Up-regulated p27Kip1 Reduces Matrix Metalloproteinase-9 and Inhibits Invasion of Human Breast Cancer Cells

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Abstract. Background: p27Kip1 is a cyclin-dependent kinase inhibitor which has been reported to be associated with invasion, metastasis and angiogenesis in malignant tumors, but its mechanism of action remains unknown. Here, it was examined whether p27Kip1 has an inhibitory effect on cancer cell invasion and correlates with matrix metalloproteinase expression (MMPs). Material and methods: The human breast cancer cell line MDA-MB-231 and MDA-MB-231 transfected p27Kip1 MDA-MB-p27 were used for the invasion assay, Western blotting and real-time quantitative RT-PCR. Results: In the invasion assay, the invasion of MDA-MB-p27 was significantly less than that of the parent cell line. In Western blotting analyses, the protein level of MMP-9 was also reduced in MDA-MB-p27. Furthermore, the activity of MMP-9 in cell culture supernatants was lower in MDA-MB-p27 as compared with with enzyme-linked immunosorbent assays. In real-time quantitative RT-PCR, the mRNA level of MMP-9 was lower in MDA-MB-p27 cells. Conclusion: Up-regulation of p27Kip1 remarkably inhibited the invasion of the breast cancer cells, in part due to the reduced expression of MMP-9. This is the first report of p27Kip1 modulating MMP-9 and indicating that p27Kip1 might play a key role in tumor cell invasion.

Abbreviations: CDK, cyclin-dependent kinase; MMPs, matrix metalloproteinases; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; TIMPs, tissue inhibitors of metalloproteinases.

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In the present study, an evaluation of the inhibitory effect on tumor invasion by up-regulated p27\(^{kip1}\) and the relationship between p27\(^{kip1}\) and the gelatinases using p27\(^{kip1}\) transfectants (MDA-MB-p27) in the human breast cancer cell line MDA-MB-231 was carried out.

Materials and Methods

**Cell culture.** Human breast cancer cell line MDA-MB-231 was cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) ( JRH Biosciences, Lenexa, KS, USA), 100 unit/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco BRL, Gaithersburg, MD, USA) at 37˚C in an atmosphere of 5% CO\(_2\) and 95% air. The transfectants were maintained in RPMI-1640 medium with 10% FBS, 100 unit/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 600 μg/ml G418 (Promega, Madison, WI, USA).

**Antibodies.** Mouse monoclonal antibody for p27\(^{kip1}\) (sc-1641) and goat polyclonal antibodies for MMP-9 (sc-6840) and actin (sc-1615) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody for pRb (554136) was purchased from Pharmingen (San Diego, CA, USA). Mouse monoclonal antibodies for MMP-9 (sc-6840) and actin (sc-2005, sc-2020; Santa Cruz Biotechnology). The binding proteins were developed with horseradish peroxidase-linked anti-mouse immunoglobulin and anti-goat immunoglobulin, respectively.

**Establishment of p27\(^{kip1}\) transfectants (MDA-MB-p27).** p27\(^{kip1}\) cDNA was obtained as described elsewhere (10). This cDNA was then ligated in the sense orientation into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), which contains the neomycin-resistance gene for selection in G418-supplemented medium. This expression vector was transfected to MDA-MB-231 using FuGENE™6 Transfection Reagent (Roche, Indianapolis, IN, USA). Neomycin-resistant colonies were then grown and selected in medium containing 600 μg/ml of G418. The pcDNA3.1 expression vector with no insert was also transfected to MDA-MB-231 as a control (MDA-MB-NEO).

**Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated from cell pellets using TRizol (Invitrogen). cDNA was synthesized from 5 μg of total RNA using a SuperScriptTMIII First-Strand Synthesis System for RT-PCR kit (Invitrogen). The following primers were used (all 5’ to 3’ direction): pcDNA3.1 forward primer GACTGGGCACAAAGCACAATCG and reverse primer TACTTTCTGGGCGAGGACGAAGGTG; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer CCACCATGGCAAATTCATGCGCA and reverse primer TCTAGACGGCCAGCTGGCAAGA. DNA amplifications were carried out in a 96-well reaction plate format in a GeneAmp 5700 Sequence Detection System. Each sample was analyzed in triplicate.

**Cell proliferation assay.** MDA-MB-231, MDA-MB-NEO and MDA-MB-p27 cells were seeded at a density of 1x10\(^5\) cells/well in a 60 mm dish (Becton Dickinson, Franklin Lakes, NJ, USA). These cells were harvested by trypsinization and counted with 0.4% tripan blue dye on days 1, 2 and 3. The medium was changed after 48 h. This assay was performed in triplicate and repeated three times.

**Cell cycle distribution.** MDA-MB-231, MDA-MB-NEO and MDA-MB-p27 cells (5x10\(^5\)) were incubated for 48 h in RPMI-1640 with 10% FBS. The cells were harvested and washed in phosphate buffered saline (PBS) three times. Analyses of the cell cycle distribution were performed by measuring the fluorescent activity of propidium iodide-stained DNA of permeabilized and fixed cells using kits (Becton Dickinson) on a FACSCalibur instrument (Becton Dickinson).

**Invasion assay.** The invasive potential of MDA-MB-p27 cells was evaluated by an invasion assay. Cells (5x10\(^5\)) were seeded in the upper chamber of 24-well Transwell chambers (8 μm diameter pores) coated with Matrigel (Becton Dickinson). The lower chamber was filled with 750 μl of RPMI-1640 with 20 μg/ml fibronectin (Asahi Technoglass Corporation, Tokyo, Japan) as a chemoattractant. After 12 h incubation, the cells in the upper chamber were gently scraped away and the invasive cells that had migrated through the membrane to the lower surface were stained with Wright’s and Giemsa’s solution (Merck Ltd, Tokyo, Japan). The invasive cells were counted in nine separate high-power microscopic fields (magnification, ×200). Each experiment was performed in triplicate wells and repeated three times.

**Western blot analysis.** Total protein extracts from samples were subjected to SDS-PAGE as previously described (25, 26). Proteins were electroblotted onto a polyvinylidene difluoride membrane (BIORAD Laboratories, Hercules, CA, USA) and probed with the respective primary antibodies at the following dilutions: p27, 1:500; pRb, 1:500; MMP-9, 1:100 and actin, 1:600. The blots were developed with horseradish peroxidase-linked anti-mouse immunoglobulin and anti-goat immunoglobulin, respectively (sc-2005, sc-2020; Santa Cruz Biotechnology). The binding proteins were visualized using a chemiluminescence detection system.
The relative amounts of p27 and MMP-9 to actin were analyzed densitometrically by the National Institutes of Health Image Program.

Enzyme-linked immunosorbent assay (ELISA). Cells (5×10⁴/well) were seeded in 6-well plates (Becton Dickinson) and incubated for 24 h in FBS-supplemented medium. After washing with PBS three times, cells were incubated in the absence of FBS for an additional 24 h. The culture supernatants from the cells were harvested under sterile conditions and frozen at −20°C before assay. Active MMP-9 contents were determined using commercial ELISA kits (RPN 2634; Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis. Data are presented as the mean±S.E of independent experiments. Statistical significance was determined by Scheffe’s protected least significant difference test. The difference between experimental groups was considered significant when the p-value was <0.05.
Results

Confirmation of the transfection and the up-regulation of p27<sup>Kip1</sup> expression in p27<sup>Kip1</sup> transfectants. Six p27<sup>Kip1</sup>-transfected clones were established (MDA-MB-p27). RT-PCR was performed to confirm the transfected vector. mRNA from the expression vector was seen in all of these clones and MDA-MB-NEO (Figure 1A). The level of up-regulated p27<sup>Kip1</sup> expression in p27<sup>Kip1</sup> transfectants was evaluated. All of the p27<sup>Kip1</sup>-transfected clones showed a higher level of p27<sup>Kip1</sup> expression than the parent. These clones were separated into three types by the amounts of p27<sup>Kip1</sup> expression. That is, the high level group of MDA-MB-p27H1 to H3, the moderate level group of MDA-MB-p27M1 and -M2, and the low level group of MDA-MB-p27L1 (Figure 1B and C). Hereinafter, key samples were indicated in tables and figures for simplifying our data. In particular, high expression p27 samples were important; two p27 high samples (-p27H1 and -p27H3) are shown on Figure 4 and Table I.

pRb Protein expression in p27<sup>Kip1</sup> transfectants. Expression of pRb protein in p27<sup>Kip1</sup> transfectants was evaluated by Western blot analysis. Dephosphorylation of pRb was not clearly recognized in MDA-MB-p27H1 and M1 compared with the parent (Figure 2A).

Growth rate and cell cycle distribution in p27 transfectants. We assessed whether transfection of p27<sup>Kip1</sup> altered the growth rate and cell cycle distribution. There was no significant difference in the growth rate between MDA-MB-p27 and the parent cell line (Figure 2B). As shown in Table I, the cell cycle distribution of MDA-MB-p27 compared with that of the parental cells (MDA-MB-231). There were no difference in the cell cycle ratio between parent cells and MDA-MB-p27 analyzed by chi-square-test. Therefore, the results of the present study were not dependent on the cell growth or activity.

Inhibition of cancer cell invasion by up-regulation of p27<sup>Kip1</sup>. The alteration of the invasive potential by the up-regulation of p27<sup>Kip1</sup> was studied with Matrigel invasion assays. The number of invasive cells was lower in MDA-MB-p27 as compared with the parent cell line (Figure 3A and B). MDA-MB-p27 cells exhibited significantly less invasive potential than the parent cells (p<0.0001; Figure 3B). The up-regulation of p27<sup>Kip1</sup> reduced the invasive potential, but this inhibitory effect did not correlate with the quantitative level of p27<sup>Kip1</sup> expression.

Alteration of MMP-9 expression by up-regulation of p27<sup>Kip1</sup>. Firstly, the expression of MMP-9 mRNA in MDA-MB-231, MDA-MB-NEO and MDA-MB-p27 was qualitatively confirmed by RT-PCR (Figure 4A). All of the cell lines showed the expression of MMP-9 mRNA. No difference in the expression of MMP-9 mRNA was found between MDA-MB-p27 and the parent cell line. The MMP-9 protein level was evaluated by Western blot analysis. It was shown that MMP-9 expression was attenuated in MDA-MB-p27 compared with the parent cell line (Figure 4B). The relative amount of MMP-9 in MDA-MB-p27H1, H3, M1 and L1 cells was 1.5, 1.8, 3.7 and 28.5%, respectively (Figure 4C). An inverse correlation was found between the amounts of p27<sup>Kip1</sup> and MMP-9 (Figure 1C and 4C).

Active MMP-9 in cell culture supernatants. Active MMP-9 production was determined in the cell culture supernatants of MDA-MB-231, MDA-MB-NEO and MDA-MB-p27 cells by ELISA. As shown in Figure 5A, the active MMP-9 contents in MDA-MB-231, MDA-MB-NEO, MDA-MB-p27H1 and L1 cells were 2.56±0.14, 2.77±0.30, 1.58±0.15 and 1.55±0.16 (ng/5 ×10<sup>4</sup> cells), respectively. Active MMP-9 was lower in MDA-MB-p27 cells than in the parent cell line (p<0.05).

Quantification of MMP-9 mRNA by real-time quantitative RT-PCR. Real-time quantitative RT-PCR was performed to study the alteration of MMP-9 mRNA by the up-regulation of p27<sup>Kip1</sup>. The expression of MMP-9 mRNA was reduced in MDA-MB-p27 cells compared to that of the parent cells (p<0.005 between MDA-MB-p27 and the parent, p<0.05 between MDA-MB-p27H1 and MDA-MB-p27L1, Figure 5B). There seemed to be an inverse correlation between the amount of MMP-9 mRNA and the level of p27<sup>Kip1</sup> protein.

Discussion

In this investigation, we established p27<sup>Kip1</sup> transfectants in the MDA-MB-231 breast cancer cell line. MDA-MB-231 has a low p27<sup>Kip1</sup> protein level and high proliferative activity.
Firstly, we evaluated the alteration of pRb protein status, the growth rate and cell cycle distribution by up-regulated p27Kip1 under stable conditions. Dephosphorylation of pRb was not recognized in p27Kip1 transfectants compared with the parent cells (Figure 2A). We expected a reduced growth rate and induction of G1 arrest in MDA-MB-p27 cells, but neither the growth rate nor cell cycle distribution were different between MDA-MB-p27 and the parent cells (Figure 2B and Table I). The reason for this is likely that the cells expressing a high protein level of transfected p27Kip1 were not selected due to apoptosis and G1 arrest. In other words, transfected p27Kip1 of the selected cells had very little function as a CDK inhibitor. Therefore, p27Kip1 transfectants were not different from the parent cells in pRb protein status, or in their growth and cell cycle distribution.

p27Kip1 is not only a negative cell-cycle regulator but is also associated with cell migration and invasion (13-15). However, the mechanism of cell migration and invasion in p27Kip1 remains unknown. The present study showed that the up-regulation of p27Kip1 had a remarkable inhibitory effect on the cancer cell invasion through Matrigel, as found in the previous report (15). Although it was reported that overexpression of S-phase kinase-associated protein 2 (Skp2), which is required for the ubiquitination and subsequent degradation of p27Kip1 (27), enhances cancer cell invasion, possibly via p27Kip1 proteolysis (15), this is the first report showing that up-regulation of p27Kip1 directly impairs cancer cell invasion through Matrigel in vitro. This result supports the notion of p27Kip1 being prognostic factor. In this study, the up-regulation of p27Kip1 inhibited cancer cell invasion, but this inhibitory effect did not appear to correlate with the protein level of p27Kip1. The threshold to which p27Kip1 inhibited cancer cell invasion might have been low.
MMPs are a family of zinc-dependent neutral endopeptidases which are capable of degrading the ECM (28). Most MMPs, including MMP-2 and MMP-9, are secreted as latent pro-enzymes (pro-MMPs) which are extracellularly activated through proteolytic cleavage (28). Cancerous cells mainly secrete MMP-1, -2, -3, -7 and -9 among the many MMPs, but the types of MMPs secreted from cancerous cells are different in each type of cancer. Up-regulation of MMP-2 and MMP-9 has been shown in various malignancies including breast cancer (17, 29) and is correlated with the invasive potential (19, 20). In this study, up-regulation of p27<sup>Kip1</sup> attenuated the protein synthesis of MMP-9 in MDA-MB-231, whereas MMP-2 was not altered by the up-regulation of p27<sup>Kip1</sup> (data not shown). In the invasion assay, up-regulation of p27<sup>Kip1</sup> remarkably reduced the number of cells invading through Matrigel. Hence, it is likely that the inhibitory effect on cancer cell invasion through Matrigel by up-regulated p27<sup>Kip1</sup> can be attributed in part to down-regulated expression of MMP-9. However, the expression of various MMPs, as well as MMP-2, and -9, had been is shown in MDA-MB-231 (30, 31). In this work, we did not assess other factors related to invasion, such as other MMPs and tissue inhibitors of metalloproteinases (TIMPs), which counterbalance the activities of MMPs. Therefore, it is not definite that reduced expression of MMP-9 alone was responsible for suppressing invasion by MDA-MB-p27 cells.

In the present study, we elucidated that up-regulation of p27<sup>Kip1</sup> reduces MMP-9 expression via reduced mRNA levels (Figures 3 and 5), but the reduced mRNA levels are not so remarkable compared with the decreased protein levels of MMP-9. Although the production of proMMPs is known to be regulated at the transcriptional level, in this study MMP-9 might be regulated in part at unknown posttranscriptional levels. Transcription of the MMP-9 gene is regulated by 670 base pairs of upstream sequence, including AP-1 (-533, -79), NF-κB (-600), Ets (-540) and Sp1 sites (-558) (32). In particular, both AP-1 and NF-κB binding sites have been reported to be important regulatory elements of the MMP-9 promoter (33, 34). p27<sup>Kip1</sup> might have reduced MMP-9 expression via these transcriptional regulations. However, the signal transduction between p27<sup>Kip1</sup> and MMP-9 is still unknown. Recent reports have shown that MMP-9 secretion by stimulant factors, such as growth factors and cytokines, is dependent on the activation of the mitogen-activated protein kinase (MAPK) signal transduction pathway and/or the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway in some cancer cell lines (35-36). The signaling required for MMP-9 secretion seems to be different according to the type of cell line and stimulant factors. MAPK is a common downstream pathway of many growth factor signal cascades and is essential for the triggering of cell proliferation and differentiation (37).
Activated Akt acts on many different substrates associated with the regulation of apoptosis and the cell cycle (38). It has been reported that these signal transduction pathways, MAPK and PI3-K/Akt, lead to the regulation of p27 (39-41). MAPK activation has been reported to cause the down-regulation of p27Kip1 (39). Akt is able to down-regulate p27Kip1 by increasing p27Kip1 proteolysis (41) or repressing p27Kip1 expression through Akt phosphorylation of a forkhead transcription factor (40). When considering the signal transduction between p27Kip1 and MMP-9, the common features in the regulation of p27Kip1 and MMP-9 become apparent in these previous reports. In other words, the MAPK and PI3-K/Akt pathways control in part the expression of both p27Kip1 and MMP-9. p27Kip1 is possibly linked with MMP-9 downstream of the MAPK and/or PI3-K/Akt pathways. However, the possibility of other types of signal transduction between p27Kip1 and MMP-9 must be considered. Therefore, further studies are needed to identify the molecular links between p27Kip1 and MMP-9.

In conclusion, we demonstrated that up-regulation of p27Kip1 has a strong inhibitory effect on tumor cell invasion. Moreover, we demonstrated that up-regulation of p27Kip1 reduces the production of MMP-9. It is suggested that reduced expression of MMP-9 was partly responsible for the impairment of invasion caused in part by the augmented p27Kip1. This is the first report showing the interaction between p27Kip1 and MMP-9. p27Kip1 is not only a negative cell-cycle regulator but also a suppressor of tumor cell invasion. This phenomenon strongly suggests that p27Kip1 is a prognostic factor in breast cancer.
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References


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