Critical Involvement of the Phosphatidylinositol 3-Kinase/Akt Pathway in Anchorage-independent Growth and Hematogeneous Intrahepatic Metastasis of Liver Cancer

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ABSTRACT

In the multistep process of metastasis, “anchorage-independent growth,” where cancer cells need to survive without cell-substratum interaction, is supposed to be important. In this study, we found that anchorage-independent growth analyzed using the soft agar colony formation assay correlated with hematogeneous intrahepatic metastasis of liver cancer cell lines and also Akt activation status. Two highly metastatic liver cancer cell lines showed high Akt activity and formed many colonies in soft agar, whereas three nonmetastatic cell lines showed less Akt activity and formed fewer colonies. Inhibition of Akt activation in the highly metastatic cell line Li7 by transfection with kinase-dead Akt or the phosphatidylinositol 3-kinase inhibitor, LY294002, resulted in formation of fewer colonies in soft agar than was the case with control cells. Moreover, in an orthotopic implantation model, this inhibition resulted in a reduced rate of hematogeneous intrahepatic metastasis. These findings indicated that anchorage-independent growth regulated by phosphatidylinositol 3-kinase/Akt pathway plays a critical role in metastasis, and that this could be a potential therapeutic target to combat metastasis.

INTRODUCTION

Cancer metastasis is a multistep process that involves cell detachment from the primary tumor, entry into the vascular or lymphatic system, dispersal through the circulation, and proliferation after extravasation in the target organs (1). Many studies have shown the importance and mechanisms of invasion, one of the first steps in metastasis. Loss of cell-cell contact caused by self-regulation of E-cadherin (2) and increased cell motility (3, 4) have been reported to be critical steps in this process. In the next step of invasion, tumor cells become detached from the substratum or each other and dispersed through the circulation, where they lose the signals for cell growth and cell survival generated by the cell-substratum interaction (5–7). Some cancer cells can survive without these signals, and this is termed AIG. However, little is known about the importance and mechanisms of AIG in the course of metastasis.

Serine/threonine kinase Akt (protein kinase B) was originally identified as a homologue of the v-Akt oncogene from a transforming retrovirus in a spontaneous thymoma of a mouse (8). This enzyme is a direct downstream effector of PI3K (9). Activation of PI3K occurs through phosphorylation of a tyrosine residue by either receptor or nonreceptor tyrosine kinase (10–14). Akt is fully activated by phosphorylation of threonine 308 and serine 473 after activation of PI3K. Activated Akt regulates multiple processes, such as apoptosis, cell proliferation, and glucose usage (15, 16). In normal epithelial cells, detachment from the extracellular matrix leads to a rapid decrease in the level of PI3K products and Akt activity, leading to apoptosis. On the other hand, constitutively activated Akt in Ras-transformed epithelial cells inhibits anoikis on removal from the matrix (17, 18). These results suggest that activated Akt induces AIG.

HCC is one of the most common and aggressive malignancies in the world. Regardless of recent advances in diagnostic methods and therapeutic approaches to HCC, the prognosis of HCC patients remains poor because of the high incidence of hematogeneous intrahepatic metastasis after initial treatment (19, 20). Intrahepatic metastasis of HCC is observed frequently in advanced cases and is thought to develop through tumor cell dispersal via the portal vein (21). In fact, portal vein invasion of HCC is a poor prognostic factor because of the risk of recurrence. Furthermore, α-fetoprotein mRNA can be detected in the circulating blood of HCC patients during the perioperative period, indicating detachment of HCC cells from tumors into the circulation. However, metastasis is not always observed in portal vein invasion-positive and/or α-fetoprotein mRNA-positive cases (22, 23). Therefore, it can be speculated that metastatic cancer cells need not only invade and enter the vascular or lymphatic system, but they must also survive during dispersal through the circulation.

In this study, a correlation was observed between hematogeneous intrahepatic metastasis and AIG, regulated by Akt activation, in an orthotopic implantation model of HCC (3). Inhibition of the PI3K/Akt pathway reduced hematogeneous intrahepatic metastasis by suppressing AIG. These results therefore suggest that inhibition of the PI3K/Akt pathway may be a possible new therapeutic strategy for suppression of metastasis.

MATERIALS AND METHODS

Cell Culture and Reagents. The human HCC cell lines, PLC/PRF/5 and HepG2, were obtained from the American Type Culture Collection. KIM-1 and KYN-2 were kindly provided by Dr. Masamichi Kojiro, and Li7 was established in our laboratory (3). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The specific PI3K inhibitor LY294002 (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO, and the same volume of solvent was added to the controls. Polyclonal antibodies to Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA).

Immunoblotting. For immunoblot analysis, cells were lysed with lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 25 mM NaF, and complete protease inhibitor cocktail tablet (Roche Molecular Biochemicals, West Germany)]. The tumor specimen was obtained at the time of autopsy, snap-frozen in liquid nitrogen, and stored at −80°C. The frozen specimen was homogenized and lysed with the same buffer. The lysate

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The abbreviations used are: AIG, anchorage-independent growth; PI3K, phosphatidylinositol 3-kinase; HCC, hepatocellular carcinoma; HA, hemagglutinin; MAPK, mitogen-activated protein kinase.

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Fig. 1. A, growth and hematogeneous intrahepatic metastasis of Li7 cells in SCID mice after orthotopic implantation. Left, macroscopic view of Li7 tumor. Arrowhead, the primary tumor that developed at the injected lobe; arrows, intrahepatic metastases in the un.injected lobe. Right, histological findings of Li7 tumor thrombi in portal vein. ×200. B, anchorage-independent growth was assessed by the colony formation assay. The number of colonies was counted under low magnification (×100) at four points on each dish. The data represent the means of two independent experiments performed in triplicate. The effect of LY294002 on AIG of Li7 cells. The data represent the means of two independent experiments performed in duplicate; bars, SD.

was centrifuged, and the supernatant was prepared. Protein concentration was determined with the Bradford reagent (Bio-Rad). Proteins were resolved by SDS-PAGE (4–12%) and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, MA). For immunoblot analysis of Akt, nonspecific 

sites on the membrane were blocked by incubation for 90 min at room temperature with 5% (w/v) nonfat dry milk in PBS. For immunoblot analysis with other antibodies, the membrane was blocked with 2% (w/v) BSA in PBS. All of the membranes were then incubated overnight at 4°C with primary antibodies in the same blocking solution. The membranes were then washed and incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Promega). Immunocomplexes were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

**Plasmids and Transfection.** Li7 cells were transfected with the plasmid, pHM6-K179A Akt1, which encodes a dominant-negative form of Akt1 mutant, or vector alone using LipofectAMINE 2000 (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Stable transfectants were then selected by incubation with geneticin (G418; Life Technologies, Inc.) and HA-tagged dominant-negative Akt protein expression. Bulk cultures without subcloning were prepared as Bulk 1 and Bulk 2. Limiting dilutions were performed for cloning, and the monoclonal clones were isolated (clones 1 and 3). All transfectants were maintained in medium containing 800 μg/ml G418. HA-tagged dominant-negative Akt proteins in the transfectants were detected by immunoblotting using rat monoclonal anti-HA antibodies (Roche Molecular Biochemicals).

**Colony Formation Assay.** Soft agar colony formation assays were carried out in six-well dishes. Cells (1 × 10⁴) suspended in 2 ml of 0.36% bactoagar (Becton Dickinson, Sparks, MD) with growth medium (RPMI 1640 supplemented with 10% fetal bovine serum) were added on a base layer of 0.72% bactoagar containing culture medium. The plates were incubated at 37°C in a 5% CO₂ incubator for 3 weeks. AIG was assessed by counting the number of colonies under low magnification (×100) at four points on each well.

**Orthotopic Implantation in Mice.** Male homozygous C.B-17 SCID/SCID mice were purchased from Charles River Japan Inc., (Tokyo, Japan) and maintained in a specific pathogen-free environment. The animals received humane care, and the studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Five- to 6-week-old mice were used. Orthotopic implantation of Li7 cells and Li7 transfectants was performed as described previously (3). Briefly, the cells were harvested from subconfluent cultures and pelleted by centrifugation, washed once, and resuspended in PBS at 1 × 10⁶ cells/ml. Mice were anesthetized by i.p. administration (0.3 ml/mouse) of a 2.5% solution of a mixture of 2,2,2-tribromoethanol (Aldrich Chemical Company Inc., Milwaukee, WI) and tert-amylalcohol (Wako Pure Chemical Industries Ltd., Osaka, Japan; 1:1), and an incision was made through the left upper abdominal transrectal line and peritoneum. The liver was carefully exposed, and 20 μl of the cell suspension (2 × 10⁶ cells) were injected into the subserosa of the liver. For in vivo treatment with...
LY294002, the mice were randomized into control (n = 10) and LY294002-treated groups (n = 10) 2 weeks after inoculation. Microosmotic pumps (type 1002; Alzet, Palo Alto, CA) were implanted into the peritoneal cavity and were primed to deliver LY294002 (100 μg in 35% DMSO/day) or 35% DMSO alone in a reservoir volume of 100 μl with a mean pumping rate of 0.22 μl/h for 2 weeks. Replacement of the pumps was performed 4 weeks after inoculation, and measurement of body weight was performed every week. Mice were sacrificed 6 weeks after inoculation, and autopsies were performed immediately. After macroscopic examination, the liver was removed, and the number of tumors in each liver was counted and measured. The liver sample was fixed in 10% formalin, cut into 2-mm-thick slices, embedded in paraffin, and processed for histological examination. Intrahepatic metastatic lesions were defined as either: (a) lesions in lobes other than the lobe that was injected; or (b) lesions that were clearly separate from the primary tumor.

**RESULTS**

Hematogeneous Intrahepatic Metastasis in HCC Cell Lines Is Correlated with AIG and Activation of Akt. Five HCC cell lines, implanted orthotopically into SCID mice, were found previously to form liver tumors: two of these cell lines, Li7 and KYN-2, resulted in hematogeneous intrahepatic metastasis (Fig. 1A), whereas the other three, PLC/PRF/5, HepG2, and KIM-1, did not (3). To investigate the correlation between metastatic potential of these cells and AIG, a soft agar colony formation assay was performed. When a suspension of 1 × 10⁴ cells was plated out, KIM-1 formed no colonies, PCL/PRF/5 formed a few colonies, and HepG2 formed fewer colonies than two other metastatic cell lines (Fig. 1B). These results suggested that these nonmetastatic cell lines formed fewer colonies than two other metastatic cell lines.

Activated (phosphorylated) Akt is known to be an antiapoptotic molecule (15, 24) and is thought to have the advantage of AIG (25–28). The expression and activation status of Akt were therefore examined. Total Akt expression was similar in all cell lines, but the level of Akt phosphorylation of the two metastatic cell lines was much stronger than that of the three nonmetastatic cell lines in the presence of serum (Fig. 1C). In Li7 cells, phosphorylated Akt was detected, even in the absence of serum. These two results suggested that Akt activation and AIG and hematogeneous intrahepatic metastasis were all correlated.

Expression of Dominant-Negative Akt and Treatment with the PI3K Inhibitor, LY294002, Suppressed AIG. To analyze the direct interaction between Akt activity and AIG, a dominant-negative K179A-Akt mutant was transfected into Li7 cells, and two monoclonal clones (Clones 1 and 3) and two bulk cultures (Bulk 1 and 2) were prepared. The growth rate of these clones was ~80% of the mock transfectants in vitro (data not shown). The colony formation assay showed that the number of colonies was ~3-fold higher in mock transfectants than that in the monoclonal clones. Suppression of colony formation was different in each bulk culture and was weaker than in the monoclonal clones (Fig. 2A).

The flavonoid derivative, LY294002, is a specific inhibitor of PI3K (29); it competitively and reversibly inhibits the ATP-binding site of PI3K, leading to suppressed activation of Akt. As expected, activation of Akt in Li7 cells was completely suppressed in the presence of 50 μM LY294002 (Fig. 2B) and was sustained for 24 h (data not shown). LY294002 has also been reported to suppress cell proliferation by G1 arrest (29). Twenty-four h after Li7 cell seeding, culture medium containing or lacking 20 μM LY294002 was replaced daily. The number of Li7 cells treated with LY294002 was much smaller than that with no LY294002 treatment (Fig. 2C). The effect of LY294002 on AIG of Li7 cells was determined in a colony formation assay. LY294002 (0, 20, or 50 μM) was added to soft agar every day for 3 weeks. Colony numbers were suppressed in accordance with an increasing concentration of LY294002 (Fig. 2D). These results showed that AIG of Li7 cells was regulated by the PI3K/Akt pathway.

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Table 1 Hematogeneous intrahepatic metastasis of dominant-negative Akt transfectants of Li7 cells

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Suppression of the PI3K/Akt Pathway Inhibited Hematogeneous Intrahepatic Metastasis in Vivo. The effect of suppression of the PI3K/Akt pathway on hematogeneous intrahepatic metastasis of Li7 tumors in vivo was then investigated, and the metastatic potential of the transfectants was evaluated in the orthotopic implantation model (Table 1). One mouse each in the Clone 3 and Bulk 2 groups had no tumor at the injection site, and these mice were therefore excluded from the experiment. The other mice produced a tumor at the site of injection (primary tumor). Expression of dominant-negative Akt in the primary tumor of each clone was confirmed by immunoblotting (Fig. 3). With respect to the maximum tumor diameter and primary tumor volume, there was no significant difference among the clones, which suggested that suppression of Akt did not affect primary tumor growth (data not shown). However, the incidence of hematogeneous intrahepatic metastasis was significantly suppressed in dominant-negative Akt transfectants compared with mock transfectants. Although a recent study showed that Akt activation promoted the invasiveness of cancer cells (30, 31), both mock and dominant-negative Akt transfectants showed infiltrative growth into the sinusoidal area at the tumor boundary in this study, and no difference in invasiveness was observed.

The effect of LY294002 on homogeneous intrahepatic metastasis of Li7 tumors was also examined. Micro-osmotic pumps were implanted into the peritoneal cavity of mice and continuously delivered the inhibitor into the peritoneal cavity. To not inhibit primary tumor growth and to see a specific effect on metastasis, a 2-week interval was left before implanting the pumps. During the experiment, LY294002-treated mice did not show any sign of general toxicity. After 6 weeks, one mouse in each group had no tumor at the injection site and these mice were excluded from the experiment. The control group was heavier in body weight than the LY294002-treated group during LY294002 treatment (Fig. 4A). With respect to the maximum tumor diameter (Fig. 4B) and primary tumor volume (Fig. 4C), LY294002-treated tumors tended to be smaller in volume, although the difference between the two groups was not significant. However, the incidence of hematogeneous intrahepatic metastasis was significantly suppressed in the LY294002-treated group compared with the control group (Table 2). Infiltrative growth into the sinusoidal area at the tumor boundary was not affected by LY294002 treatment, similar to dominant-negative Akt transfectants.

DISCUSSION

Cancer metastasis is a multistep process. During detachment from the tumor and dispersal through the circulation, it is speculated that some cancer cells die attributable to apoptosis because of the loss of survival signals, and that only cancer cells that are resistant to apoptosis survive and form metastases. In other words, AIG leads to metastasis formation (32–34). Our results support the correlation between AIG and metastasis. In this study, the number of colonies in soft agar was correlated to the level of Akt activation. Furthermore, inhibition of the PI3K/Akt pathway by dominant-negative K179A-Akt transfection and LY297002 treatment suppressed colony formation in vitro and hematogeneous metastasis in vivo. Therefore, Akt activation appears to be critical to AIG and hematogeneous metastasis.

Activated Akt is known to be an antiapoptotic molecule, and multiple mechanisms inhibiting apoptosis through the PI3K/Akt pathway have been reported (15, 16). Akt phosphorylates and inactivates various components of the apoptotic machinery such as BAD (35), caspase 9 (36), and forkhead transcription factors FKHL1, FKHR, and AFX (37). Moreover, it was shown recently that phosphorylated Akt activates the transcriptional activity of nuclear factor-κB, which is a family of transcriptional factors (38, 39).

Generally, Akt is activated by interaction of cells with the extracellular matrix, and this is mainly mediated by integrin receptors on the cell surface (27, 40, 41). Furthermore, some kinds of growth factor, such as platelet-derived growth factor (12) or insulin-like growth factor I (13) can also activate the PI3K/Akt pathway, and the addition of these growth factors to cells deprived of matrix contact can be sufficient to maintain activation of the PI3K/Akt pathway.

At present, it is not clear why Li7 and KYN-2 had higher Akt activity than the other three HCC cell lines. Expression of PTEN, which is a multifunctional phosphatase capable of dephosphorylating the same sites in membrane phosphatidylinositols phosphorylated by PI3K (42), was similar in all HCC cell lines.4

In the present study, metastasis could be suppressed not only by stable transfection of dominant-negative Akt but also by administration of a PI3K inhibitor. At present, no specific inhibitor for Akt is available, and the PI3K inhibitor, LY29402, is widely used. It can be speculated that LY294002 inhibits some other downstream effectors of PI3K in addition to Akt. The MAPK cascade and small G protein, Rac/cdc42, have been reported to be downstream of Akt (14). The rate of inhibition of metastasis with LY294002 was a little better than with dominant-negative Akt transfectants. Moreover, in vitro growth was more severely suppressed in cells treated with LY294002 than in the dominant-negative Akt transfectants. In Li7 cells, LY294002 inhibited activation of the MAPK cascade but did not inhibit activation of the Rac cascade.4 This result means that suppression of metastasis by LY294002 may be caused by inhibition of both Akt activation and MAPK activation.

These data suggest that a PI3K/Akt inhibitor may be used therapeutically to suppress metastasis. The side effects of in vivo administration of LY294002 are unknown, although no adverse reactions were evident in our study. The pharmacokinetics and pharmacodynamics of LY294002 are also poorly understood. Further work is required to determine the optimal dose and route of LY294002 administration. It is also expected that more specific inhibitors of Akt will be developed.

In conclusion, inhibition of the PI3K/Akt pathway was found to reduce metastasis of HCC by suppressing AIG. Inhibition of this pathway may be a potential new therapeutic strategy to suppress metastasis.

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4 Unpublished observation.

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