Detection of the p53 Regulator Murine Double-Minute Protein 2 in Rheumatoid Arthritis

ELLIOTT TARANTO, JIN RONG XUE, DEREK LACEY, PAUL HUTCHINSON, MALCOLM SMITH, ERIC F. MORAND, and MICHELLE LEECH

ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is characterized by hyperplasia of synovial lining tissue, which is involved directly in the damage of cartilage and bone. One of the factors thought to contribute to this synovial lining hyperplasia is dysregulation of, or functional abnormality in, the tumor suppressor protein p53. The protein known as murine double-minute protein 2 (MDM2) is the major negative regulator of p53, and in tumors contributes to increased cell proliferation. The detection of MDM2 in rheumatoid synovium has not previously been described. We investigated whether this protein is detectable in cells and tissues derived from patients with RA.

Methods. Expression of MDM2 protein was examined in fibroblast-like synoviocytes (FLS) by methods including permeabilization flow cytometry, immunofluorescence, and Western blotting, and in synovial tissues using immunohistochemistry. The proliferative capacity of these cells was also examined using ^{3}H/thymidine incorporation. Cell cycle analysis was performed by propidium iodide incorporation.

Results. MDM2 was detected in RA FLS and synovial tissues. MDM2 protein was identified in CD14-positive and CD14-negative synovial lining cells and CD14-positive sublining cells. RA FLS exhibited faster proliferative rates and higher levels of MDM2 expression than FLS derived from patients with osteoarthritis (OA). Both OA and RA FLS were found to be in similar phases of the cell cycle at the time of MDM2 protein analysis.

Conclusion. The abundant expression of MDM2 in RA may be a contributing factor to the hypoapoptotic phenotype of lining tissue through its capacity to downregulate p53 levels and effects. Further studies are required to determine the relationship between this cell-cycle protein profile, tissue hyperplasia, and the functional abnormality of p53 in RA. (J Rheumatol 2005;32:424–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS | MURINE DOUBLE-MINUTE PROTEIN 2
p53 | ARTHRITIS | CELL CYCLE

The synovial lesion of rheumatoid arthritis (RA) is characterized by recruitment of blood-derived leukocytes as well as synovial lining hyperplasia where the usually single or double cell lining layer evolves to a multilayered hyperplastic structure. In RA and animal models of inflammatory arthritis, invasive and destructive hyperplastic synovial lining tissue is identified histologically. Proliferating cell nuclear antigen (PCNA)-positive cells are identified in the superficial lining layer in RA[1], and expression of proto-oncogenes, including c-myc and ras, has been identified in fibroblast-like synoviocytes (FLS) attached to cartilage and bone. RA FLS exhibit autonomous invasive capacity into cartilage implants in SCID mice[2]. Evidence of apoptosis defects in RA tissue suggests another mechanism contributing to tissue hyperplasia[3]. While cells with identified DNA fragmentation are detected in RA, morphologic evidence of completed apoptosis is consistently lacking[4,5]. Both pro- and anti-apoptotic regulatory proteins are expressed in RA, with no clear pattern to date of an imbalance or functional abnormalities in these proteins to explain dysregulated apoptosis.

The tumor suppressor and cell-cycle regulatory protein p53 has been a focus of major interest in the investigation of dysregulated synoviocyte growth in RA. The protein known as murine double-minute protein 2 (MDM2) is the major negative regulator of p53[6]. Although expression of a MDM2-like protein has been reported in RA FLS transfected with the tumor necrosis factor (TNF) receptor p55 gene[7], MDM2 expression in RA tissue has not been definitively described. We believe detection of MDM2 in synovial tissue is of significant interest as a prelude to examining its possible contribu-

From the Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, Clayton, Melbourne; and Repatriation General Hospital, Daw Park, Adelaide, Australia.

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E. Taranto, BSc (Hons); J.R. Xue, MD; D. Lacey, BSc (Hons), PhD; P. Hutchinson, MSc, Centre for Inflammatory Diseases, Monash University Department of Medicine; E.F. Morand, MBBS (Hons), FRACP, PhD, Deputy Director, Rheumatology and Immunology, Southern Health, Centre for Inflammatory Diseases, Monash University Department of Medicine; M. Leech, MBBS (Hons), FRACP, PhD, Consultant Rheumatologist, Southern Health, Centre for Inflammatory Diseases, Monash University Department of Medicine; M. Smith, MD, PhD, Repatriation General Hospital.

Address reprint requests to E. Taranto, Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, Locked Bag No. 29, Clayton, Melbourne, 3168 Australia.
E-mail: elliott.taranto@med.monash.edu.au

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tion to p53-dependent and independent growth dysregulation in this setting.

MATERIALS AND METHODS

Isolation and culture of FLS. FLS were obtained from synovium of RA patients undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology (ACR) criteria for the classification of RA[8]. FLS were isolated using enzyme digestion and cultured in RPMI/10% fetal calf serum (FCS; ICN, Melbourne, Australia). In brief, a single-cell suspension was obtained by digesting minced synovial tissue with 2.4 mg/ml Dispase (grade II, 5 U/mg; Boehringer Mannheim, Melbourne, Australia), 1 mg/ml collagenase (type II; Sigma, Melbourne, Australia), and DNase (type I; Boehringer Mannheim). FLS were propagated in 10 cm culture plates in RPMI (ICN Biomedicals, Cincinatti, OH, USA)/10% FCS at 37°C in a 5% CO_2 humidified incubator. Cells beyond third passage were more than 99% CD45-negative. Cells were used between passages 4 and 9. In each group of experiments, n refers to the number of individual human donor FLS used.

Detection of intracellular MDM2 and p53 in RA FLS by permeabilization flow cytometry. Cultured human synovial fibroblasts were washed with Hanks' balanced salt solution (HBSS) and removed from culture dish after exposure to trypsin EDTA. Cells were subsequently washed and fixed in 2% paraformaldehyde. Cells were washed again and then permeabilized in 0.1% Triton X (Sigma). Cells were resuspended in phosphate buffered saline (PBS)/0.1% sodium azide/0.1% bovine serum albumin (BSA) and incubated with specific mAb to MDM2 (C-18), p53 (DO-1), or isotype-matched negative control antibodies (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min. Cells were washed, resuspended in PBS/0.1% sodium azide/0.1% BSA and incubated 30 min with fluorescein isothiocyanate (FITC) conjugated secondary antibody (Silenus, Melbourne, Australia). Cells were washed and analyzed, then examined on a Cytomation Mo-flo flow cytometer (Cytomation, Fort Collins, CO, USA). At least 5000 events were analyzed for each sample.

Detection of MDM2 in FLS by Western blotting. FLS from 3 different OA and 3 different RA donors were compared in terms of the expression of MDM2 using Western blotting with a mAb specific for MDM2 (C-18; Santa Cruz Biotechnology). Cells were washed with cold PBS and then lysed with 2× SDS sample buffer. The protein samples were boiled for 10 min and stored at –20°C. Samples were subjected to 10% Tris Glycine iGel SDS/polyacrylamide gel electrophoresis (Gradipore, Sydney, Australia), transferred to a Hybond C membrane, and analyzed using the ECL detection system (Amersham).

Immunofluorescent detection of MDM2 in FLS. Three different OA and RA donor FLS taken from confluent culture were seeded on 24 well tissue culture plates at a density of 2.5×10^4 cells per well for 24 h. Adherent cells were then washed with HBSS and fixed in 2% paraformaldehyde. Cells were again washed and then permeabilized in 0.1% Triton X (Sigma) before being washed again in PBS/0.1% sodium azide/0.1% BSA and incubated with specific mAb to MDM2 (C-18) or isotype matched negative control antibodies (Santa Cruz Biotechnology) for 30 min. Unbound antibody was removed by washing in PBS/0.1% sodium azide/0.1% BSA. Cells were then incubated for 30 min with FITC conjugated secondary antibody (Silenus) before being washed and analyzed under UV microscope.

MDM2 detection in rheumatoid synovial tissue and FLS cytospin. RA FLS cytospin preparations were obtained as follows: cultured human synovial fibroblasts were removed from plates by trypsinization. Cells were then washed in RPMI, counted using a Nebauer hemocytometer, and centrifuged at 700 rpm for 5 min onto Superfrost Plus microscope glass slides using a cytospin centrifuge (Shandon, Pittsburgh, PA, USA).

Synovial tissue samples were obtained from individuals meeting the ACR criteria for the classification of RA by arthroscopy[8]. MDM2 was detected in RA synovial tissue and FLS using 3-layer immunohistochemistry. Briefly, sections were incubated with mAb specific for MDM2 (C-18) and CD14 (Dako, Carpinteria, CA, USA). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol, and sections were then stained using rabbit anti-mouse IgG and mouse peroxidase antiperoxidase complexes (Dako) and diaminobenzidine tetrahydrochloride-H_2O_2 solution. In the case of MDM2 staining, primary antibody was followed by sequential incubation with swine-anti-rabbit immunoglobulin and rabbit PAP conjugate.

DNA content analysis. For assessment of the cell cycle, 4 donor OA and RA FLS were plated in culture dishes 2.5×10^4 in RPMI supplemented with 10% FCS. Cells were removed from plates by trypsinization and subsequently fixed with 70% ethanol, washed, and incubated with ribonuclease (100 mg/ml; Sigma) for 30 min. After centrifugation, cells were resuspended in propidium iodide (100 mg/ml; Sigma) in PBS, then analyzed by flow cytometry. At least 5000 events were analyzed for each sample. Results are expressed as percentage of cells cycling, i.e., cells not in G0/G1.

Fibroblast proliferation. Donor FLS derived from 5 different OA and 5 different RA patients were compared. All FLS were at the same passage (P6) and confluence prior to being seeded for the experiment. Multiple duplicates of each donor were used in each experiment. Cells were trypsinized from fully confluent plates and seeded onto a 24 well plate at low density (1×10^5 cells/well) in RPMI (10% FCS) and incubated for 24 h at 37°C. Cells were then washed twice with HBSS before starving in 500 µl RPMI (0.1% BSA) at 37°C for 24 h. Cells were then cultured for 54 h in 10% FCS. Cells were pulsed with 1 µCi/ml ^{3}H/thymidine in 50 µl RPMI for 18 h at 37°C. Radioactivity content was determined by scintillation counting using a Wallac 1409 liquid scintillation counter (^{3}H/thymidine, 60 s/tube). DNA synthesis was estimated by measurement of ^{3}H/thymidine incorporation in test fibroblasts. Results were expressed as average cpm with standard error of the mean.

Data analysis. Delta mean fluorescence intensity (δMFI) was calculated by subtracting the mean fluorescence of the samples stained with an isotype matched negative control antibody from that of the samples stained with specific antibodies. Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student t test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of MDM2 in RA. MDM2 was detected in RA synovial tissue derived from 4 different donors using 3-layer immunohistochemistry. MDM2-positive cells were seen in synovial lining and sublining regions in each case (a representative section is shown in Figure 1A). CD14-positive and CD14-negative synovial lining cells were positive for MDM2 staining, indicating expression in both fibroblast-like and macrophage-like synoviocytes (Figures 1C, 1D). MDM2 was also detected in CD14-positive macrophages in the sublining layer. Using 3-layer immunohistochemistry of cytospin preparations of RA FLS, prominent immunostaining of MDM2 was observed (Figure 2A). Under high power, prominent nuclear localization of MDM2 was seen in the majority of RA FLS (Figure 2B).

Detection of p53 protein in FLS. We next sought to compare the expression of MDM2 protein with the expression of p53. In contrast to the high level of MDM2 expression observed in RA FLS relative to OA FLS, p53 was detected at very low level, with no difference observed between RA and OA FLS growing in standard culture conditions (Figure 3).

Comparison of MDM2 expression in RA FLS and OA FLS. Levels of MDM2 protein expression were compared in FLS derived from each of 4 different OA donors and 4 different RA donors. MDM2 protein expression was increased in RA FLS

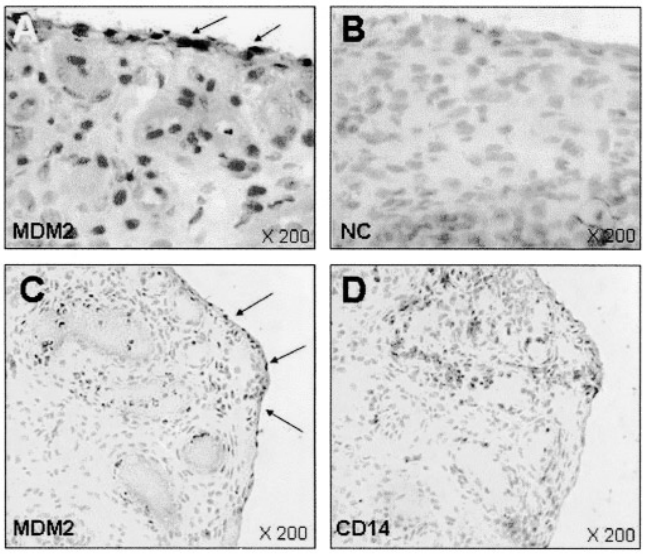

Figure 1. A. Immunohistochemistry of RA synovial tissue stained with specific mAb for MDM2. MDM2-positive cells are seen in the synovial lining (original magnification ×200). B. Immunohistochemistry of RA synovial tissue stained with isotype matched negative control IgG (original magnification ×200). C. Distribution of MDM2 staining in RA synovial lining and sublining regions. MDM2 expression is detectable in almost all lining cells and in a subpopulation of sublining cells. D. Distribution of CD14-positive cells in RA synovial tissue. CD14 staining in lining tissue is discontinuous. In the sublining, CD14 staining closely approximates MDM2 staining (original magnification ×200).

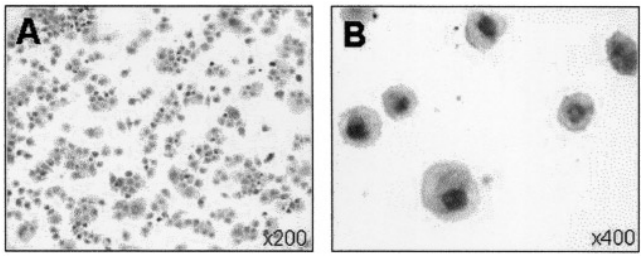

Figure 2. A. Cytospin of RA FLS immunostained using specific mAb for MDM2 showing prominent cellular staining of MDM2 (original magnification ×200). B. Cytospin of RA FLS immunostained using specific mAb for MDM2, high power view, showing nuclear localization of MDM2 (original magnification ×400).

compared with OA FLS (mean ± SEM, 37.2 ± 5.6 vs 11.0 ± 1.5, respectively; $p < 0.05$) as determined by flow cytometry (Figure 4A). Higher MDM2 protein expression in RA FLS compared with OA FLS was further confirmed using Western blotting (Figure 4B) and immunofluorescence (Figures 4C, 4D), where increased expression and nuclear localization of MDM2 was observed in RA FLS.

Comparison of cell cycle and proliferative rates in RA FLS and OA FLS. Cell cycle analysis was undertaken to determine if the observed differences in MDM2 expression between OA and RA donor FLS were the result of cell cycle phase differences. Results indicate that the percentage of cells cycling was similar between donors (Figure 5).

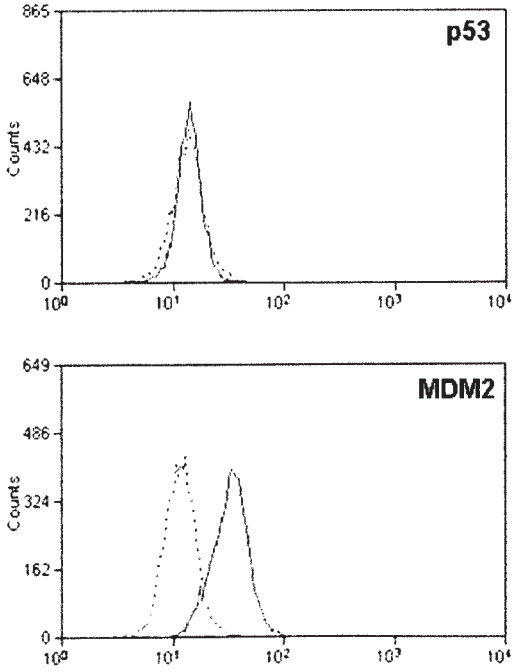

Figure 3. Flow cytometric detection of p53 and MDM2 in RA and OA FLS. A 5% fluorescence cutoff was established with negative control mAb. Abundant expression of MDM2 with higher expression seen in RA FLS (solid line) compared with OA FLS (broken line). In contrast, p53 was detected at low level in both RA and OA FLS. Each histogram is representative of FACS analysis of FLS derived from 4 different RA and 4 different OA donors.

Under standard cell culture conditions FLS derived from RA patients exhibited faster growth rates than those derived from patients with OA in that when seeded at the same density they achieved confluence faster and required passaging more frequently. To confirm and quantify this, formal assessment of proliferative status was undertaken. This was accomplished using a proliferation assay measured by tritiated thymidine incorporation. Results show significantly higher levels of tritiated thymidine incorporation in RA FLS, a mean of 3884.9 ± 86.6 cpm compared with the much less proliferative OA FLS yielding a mean of 1587.8 ± 64.5 cpm ($p < 0.05$; Figure 6). The low SEM in each case also highlights consistent counts between donors. The very low counts observed in the OA FLS reflect the normally slower doubling time of mature fibroblasts as well as the low seeding density in conjunction with 24 h starvation.

DISCUSSION

The p53 protein plays a key role in cell growth and cell cycle control. The apparent lack of apoptosis in RA has implicated dysfunctional p53 as a potential cause of synovial lining hyperplasia and growth dysregulation. In normal cells, p53 is induced in response to genotoxic stresses, genomic threat, or DNA damage. Its accumulation, stabilization, and activation results in cell cycle arrest or apoptosis, thereby deleting dam-

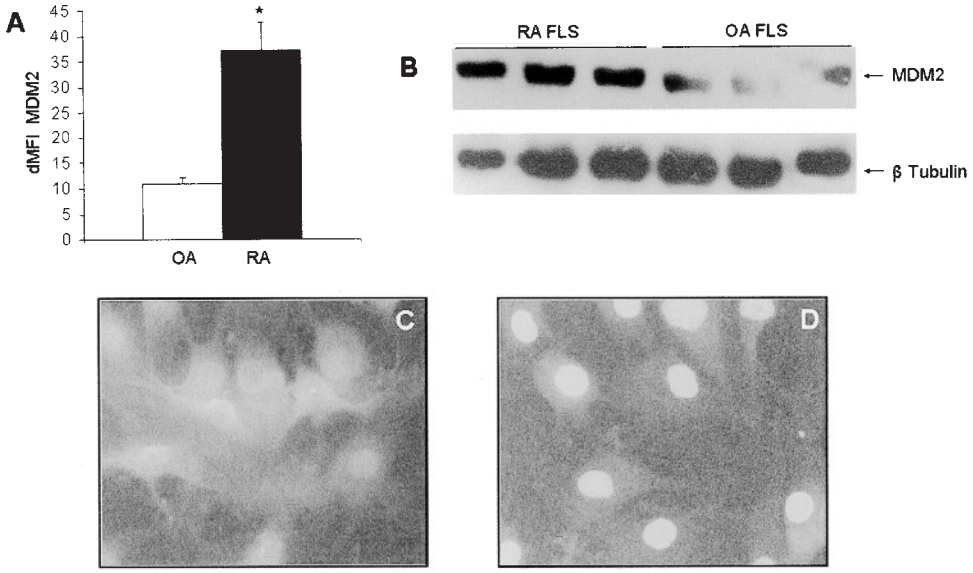

Figure 4. A. Flow cytometric detection of MDM2 in RA and OA FLS. Four different patient donors are compared in each group. Increased mean fluorescence intensity (MFI) for MDM2 staining was observed in RA FLS compared with OA FLS (*p < 0.05). B. Comparison of MDM2 expression by Western blotting in 3 different RA FLS donors (lanes 1–3) and 3 different OA FLS donors (lanes 4–6) with ß-tubulin lower band as loading control in each case. C. Immunofluorescent detection of MDM2 in OA FLS. A representative example of MDM2 staining in 3 different OA FLS donors. D. A representative example of immunofluorescent detection of MDM2 in 3 different RA FLS donors.

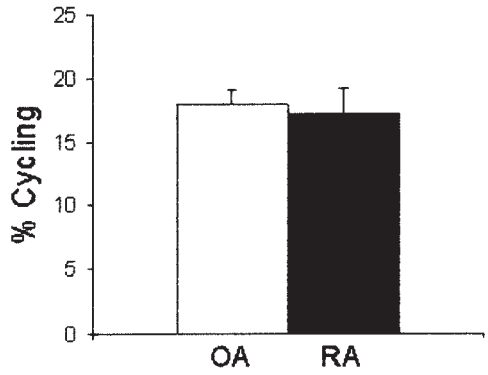

Figure 5. Cell cycle analysis of 4 different OA and RA patient donors was compared. No statistically significant difference in the percentage of cycling cells could be detected between these cell types.

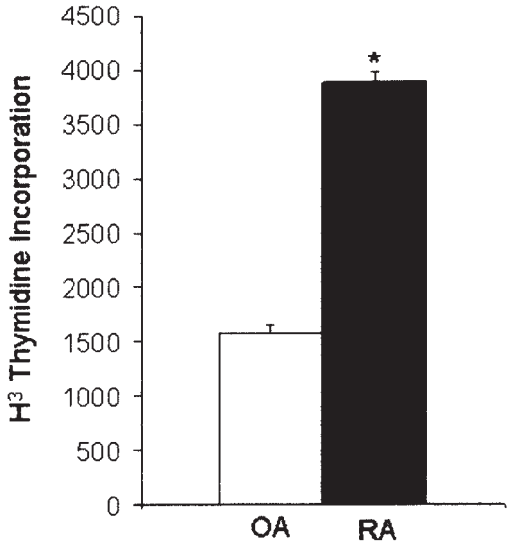

Figure 6. Proliferation of RA FLS and OA FLS was quantified using ^{3}H-thymidine incorporation. Five different patient donors were compared in each group. Increased proliferation was observed in RA compared with OA FLS (*p < 0.05).

aged cells or allowing time for genomic repair[9]. Reduced or dysfunctional p53 would therefore predispose to a proliferative, hypoapoptotic, or hyperplastic phenotype. In the tumor setting, mutations in p53 are ascribed a causative role in tumor formation, directly via growth dysregulation and indirectly via predisposition to further unrepaired genomic damage/mutation. Overexpression of p53 and functional p53 mutations has been detected in cells and tissue derived from RA[11]. Increased expression of wild-type (wt) p53 has also been described in several other inflammatory diseases[12]. Difficulty clarifying the contribution of p53 mutations to synovial hyperplasia has resulted largely from discrepancies in the detection rate of p53 mutations within tissues and FLS derived from patients with RA[13]. Fluctuations in the levels or function of wt p53 unrelated to mutation could also have an influence on synovial proliferative capacity. The levels of wt p53 have not been the focus of investigation in the past; this may relate to the difficulty of detection of wt p53 in synovial tissue. In normal cells, wt p53 protein is barely detectable because of its short half-

life. The role of functional wt p53 has recently been examined in collagen induced arthritis, where more severe arthritis and earlier onset was observed in p53–/– mice[14].

p53 has been described as a nodal point in the control of cell growth. Its capacity to restrain growth and induce apoptosis in response to damage or threat is mediated through a vast and growing number of identifiable transcriptional and nontranscriptional targets. It is known from the cancer biology literature that p53 mediated apoptosis and growth arrest can be blocked by the overexpression of several cellular or viral proteins. Thus while p53 itself is the most commonly studied target in the investigation of dysregulated growth, alterations in any upstream or downstream factor may render the p53 mechanism ineffective.

In normal nonstressed cells, p53 is maintained at very low concentrations via its interaction with its major regulatory protein MDM2. This protein continuously targets p53 for proteasomal degradation via its capacity to ubiquitinate the p53 molecule[15]. At the same time, MDM2 is well described as a positive transcriptional target of p53. This negative regulatory feedback loop has now been well characterized and provides a mechanism for automodulation of p53 levels[6]. It is similarly known that MDM2 transactivation can occur independent of p53. In p53 null mice, for example, levels of MDM2 in tissues were similar to those in normal mice[16]. The p53 family member p73 may transactivate MDM2 in the absence of p53[17]. In addition to direct effect on p53 levels, MDM2 has been identified to interfere with p53 transcriptional activity[18], and its capacity for independent oncogenic potential is a subject of investigation. A further layer of complexity relates to the observation that mutant p53 can stabilize and increase the function of MDM2, thus further compromising any residual wild-type p53 function, and suggesting a possible mechanism underlying the dominant negative effect of single-strand p53 mutations[19]. The p53-MDM2 module therefore serves as a major integrator in response to environmental or cellular stress.

We describe the detection of MDM2 in RA FLS and synovial tissue. Its prominent expression in lining synoviocytes may be particularly relevant given the observation that p53 mutations and functional abnormalities may be confined to discrete but pathologically important areas of synovium such as the cartilage-pannus junction[7,11]. In this study we found very low detection of p53 in OA and RA FLS using flow cytometry. Previous studies identified clear expression of p53 in RA as well as OA and reactive arthritis[20,21]. Our findings of low p53 in these cultured synoviocytes may relate to choice of antibodies as well as patient selection. Previous studies have shown that patient selection may be critical for interpretation of results with respect to p53 expression in RA[22]. Nevertheless, the expression of a negative regulator of p53 such as MDM2 in the synovial lining regions may be a contributory factor to the hypoapoptotic phenotype and invasive potential of lining tissue. This may be by direct interference with p53 transactivation function, as discussed above. The capacity to downregulate wt p53 levels in these regions may have specific implications in terms of undermining the protective effect of wt p53 repression of matrix metalloproteinase expression[23].

MDM2 is a focus of intense interest in cancer biology. While an MDM2–like protein has been described in RA FLS transfected with tumor necrosis factor receptor p55[7], its expression has not previously been characterized in RA FLS and tissue. This molecule is intrinsically capable of downregulating p53 levels and function, and is therefore hypothetically capable of contributing to tissue hyperplasia in the setting of inflammatory stress. The contribution of antiapoptotic molecules including PTEN and sentrin to tissue hyperplasia in RA has recently been described[24,25]. Ours is the first description of MDM2 expression in macrophage-like CD14-positive lining and sublining cells in the setting of RA. MDM2 expression has been described in cells of monocyte lineage specifically in the setting of hemopoietic malignancy, where MDM2 is ascribed a functional role in neoplasia, aggressiveness, and resistance to antiproliferative therapies[26].

The abundant expression of MDM2 detected in cultured RA FLS was in contrast to the lower levels of expression in OA FLS. DNA content analysis of these cells revealed no significant difference in the number of cycling cells, indicating that differences observed in MDM2 expression were not caused by cycle phase differences. In spite of this, increased tritiated thymidine incorporation indicates that RA FLS do proliferate faster than OA FLS. This discrepancy may be explained by the duration of the thymidine incorporation assay, where both increased cell division and decreased apoptosis could contribute to thymidine incorporation. Additionally, cell cycle analysis provides a "snapshot" of the percentage of cells cycling, regardless of the absolute number, whereas thymidine incorporation more accurately reflects the total number of cells present at the end of the experiment.

The capacity of MDM2 to interfere with p53 function and to reduce p53 levels may implicate it as one of many factors that could block effects of p53 in RA synovium. Our observation of increased MDM2 in RA synoviocytes has not been mechanistically linked to low levels of p53 and p53-induced genes or to inhibition of p53 function. In our study, the detection of MDM2 in RA synovial tissue forms an important prelude to an assessment of its role in observed functional abnormality of p53 in RA. The detection of higher levels of MDM2 and faster proliferative rates in RA FLS compared with OA FLS provides preliminary evidence for a hypothetical link between high MDM2 and tissue hyperplasia in RA.

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