J. Biochem. **124**, 1153-1162 (1998)

Establishment and Characterization of a Novel Human Rheumatoid Fibroblast-Like Synoviocyte Line, MH7A, Immortalized with SV40 T Antigen

Keiji Miyazawa,*[,1] Akio Mori,[†] and Hirokazu Okudaira[‡]

**Central Research Laboratories, Kissei Pharmaceutical Co., Ltd., Nagano 399-8304;* [†]*Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, Kanagawa 228-8522; and the* [‡]*Department of Medicine and Physical Therapy, Faculty of Medicine, The University of Tokyo, Tokyo 113-0033*

Received for publication, July 3, 1998

A novel immortalized rheumatoid fibroblast-like synoviocyte (FLS) line, MH7A, was established by stably transfecting FLS cells with SV40 T antigen gene. MH7A cells expressed SV40-specific small t and large T antigens as well as an elevated level of p53 protein. They have already reached over 150 population doublings through culture crisis, and have been growing rapidly compared with the parental FLSs. Constitutive activation of p42/p44 mitogen-activated protein (MAP) kinase was detected in MH7A cells. Serum requirements for the growth of MH7A were markedly decreased compared with those for the parental FLSs. MH7A cells were stained positively for interleukin (IL)-1R, intercellular adhesion molecule-1 (ICAM-1), CD16, CD40, CD80, and CD95. IL-1β enhanced the production of IL-6 and stromelysin-1, and the surface expression of ICAM-1, in a manner similar to that in the parental FLSs. SB203580, a specific inhibitor of p38 MAP kinase, significantly inhibited IL-1β-induced IL-6 and stromelysin-1 production by both parental FLSs and MH7A cells; although PD098059, an inhibitor of the p42/p44 MAP kinase pathway, did not affect it. Our results clearly indicate the usefulness of MH7A cells for investigating the regulation of rheumatoid FLSs and the IL-1 signal transduction pathway to develop future RA therapy.

Key words: fibroblast-like synoviocyte, immortalization, MAP kinase, rheumatoid arthritis.

Fibroblast-like synoviocytes (FLSs) derived from patients with rheumatoid arthritis (RA) exhibit characteristics of inflammatory cells and are critically involved in several aspects of rheumatoid pathophysiology (*1*). Firstly, they proliferate in an anchorage-independent manner upon stimulation with cytokines and growth factors, including interleukin (IL-1) and PDGF (*2*). Secondly, they secrete matrix metalloproteinases (MMP) such as stromelysin-1 (MMP-3), enzymes essentially involved in the degradation of cartilage (*3-7*). Thirdly, FLSs are involved in the infiltration and activation of other immune cells in the synovial tissue by secreting various cytokines including IL-6, or by expressing adhesion molecules including intracellular adhesion molecule-1 (ICAM-1) (*8*). FLSs, therefore, seem to be the crucial regulators of joint inflammation and destruction in RA.

Rheumatoid FLSs can be expanded and maintained *in vitro* for approximately 10 passages. However, they gradually lose the above-described rheumatoid characteristics upon serial passage. In addition, their growth potential *in vitro* appears to be limited. Source material (human rheumatoid pannus) is not readily available, as it is usually obtained from RA patients undergoing total joint replacement. In an attempt to overcome these shortcomings, we sought to develop an immortalized synoviocyte line from primary synoviocyte cultures. Although characterization of SV40 T antigen transfected synoviocyte lines has already been made by several investigators (*9-11*), these cell lines were still not immortalized as a rare subpopulation of cells is able to escape crisis to become immortal. More recently, an SV40 T antigen immortalized human FLS line (K4IM) was established by Haas *et al.* (*12*), but unfortunately it was not derived from patients with RA and had lost the receptor for IL-1, a potent inflammatory mediator. In addition, regulation of p42/p44 and p38 mitogen-activated protein (MAP) kinases in rheumatoid FLSs and its immortalized FLSs has not been reported.

Our present study achieved the first successful immortalization of FLSs obtained from RA patients by transfecting the cells with SV40 large T antigen. Constitutive activation of p42/p44 MAP kinase was detected in MH7A cells and seemed to be associated with its transforming property. The IL-1 type-I receptor was functionally expressed in the immortalized MH7A cells, as IL-1β induced

[1] To whom correspondence should be addressed at: Central Research Laboratories, Kissei Pharmaceutical Co., Ltd., 4365-1 Kashiwabara, Hotaka Minamiazumi, Nagano 399-8304; Tel: +81-263-82-8820, Fax: +81-263-82-8826

Abbreviations: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; IL, interleukin; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; MAP, mitogen-activated protein.

production of IL-6 and stromelysin-1, and the expression of ICAM-1. p38 MAP kinase was found to be involved in IL-6 and stromelysin-1 production, whereas p42/p44 MAP kinase was not. These results suggest that MH7A cells are a useful tool for investigating the regulation of the IL-1 signal transduction pathway in rheumatoid FLS and may be beneficial for the development of future RA therapy.

MATERIALS AND METHODS

Reagents—Recombinant human IL-1β, IL-6, and IFN-γ were obtained from Genzyme (Cambridge, MA). Anti-CD4 (Leu-3a)-FITC, anti-CD11c (Leu-M5)-PE, anti-CD14 (Leu-M3)-FITC, anti-CD16 (Leu-11a)-FITC, anti-CD18 (LFA-1β)-FITC, anti-CD40-FITC, and anti-CD64-FITC antibodies were obtained from Nippon Becton Dickinson Company (Tokyo). Anti-CD54 (Leu-54)-PE, anti-CD80-FITC, anti-CD121a (IL-1R type I), and anti-HLA-DR-FITC antibodies were purchased from Pharmingen (San Diego, CA). The compound PD098059 was purchased from Calbiochem (La Jolla, CA). The compound SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] was synthesized by the Central Research Laboratories, Kissei Pharmaceutical (Nagano) according to the procedure reported by Adams *et al.* (*13*). The preparation was determined to be >95% pure on the basis of high performance liquid chromatography and NMR analysis (data not shown).

Cells—Synovial tissue samples were obtained from patients with rheumatoid arthritis (RA) undergoing total joint replacement. All RA patients were evaluated by a certified rheumatologist and were diagnosed as having RA, according to the criteria of the American College of Rheumatology (*14, 15*). Written informed consent was obtained from each patient. In this study, most experiments were performed using synoviocytes derived from a 53-year-old female RA patient. Each tissue specimen was minced, and digested with 4 mg/ml collagenase for 2 h at 37°C. The cells were then plated in RPMI 1640 (Nikken, Kyoto) supplemented with 10% fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, New York). When the cells had grown to confluence, they were treated with trypsin/EDTA and split at a 1:4 ratio. For experiments, FLSs were used at passages 3 to 10.

Plasmid Construction—The DNA fragment carrying SV40 early region was obtained by digestion of pMT1 (*16*) with *Pvu*II and *Eco*RI. After creating *Bgl*II restriction sites at the ends of the SV40 early region, the fragment was inserted between the *Bam*HI sites of pSV2-neo (*17*) to construct pMT1-neo containing the SV40 T antigen gene and the gene for G418 resistance.

Transfection of Rheumatoid Synoviocytes with SV40 T Antigen Gene—The immortalized rheumatoid FLS line MA7A was established by transfection of FLSs with plasmid DNA containing the SV40 T antigen gene. Briefly, rheumatoid synoviocytes were harvested, and resuspended in RPMI 1640 medium to give a cell concentration of 2×10^6 cells/ml. A 500-μl volume of cell suspension and 10 μg of pMT1-neo plasmid were placed in a Bio-Rad 4-mm cuvette, and electroporation (Bio-Rad Gene Pulser, 0.29 kV, 960 μF) was then performed. The transfected cells were cultured in RPMI1640 medium supplemented with 20% FBS. A 100-μl aliquot of the cell suspension was then inoculated into a 96-well culture plate and incubated at 37°C in a humidified atmosphere with 5% CO_2. From 2 d after the transfection, cells were grown in the presence of 400 μg/ml G418 (GIBCO). No more than ten wells/plate were positive for growth, and such growth appeared to have originated from a single cell.

Measurement of DNA Synthesis—Cells were grown to subconfluence in 96-well tissue culture dishes, and the growth was arrested by incubation for 48 h in a serum-free medium consisting of RPMI 1640 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (ITS). The RPMI 1640/ITS medium was employed to maintain the FLSs in a quiescent but not catabolic state, which condition resembles that of healthy cells (*18*). The RPMI 1640/ITS medium was then removed, and fresh RPMI 1640 containing PDGF-BB was added to the quiescent cells. The cells were subsequently incubated with [^{3}H]-thymidine (46 kBq/ml) for 24 h. Next, ice-cold 10% trichloroacetic acid was added to each well, and the plates were kept at 4°C for 10 min. Trichloroacetic acid-insoluble materials were subsequently harvested onto Unifilter plates (GF/B 96, Packard) with a cell harvester. [^{3}H]-Thymidine incorporation was determined by scintillation counting.

Immunohistochemical Studies—Cells were grown on a chamber slide for 2 d, and were stained with Giemsa or by immunohistochemical techniques as described below. The culture medium was removed, and the cells were fixed for 20 min at room temperature in 95% ethanol. After fixation, the cells were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 20.

For the detection of SV40 large T antigen, nonspecific binding was blocked for 20 min at room temperature with 10% normal goat serum (Vector Laboratories, Burlingame, CA) diluted in PBS. Antibodies were diluted in PBS containing 1% bovine serum albumin (BSA). The optimal dilution for the mouse anti-SV40 T antigen monoclonal antibody (Cat. #DP02; CALBIOCHEM, Cambridge, MA) was determined to be 1:20. Cell-bearing slides were incubated with the primary antibody for 60 min at room temperature, and washed in PBS containing 0.05% Tween 20. The slides were subsequently incubated for 30 min with goat anti-mouse FITC-conjugated antibody (1:200) (Vector Labs.), washed 3 times in PBS plus 0.05% Tween 20, and mounted in VECTASHIELD mounting medium for fluorescence H-1000 (Vector Labs.). The negative control slides were incubated with prediluted nonimmune mouse IgG1 (1:500) (Vector Labs.) as a source of irrelevant antibody.

For the detection of p53 and RB, nonspecific binding was blocked for 20 min at room temperature with 10% normal horse serum (Vector Labs.) diluted in PBS. Antibodies were diluted in PBS containing 1% BSA. The optimal dilutions for mouse anti-human p53 monoclonal antibody (DAKO JAPAN, Tokyo) and mouse anti-human RB monoclonal antibody (PharMingen) were determined to be 1:50 and 1:300, respectively. The slides were incubated with the primary antibody for 60 min at room temperature, and then washed in PBS containing 0.05% Tween 20. The slides were subsequently incubated for 30 min with biotinylated horse anti-mouse secondary antibody (1:500) (Vector Labs.), and washed 3 times in PBS plus 0.05% Tween 20. Specific binding was amplified by ABC solution according to

the manufacturer's instructions (Vectastain Elite; Vector Labs.). The reaction product was visualized with 3-amino-9-ethyl carbazole (DAKO). The negative control was treated with prediluted nonimmune mouse IgG1 (Vector Labs.) as a source of irrelevant primary antibody.

Immunofluorescence Flow Cytometry—Cells were grown to confluence in 75-cm² tissue culture flasks, and were then incubated for 24 h with RPMI 1640 containing 10% FBS in the presence or absence of 1 ng/ml IL-1β or 200 units/ml IFN-γ. The cells were incubated on ice for 60 min with the monoclonal antibodies for CD4, CD11c, CD14, CD16, CD18, CD40, CD64, CD80, CD95, IL-1R type-I, ICAM-1, and HLA-DR. Isotype-matched control antibodies were also included. Then, cells were washed twice in PBS and analyzed with a FACScan flow cytometer (Nippon Becton Dickinson Company, Tokyo). For each sample, 5000 cells were counted. Data were plotted as the number of cells *versus* the log fluorescence intensity.

Quantification of IL-6—IL-6 was measured by a sandwich ELISA as described previously (*19*), using anti-human-IL-6 monoclonal antibody (2 μg/ml; R&D Systems, Minneapolis, MN) as the capture antibody and biotinylated purified anti-human-IL-6 polyclonal antibody (2 μg/ml; R&D) as the second antibody. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA). The minimum detection limit of the assay was 6.25 pg/ml.

Measurement of Stromelysin-1—Cells at confluence were cultured for 24 h in RPMI 1640 medium supplemented with 10% FBS. The culture supernatants were kept frozen until used. The concentration of stromelysin-1 was measured by using a specific ELISA kit (Fuji Chemical Industries, Toyama). The minimum detection limit of the assays was 12.5 ng/ml.

Western Blotting—Cellular extracts (20 μg/lane) were run on a 12% SDS-polyacrylamide gel following denaturation by boiling for 5 min in SDS loading buffer. After electrophoresis, proteins were transferred onto PVDF membranes by electroblotting (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) in 25 mM Tris, 192 mM glycine, 5% methanol at 15 V for 90 min. Membranes were probed with the following primary antibody overnight at 4°C: mouse monoclonal anti-SV40 small t antigen (Cat. #DP14; CALBIOCHEM, Cambridge, MA). After incubation for 1 h at room temperature with anti-mouse secondary antibody conjugated with horseradish peroxidase, specific bands were revealed by using ECL Western blotting detection reagents (Amersham). Blots were exposed to high-performance chemiluminescence film (Hyperfilm ECL; Amersham) for 0.5-2 min for detection.

Analysis of In Vivo p42/p44 and p38 MAP Kinase Activities—Cells were lysed on ice in 1 ml of cold lysis buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM Na_2EDTA, 0.5% NP40, 50 mM NaF, 0.5 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF]. Insoluble nuclear material was pelleted by centrifugation at 14,000 × *g* for 10 min at 4°C, and the supernatant (100 μg of total protein) was precleared with 30 μl of protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. Two microliters of rabbit polyclonal anti-p42/p44 or anti-p38 MAP kinase antibody (Santa Cruz, CA) was added to the precleared lysates, and incubated for 2 h at 4°C. The mixtures were further incubated for 1 h at 4°C after the addition of 15 μl of protein A-Sepharose beads. The immunoprecipitates were then washed twice with lysis buffer and twice with kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 10 mM $MgCl_2$, 2 mM DTT, and 0.1 mM Na_3VO_4] and resuspended in 30 μl of kinase assay buffer containing 1 μg of myelin basic protein (MBP) for p42/p44 MAP kinase assay or GST-ATF-2 fusion protein (Santa Cruz) for p38 MAP kinase assay, 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The reactions were incubated at 30°C for 30 min, terminated by the addition of 30 μl of 2×SDS-sample buffer containing 20 mM DTT, and then boiled for 5 min. The phosphorylation of the substrate proteins was examined by SDS-polyacrylamide gel electrophoresis (16 or 12% gel) followed by autoradiography.

Analysis of In Vivo MAPKAP Kinase-2 Activity—MAPKAP kinase-2 activity in human FLS lysates was measured by use of a MAPKAP kinase-2 immunoprecipitation assay kit obtained from Upstate Biotechnology (Lake Placid, NY). Briefly, cells were lysed in cold lysis buffer as described above. After centrifugation and normalization of the protein content, the extracts were mixed with 2 μg of sheep anti-MAPKAP kinase-2 antiserum for 2 h at 4°C. The MAPKAP kinase-2 and anti-MAPKAP kinase-2 immune complexes were precipitated with protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). The immunoprecipitates were then washed twice with lysis buffer and twice with kinase buffer, and resuspended in 30 μl of kinase assay buffer containing 100 μM substrate peptide KKLNRTLSVA (*20*), 50 μM ATP, and 10 μCi of [γ-^{32}P]ATP. The reactions were incubated at 30°C for 30 min. An aliquot of the reaction mixture was removed and blotted onto p81 phosphocellulose paper. The papers were washed twice in 1% acetic acid and twice in water, and then measured in a scintillation counter.

Statistics—Statistical analysis was performed by ANOVA and Scheffe's *F* test using the StatView 4.0 software program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered to be significant.

RESULTS

Establishment of Immortalized Human Rheumatoid FLS Line MH7A—Rheumatoid FLSs were transfected with plasmids carrying the SV40 T antigen gene and bacterial neomycin phosphotransferase gene, the latter of which confers resistance to the antibiotic geneticin (G418), and cultured in the presence of 400 μg/ml G418. No more than ten wells/plate were positive for growth, and such growth in each positive well appeared to have originated from a single cell. FLS clones were expanded into 75-cm² flasks containing 20 ml of RPMI 1640 supplemented with 10% FBS and 400 μg/ml G418, and subcultured at a 1:2 to 1:10 split ratio when the cells became confluent until they entered the crisis period (Fig. 1). During the crisis period, the cultures were kept by feeding weekly to maintain the living cells at a sufficient cell density. After several weeks, clusters of rapidly growing cells became visible, and were trypsinized and passaged at split ratios of 1:2, 1:4, or 1:8 according to their growth speed. Accumulated population doublings (PDLs) were calculated according to the following formula: PDLs = log (number of cells recovered/number seeded)/log 2.

SV40 T antigen-transformed FLSs derived from a 53-year-old female RA patient and the subsequently immortalized MH7A cells contained nuclear SV40 large T antigen protein as assessed by immunohistochemical staining (Fig. 2A). The level of p53 protein expression in both the SV40 T antigen-transformed FLSs and the immortalized MH7A cells was significantly higher than that in the parental FLSs (Fig. 3B). In contrast, the level of RB protein expression was not significantly different between parental and transfected FLSs (Fig. 3C). Nontransformed FLSs were uniformly negative for SV40 T antigen expression (data not shown), and unable to grow in the presence of 400 μg/ml G418.

Requirement of Growth Factors for MH7A Cells Compared with That for Parental FLSs—MH7A cells exhibited a spindle-like shape (Fig. 2B). They grew in RPMI 1640 supplemented with only 0.1% FBS, whereas a serum concentration ranging from 5 to 10% was required for

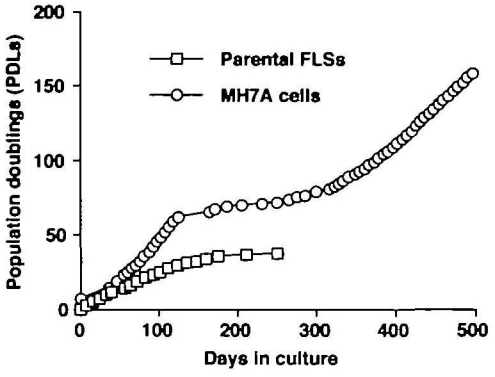

Fig. 1. **Cumulative growth curves for human rheumatoid FLSs transfected or not with a plasmid carrying the SV40 T antigen gene.** Estimations of population doublings of the parental FLSs (squares) and MH7A cells (circles) were started from the day after the transfection.

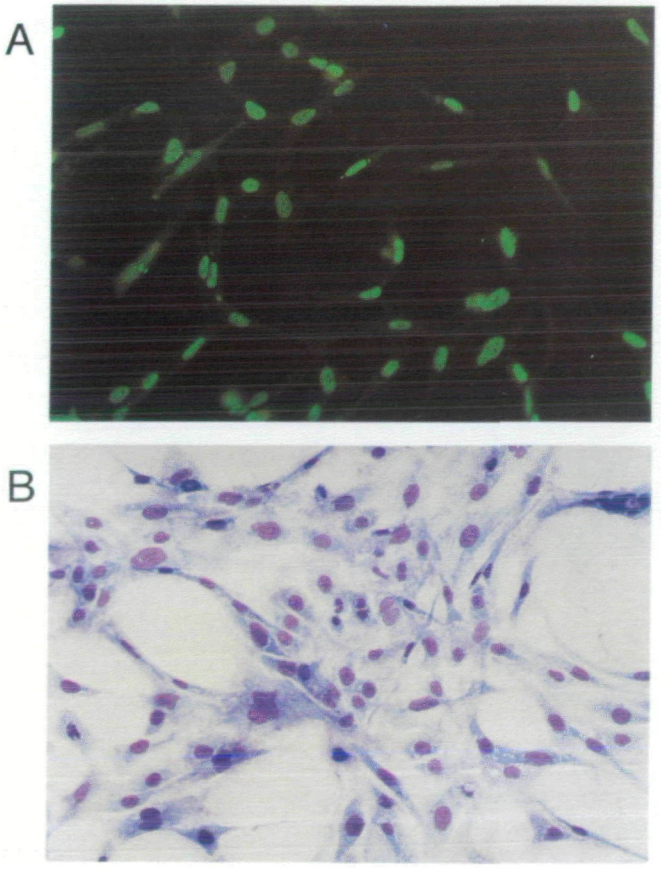

Fig. 2. **Morphology and SV40 large T antigen expression in MH7A cells.** MH7A cells were probed with anti-SV40 large T antigen antibody (A). Exponentially growing culture (B): note the spindle-like MH7A cells. Cells were stained with Giemsa staining (magnification × 200).

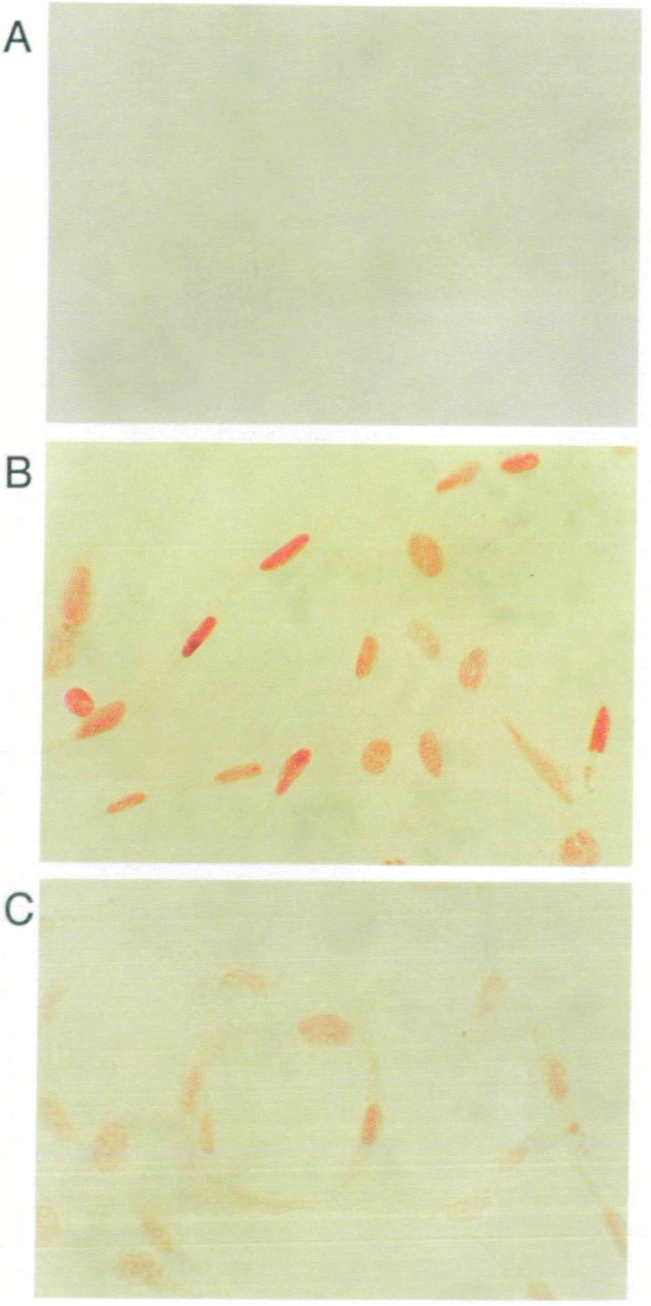

Fig. 3. **p53 and RB proteins expressed in MH7A cells.** MH7A cells were probed with nonimmune IgG (A), anti-p53 (B), and anti-RB (C) antibodies. Note that both p53 and RB proteins were localized in the nucleus of MH7A cells.

optimum growth of the parental rheumatoid FLSs. At serum concentrations of 1 and 10%, MH7A cells grew faster and at a higher density compared with the parental FLSs (Fig. 4A). Fully confluent parental cell cultures showed a density of approximately 15,000 cells/cm², whereas confluent MH7A cells were much more numerous—approximately 70,000 cells/cm².

PDGF-BB is a potent mitogen for FLSs (*21*). The growth of parental FLSs was arrested by incubating them for 48 h in a serum-free medium. The cells were then cultured in fresh RPMI 1640 medium containing various concentrations of PDGF-BB with [³H]thymidine (46 kBq/ml) for 24 h. PDGF-BB stimulated DNA synthesis of rheumatoid parental FLSs in a concentration-dependent manner (Fig. 4B). In contrast, MH7A cells kept proliferating and did not stay in a quiescent state (G_0/G_1 phase) under the serum-free condition (Fig. 4B). The addition of PDGF-BB did not significantly affect DNA synthesis (S phase) of the MH7A cells.

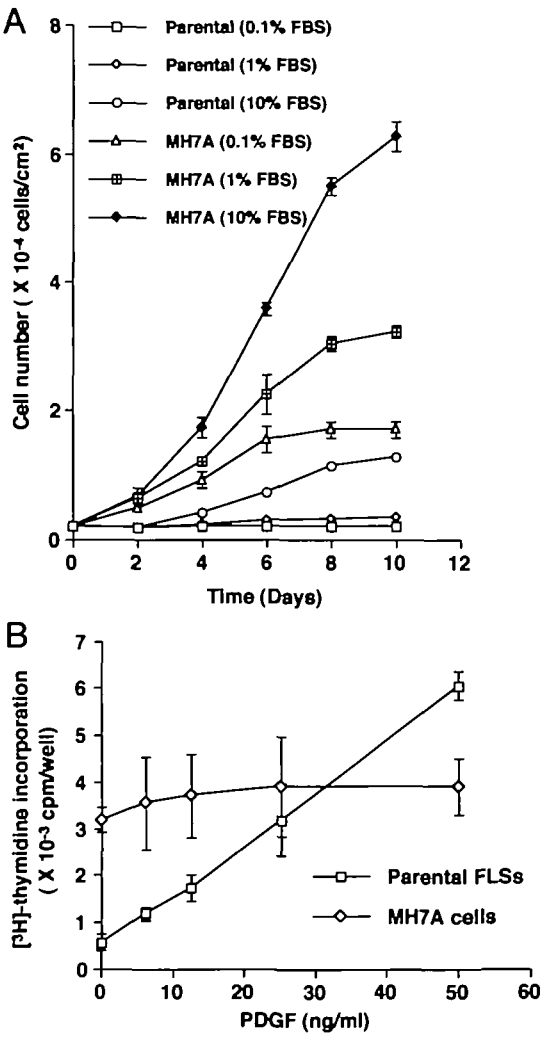

Fig. 4. **Growth factor requirements of MH7A cells and the parental FLSs.** A, parental FLSs and MH7A cells were plated at a density of 2×10^3 cells/cm² in RPMI 1640 with 10% FBS. After 24 h, the culture medium was replaced by RPMI 1640 medium containing various concentrations of FBS as indicated. The number of viable cells per well was calculated at 2, 4, 6, 8, and 10 days. Data are expressed as the mean ± SD of triplicate cultures. B, growth of MH7A cells and the parental FLSs was arrested by incubating them in RPMI 1640/ITS medium for 48 h. The cells were then cultured in fresh RPMI 1640 medium containing various concentrations of PDGF-BB with [³H]thymidine (46 kBq/ml) for 24 h. Data are expressed as the mean ± SD of sextuplicate cultures.

Immunophenotyping of MH7A Cells—Expression of various cell-surface molecules on the parental FLSs and MH7A cells was next determined by FACS analysis (Table I). IL-1 type-I receptor (CD121a) was constitutively expressed on the parental FLSs and MH7A cells. ICAM-1 (CD54) was spontaneously expressed on the parental FLSs and MH7A cells, and was further upregulated by IL-1β (Fig. 5), or IFN-γ stimulation (data not shown). HLA-DR was not detectable on the resting parental FLSs and MH7A cells, but was induced upon stimulation with IFN-γ. CD16, CD40, and CD95 were constitutively expressed on both parental FLSs and MH7A cells, and were not affected by IL-1β or IFN-γ. CD80 expression was detectable on MH7A cells, and was slightly enhanced upon IL-1β or IFN-γ stimulation; whereas it was not detectable on the parental FLSs, even with stimulation. No surface expression of CD4, CD11c, CD14, CD18, or CD64 was detectable on parental FLSs or MH7A cells with or without IL-1β or IFN-γ stimulation.

Effects of PD098059, SB203580, and Dexamethasone on the Surface Expression of ICAM-1 and on the Production of IL-6 and Stromelysin-1 by MH7A Cells and Parental FLSs—As the responsiveness to IL-1β to produce IL-6 and stromelysin-1 and to express ICAM-1 was retained in the immortalized MH7A cells, we analyzed the effects of PD098059, a selective inhibitor of the p42/p44 MAP kinase pathway (*22*); SB203580, a highly specific inhibitor of p38 MAP kinase (*23*); and dexamethasone on MH7A cells and the parental FLSs.

FLSs were stimulated with IL-1β for 24 h in RPMI 1640 supplemented with 10% FBS and then examined for ICAM-1 by FACS analysis. As shown in Fig. 5, dexamethasone (10^{-6} M) significantly inhibited IL-1β-induced ICAM-1 expression on the parental FLSs and MH7A cells. In contrast, PD098059 (50 μM) and SB203580 (10^{-5} M) did not affect it. CD80 expression on MH7A cells was regulated as well as the ICAM-1 expression (data not shown).

FLSs were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, and the resulting supernatants were tested

TABLE I. **Surface expression on MH7A cells and the parental FLSs.**[a]

Surface molecule	Parental FLSs	MH7A cells
CD4	−	−
CD11c	−	−
CD14	−	−
CD16 (FcγRIII)	+	+
CD18	−	−
CD40	+	+
ICAM-1 (CD54)	+	+
CD64 (FcγRI)	−	−
CD80 (B7-1)[b]	−	+
CD95 (Fas)	+	+
IL-1R type-I (CD121a)	+	+
HLA-DR[c]	+	+

[a]FACS analysis for each surface molecule was performed. Intensity of staining was judged as − or +: −, negative staining; +, positive staining. [b]After 24-h stimulation with IL-1β (1 ng/ml) or IFN-γ (200 units/ml). [c]After 24-h stimulation with IFN-γ (200 units/ml).

for IL-6 and stromelysin-1 by their specific ELISAs. Preliminary experiments were performed and indicated that IL-1β stimulated production of the IL-6 and stromelysin-1 in a concentration-dependent manner with a submaximal stimulation at 1 ng/ml (data not shown). As shown in Fig. 6, exposure of parental FLSs and MH7A cells to IL-1β (1 ng/ml) induced IL-6 and stromelysin-1 production. SB203580 (10^{-5} M) and dexamethasone (10^{-6} M) significantly inhibited IL-1β-induced IL-6 and stromelysin-1 production. In contrast, PD098059 (50 μM), an inhibitor of MEK1 and MEK2, did not affect IL-1β-induced synthesis of either one.

IL-1β-Induced p38 MAP Kinase Activity in MH7A Cells and Parental FLSs—p38 MAP kinase is activated by dual-specificity kinases that phosphorylate the threonine and tyrosine residues in its TGY motifs (*24*). p38 MAP kinase was immunoprecipitated from the cellular extracts with specific anti-p38 MAP kinase antibody, and *in vitro* kinase assays were performed using GST-ATF-2 fusion protein as a substrate. As shown in Fig. 7A, treatment of MH7A cells or parental FLSs with IL-1β increased their p38 MAP kinase activity.

In Vivo Activation of MAPKAP Kinase-2 in IL-1β-Induced MH7A Cells and Parental FLSs—MAPKAP kinase-2 is a substrate of p38 MAP kinase (*25*). To determine whether MAPKAP kinase-2 in MH7A cells and parental FLSs is activated by IL-1β, we immunoprecipitated MAPKAP kinase-2 from lysates of unstimulated or stimulated FLSs, and then performed *in vitro* kinase assays using the peptide KKLNRTLSVA as a substrate (*20*). As shown in Fig. 7B, MAPKAP kinase-2 was significantly activated by IL-1β and was inhibited by SB203580, but not by PD098059 or dexamethasone.

Constitutive Activation of p42/p44 MAP Kinase in MH7A Cells—p42/p44 MAP kinase is activated by dual-specificity kinases, MEK1 and MEK2 (*22*). p42/p44 MAP kinase was immunoprecipitated from the cellular extracts

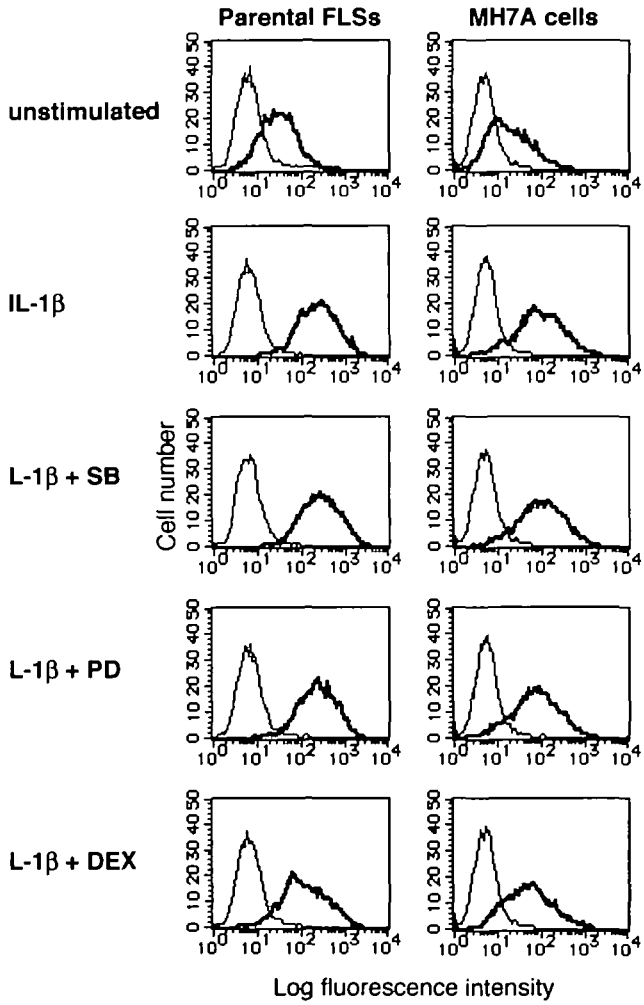

Fig. 5. **Effects of IL-1β alone or in combination with PD098059, SB203580, or dexamethasone on ICAM-1 expression of MH7A cells and parental FLSs.** Cells were incubated in 75-cm^2 culture flasks at a density of 1×10^4 cells/cm^2 for 24 h in the absence or presence of IL-1β (1 ng/ml). PD098059 (PD), SB203580 (SB), or dexamethasone (DEX) was added to IL-1β-containing cultures from the start of each culture. A minimum of 5×10^3 cells were counted for each sample with a FACScan. The fluorescence intensity is shown in log intervals. Staining patterns with specific antibodies (thick lines) and isotype-matched control antibodies (thin lines) are shown. Data are representative of three independent experiments.

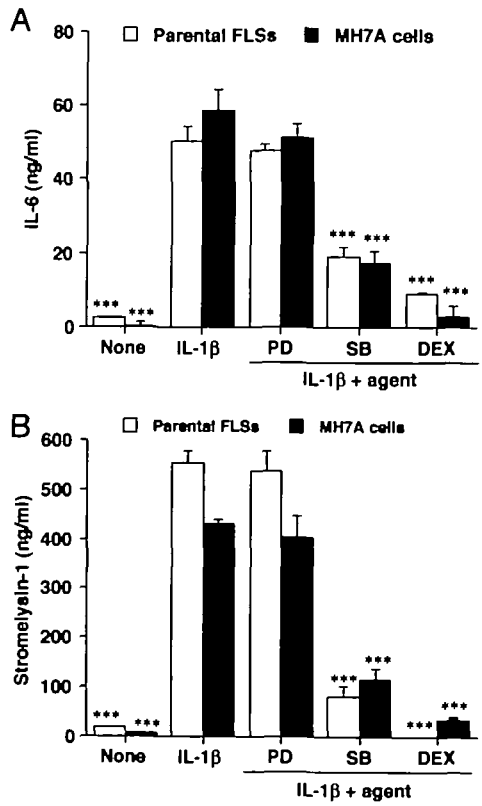

Fig. 6. **Effects of IL-1β, PD098059, SB203580, and dexamethasone on IL-6 and stromelysin-1 production by parental FLSs and MH7A cells.** Cells were incubated in 24-well culture plates for the IL-6 assay and in 6-well culture plates for the stromelysin-1 assay at a density of 1×10^4 cells/cm^2 for 24 h in the absence or presence of IL-1β (1 ng/ml). PD098059 (PD), SB203580 (SB), and dexamethasone (DEX) were added from the start of each culture. IL-6 (A) and stromelysin-1 (B) concentrations in the culture supernatants were assayed by specific ELISAs. IL-6 and stromelysin-1 concentrations in the culture medium (10% FBS/RPMI 1640) without cells was below the detection limit. Data are expressed as the mean $\pm$ SD of quadruplicate cultures. $^*p<0.05$, $^{***}p<0.001$ *vs.* the value in the presence of IL-1β alone.

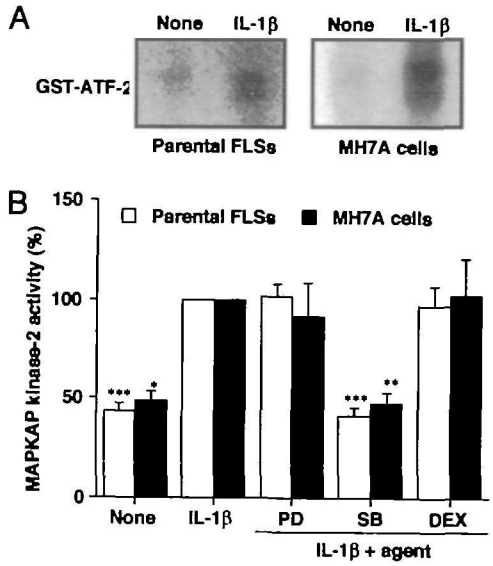

Fig. 7. ***In vivo* activation of p38 MAP kinase and MAPKAP kinase-2 by IL-1β in MH7A cells and the parental FLS.** A, cells were stimulated with IL-1β (1 ng/ml) for 30 min. p38 MAP kinase was immunoprecipitated from cell lysates, and *in vitro* kinase assays were performed using 10 μCi of [γ-^{32}P]ATP and 1 μg of GST-ATF-2 fusion proteins as a substrate. Data are representative of two independent experiments. B, cells were incubated in the absence or presence of PD098059 (PD), SB203580 (SB), or dexamethasone (DEX) for 1 h, and treated with IL-1β (1 ng/ml) for 30 min. MAPKAP kinase-2 was immunoprecipitated from cell lysates, and *in vitro* kinase assays were performed using 10 μCi of [γ-^{32}P]ATP and the substrate peptide KKLNRTLSVA (*20*). Data are expressed as the mean ± SD of triplicate cultures. The 100% value is for the cells stimulated with IL-1β alone. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* the value in the presence of IL-1β alone.

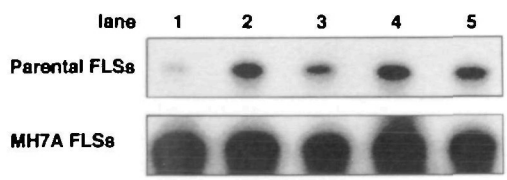

Fig. 8. **Effects of IL-1β, PD098059, SB203580, and dexamethasone on *in vivo* activation of p42/p44 MAP kinases in the parental FLSs and MH7A cells.** Cells were preincubated in the absence or presence of each agent for 1 h and then stimulated with IL-1β for 30 min. p42/p44 MAP kinases were immunoprecipitated from cell lysates, and *in vitro* kinase assays were performed using 10 μCi of [γ-^{32}P]ATP and 1 μg of myelin basic protein (MBP) as a substrate. Data are representative of two independent experiments. Lane 1, none; lane 2, IL-1β; lane 3, IL-1β and PD098059; lane 4, IL-1β and SB203580; lane 5, IL-1β and dexamethasone.

with specific anti-p42 and anti-p44 MAP kinase antibodies, and *in vitro* kinase assays were performed with MBP used as a substrate. As shown in Fig. 8, p42/p44 MAP kinase activity in the parental FLSs was clearly induced by IL-1β (lane 2). The activities were significantly inhibited by PD098059 (lane 3), but not by SB203580 (lane 4) or dexamethasone (lane 5). In contrast, p42/p44 MAP kinases were constitutively activated in MH7A cells, and were not affected by the treatment with IL-1β, PD098059, SB203580, or dexamethasone.

Expression of SV40 Small t Antigen in MH7A Cells—

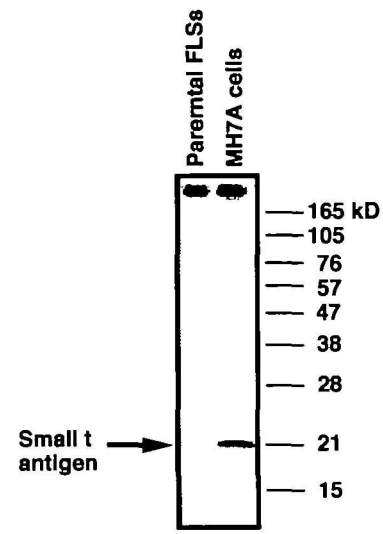

Fig. 9. **Expression of SV40 small t antigen by MH7A cells.** Cell lysates (20 μg/lane) were fractionated by SDS-PAGE and then transferred to PVDF membranes. Expression of SV40 small t antigen was analyzed using a specific antibody for SV40 small t antigen. Data are representative of two independent experiments.

p42/p44 MAP kinase is deregulated by SV40 small t antigen (*26*). Expression of SV40 small t antigen in MH7A cells was determined by Western blotting. As shown in Fig. 9, the 20.5 kDa SV40 small t antigen was constitutively expressed in MH7A cells. The 94-kDa SV40 large T antigen was also detected by Western blotting using a mouse monoclonal antibody specific for this antigen (data not shown).

DISCUSSION

We have described the first successful immortalization of rheumatoid FLSs by transfection of the cells with the SV40 T antigen gene. The immortalized MH7A cells expressed SV40-specific small t and large T antigen, and contained constitutively activated p42/p44 MAP kinases (Figs. 2 and 8). They have been growing rapidly to a higher density than the parental FLSs (Fig. 4). IL-1β induced the production of IL-6 and stromelysin-1, as well as the surface expression of ICAM-1, by MH7A cells in a manner similar to that seen for the parental FLSs (Figs. 5 and 6). p38 MAP kinase was involved in IL-1β-induced IL-6 and stromelysin-1 production (Figs. 6 and 7), whereas p42/p44 MAP kinase was not involved (Figs. 6 and 8). In fact, p38 MAP kinase was activated by IL-1β (Fig. 7).

RA is a chronic inflammatory disease characterized by the proliferation of the synovial membrane into a highly vascularized tissue known as a pannus. The pannus consists of several distinct cell types including resident FLSs and infiltrating mononuclear cells capable of producing inflammatory cytokines such as IL-1, TNF-α, and IL-6 (*27*). Several studies including ours have demonstrated that rheumatoid FLSs produce IL-6 upon stimulation with IL-1 or TNF-α (*8, 28*). IL-6 induces the production of acute-phase proteins by hepatocytes (*29*). It also facilitates differentiation of B cells and may contribute to the production of rheumatoid factors (*30*). In addition, IL-6 is involved in FcγRI expression on monocytes through the

induction of STAT family factors (*31*). Excessive production of IL-6 seems to be related to the immunological abnormalities associated with RA. Upon stimulation with IL-1, rheumatoid FLSs secrete stromelysin-1, which plays an important role in the degradation of the major macromolecular components of cartilage tissue, *i.e.*, collagen and proteoglycan (*5*).

Our knowledge relating to rheumatoid FLSs has largely been obtained from the isolation and *in vitro* culture of FLSs derived from RA patients. Rheumatoid FLSs, however, pose several problems that constrain their usefulness as *in vitro* models, *i.e.*, their limited source and growth potential, the requirement of a high serum concentration for growth, and lot-to-lot variability in functional assays. Therefore, we sought to establish an FLS line derived from RA patients, and succeeded in immortalizing FLSs obtained from an RA patient by transfecting the cells with the SV40 large T-antigen gene. The novel rheumatoid FLS line MH7A has now reached over 150 population doublings while passing through the culture crisis (Fig. 1). In contrast, nontransfected parental FLSs could not be passaged more than 10 times before losing their spindle-like morphology and many functional activities. Moreover, MH7A cells can be maintained under low serum concentrations, and can grow to cell densities 5 to 6 times greater than those of parental FLSs under the same culture conditions (Fig. 4). The expression of T antigen in our immortalized FLS line, MH7A, was associated with elevated cellular p53 protein expression (Fig. 3B). The level of p53 expression was very low in the parental FLSs and apparently very high in both the transformed and immortalized FLSs, whereas the level of Rb expression was quite equivalent between the parental FLSs and MH7A cells (Fig. 3C). The most important contribution of the SV40 large T antigen during the process of immortalization may be its ability to bind to p53 molecules, thus considerably stabilizing the protein by increasing its effective concentration (*32*). Recently, the involvement of p53 and Rb in regulating the M1 mechanism of human fibroblasts has been elucidated (*33, 34*).

The principal question in this work was whether or not MH7A cells would maintain the functional characteristics of the original rheumatoid FLSs. We found that MH7A cells maintained the IL-1 type-I receptor (CD121a), ICAM-1, CD16, CD40, CD80, and CD95 (Fas) (Table I). HLA-DR and that ICAM-1 expression was upregulated by IFN-γ. IL-1 is a potent inflammatory cytokine and one of the strongest inducers of IL-6, stromelysin-1, and ICAM-1 (*5, 8, 35*). MH7A cells retained their responsiveness to IL-1β to produce IL-6, and stromelysin-1 (Fig. 6), and to express ICAM-1 (Fig. 5). The capacity to produce large amounts of stromelysin-1 is considered one of the typical characteristics of rheumatoid synovial FLSs (*5-7*). Our present findings thus clearly indicate that MH7A cells retain several aspects of the original FLS phenotype.

The IL-1 signal transduction pathway in MH7A cells and parental FLSs was next characterized. IL-1 acts through its type-I receptor to induce gene expression (*36*). IL-1-responsive genes differ depending upon the cell type (*37*). Well-characterized transcription factors such as activator protein (AP)-1, nuclear factor (NF)-κB, and C/EBPβ have been implicated in the regulation of IL-1-responsive genes (*38*). In fact, NF-κB binding activity to the human IL-6 promoter gene was significantly upregulated in MH7A cells upon stimulation with IL-1β (*19*). The activity of these transcription factors is regulated by phosphorylation (*39, 40*). IL-1 activates several distinct protein kinases including the three different kinds of MAP kinases, *i.e.*, p42/p44, p38 MAP kinases, c-jun NH_2-terminal kinase (JNK), and a β casein kinase (*38*). In the present study, p42/p44 and p38 MAP kinases were analyzed. p42/p44 MAP kinase was constitutively activated in MH7A cells (Fig. 8). It is reported that activation of p42/p44 MAP kinase pathway and stimulation of cell growth are dependent on the interaction between small t antigen and protein phosphatase 2A (*26*). As expected, SV40 small t antigen was clearly expressed in MH7A cells (Fig. 9). SV40 small t antigen is essential for the morphological transformation of human fibroblasts (*41*), suggesting that small t antigen may be involved in the p42/p44 activation. In the parental FLSs, p42/p44 MAP kinases were induced by IL-1β, and significantly suppressed by a selective inhibitor of MEK1 and MEK2 (Fig. 8). Neither IL-6, stromelysin-1, nor ICAM-1 synthesis by the parental FLSs or MH7A cells was affected by PD098059 (Figs. 5 and 6), indicating that the p42/p44 MAP kinase pathway is not involved in the production of IL-6, stromelysin-1, or ICAM-1 by rheumatoid FLSs.

p38 MAP kinase was activated by IL-1β (Fig. 7A). MAPKAP kinase-2, a substrate of p38 MAP kinase, was also activated by IL-1β (Fig. 7B), clearly indicating that p38 MAP kinase activity is induced in response to IL-1β *in vivo*. The p38 MAP kinase inhibitor SB203580 inhibited the induction of p38 MAP kinase activity (Fig. 7B) and at the same time significantly suppressed IL-6 and stromelysin-1 production by the parental FLSs and MH7A cells stimulated with IL-1β (Fig. 6). These results strongly implicate the critical role of p38 MAP kinase in the IL-1 signal leading to the synthesis of IL-6 and stromelysin-1 in rheumatoid FLSs.

Glucocorticoids have long been considered the most effective treatment for RA. Dexamethasone significantly inhibited the production of IL-6 and stromelysin-1 and the expression of ICAM-1 upon stimulation of MH7A cells with IL-1β in a way similar to that observed for the parental FLSs (Figs. 5 and 6). This finding suggests that MH7A cells retain functional glucocorticoid receptors. IL-1-inducible and glucocorticoid-sensitive molecules expressed in the rheumatoid MH7A line warrant further investigation.

Our immortalized rheumatoid FLS line, MH7A, expressed constitutively active p42/p44 MAP kinase, proliferated faster than the parental FLSs in the absence of growth factors, and still responded to the IL-1 signal. Rheumatoid FLSs respond to factors produced by macrophages and T cells, or alternatively, are irreversibly altered in rheumatoid status to autonomous activation—for RA FLSs, under some circumstances, proliferate in an anchorage-independent manner (*2*). Our present findings extend our knowledge concerning the pathophysiology of the rheumatoid synovium and may support the concept that infectious viruses such as Epstein-Barr virus (EBV), human T cell lymphotropic virus type I (HTLV-I), parvovirus, and cytomegalovirus might contribute to the pathogenesis of RA (*42, 43*). The MH7A cell is expected to prove useful for research on rheumatoid FLSs, particularly on the IL-1 signal transduction pathway, and may greatly facilitate our understanding of the pathogenesis of RA and thereby aid future therapeutic development.

We thank Dr. Takeo Ohmura (Wakamoto Pharmaceutical Co., Ltd.) for providing us with the pMT1 plasmid.

REFERENCES

1. Firestein, G.S. (1996) Invasive fibroblast-like synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* **39**, 1781-1790
2. Lafyatis, R., Remmers, E.F., Roberts, A.B., Yocum, D.E., Sporn, M.B., and Wilder, R.L. (1989) Anchorage-independent growth of synoviocytes from arthritic and normal joints: stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-β and retinoids. *J. Clin. Invest.* **83**, 1267-1276
3. Dayer, J.M., Krane, S.M., Russel, G.G., and Robinson, D.R. (1976) Production of collagenase and prostaglandins by isolated adherent synovial cells. *Proc. Natl. Acad. Sci. USA* **73**, 945-949
4. Chin, J.R., Murphy, G., and Werb, Z. (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. *J. Biol. Chem.* **260**, 12367-12376
5. MacNaul, K.L., Chartrain, N., Lark, M., Tocci, M.J., and Hutchinson, N.I. (1990) Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinase-1 in rheumatoid human synovial fibroblasts: synergistic effects of interleukin-1 and tumor necrosis factor-alpha on stromelysin expression. *J. Biol. Chem.* **265**, 17238-17245
6. Taylor, D.J., Cheung, N.T., and Dawes, P.T. (1994) Increased serum proMMP-3 in inflammatory arthritides: a potential indicator of synovial inflammatory monokine activity. *Ann. Rheum. Dis.* **53**, 768-772
7. Manicourt, D.-H., Fujimoto, N., Obata, K., and Thonar, E.-M.A. (1995) Levels of circulating collagenase, stromelysin-1, and tissue inhibitor of matrix metalloproteinase 1 in patients with rheumatoid arthritis. *Arthritis Rheum.* **38**, 1031-1039
8. Guerne, P.A., Zuraw, B.L., Vaughan, J.H., Carson, D.A., and Lotz, M. (1989) Synovium as a source of interleukin 6 in vitro. *J. Clin. Invest.* **83**, 585-592
9. Lamaire, R., Flipo, R.M., Monte, D., Dupressoir, T., Duquesnoy, B., Cesbron, J.Y., Janin, A., Capron, A., and Lafyatis, R. (1994) Synovial fibroblast-like cell transfection with the SV40 large T antigen induces a transformed phenotype and permits transient tumor formation in immunodeficient mice. *J. Rheumatol.* **21**, 1409-1419
10. Shimozato, O., Watanabe, N., Goto, M., and Kobayashi, Y. (1996) Cytokine production by SV40-transformed adherent synovial cells from rheumatoid patients. *Cytokine* **8**, 99-105
11. Zhang, H.G., Blackburn, W.D.J., and Minghetti, P.P. (1997) Characterization of a SV40-transformed rheumatoid synovial fibroblast cell line which retains genotypic expression patterns: a model for evaluation of anti-arthritic agents. *In Vitro Cell. Dev. Biol. Animal.* **33**, 37-41
12. Haas, C., Aicher, W.K., Dinkel, A., Peter, H.H., and Eibel, H. (1997) Characterization of SV40T antigen immortalized human synovial fibroblasts: maintained expression patterns of EGR-1, HLA-DR and some surface receptors. *Rheumatol. Int.* **16**, 241-247
13. Adams, J.L., Gallagher, T.F., Lee, J.C., and White, J.R. (1993) Vol. WO 93/14081
14. Arnett, F.C., Edworthy, G.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kapkan, S.R., Liang, M.H., Luthra, H.S., Medsger, T.A. J., Mitchell, D.M., Neustadt, D.H., Pinals, R.S., Schaller, J.G., Sharp, J.T., Wilder, R.L., and Hunder, G.G. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* **31**, 315-324
15. Altman, R., Asch, E., Bloch, D., Bole, G., Borenstein, D., Brandt, K., Christy, W., Cooke, T.D., Greenwald, R., Mochberg, M., Howell, D., Kaplan, D., Koopman, W., Longley, S., Mankin, H., McShane, D.J., Medsger, T., Meenan, R., Mikkelsen, W., Moskowitz, R., Murphy, W., Rothschild, B., Segal, M., Sokoloff, L., and Wolfe, F. (1986) Development of criteria for the classification and reporting of osteoarthritis. *Arthritis Rheum.* **29**, 1039-1049
16. Sugano, S. and Yamaguchi, N. (1984) Two classes of transformation-deficient, immortalization-positive simian virus 40 mutants constructed by making three-base insertion in the T antigen gene. *J. Viol.* **52**, 884-891
17. Southern, P.J. and Gerg, P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Gen.* **1**, 327-341
18. Libby, P. and O'brien, K.V. (1983) Culture of quiescent arterial smooth muscle cells in a defined serum-free medium. *J. Cell. Physiol.* **115**, 217-223
19. Miyazawa, K., Mori, A., Yamamoto, K., and Okudaira, H. (1998) Transcriptional roles of C/EBPβ, NF-$\varkappa$B, and CBF1 in IL-1β-induced IL-6 synthesis by human rheumatoid fibroblast-like synoviocytes. *J. Biol. Chem.* **273**, 7620-7627
20. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A.R. (1994) Identification of a novel protein kinase cascade stimulated by chemical stress and heat shock which activates MAP kinase-activated protein MAPKAP kinase-2 and induces phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037
21. Remmers, E.F., Lafyatis, R., Kumkunian, G.K., Case, J.P., Roberts, A.B., Sporn, M.B., and Wilder, R.L. (1990) Cytokines and growth regulation of synoviocytes from patients with rheumatoid arthritis and rats with streptococcal cell wall arthritis. *Growth Factors* **2**, 179-188
22. Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., and Saltiel, A.R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase, MAP kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494
23. Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., and Lee, J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homolog which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229-233
24. Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McMulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., and Young, P.R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739-746
25. Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039-1049
26. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993) The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. *Cell* **75**, 887-897
27. Feldmann, M., Brennan, F.M., and Maini, R.N. (1996) Role of cytokines in rheumatoid arthritis. *Annu. Rev. Immunol.* **14**, 397-440
28. Miyazawa, K., Mori, A., Yamamoto, K., and Okudaira, H. (1998) Constitutive transcription of the human IL-6 gene by rheumatoid synoviocytes: Spontaneous activation of NF-$\varkappa$B and CBF1. *Am. J. Pathol.* **152**, 793-803
29. Hirano, T., Matsuda, T., Uruner, M., and Kishimoto, T. (1988) Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. *Eur. J. Immunol.* **18**, 1797-1801
30. Akira, S., Taga, T., and Kishimoto, T. (1993) Interleukin-6 in biology and medicine. *Adv. Immunol.* **56**, 1-78
31. Sengupta, T.K., Chen, A., Zhong, Z., Darnell, J.E., and Ivashkiv, L.B. (1995) Activation of monocyte effector genes and STAT family transcription factors by inflammatory synovial fluid is independent of interferon γ. *J. Exp. Med.* **181**, 1015-1025
32. Banerjee, A., Srivatsan, E., Hashimoto, T., Takahashi, R., Xu, H.-J., Hu, S.-X., and Benedict, W.F. (1992) Immortalization of fibroblasts from two patients with hereditary retinoblastoma. *Anticancer Res.* **12**, 1347-1354
33. Hara, E., Tsurui, H., Shinozaki, A., Nakada, S., and Oda, K. (1991) Cooperative effect of antisense-Rb and antisense-p53

oligomers on the extension of life span in human diploid fibroblasts, TIG-1. *Biochem. Biophys. Res. Commun.* **179**, 528-534
34. Shay, J.W., Pereira-Smith, O.M., and Wright, W.E. (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* **196**, 33-39
35. Chin, J.E., Winterrowd, G.E., Krzesicki, R.F., and Sanders, M.E. (1990) Role of cytokines in inflammatory synovitis: the coordinate regulation of intracellular adhesion molecule 1 and HLA class I and class II antigens in rheumatoid synovial fibroblasts. *Arthritis Rheum.* **33**, 1776-1786
36. Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, F., Fe, F., Mantovani, A., Shanebeck, K., Grabstein, K.H., and Dower, S.K. (1993) Interleukin-1 signaling occurs exclusively via the type I receptor. *Proc. Natl. Acad. Sci. USA* **90**, 6155-6159
37. Dinarello, C.A. (1996) Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095-2147
38. O'Neill, L.A.J. (1995) Towards an understanding of the signal transduction pathways for interleukin 1. *Biochim. Biophys. Acta* **1226**, 31-44
39. Wulczyn, F.G., Krappmann, D., and Scheidereit, C. (1996) The NF-κB/Rel and IκB gene families: mediators of immune response and inflammation. *J. Mol. Med.* **74**, 749-769
40. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA* **90**, 2207-2211
41. Ronde, A.D., Sol, C.J.A., Strien, A.V., Schegget, J.T., and Noordaa, J.V.D. (1989) The SV40 small T-antigen is essential for the morphological transformation of human fibroblasts. *Virology* **171**, 260-263
42. Murayama, T., Tsuchiya, N., Jisaki, F., Ozaki, M., Sakamuro, D., Hirai, K., Shimizu, S., Ito, K., Matsushima, K., and Furukawa, T. (1994) Elevated cytokine levels in synovial fluid of rheumatoid arthritis correlates with the presence of cytomegalovirus genome. *Autoimmunity* **17**, 333-337
43. Harris, E.D. (1990) Rheumatoid arthritis: Pathophysiology and implication for therapy. *N. Engl. J. Med.* **322**, 1277-1289