

Study of P53 in Peripheral Blood and Synovial Mononuclear Cells of Rheumatoid Arthritis and Osteoarthritis Patients and Its Relation to The Degree of Disease Activity

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Over expression of P53 has been described in many inflammatory conditions including rheumatoid arthritis (RA) and osteoarthritis (OA) as a protective mechanism to induce apoptosis of synovial cells. Lack of P53 function through mutation in human synoviocytes increases the development of normal synovial fibroblasts into transformed aggressive synovial fibroblasts. P53 levels were determined in supernatant of cultured mononuclear cells (MCs) isolated from peripheral blood (PBMCs) of patients with RA (n=10) and OA (n=10) as well as 10 normal healthy controls (C). P53 levels were also determined in supernatants of MCs isolated from synovial fluid (SFMCs) of RA and OA patients. Results of this work revealed that P53 level was significantly higher in PBMCs supernatant of RA group than those of both (C) and (OA) groups (P = 0.022). P53 level was non-significantly higher in SFMCs supernatant of RA than OA group. Significantly higher levels of P53 was detected in SFMCs culture supernatant than that of PBMCs within each RA (P = 0.003) and OA (P = 0.001) group. Results also showed a significantly positive correlation between P53 levels (in both PBMCs and SFMCs) and the disease activity score (DAS) in RA group (P= 0.01, P = 0.02 respectively) while insignificantly positive correlations between P53 level (in both PBMCs and SFMCs) and radiological grading of OA group were obtained. These results indicate that mutations and consequent dysfunction of P53 gene may result in chronic inflammation and hyperplasia in RA patients. In conclusion, gene therapy targeting P53-dependent pathway could be a promising therapy for RA and OA diseases.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which is mainly characterized by synovial hyperplasia, angiogenesis, pathological immune phenomenon and progressive destruction of the affected joint (El Hallous et al., 1997 ; Franz et al., 1998). Various cell types are involved in the pathogenesis of RA including T lymphocytes, antigen presenting cells, and endothelial cells. RA synovial fibroblasts are able to destroy articular cartilage independent of inflammation (Seemayer et al., 2001).

Osteoarthritis (OA) is an insidious, slowly advancing articular disease resulting in progressive softening, ulceration and focal disintegration of the articular cartilage, osteophyte formation, subchondral bone collapse and sclerosis, which produce variable clinical disability of the affected joint (Mankin et al., 1989). Both RA and OA

exhibit active angiogenesis and up-regulation of angiogenic factors in the synovium (Giatromanolaki et al., 2001).

Apoptosis is a key mechanism that regulate tissue composition and homeostasis. Apoptosis of synovial cells shows alterations described in residential synoviocytes as well as inflammatory cells and is associated with the pathogenesis of RA. These changes contribute hallmarks of synovial cell activation and contribute to both chronic inflammation and hyperplasia (Bair et al., 2003). The degree of chondrocyte apoptosis is closely related to cartilage destruction and that chondrocytes in RA more readily undergo apoptosis than those in OA (Yatsugi et al., 2000). Apoptosis in RA is strongly associated with expression of the tumor suppressor gene P53 (Pozza et al., 2000).

P53 is mapped to chromosome 17. In the cell, P53 protein binds DNA, which in turn stimulates another gene to produce a protein called P21 that interacts with a cell division-stimulating protein (cd K₂). When P21 is complexed with cdK₂, the cell cannot pass through to the next stage of cell division. Mutant P53 does not bind DNA effectively, so the cell division goes without control (Schmitt et al., 1999 and Chou et al., 2001). Such mutation is suggested by Yamanashi et al. (2002) to be found in rheumatoid synovium perhaps a consequence of reactive oxygen and nitric oxide being produced during inflammation.

The dysfunction of P53 may play a role in the proliferation of synovial tissues in RA (Inazuka et al., 2000). P53 is suggested by Sun et al. (2002) and Toubert et al. (2000) to be expressed in RA fibroblast-like synoviocytes (FLSs), and its over expression is a characteristic feature of RA. Accordingly, we aimed in this study to investigate the possible role of this gene among rheumatoid arthritis and osteoarthritis patients and its relation to the degree of disease activity. P53 suppressor gene concentration was quantitatively measured in culture supernatant of MCs isolated from peripheral blood and synovial fluid (SF) of both RA and OA patients.

Subjects and Methods

Subjects

The study was conducted on three groups of consenting human subjects:

Group 1 comprised 10 female patients with RA chosen according to the American College of Rheumatology (ACR) criteria and were all sero-positive for RF (Arnett et al., 1987).

The second group consisted of 10 female patients with OA. They had at least 2 or more clinical symptoms, had grade 2 or more radiological changes according to the following criteria (Won et al., 2000); females ranging in age from 45 to 65 years, primary knee osteoarthritis with criteria of i and ii being met, with pain and/or tenderness of involved joints.

Radiographic evaluation shows Grade 2 or higher according to the following:

Grade 0: No features of OA, Grade 1: Minute osteophytes, doubtful significance, Grade 2: Definite osteophytes, unimpaired joint space, Grade 3: Moderate diminution of joint space, Grade 4: Joint space greatly impaired with sclerosis of subchondral bone.

The third group consisted of ten age and sex matched normal healthy individuals as a group of control.

Methods

The study subjects were clinically examined with stress on pain, number of tender joints, number of swollen joints, manifestations of inflammation, limitation of motion of affected joint or the presence of deformity. C-reactive protein level was determined following Deodhar et al., 1989. For RA group; disease activity was measured using the following disease activity score DAS28 – 3(CRP) (Leeb et al., 2005).

$$\text{DAS28} - 3(\text{CRP}) = \{0.56 \times \text{SQRT}(\text{TJC } 28) + 0.28 \text{SQRT}(\text{SJC } 28) + 0.36 \times \ln(\text{CRP} + 1)\} \times 1.01 + 1.15$$

Where SQRT = square root, TJC = tender joint count, SWC = swollen joint count and CRP = C reactive protein.

Isolation of peripheral blood and synovial fluid mononuclear cells (PBMCs and SFMCs)

Heparinized blood samples were obtained from all subjects under study. Samples of synovial fluid were taken through aspiration from the knee joint of patients with RA and OA. Peripheral blood and synovial fluid mononuclear cells (PBMCs and SFMCs) were separated according to Perper et al. (1989) by density gradient centrifugation over Ficoll-Hypaque under aseptic conditions. The cells were adjusted at 1X10⁶ cell/ml, then incubated for 24 h at 5% CO₂ humid incubator at 37 °C. After incubation, the cell cultures were centrifuged at 1800 rpm for 10 minutes and the supernatants were harvested and stored at -70 °C until determination of P53 performed.

Determination of P53

P53 protein concentration was quantitatively determined in all samples of PBMCs and SFMCs culture supernatant using a solid phase standard P53 ELISA kit (Diaclone). A monoclonal antibody specific for P53 was coated onto the wells of the microtiter strip. Samples, including standards of known P53 concentrations (from 100 to 3.12 U/ml) and unknowns were pipetted into these wells. During the first incubation, the P53 antigen was added to wells. After washing, a biotinylated monoclonal antibody specific

for P53 was incubated. The enzyme was then added, after incubation and washing to remove all unbound enzyme, a substrate solution which acts on the bound enzyme was added to induce a coloured reaction product. The colour absorbance was read using an ELISA reader at 450 nm. The P53 concentration of each sample was determined by extrapolating optical density values to P53 concentrations using a standard curve generated by plotting the average absorbance on vertical axis versus the corresponding P53 standard concentration on the horizontal axis.

Statistical analysis

Results of the present work were statistically analyzed using SPSS version 10, P-value < 0.05 was considered significant. Also correlation between variables within each group was tested by Pearson's correlation coefficient.

Results

Age and duration of illness of RA and OA patients, clinical data of OA, and clinical data of RA were represented in tables 1, 2, 3 respectively.

Table 1. Age and duration of illness of patient groups:

	RA	OA
Age		
Range	50-64	49-65
Mean \pm S.D.	54.7 \pm 4.35	57.5 \pm 5.72
Duration		
Range	3-12	5-12
Mean \pm S.D.	7.1 \pm 3.25	7.7 \pm 2.41
*P		NS

RA; rheumatoid arthritis

OA; Osteoarthritis

P is significant at the level < 0.05.

Table 2. Clinical data of Osteoarthritis patients.

	Frequency	
	No.	%
Clinical symptoms *		
+	5	50.0
++	3	30.0
+++	2	20.0
Radiological grading**		
2	3	30.0
3	5	50.0
4	2	20.0

*Clinical Symptoms:

- 1) Pain in involved joints
 - 2) Tenderness of involved joints
 - 3) Limitation of motion
 - 4) Swelling of joints
- + indicate 2 symptoms
++ indicate 3 symptoms
+++ indicate 4 symptoms

**Radiological grading:

- Grade 0: No features of OA
Grade 1: Minute osteophyte, doubtful significance
Grade 2: Definite osteophyte, unimpaired joint space
Grade 3: Moderate diminution of joint space
Grade 4: Joint space greatly impaired with sclerosis of subchondral bone.

Table 3. Clinical data of Rheumatoid arthritis group.

Number of patients	*Pain	Morning stiffness	Deformity
1	Grade 1	About ¼ hour	Fusiform swelling
2	Grade III	More than 1 hour	Elbow flexion deformity, ulnar deviation, swan neck, z, deformities
3	Grade IV	More than 1 hour	Fusiform swelling, knee effusion, ulnar deviation, swan neck, boutonniere, deformities, temporo-mandibular affection.
4	Grade II	About ¼ hour	Ulnar deviation
5	Grade I	About ¼ hour	Swan neck deformities
6	Grade III	More than 1 hour	Atlanto-axial affection, Ulnar deviation, swan neck, z, fusiform deformities
7	Grade II	Around ½ hour	Fusiform swellings
8	Grade II	Around ½ hour	Synovial thickening at wrist
9	Grade 1	About ¼ hour	No deformity
10	Grade I	About ½ hour	No deformity

*Pain: grade 1: Patient said the joint is tender
 grade II: The patient express pain during examination
 grade III: The patient withdraw his joint on examination
 grade IV: The patient did not allow any one to touch his joint

Serum level of C-reactive protein in both RA and OA group was significantly higher than that of controls (Table 4).

The disease activity score (DAS) in RA group was represented in table 5.

Table 4. C-reactive protein levels in sera of the study subjects.

CRP	Control	RA patients	OA patients
Range	4-10	8-25	10-28
Mean ± S.D.	6.7 ± 1.89	13.9 ± 6.03	15.4 ± 5.68
*P		< 0.001	
LSD		Control # RA, OA	

RA; rheumatoid arthritis

OA; Osteoarthritis

P is significant at the level < 0.05.

Table 5. Disease Activity Score [DAS28 – 3(CRP)] in Rheumatoid arthritis group.

DAS28	TJC	SJC	CRP	DAS28-3 (CRP)
Range	1-10	1-7	8-25	3.15-5.0
Mean ± S.D.	5.3 ± 2.63	3.8 ± 1.99	13.9 ± 6.03	3.99 ± 0.68
P			0.001	
LSD			CRP # other gp.	

TJC= Tender joint count, SJC= swelling joint count, CRP= C-reactive protein

Start remission if DAS 28-3(CRP) < 2.6

Low disease activity if DAS 28-3(CRP) < 3.2

High disease activity if DAS 28-3(CRP) >5.1

*P is significant at the level < 0.05.

Results also revealed that P53 concentration in PBMCs culture supernatant was significantly increased ($P < 0.05$) in RA group (8.78 U/ml) than in both OA and control groups (6.69 and 6.1 U/ml) (Table 6).

Table 6. P53 concentration (U/ml) in supernatants of cultured PBMCs of Rheumatoid arthritis (RA), Osteoarthritis (OA) and control (C) groups.

	RA	OA	C
Mean \pm S.D	8.781 \pm 2.531	6.693 \pm 2.430	6.150 \pm 1.636
*P		0.05	
LSD		RA # OA, C	

*P is significant at the level < 0.05 .

P53 concentration in SFMCs culture supernatant of RA group (14.38 U/ml) showed non-significant increase when compared with that of OA group (12.56 U/ml) (Table 7). Within each of RA and OA groups, P53 concentration was significantly increased in SFMCs than that of PBMCs (Table 8).

Table 7. P53 concentration (U/ml) in supernatant of cultured SFMCs of Rheumatoid arthritis (RA), Osteoarthritis (OA) groups.

	RA	OA
Mean \pm S.D	14.38 \pm 1.61	12.56 \pm 3.41

RA; rheumatoid arthritis, OA; Osteoarthritis

Table 8. Statistical comparison of the concentrations of SFMCs-P53 and PBMCs-P53 in Rheumatoid arthritis (RA), Osteoarthritis (OA) groups.

	Mean values of P53 concentration		*P-value
	SFMCs	PBMCs	
RA	14.38	8.78	0.01
OA	12.56	6.69	0.001

SFMCs= Synovial mononuclear cells, PBMCs= Peripheral blood mononuclear cells.

*P is significant at the level < 0.05 .

Positive correlations have been found between P53 levels (in both PBMCs and SFMCs) and disease activity score (DAS) of RA group and radiological grading of OA grade (Table 9).

Table 9. Correlation between P53 levels in PBMCs and SFMCs and disease activity score (DAS) of Rheumatoid and the radiological grade of Osteoarthritis patients.

	r	*P
DAS of RA # P53 in PBMCs	0.65	< 0.01
DAS of RA # P53 in SFMCs	0.52	< 0.05
Radiological grade of OA # P53 in PBMCs	0.23	NS
Radiological grade of OA # P53 in SFMCs	0.26	NS

r = Correlation coefficient.

SFMCs= Synovial mononuclear cells, PBMCs= Peripheral blood mononuclear cells.

*P is significant at the level < 0.05 .

Discussion

Continued oxidative stimulation can eventually cause mutations in various genes including P53. (Tak et al., 2000). Lack of P53 function through mutations in human synviocytes increases the development of normal synovial fibroblasts into transformed appearing aggressive synovial fibroblasts (Pap et al., 2001). Several studies have demonstrated somatic mutations in P53 gene in synovial tissues of RA patients requiring joint replacement therapy (Inazuka et al., 2000). Over expression of P53 in tissues was originally thought to be a surrogate marker for mutations. RA synovial fibroblasts are affected most prominently and their resistance to apoptosis has been linked closely to the progressive destruction of articular cartilage. (Yamanishi et al., 2002). The prevalence of OA is increased in aged individuals and chondrocyte apoptosis is increased on average 2-4 folds in OA. A direct correlation between chondrocyte apoptosis and cartilage degradation secondary to OA has been demonstrated (Sharif et al., 2004). Hydrostatic pressure is a strong inducer of apoptosis in OA through upregulation of P53 (Islam et al., 2002).

Our results revealed that P53 concentration in PBMCs of both RA and OA groups was greater than that of control group and it was significantly higher in RA group than in OA group. This can be explained by the presence of chronic inflammation which may be a key stimulus for the upregulation of P53 as a protective mechanism to induce apoptosis, and that chondrocytes in RA more readily undergo apoptosis than those in OA (Yatsugi et al., 2000). This result is supported by many studies demonstrating that elevated levels of several inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, IL-12, IL-17, IL-18, tumor necrosis factor α (TNF α), interferon- α (IFN α) and granulocyte-

monocyte-colony stimulating factor (GM-CSF) are involved in almost all aspects of articular inflammation and destruction detected in both RA and OA patients (Dai et al., 2004; Kehlen et al., 2000). Our results also showed that P53 concentration increased in SFMCs than PBMCs of both RA and OA that appeared to be due to synovial hyperplasia in both groups.

The higher P53 levels in RA group compared to OA group in our study is a finding that is in agreement with that of Tak et al., (1999) who showed that expression of P53 in RA was considerably higher than in OA synovial specimens. They also reported in another study (Tak et al., 2000) that elevated P53 has been described in many other inflammatory diseases suggesting that P53 induction is a normal component of inflammation. Over expression of P53 was also detected in RA synovium by Firestein et al. (1996) who suggested that cultured rheumatoid synovial fibroblasts probably contribute to this expression. In addition, Chou et al. (2001) found a significant increase of P53 in RA synovial tissues compared with OA and when expression of P53 protein was comparable for two autoimmune diseases; juvenile chronic arthritis (JCA) and RA, P53 protein was detectable in all RA samples (Taubert et al., 2000). Chronic inflammation in RA was suggested by Tak et al. (2000) to be a key stimulus for the upregulation of P53 and the number of apoptotic cells detected in RA synovium. Klimiuk et al. (2003) showed that the serum P53, sTNF- α concentrations were higher in all patients with RA in comparison with OA under their study. TNF- α which is the most important inflammatory cytokine, was detected at biologically significant levels in RA synovial tissue and fluid, but not in OA synovia (Matsuno et al., 2002). Moreover, significantly high levels of IL-6 were detected in SF and serum of RA patients than in patients with OA (Swaak et

al., 1988). High levels of active TGF- β have been detected by Brennan et al. (1990) in the affected joints of OA and RA patients where it may have profound effects on chondrocyte, synoviocyte, osteoclast and osteoblast metabolism. TNF- α and IL-6 in particular, were suggested by Manigurt et al. (2000) to be involved in some way in the upregulation of degenerative processes in the affected joint in RA patients. In addition, IL-6 and IL-1 were reported to stimulate metalloproteases in RA synovial fibroblasts and their levels increased in the synovial fluid of patients with arthropathies including both RA and OA (Ito et al., 1992 and Gverne et al., 1989). Moreover, P53 alters the cytokine balance in inflammation by suppressing proinflammatory cytokines. P53 protein distribution in synovial tissue typically includes both sublining and intimal lining cells. Regarding the increased inflammation in RA than in OA patients which is reported in this study, Farahat et al. (1993) and Koch et al., (1991) suggested that this inflammation results in greater amount of cytokine production in RA compared to OA that are in keeping with the higher endothelial cell expression in RA synovial membranes. They also suggested that this difference in cytokine production are quantitative rather than qualitative.

In RA and OA groups, the level of P53 showed positive correlation with disease activity score and radiological grade of OA. The increased inflammation associated with higher activity scores causing higher expression of P53 to parallel increasing apoptosis in inflamed synovial tissue as P53 is an important homeostatic protein that has anti-inflammatory effects (Muller-ladnen 2000). Supporting our data, Holt et al. (1992) showed significantly higher concentration of IL-1 β , IL-6 and TNF α in SF of RA than in OA patients which was related to leucocyte counts in SF and disease activity. It is also found to be associated with decreased Fas mediated apoptosis (El Hallous et al., 1997). The

hypothesis that P53 protects patients with inflammatory diseases, particularly RA and OA, has been extensively discussed. P53 performs this protection by down regulation of inflammation, inducing cell cycle arrest and apoptosis leading to limitation of synoviocyte hyperplasia, synovitis and joint destruction rather than antigen specific responses (Pozza et al., 2000). Akaika et al (2003) suggested that induction of P53 may potentially trigger H₂O₂-induced apoptosis processes in RA synovial cells. Chou et al (2001) also reported that apoptosis in RA is strongly associated with the expression of Fas and P53. In joint of patients with active RA, few apoptotic cells are detected and experimental data suggested that enhanced apoptosis within the joint might be therapeutically beneficial. (Liu et al., 2003). Joint destruction and deformities were found in 3 patients with RA that were associated with high P53 levels. In spite of the protective role of P53, its over expression in the synovium of RA might indicate the presence of P53 gene mutations associated with a lack of P53 function. The altered rates of apoptosis of RA synovial cells results in hyperplasia of synovial tissue and increased invasiveness and joint destruction (Baier, 2003). Over expression of P53 in the synovium of patients with RA and OA in response to chronic inflammatory stimuli is correlated to the severity of the disease. This is due to mutations in this gene which was documented by many workers. (Albano et al., 2001; Sun and Cheung, 2002). In some patients with RA, dysfunction of P53 might play a role in the proliferation of the synovial tissue (Inazuka et al., 2000). Recent studies indicated that DNA damage and somatic mutation in the P53 gene can occur because of genotoxic stress in many tissues including synovium (Yamanishi et al., 2002). A relatively low percentage of cells containing P53 mutations can potentially affect neighboring cells and enhance inflammation through the elaboration of

proinflammatory cytokines. In addition, suppression of P53 function in human synoviocytes has been documented by Aupperle et al. (1998) to increase proliferation and invasiveness, and mutant cells that arise in the RA joint could contribute to increased cytokine production. Moreover, Collins et al. (1992) and Ogawa et al. (2001) confirmed the defect in apoptosis and P53 expression in the synovial fibroblasts undergoing hyperplasia in RA patients.

In conclusion; the development of novel therapeutic strategies including gene therapy (Pap et al., 1999) targeting P53-dependent pathway may become the future promising therapy for these disabling diseases RA and OA.

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