD14+ cell differentiation co-culture with RA-NLC

1) NCD14+ cells differentiated from CD14+ monocytes

To examine the effects of RA-NLCs on the differentiation of monocytes, CD14+ cells isolated from peripheral blood were cultured with RA-NLCs. After 3 to 4 weeks, those cells (NCD14+ cells) showed abundant cytoplasm and an off-center nucleus, and over 97% of NCD14+ were tartarate- resistant acid phosphates (TRAP) positive (**Fig 22**). RA-NLC supported such NCD14+ cell differentiation of peripheral blood CD14+ cells not only from RA patients but also from normal control subjects (43).

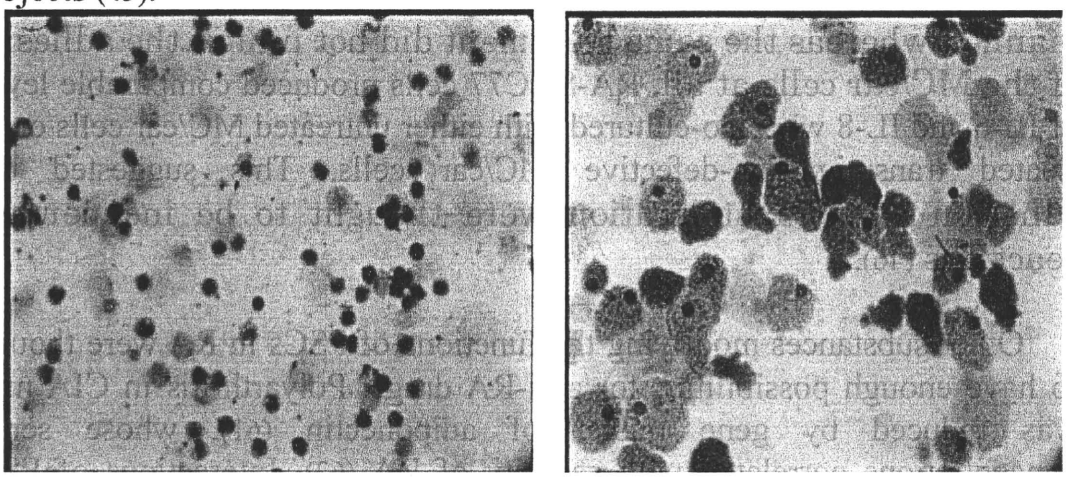


Fig 22. CD14+ cells cultured for 3 to 4 weeks with RA-NLCs (right) or without RA-NLCs (left).

2) RANKL independent differentiation of osteoclast-like cells

It was noteworthy that NCD14+ monocytes (**Fig 23a**) differentiated to TRAP+ multinucleated giant cells (MultiNCs) (**Fig 23b**) without RANKL (receptor activator of nuclear factor-κ β ligand) in the presence of IL-3, IL-5, IL-7, or GM-CSF (43). Such differentiation to MultiNCs was observed within 1 day of cultures, and inhibited by respective monoclonal antibody to each cytokine. Subsequently, LIGHT, a member of the tumor necrosis factor (TNF) superfamily, was also found to induce the differentiation of NCD14+ cells to TRAP+ MultiNC, but not of freshly isolated CD14+ monocytes (58,60).

These MultiNCs showed remarkable bone-resorbing activity similar to osteoclasts by culturing on dentine slices or osteologic disks (43). NCD14+ cells as well as multinucleated cells (MultiNCs) in RA synovial tissue at the bone-cartilage interface showed strong expression of MMP-2 and MMP-9, and was thought to induce joint degeneration in vitro (48). NLCs , on the other hand, cocultured with CD14+ cells produced high levels of IL-6 and IL-8 (51), just as cocultured with B cells.

These NCD14+ cells and osteoclast-like MultiNCs were found both in synovial fluid (49) and in synovial tissue (60) at characteristically high ratio in RA, and the numbers of nuclei and the area absorbed on detine slice were remarkable in severe disease subsets of RA.

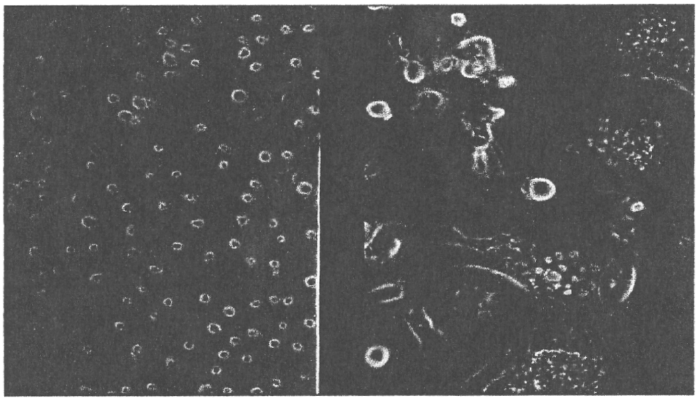


Fig.23a Fig23b

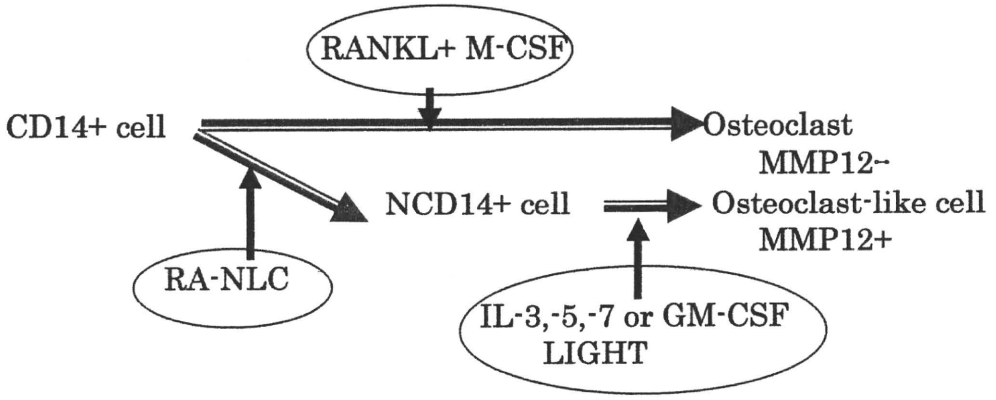
Figure 23. NCD14+ monocytes (Fig 23a) differentiated to multinucleated giant cells (MultiNCs) (Fig 23b) without RANKL

3) Two Different differentiation pathways to multinucleated bone resorbing giant cells

Between fresh CD14+ cells and NCD14+ cells, we compared the abilities to differentiate into TRAP+ MultiNCs in response to M-CSF plus RANKL (52). In fresh CD14+ cultures MultiNCs were induced in 7 days, although in NCD14+ cultures a longer culture period (7- 14 days) was

required (52). We also compared the same abilities between 2 kinds of CD14+ cells in response to LIGHT (60). Fresh CD14+ or NCD14+ cells were cultured for 6 days with RANKL and/or LIGHT in the presence of M-CSF. CD14+ monocytes were differentiated into TRAP+ MultiNCs with RANKL, but not with LIGHT. Conversly, NCD14 monocytes were strongly differentiated into TRAP positive MultiNCs when treated with LIGHT. The combination of RANKL and LIGHT had little effect on MultiNC formation. Process of differentiation to MultiNCs were thought to be different between freshly isolated CD14+ cell and NCD14+ cell (**Fig 24**).

Figure 24. Differentiation of CD14+ cells to osteoclast or osteoclast-like cells

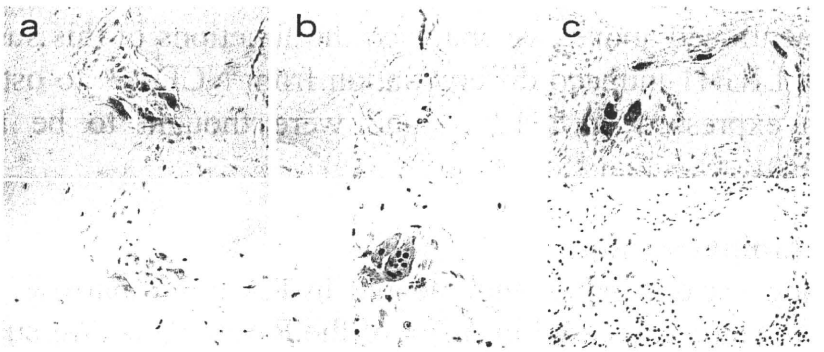


The MultiNCs differentiated from NCD14+ monocytes showed phenotypical and functional characteristics of osteoclasts. They showed the expression of osteoclast markers such as cathepsin K, actin- ring formation, and the ability to resorb bone. Those osteoclast-like cells expressed MMP-2, MMP-9, and MMP-12, but RANKL-induced osteoclast-like cells expressed MMP-2, MMP-9 as well as other osteoclast markers, but not MMP-12 (60).

As we suspected the possibility that those two different multinucleated giant cells could be discriminated in vivo by the expression of MMP-12, we observed the erosive bone area of RA patients comparing with those of OA patients. As shown in **Fig 25**, both MMP 12+ and MMP 12- MultiNCs expressing TRAP+ were present in the affected bone areas all RA patients examined. The ratios of MMP 12+ TRAP+ MultiNCs to whole TRAP+ MultiNCs in RA patients were between 52.5 and 2.2%. By contrasts, no MMP 12+ MultiNCs were observed in OA patients. These MMP12+ osteoclast-like MultiNCs were thought to be differentiated from the RA-specific pathways, and have effects on bone resorbtion in addition to the MMP12- osteoclasts.

Fig 25;

Expression of TRAP and MMP12 in the bone-resorbing area of RA patients
(Yamane et al)



Such additive number of osteoclasts (29) and/or osteoclast-like cells were thought to induce severe osteoporosis in RA patients, which were also observed in iliac bone (**Fig 26**) as well as well-known spine and femoral neck..

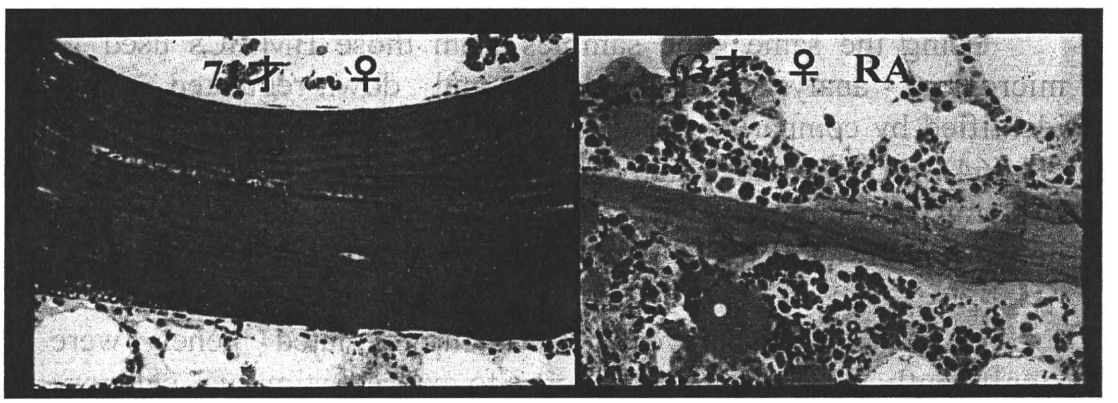


Fig 26 Trabecular bone in iliac bone of non-RA (left; 71 yrs old lady) and RA (right; 63 yrs old lady). Histological specimen was made by Dr T Ito.

Ⅶ. RA specific gene expressions in bone marrow of RA patients

As we had enough evidences suggesting that bone marrow to be the important pathophysiological site inducing RA, we analyzed genes of MNCs from iliac bone marrow (BMMCs) of RA. Nine patients with RA and 10 patients with OA were enrolled in the study. Total RNA were extracted from those BMMCs and used for DNA microarray analysis Gene expression profiles (GEPs).

1) Augmented gene expression in the immune response

We have comprehensively identified the genes whose expression was augmented in BMMC from RA patients as compared with BMMC from OA patients We identified RA-associated genes in bone marrow cells using a cDNA subtraction technique (55), and selected several genes such as LIGHT (58,60), AREG (59) and others which were somehow related to

immune responses, and increased in many of RA patients.

i)Light (TNFRSF3);

As mentioned above, we analyzed the functions of this substance and found that LIGHT-induced differentiation from NCD14+ to osteoclast-like MultiNCs expressed MMP12+, and were thought to be RA-specific MultiNC (60).

ii)AREG (amphiregulin);

Among various genes augumented in RA bone marrow, AREG was most significantly increased in many of the RA patients. We subjected it to further analysis and found that AREG-epidermal growth factor receptor signaling was highly activated in synovial cells isolated from RA patients, but not in OA synoviocytes (59). We have been further analyzing this especially in relation to synoviolin (56).

2) Abnormal networks of immune response in bone marrow of RA

Using the same gene samples from those BMMCs used for DNA microarray analysis, up-regulated and down-regulated genes were identified by comparing the gene expression profiles of RA with those of OA (64). Bioinformatics analysis was performed using Expression Analysis Systemic Explorer (EASE) 2.0 based on gene ontology followed by network pathway analysis with Ingenuity Pathways Analysis (IPA) 7.5.

EASE analysis revealed that up-regulated genes were most significantly classified into "response to external stimulus" category, which included functional category of "immune response". Abnormality in "Cell Organization and Biogenesis", and "Signal Transduction"were also overrepresented. Down-regulated genes were dominantly classified into "Cell Proliferation", which included "Mitotic Cell Cycle", "DNA Replication and Chromosome cycle", and "DNA metabolism". Most of the genes in these three functional categories were overlapped with each other.

Following IPA analyses, 4 networks were represented by the 61 up-regulated genes. The first network that with interferon (IFN) α, IFN β, and mHC class I (family) etc. at the center included 5 IFN-inducible molecules, a cluster of human leukocyte antigen (HLA), i.e. HLA-E, HLA-F, HLA-G, and tapasin (TAP) etc. This network was relevant to antigen presentation, cell-mediated immune response, and humoral immune response. The remaining 3 networks were also related to cell-mediated immune response.

Abnormal functional networks of "immune response" and "cell cycle" were identified in BMMCs of RA compared to OA. IFN signaling as well as antigen presentation pathway were found activated in BM of RA patients. These results showed bone marrow to contribute to the pathogenesis of RA.

Ⅷ. Natural course of joint destruction of RA

In studying the pathophysiological changes of BMMCs in RA patients as mentioned above, we noticed that those cellular changes related to the natural course of RA which we reported as below in 1998 (3).

Fig 27

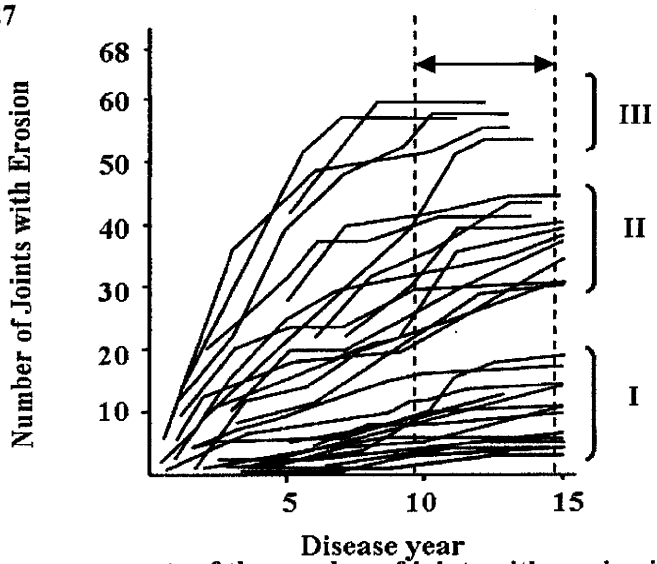


Fig 27. Serial measurements of the number of joints with erosion in 30 typical cases examined. The courses of erosion after 10 to 15 years of RA fell into 3 groups (Ⅰ , Ⅱ , Ⅲ).

We assessed the yearly changes of the number of joints with erosion (NJE) in a total of 68 joints in RA patients whose serial radiological records for over 10 years were available. The natural courses of joint destruction in RA patients separated into 3 groups according to NJE present at 10 years or more of RA (Figure 27). Group I was the subset with least erosive disease (LES), group II the subset with more erosive disease (MES), and group III the subset with mutilating disease (MUD).

In group I, the NJE of patients increased gradually during the first 5 years of RA, did not increase rapidly thereafter, and the total NJE < 20 at 15 years of RA. In group III, the most severely involved group, the NJE increased rapidly up to approximately 50 or more during the first 10 years of RA. In group II, the NJEs were more than those of group I, but less than those of group III. Their NJEs often continued to increase even after 15 years up to the similar levels of group III. At 10- 15 years of RA, the mean

number of joints with erosion was 10.9 in LES, 32.2 in MES, and 53.5 in MUD. Finally at 20 years or more of RA, RA patients were classified to the mild disease (group I) and the severe disease (group II and III) based on NJEs.

Even in the early disease years they were highly significant in the rapidity of carpal bone destruction as assessed by the yearly reduction of carpal height ratio ($P<0.001$) (3), and in the serum C1q level using polyclonal anti-C1q antibody ($P<0.001$) (2, 3).

Their clinical aspects were as follows. In LES, erosive articular changes were primarily limited to the peripheral smaller joints, and their abilities of daily lives (ADLs) were only modestly limited. In MES, the larger axial joints were also gradually involved as well as the peripheral smaller joints, and their ADLs were getting severely involved. In MUD, almost all joints were damaged during 10 years of their diseases, and their ADLs were severely limited. Differences of extension of their joint destructions between the 3 groups were getting apparent year by year, and clearly different after 10 years of RA.

Clinical and pathological findings and the effects of various therapies were recognized to have close relation with these 3 disease subsets of RA (20, 21, 26, 27, 32, 34, 41). It was amazing for us that the pathophysioligical changes of bone marrow cells mentioned above showed close relation with the clinical courses of RA patients.

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関節リウマチの病因に迫る：

腸骨骨髄の場で、

ナース様細胞と CD14(+)細胞を焦点にあてて。

厚生労働科学研究 報告書
（平成 22 年度まで）

関節リウマチ骨髄血中の疾患誘導因子解明と根治療法開発研究班
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サマリー

現在も多くの未解明部分を残す関節リウマチ(RA)の病因・病態に関して、我々は従来の免疫亢進機序解明研究から視点を変えて、RAによる関節破壊機序解明を主課題に研究を進めた。造血組織である腸骨骨髄での白血球全体の機能亢進の中で特にCD14(+)細胞（単球・マクロファージ系細胞）を焦点に当ててRA病態解明研究を進め、病因にも迫ってきた。多くの研究結果から、我々はRAの最重要病巣は腸骨骨髄と考えるに到った。主病巣と考えられてきた関節滑膜は局所病態の重症化（増悪）に重要な役割を担うが、腸骨骨髄の異常活性化に引き続いて造られる（二次的な）局所病巣と考えている。

RA患者の腸骨骨髄では、白血球細胞が一様に増殖・活性化し病的細胞が造られている。病的細胞は末梢血を経て関節部骨髄にも集まり、関節内に入り滑膜病巣を形成する。例えば異常なCD14(+)CD15(+)骨髄球系細胞を追跡すると、腸骨骨髄内で分化・形成されて末梢の関節部骨髄に現れていた。活性因子も腸骨骨髄中には関節内より著しい亢進を認めた。我々がRA病巣に見出した“ナース様細胞”はRAの免疫機能亢進や骨・軟骨破壊機能亢進を導く白血球系細胞個々の維持・活性化の鍵をにぎる細胞であり、RAの腸骨骨髄で造られ関節部骨髄を経て滑膜組織に加わるようだ。多様な滑膜細胞機能を説明できる。このようなRA腸骨骨髄病態の重要性を示す研究結果に加えて、RA骨髄細胞遺伝子の帰納法的解析結果から免疫亢進は腸骨骨髄からの発進らしいという研究結果が示された。「RAの原因病巣は腸骨骨髄にある」という仮説がより強くなった。

RAによる骨・軟骨破壊の面から考えるときに、CD14(+)細胞が根幹的な“悪玉細胞”のようだ。通常の骨代謝として骨吸収に働く破骨細胞と別個に、RA特異的に分化・機能して高度な骨吸収を導く“破骨細胞様細胞”を見出した。これはRAのナース様細胞との接触によってCD14(+)細胞から誘導される細胞で高度な骨破壊や骨粗鬆症の原因になっている。本報告書の最終項に記載した臨床的研究、RA患者の全身関節破壊の長期経過観察から判別される重症RAにおける重篤な骨・関節破壊機序が本研究で説明できる。CD14(+)細胞は更にT細胞やB細胞のつくる微小環境に影響を受けながら、それらの細胞に働き自己抗体産生やリウマチ因子産生などのRA特異的な免疫反応亢進を誘導することも分かった。CD14(+)細胞を“悪玉細胞”に誘導する“ナース様細胞”はRAだけでなく白血病患者の血中にも見出されたと米国の研究グループにより報告された。RAを血液疾患と同じ場（腸骨骨髄）の疾患として広い視野で考えるべきことを教えられた。彼らは“ナース様細胞”の出現にはCD14(+)細胞が関連すると発表し研究を進めているが、これはRAの病因に直結するものとして我々も解明に迫っている。

2010年11月　　主任研究者　越智隆弘

目　次