Analysis of p53 tumour suppressor gene somatic mutations in rheumatoid arthritis synovium

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Abstract

Objective. In order to study the role of the p53 tumour suppressor gene in the proliferation of rheumatoid arthritis (RA) synovium, we analysed the mutation of p53 in the synovial fibroblast-like type B synoviocyte from RA patients.

Methods. Synovial fibroblast-like type B synoviocytes were prepared from the synovial tissues from nine Japanese patients with RA. The p53 cDNA region from exons 4–11 was screened for mutations by the streamlined mutation detection method in which polymerase chain reaction (PCR) products are post-labelled and are analysed by automated capillary electrophoresis using single-strand conformation polymorphism conditions, followed by direct sequencing of the subclones of the PCR products.

Results. p53 mutation with possible functional alteration was detected in four of the nine RA patients (44.4%). Of a total of 262 p53 cDNA subclones, 10 subclones were carrying 10 p53 mutations, eight of which were associated with amino acid alterations or protein truncation. Of the p53 functional mutations, a substitution of Gly at amino acid residue 245 to Asp (G245D) was identified in two patients in three subclones. G245D was the first mutation that was recurrently identified in different RA individuals. G245D is also one of the relatively common mutations in human cancers.

Conclusions. In some patients with RA, dysfunction of p53 might play a role in the proliferation of the synovial tissue. G245D mutation might especially need further study as it is the first recurrently identified p53 mutation in RA and is also one of the frequently identified mutations in human cancers.

Key words: p53 tumour suppressor gene, Rheumatoid arthritis, Synoviocytes, G245D.

Rheumatoid arthritis (RA) is characterized by chronic inflammation and destruction of cartilage and bone of systemic joints. Although the pathogenesis of RA is still unknown, the invasion by proliferated synovial tissue is considered to be an important factor in the development of RA [1]. Among the various cell types in the hyperplastic synovial tissue, fibroblast-like type B synoviocytes have drawn much attention as they exhibit transformed properties that may explain the invasive characteristics of RA synovium. The fibroblast-like type B cells can grow in vitro in an anchorage-independent manner and without contact inhibition [2]. More importantly, their tumour cell-like properties have been shown in vivo in experimental systems. The fibroblast-like type B cells co-implanted with normal human cartilage into severe combined immunodeficient (SCID) mice invaded the cartilage and kept their transformation-appearing cellular shape [3]. RA synovium is thus able to be considered as a locally invading tumour. Therefore, as in the case of malignant tumours in other tissues, dysfunction of oncogenes and/or tumour suppressor genes might also be involved in the pathogenesis of RA. In fact, over-expression of oncogenes, such as myc, ras, fos and egr-1, is demonstrated in RA synovium [4]. In addition, dysfunction of the tumour suppressor gene has also been suggested. The p53 tumour suppressor gene is over-expressed in the intimal lining of RA synovium, as well as in the cultured fibroblast-like type B cells from RA patients [5, 6]. As normal p53 is hardly detected due to the very short half-life of less than 30 min and mutation in p53 often prolongs its half-life, thereby permitting its detection [7], the p53 molecule in the RA synovium is likely to be mutated. Considering that the p53 tumour suppressor gene is mutated in more than half of human tumours [8] and plays a critical role in cell cycle arrest and apoptosis [9, 10], the ‘transformed’ characteristics...
of RA synovium might be at least in part caused by the defects in the p53 gene.

Recently, the somatic mutations in the p53 tumour suppressor gene have been demonstrated in RA synovium [11, 12]. All the mutations are caused by point mutations, resulting in variable distribution, both in sites and patterns. In order to understand the role of p53 mutation in the growth of RA synovium in more detail, we looked for somatic mutations in the p53 gene in fibroblast-like synoviocytes from nine Japanese patients with RA.

Patients and methods

Synovial tissues were collected at the time of joint replacement from nine patients with RA and one osteoarthritis (OA) patient. All the RA patients had been diagnosed as definite RA by the 1987 American College of Rheumatology (ACR) criteria [13]. The tissue was minced, dispersed with collagenase and trypsin, and cultured. After 1 day of culture, non-adherent cells were removed, and adherent cells were cultivated, leading to the exclusive growth of fibroblast-like type B synoviocytes. At confluence, the cells were trypsinized, split at a 1:3 ratio, and recultured. Synoviocytes from passages 2–4 were used in the experiments. A breast cancer cell line MCF7 was used as a negative control for the somatic mutations of the p53 gene.

Total RNA was extracted from the cultured synoviocytes using ISOGEN (Nippongene, Tokyo, Japan). Reverse transcription was performed in 15 μl of reaction mixture with 8 μg of total RNA and random hexadeoxynucleotides at 37°C for 60 min using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Piscataway, NJ, USA). Two overlapping p53 cDNAs containing the regions from codons 102–249 (exons 4–7) or from codons 245–393 (exons 7–11) were amplified from the first-strand cDNA by polymerase chain reaction (PCR). The total volume of the reaction mixture was 10 μl containing 0.5 μM of each of the two 5’ phosphorylated primers (Table 1), 0.2 mM of each of the four nucleotides, 1 μl of the first-strand cDNA, 0.025 U/μl of PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA), 5% dimethylsulphoxide (DMSO), 20 mM Tris–HCl (pH 8.8), 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, and 0.1 mg/ml nuclease-free bovine serum albumin (BSA). The PCR reaction was performed using a Gene Amp 2400 cycler (PE Applied Biosystems, Foster City, CA, USA). The temperature profile was an initial 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 55°C, 60 s at 72°C, and a final 5 min at 72°C. The amplified p53 cDNAs were then subcloned into plasmid pBluescript SK+ (Stratagene), and transferred into Escherichia coli, DH5α. The transformed cells were grown on a LB plate containing 0.1 mg/ml ampicillin. At least 10 subclones of each cDNA except for patient 3 were isolated using an automated plasmid isolation system PI-100 (Kurabo, Osaka, Japan).

The screening for p53 mutations was performed using the streamlined mutation detection method in which PCR products are post-labelled with two different fluorescent dyes in one tube, and analysed by automated capillary electrophoresis (PLACE) using single-strand conformation polymorphism (SSCP) conditions as described previously [14]. Briefly, subclones were amplified by PCR, treated with the Klenow fragment of DNA polymerase I to exchange the 3’ terminal residues for fluorescent nucleotides, R110-dUTP or R6G-dCTP (PE Applied Biosystems), added to formamide, and heat denatured at 90°C for 3 min. Electrophoresis was performed using a Prism 310 CE system (PE Applied Biosystems). The effective length of the capillary for detection was 36 cm. The separation matrix was 5% performance optimized polymer (PE Applied Biosystems) in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) containing 10% glycerol. The running conditions were 15.0 kV at 25°C. In this analysis, mutations were detected as a difference in the retention time of the fluorescent products between the reference (a subclone with a normal sequence) and the samples, because the strands of the samples have different conformation if there are sequence changes and therefore migrate differently during electrophoresis [15]. The Δ detection time was estimated as the shift in the retention time of the samples from the average of the reference. The presence of mutations was suspected when the Δ detection time was three times larger than the standard deviation of the reference. We previously analysed 30 mutations in three independent sequence contexts and found that 97% (29 of 30) of them can be detected based on the same criteria [14].

The subclones that were suspected of the presence of mutation were subjected to further analysis by nucleotide sequencing. Sequencing of cDNA subclones was performed using the Big-Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The products were analysed using a Prism 310 CE system following the instructions for this system.

Results

In order to screen for mutations, a total of 264 p53 cDNA subclones (116 for codons 102–249 and 146 for codons 245–393) were analysed by SSCP in nine RA
patients. Based on the criteria for detection time described previously, 21 subclones were chosen and were subsequently subjected to sequencing analysis. Eleven of the 21 subclones did not have any mutations, while the remaining 10 subclones were carrying 10 mutations. Five (patients 1, 2, 6, 7 and 8) of the nine RA patients had mutations in their p53 subclones (Table 2). Five functional mutations (A161V, G245D, R267Q, A347fs, G375X) were identified in four patients and the former four mutations have been registered in the International Agency for Research on Cancer (IARC) p53 somatic mutation database [16], suggesting their contribution to tumorigenesis. Of these mutations, the mutation of G245D has been registered most frequently in the database; 62 independent reports out of a total of 9378 p53 mutations in human tumours registered so far (March 1999). Patient 1 displayed six mutations in total, two of which were the G245D mutation. The G245D mutation was also detected in patient 7. Patient 2 had a frameshift mutation (A347fs) and patient 8 was carrying a nonsense mutation (G375X), both of which lead to truncation of the p53 protein.

However, most of the mutations were identified only once, except in the case of G245D. Silent mutations were detected at a frequency comparable to functional mutations. Furthermore, no significant differences in frequency were found between most of our RA patients and the control cell line MCF7 because we detected a silent mutation in two of 29 subclones of MCF7, which should have occurred during cDNA synthesis or cultivation. The synoviocytes from an OA patient were also studied for p53 mutation and only one of 34 subclones was positive for mutation (data not shown). The frequency was as low as that of the control cell line MCF7. It is suggested that some of the mutations in our study, especially those that have not been registered in the IARC p53 somatic mutation database, may be caused by artefactual mutations.

**Discussion**

The p53 tumour suppressor gene encodes a nuclear phosphoprotein with cancer-inhibiting properties, and is mutated in more than half of human cancers [8]. The p53 protein is induced by many genotoxic stresses which is followed by cell cycle arrest and apoptosis of the injured cells [17]. Most p53 mutations in the mutational hot spots of human cancer function in a dominant-negative fashion [18], which interfere with the function of normal protein, culminating in the monoclonal expansion of the cells with p53 mutation and the development of cancer. Disruption of p53 function by a retroviral vector also results in the increased proliferation and invasiveness and impaired apoptosis of the fibroblast-like synoviocytes [19].

Here, we studied exons 4–11 for mutation, which include most of the hot spots of the p53 gene [20]. Five p53 somatic mutations with alterations at the amino acid level were identified in four of nine (44.4%) RA patients in Japan. These mutations (A161V, G245D, R267Q, A347fs, G375X) are likely to cause functional alterations because the former four have been registered in malignancies in other tissues and the latter, although not found in other tumours, causes truncation of the p53 protein. It is strongly suggested that these mutations are not caused by artefactual events during cDNA synthesis or cell culture, considering the high number of transition mutations and identity with mutations found in the p53 database. All the mutations but G245D were identified only once in the subclones, while G245D was identified in two subclones in patient 1 and in one clone in patient 7. The rate of RA patients

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**Table 2. p53 mutations identified in RA synoviocytes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutated/total subclones</th>
<th>Amino acid at mutation site</th>
<th>Sequence change</th>
<th>Mutated/total subclones</th>
<th>Amino acid at mutation site</th>
<th>Sequence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/13</td>
<td>GCC &gt; GTC</td>
<td>A161V G245D</td>
<td>4/32</td>
<td>GGG &gt; GAC</td>
<td>G244G G245D R267Q G279G</td>
</tr>
<tr>
<td>2</td>
<td>0/12</td>
<td>I332I ATC</td>
<td>0/29 A347fs</td>
<td>16/16</td>
<td>CAG &gt; GAT</td>
<td>A355A GCT &gt; GCC</td>
</tr>
<tr>
<td>3</td>
<td>0/9</td>
<td>0/5</td>
<td>0/19 n.a.</td>
<td>10/15</td>
<td>GAG &gt; GCA</td>
<td>1/20 G375X</td>
</tr>
<tr>
<td>4</td>
<td>0/13</td>
<td>0/10</td>
<td>0/29 I332I</td>
<td>15/15</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>5</td>
<td>0/15</td>
<td>0/10</td>
<td>n.a.</td>
<td>0/10</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>6</td>
<td>0/18</td>
<td>0/10</td>
<td>n.a.</td>
<td>0/10</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>7</td>
<td>0/11</td>
<td>0/10</td>
<td>n.a.</td>
<td>0/10</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>8</td>
<td>0/15</td>
<td>0/10</td>
<td>n.a.</td>
<td>0/10</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>9</td>
<td>0/10</td>
<td>0/10</td>
<td>n.a.</td>
<td>0/10</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
<tr>
<td>MCF7</td>
<td>0/35</td>
<td>0/35</td>
<td>n.a.</td>
<td>0/35</td>
<td>ATC &gt; ATT</td>
<td>I332I</td>
</tr>
</tbody>
</table>

Amino acid residues are shown in one letter code.

n.a., not analysed; MCF7, a breast cancer cell line used as a negative control for sequence variations.
carrying the p53 mutation is difficult to estimate as none of the two previous reports (one from the USA and the other from Europe) as well as our study from Japan studied the entire coding region [11, 12]. However, considering that the rates in the three studies are between 15 and 44.4%, the p53 mutation is not uncommon in the synovium of RA irrespective of ethnicity. On the other hand, the synoviocytes carrying the p53 mutations were not the majority in the RA synovium, as shown in our study. By using PLACE–SSCP followed by direct sequencing of the subclones, we showed that the percentage of the mutated to the total sequenced subclones was less than 10% (Table 2). The lack of predominance of the mutated clone was also described in the previous two studies, using RNA mismatch detection assay [11] or PCR–SSCP analysis [12]. These findings indicate that the proliferation of RA synoviocytes cannot be explained by p53 mutation alone. However, G245D mutation was independently identified in three subclones prepared from two RA patients. This was the first p53 mutation that was recurrently identified in different individuals with RA. G245D is one of the frequently identified mutations of the p53 gene in human cancers, suggesting that G245D might cause functional alteration in the p53 protein and contribute, at least in part, to the proliferation of RA synovium. In order to clarify the role of G245D in the synovial growth, the frequency of G245D mutation should be studied in the synoviocytes isolated from the invasive pannus.

Differences in the pattern of p53 mutations in human cancers from different tissues are supposed to reflect the effects of specific carcinogens. In the case of liver cancer, G/T transversion is preferentially led by tobacco smoke [21], while in the case of skin carcinoma, C/T transitions at the site of adjacent pyrimidines are detected predominantly, indicating that the mutagen is ultraviolet radiation [22]. Nitric oxide (NO) is an important mediator of immune and inflammatory responses, and increased production of NO has been observed in the synovium of RA patients [23, 24]. In the present study, all the mutations but one deletion mutation in patient 2 were G/A and T/C transitions, consistent with the hypothesis that a genotoxic environment with NO in the rheumatoid joint leads to somatic mutation in the key regulatory genes like p53 [11].

As only a minor cell population of RA fibroblasts was carrying p53 mutations, dysfunction of p53 might not play a major role in the pathogenesis in most of the RA patients. However, before concluding the extent of p53 involvement in RA, further study is needed, such as the search for p53 mutation in the invasive pannus of RA. Moreover, in some RA patients such as patient 1 (Table 2), p53 mutation was observed at relatively high frequency, suggesting that p53 mutation might be involved in the growth of the RA synovial tissues. G245D mutation might especially need further investigation as it is the first recurrently identified p53 mutation in RA and is also one of the frequently identified mutations in human cancers. It would be of interest to increase the sample size and to study G245D mutations in RA synovium.

References