ANTICANCER RESEARCH *25*: 1983-1990 (2005)

Cell Cycle Arrest and Apoptosis Induced by SART-1 Gene Transduction

MAMI HOSOKAWA[1,2], RITSUKO KADOTA[2], SHIGEKI SHICHIJO[3], KYOGO ITOH[3], IGOR DMITRIEV[4], VICTOR KRASNYKH[4], DAVID T. CURIEL[4], YOICHI TAKUE[1], HIRO WAKASUGI[5], SHIGEMITSU TAKASHIMA[2] and YUJI HEIKE[1,2,5]

[1]*Blood and Stem Cell Transplantation Unit, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045;*
[2]*Department of Clinical Research, National Shikoku Cancer Center, 13-Horinouchi, Matsuyama, Ehime 790-0007;*
[3]*Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy and Department of Immunology, Kurume University School of Medicine, Asahi-machi 67, Kurume, Fukuoka 830-0011, Japan;*
[4]*Gene Therapy Center, University of Alabama at Birmingham, BMR2 502, 901 19th Street South, Birmingham, AL 35294-2172, U.S.A.;*
[5]*Pharmacology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*

Abstract. *The biological function of the SART-1 gene product is demonstrated and its potential as a target for cancer gene therapy is discussed. Materials and Methods: The SART-1 gene was transduced by a recombinant adenovirus vector and its expression was promoted by a CMV promoter. Results: The transduction efficiency by recombinant adenoviruses in A549 and MCF-7 cells was determined using a vector expressing luciferase, which showed high expression in the cells. Cell count analysis using Trypan-Blue dye exclusion showed that SART-1 gene transduction inhibited cell growth. Flow cytometry analysis suggested that SART-1 gene transduction induced cell cycle arrest followed by apoptosis. Western blot analysis confirmed that the apoptosis pathway was activated by SART-1 gene transduction. Conclusion: These results show that SART-1 gene transduction induces cell cycle arrest leading to apoptosis and suggest the possibility of gene therapy against cancer. In addition, SART-1 is known to be a tumor antigen in a range of cancers recognized by T cells, thus a potential strategy would be the combination of suicide gene therapy with immuno-gene therapy.*

Since the MAGE-1 gene product was identified as a tumor rejection antigen by Boon *et al.*, numerous tumor rejection antigens recognized by cytotoxic T lymphocytes (CTLs) have been identified (1-3). Computer software to predict antigen peptide sequences from cDNA databases has recently become available and researchers seeking tumor antigens have reported that some identified sequences are active *in vivo*. SART-1 is ubiquitously expressed in various cancers, including breast, esophagus, lung and uterine cancers (4-10). SART-1 encodes both the SART-1_{259} antigen, expressed in the cytosol of epithelial cancers and the SART-1_{800} antigen, expressed in the nuclei of most proliferating cells (11-15). Peptides derived from the gene are known to be tumor-derived antigens recognized by HLA A2601- and A2402-restricted CTLs. There have been several reports on SART-1-derived peptides capable of inducing CTLs (16, 17). The HLA-A26 allele is found in 22% of the Japanese population, 17% of Caucasians and 16% of Africans, while the HLA-A24 allele is found in 60% of Japanese, 20% of Caucasians and 12% of Africans (18). Therefore, SART-1 may be an ideal target molecule in specific immunotherapy for cancer patients. However, in contrast to its immunological properties, the function of SART-1 has not been elucidated.

Gene transfer into mammalian cells using viral vectors provides a powerful tool for gene therapy. Vectors include retroviruses derived from the Mouse Moloney Leukemia virus, human immunodeficiency virus and herpes virus (19, 20). With regard to safety, efficiency and specificity, adenoviruses are superior to other vectors. Adenovirus vectors are able to transduce genes into non-replicating / poorly-replicating cells, while other viral vectors, including retrovirus vectors, require cell proliferation for sufficient gene transduction. This suggests that adenovirus vectors have many advantages when used as vehicles for gene transfer (21).

The RGD-fiber-modified recombinant adenovirus, in which the Arg-Gly-Asp (RGD)-containing peptide is incorporated into the HI-loop of the fiber knob domain, exhibits high transduction and expression efficiency when

Correspondence to: Yuji Heike, MD, PhD, Pharmacology Division, National Cancer Center Research Instiute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3547-5228, e-mail: yheike@ncc.go.jp

Key Words: Tumor rejection antigen, SART-1, cell cycle, apoptosis.

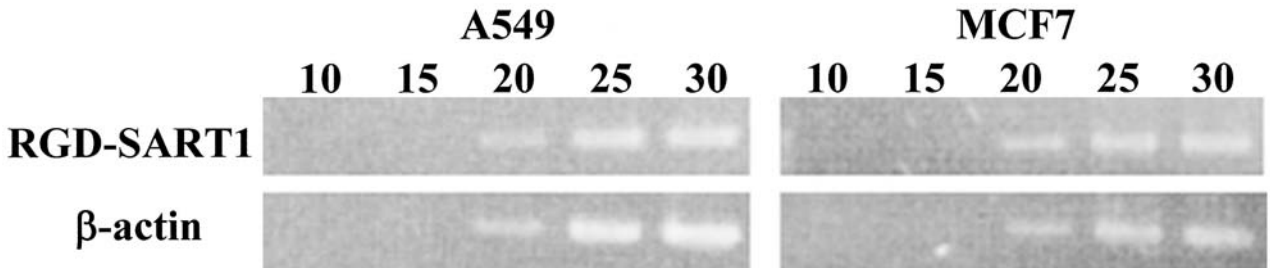


Figure 1. *Expression of SART-1 mRNA after Ad-RGD-SART-1 treatment. Cells were treated with Ad-RGD-SART-1 at 30 M.O.I for 24 h. RNA from the cells was then isolated and SART-1 mRNA was quantified by RT-PCR. The data indicate the mean and standard deviation of three independent results.*

compared with conventional recombinant adenoviruses, increasing transduction efficiency into cancer cells by two to three orders of magnitude (22).

In our experiments, the SART-1 gene was transduced into two different cancer cell lines using the RGD-fiber-modified recombinant adenovirus (Ad-RGD-SART-1) and the biological functions of the gene product, particularly on the cell cycle, cell viability and cell cycle/apoptosis-associated proteins, were analyzed. The possibility of SART-1 gene therapy is also discussed.

Materials and Methods

Cells. 293 cells (human kidney epithelial cells transformed with adenovirus 5 DNA), MCF7 cells (human breast cancer cells) and A549 cells (human non-small cell lung cancer cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 293 and MCF7 cells were cultured in DMEM/F12 supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA, USA), penicillin (100 U/ml) (Invitrogen Corp.) and streptomycin (100 mg/ml) (Invitrogen Corp.), in a humidified 5% CO_2 atmosphere at 37˚C. The A549 cells were cultured in RPMI1640 supplemented with 10% v/v heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml), in a humidified 5% CO_2 atmosphere at 37˚C.

All experiments using recombinant adenovirus were performed in a biological safety cabinet (Level 2, Sanyo, Tokyo, Japan), in accordance with institutional regulations.

Preparation of Ad-RGD-SART-1 and Ad-RGD-Luc. Ad-RGD-SART-1 and Ad-RGD-Luc are E1-deleted recombinant adenoviruses that encode SART-1 and luciferase, respectively, under the control of a hybrid promoter consisting of a cytomegalovirus (CMV). They also contain fibers with the RGD motif in the HI loop. Purification and concentration of the recombinant adenoviruses was performed using conventional CsCl gradient methods and an ultracentrifuge (Beckman, Fullerton, CA, USA), as described previously (23). Purified adenovirus was dialyzed against phosphate-buffered saline (PBS) without calcium and magnesium (PBS(-)) containing 10% glycerol, at 4˚C for 12 h. Dialyzed viral solutions were either used immediately or stored at –130˚C until used.

The number of viral particles within each solution was determined according to the following formula:

1OD260 = 7x10e11 viral particles.

The multiplicity of infection (M.O.I) was determined by plaque assay using 293 cells.

Evaluation of SART-1 gene expression by RT-PCR. Transfer and expression of SART-1 by Ad-RGD-SART-1 was tested at 30 M.O.I. Twenty-four h after transduction, the A549 and MCF7 cells were lysed and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). The expression levels of SART-1 mRNA were evaluated by reverse transcription polymerase chain reaction (RT-PCR) using specific primers (5'-TCACTTGGTGATGG TGTTCG-3' and 5'-AAGCAGCTGGAGAAGGGACG-3'). The expression of β-actin mRNA was also evaluated as an internal control using specific primers (5'-CTAGAAGCATTTGCGG TGGA-3' and 5'-ATGGATGATGATATCGCCGC -3'). Amplification was performed with 30 cycles of 1 min at 95˚C, 1.5 min at 57˚C and 2 min at 72˚C, with samples being taken every 5 cycles. Amplified DNA was electrophoresed in 1% agarose gels and PCR bands were detected and quantified.

Effects of SART-1 gene transduction on cell growth. To determine the effects of SART-1 transduction on cell growth, the viable cells were counted at 24, 48 and 72 h after treatment with 30 M.O.I of Ad-RGD-SART-1 and Ad-RGD-Luc. The percentage of cell growth was calculated using the following formula:
Number of viable cells treated with virus on day X / Number of viable cells without virus on day X x100(%).

Analysis of cell cycle by flow cytometry. The cells were treated with 30 M.O.I of Ad-RGD-SART-1 or Ad-RGD-Luc and were cultured for 24 or 48 h. Cells in 6-well plates were collected by Trypsin-EDTA (Invitrogen Corp.) treatment and were re-suspended in culture medium. The re-suspended cells were washed with PBS(-), and were fixed with 70% ethanol at 4˚C for 48 h. The fixed cells were incubated in lysis buffer containing 0.1% TritonX-100 and 0.1% RNaseA at 4˚C for 24 h. To evaluate DNA content, propidium iodide (P.I.) solution was added to the samples (final concentration; 25 μg/ml). P.I. fluorescence of nuclei was measured with a FACScan (Becton Dickinson Co., Franklin Lakes, NJ, USA) and data were obtained from 10^4 cells per sample.

Analysis of cell cycle and apoptosis by Western blot analysis. The cells were treated with Ad-RGD-SART-1 or Ad-RGD-Luc at 30 M.O.I for 1 h, followed by additional cultures. At 0, 24, 48 and 72 h after transduction, the cells were washed twice with PBS(-), scraped off the plate and lysed in cell lysis buffer (1% SDS, 1 mM sodium ortho-vanadate, 10 mM Tris-HCl, pH 7.4). Twenty μg of lysed material was

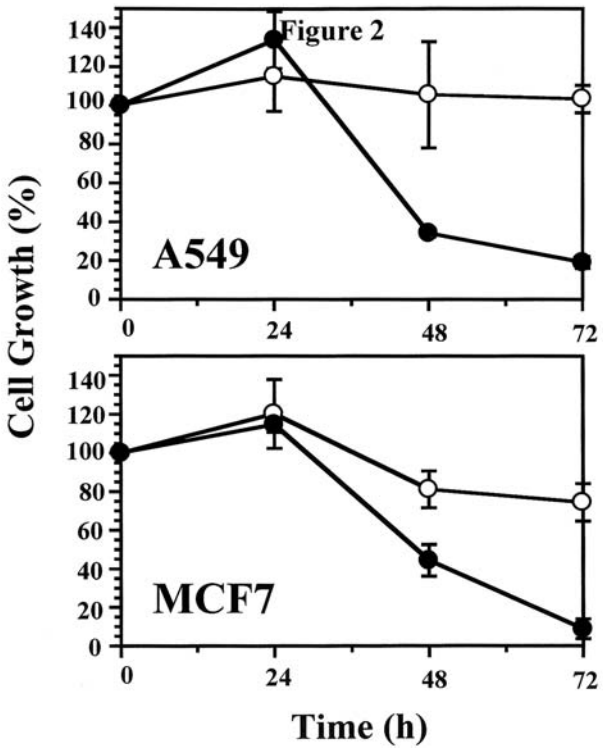


Figure 2. *Effects of SART-1 gene transduction on cell growth. To determine the effects of SART1 gene transduction on cell growth, the viable cells after treatment with 30 M.O.I of Ad-RGD-SART1 (●) and Ad-RGD-Luc (○) were counted. Twenty-four hours after infection, cells were cultured for a further 72 h. Each point represents the mean and standard deviation of triplicate cultures.*

electrophoretically separated on 7.5%, 10% or 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Primary antibodies supplied with Cell Cycle Sampler Kits I and II, and Apoptosis Sampler Kits I and II (Transduction Laboratories, Lexington, KY, USA) were used. The membranes were treated with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions in order to analyze protein expression.

Results

Transduction efficiency of adenovirus. In preliminary experiments, we tested the transduction efficiency of Ad-RGD in A549 and MCF7 cells using Ad-RGD-Luc (Data not shown). The results suggested that transduction at 30 M.O.I induced sufficient gene expression in both cells. Figure 1 shows the expression of SART-1 mRNA in the cells. The expression of SART-1 mRNA in the MCF7 and A549 cells was detected at 20, 25 and 30 cycles, but was not detected in the Ad-RGD-Luc-treated cells. SART-1 expression in Ad-RGD-SART-1 was also detected by Northern hybridization (Data not shown).

Cell growth inhibition by SART-1 gene transduction. Figure 2 shows the growth inhibition induced by SART-1 gene transduction. SART-1 gene transduction at 30 M.O.I induced strongly inhibited cell growth in a time-dependent manner. The growth inhibition rates of the A549 and MCF7 cells at 72 h after infection were 81.0% and 91.2%, respectively.

Cell cycle inhibition after SART-1 gene transduction. At 24 and 48 h after infection, the cells were collected and cell cycle status and apoptosis were analyzed by flow cytometry. Figure 3 shows that G1 and G2 arrest followed by cell death was observed after Ad-RGD-SART-1 treatment.

Western blot analysis of molecules associated with cell cycle and apoptosis. The expression of 24 cell cycle-related proteins and 22 apoptosis-related proteins was evaluated by Western blot analysis. Figure 4 shows the results for proteins influenced by treatment with Ad-RGD-SART-1. Figure 5 summarizes the results for cell cycle (A549, Figure 5A; and MCF7, Figure 5B) and apoptosis (A549, Figure 5C; and MCF7, Figure 5D) cascades. The expressions of Cyclin A, Cyclin B, CDK2, Rb and Rb2 increased, while the expressions of CDC25B, Kip1/p27, Mad2, p53 and RBBP decreased in A549 cells treated with Ad-RGD-SART-1 (Figure 5A). In MCF7 cells, Cyclin B, Rb and Rb2 increased or stayed at the same level, and Kip1/p27, Mad2 and p53 decreased (Figure 5B). Among apoptosis-related proteins, Bax, BRAUCE, Fas Ligand, hILP, PARP, p53, RIP and TRADD decreased in A549 cells treated with Ad-RGD-SART-1 (Figure 5C). In MCF7 cells, Bax, Bcl-2, hILP, PARP, p53, RIP and TRADD decreased (Figure 5D).

Discussion

Although numerous clinical trials of cancer gene therapy have been performed, there have been no reports of their clinical usefulness to date. The reasons that gene therapy is currently unable to yield clinical benefits are: i) insufficient gene transduction efficiency of vectors; and ii) insufficient therapeutic potency of the transduced gene products. For example, gene therapy using cell cycle-associated proteins, such as p53, and gene therapy using prodrugs, such as herpes simplex virus thymidine kinase, require high transduction efficiency to induce their therapeutic effects. There are no vector systems that exhibit sufficient transduction efficiency to provide therapeutic benefits. Although these strategies may result in local control of the tumor, there is no therapeutic benefit against metastatic lesions. Another cancer gene therapy strategy is immuno-gene therapy, using gene-modified cytotoxic T lymphocytes or administration vaccines in the form of gene-modified tumor cells or

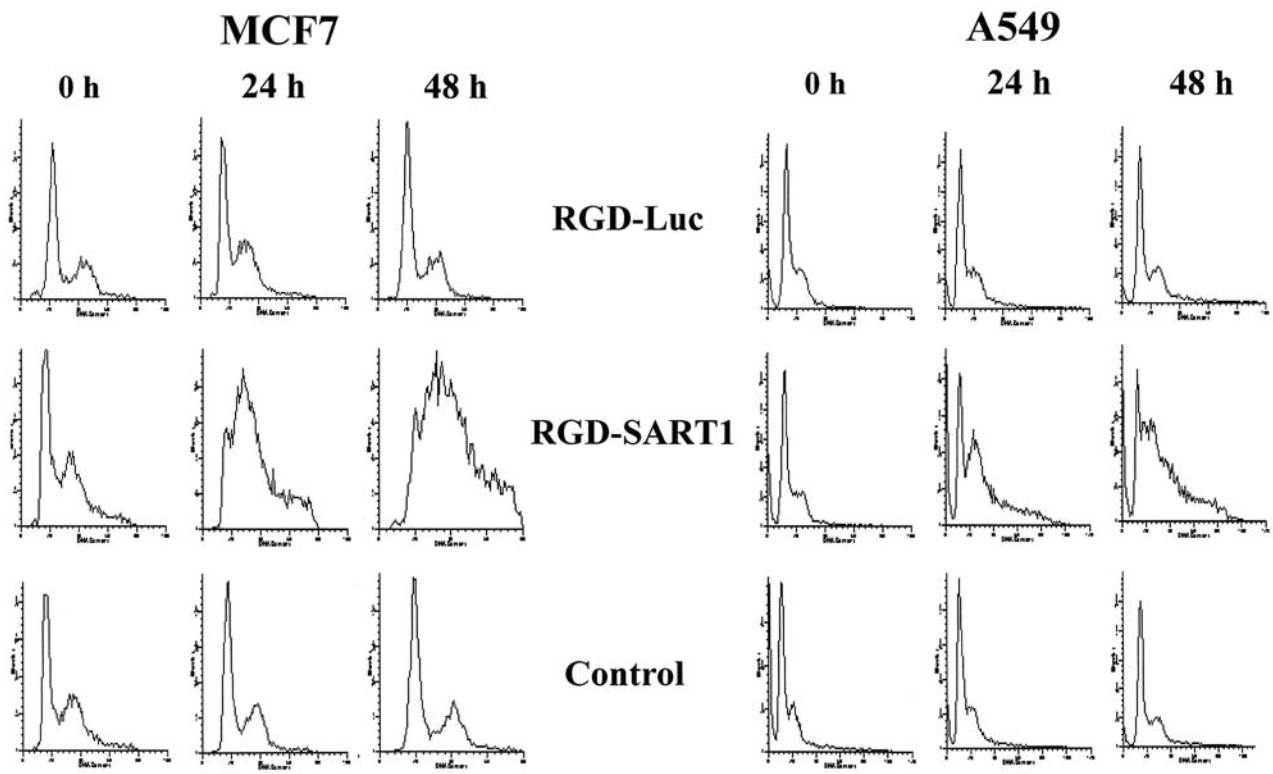


Figure 3. *Analysis of cell cycle by flow cytometry. The histogram shows the cell cycle and cell death status after treatment with Ad-RGD-SART-1 and Ad-RGD-Luc. At 24 and 48 h after infection, both the floating cells and adherent cells were collected in a single tube. For determination of DNA content, propidium iodide (PI) solution was added to the sample, as described in Materials and Methods.*

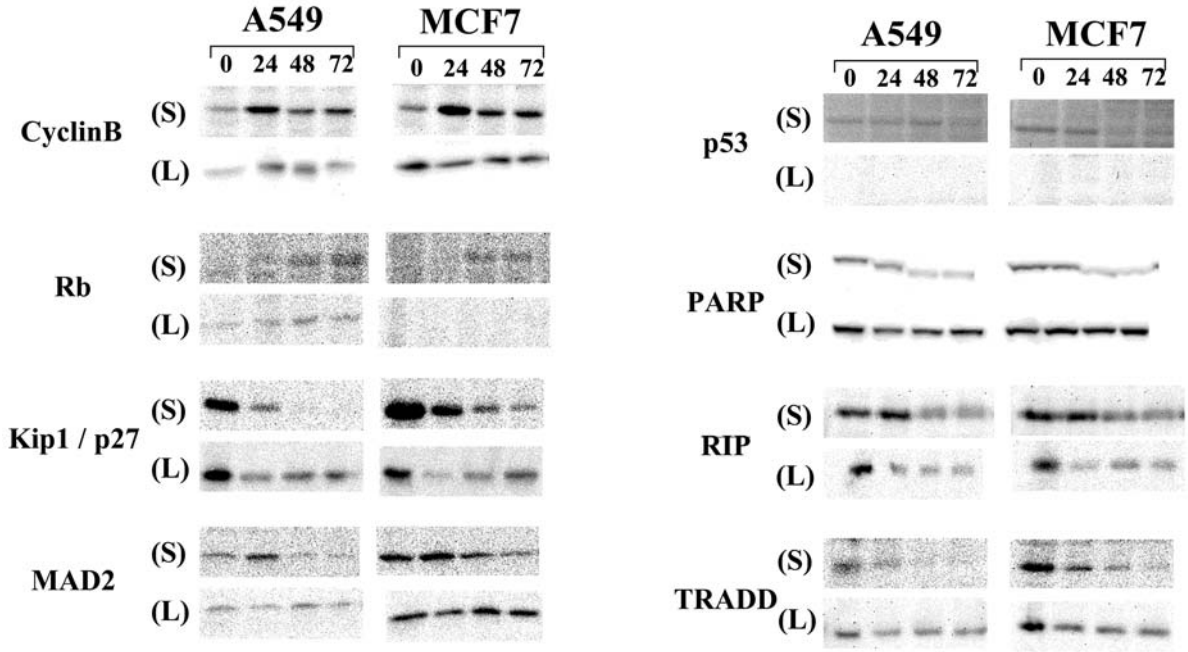
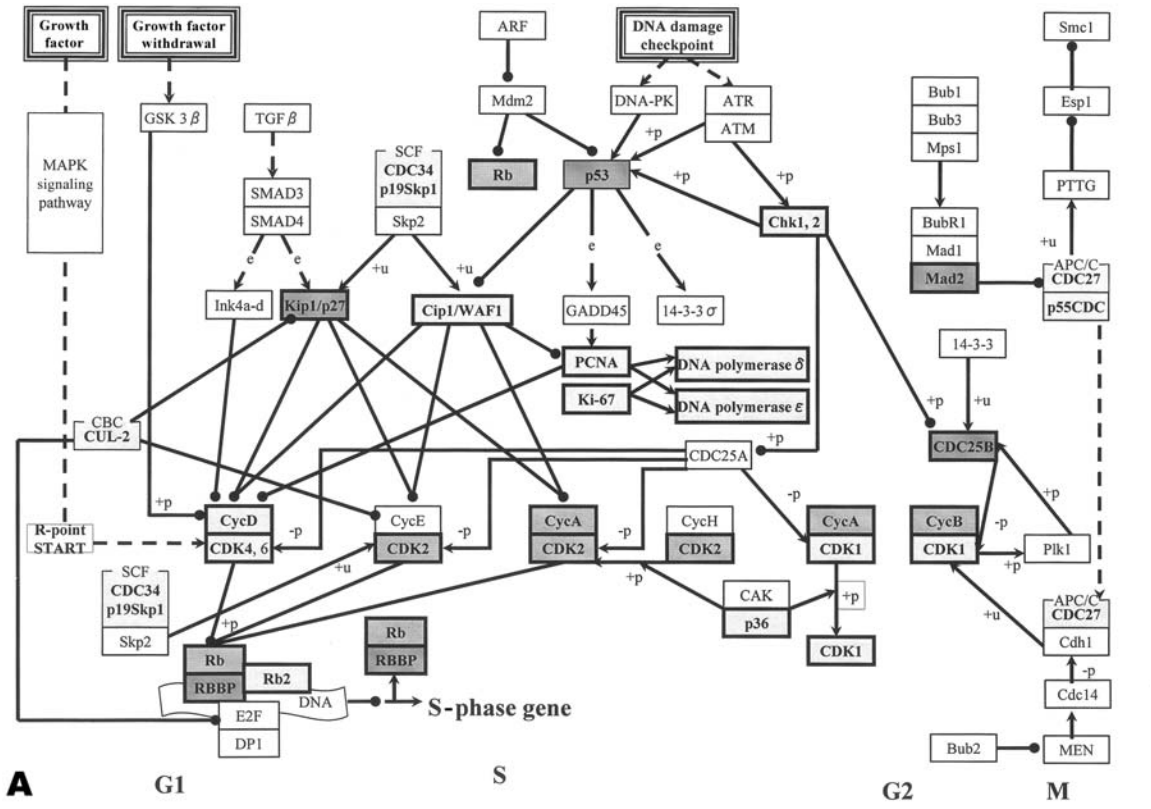


Figure 4. *Analysis of proteins associated with cell cycle and apoptosis by Western blotting. Cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were treated with 24 anti-cell cycle-related protein antibodies and with 22 anti-apoptosis-related protein antibodies. Proteins that were increased or decreased after Ad-RGD-SART-1 treatment in A549 and MCF7 cells are shown. (S); SART-1, (L); Luciferase. M.O.I: 30.*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Growth factor withdrawal
MAPK signaling pathway
GSK 3β
TGFβ
SMAD3
SMAD4
ARF
Mdm2
DNA damage checkpoint
DNA-PK
ATR
ATM
SCF CDC34 p19Skp1 Skp2
Rb
p53
Chk1, 2
Ink4a-d
Kip1/p27
Cip1/WAF1
GADD45
14-3-3 σ
PCNA
Ki-67
DNA polymerase δ
DNA polymerase ε
CBC CUL-2
CDC25A
CycD CDK4, 6
CycE CDK2
CycA CDK2
CycH CDK2
CycA CDK1
CAK p36
R-point START
Rb RBBP Rb2
E2F DP1
DNA
S-phase gene
Bub1 Bub3 Mps1
BubR1 Mad1 Mad2
Smc1
Esp1
PTTG
APC/C CDC27 p55CDC
14-3-3
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CycB CDK1
Plk1
Cdh1
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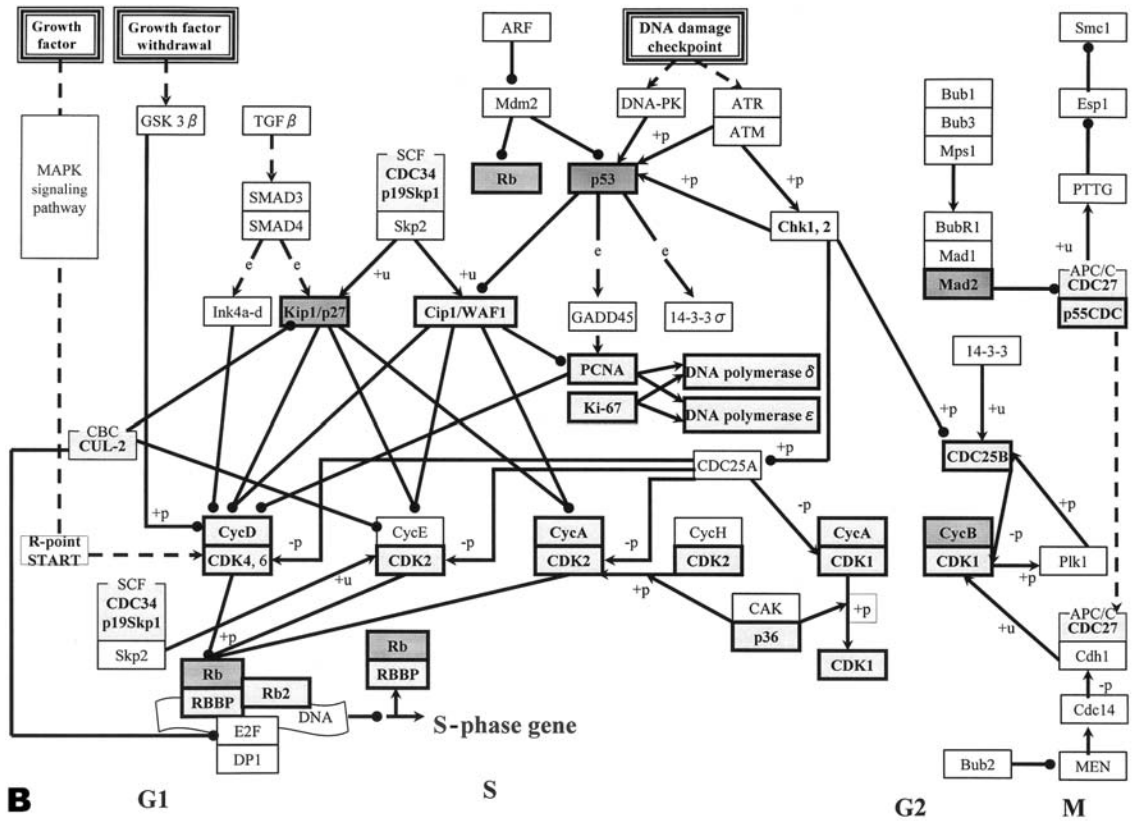
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Growth factor withdrawal
MAPK signaling pathway
GSK 3β
TGFβ
SMAD3
SMAD4
ARF
Mdm2
DNA damage checkpoint
DNA-PK
ATR
ATM
SCF CDC34 p19Skp1 Skp2
Rb
p53
Chk1, 2
Ink4a-d
Kip1/p27
Cip1/WAF1
GADD45
14-3-3 σ
PCNA
Ki-67
DNA polymerase δ
DNA polymerase ε
CBC CUL-2
CDC25A
CycD CDK4, 6
CycE CDK2
CycA CDK2
CycH CDK2
CycA CDK1
CAK p36
R-point START
Rb RBBP Rb2
E2F DP1
DNA
S-phase gene
Bub1 Bub3 Mps1
BubR1 Mad1 Mad2
Smc1
Esp1
PTTG
APC/C CDC27 p55CDC
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CycB CDK1
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Cdh1
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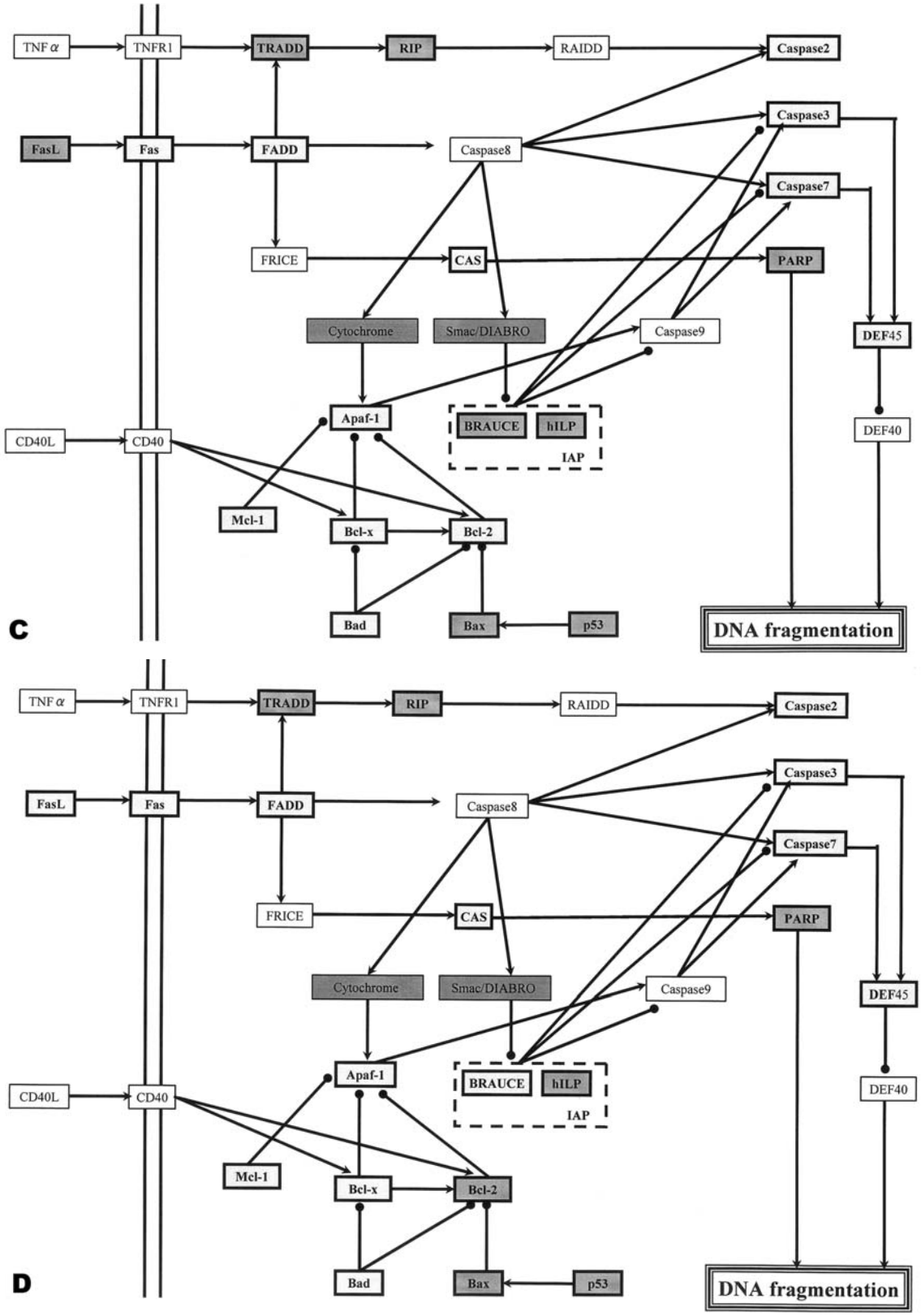

Figure 5. *Pathways of cell cycle and apoptosis induced by Ad-RGD-SART-1. (A): cell cycle pathway of A549, (B): cell cycle pathway of MCF7, (C): apoptosis pathway of A549, (D): apoptosis pathway of MCF7. Molecules marked with color (yellow, green and blue) were analyzed in the study. Yellow indicates "enhancement", green indicates "same level" and blue indicates "suppression".*

dendritic cells. Although these therapies induce general anti-tumor effects that yield some therapeutic benefit against metastatic sites, the potency is insufficient to control large tumors in advanced stages.

In this study, we used an RGD-fiber-modified adenovirus, Ad-RGD, to achieve high transduction efficiency. Previous reports have suggested that RGD-fiber-modified adenoviruses show higher transduction efficiency in freshly isolated human cells and various cell lines when compared with normal fiber adenoviruses. We confirmed that the transduction and expression levels of the RGD-fiber-modified adenoviruses were higher in the MCF-7 and A547 cell lines in preliminary experiments (unpublished data). The transduction and expression of SART-1 was evaluated by RT-PCR and a higher or equivalent expression of SART-1 mRNA was detected. Strong cell growth inhibition caused by cell cycle inhibition was observed after SART-1 gene transduction, which led to apoptosis (Figures 2-5). These data suggest that RGD-fiber-modified adenovirus vectors overcome the problem of low gene transduction efficiency.

SART-1 was identified as one of the tumor antigens recognized by established T cells. Our data also suggest that SART-1 gene transduction induced cell cycle inhibition, resulting in apoptosis (Figures 2-5). Precise analysis of cell cycle- and apoptosis-related molecules suggested the mechanism behind the SART-1 inhibition of the cell cycle and apparent induction of apoptosis (Figures 4 and 5). Our data showed that Kip1/p27, Mad2 and p53, which are cell cycle-related proteins, decreased in both cell lines (Figures 5A and C). Expression of p53 and Bax, which are apoptosis-related proteins, also decreased after SART-1 transduction in both cell lines (Figures 5B and D).

Previous reports suggest that enhancement of p53 and Adp27 (Kip1) expression inhibit cell proliferation. Cell cycle analysis has demonstrated that accumulation of cells in the G0/G1-phase at 24-120 h after transduction of the p53 or p27/kip1 genes is associated with an increase in early apoptosis (24-26). However, in our study, such molecules were decreased after SART-1 transduction, even though cell cycle arrest and apoptosis were induced by SART-1 gene transduction. Moreover, other apoptosis- and cell cycle-related proteins also exhibited inverse reactions or were not influenced by SART-1 gene transduction. Those results suggest that SART-1 induced cell cycle arrest and apoptosis *via* different pathways.

Based on the present results, two applications of SART-1 in cancer gene therapy are possible; SART-1 may be used as a cancer antigen, as reported previously (27, 28), or SART-1 can induce cell cycle arrest leading to apoptosis. The combination of these two concepts *via* transduction of a single gene is a novel approach for local and systemic therapy, and further studies are currently underway.

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Received March 8, 2005
Accepted April 4, 2005