The in Vitro and in Vivo Effects of 2-(4-Morpholinyl)-8-phenyl-chromone (LY294002), a Specific Inhibitor of Phosphatidylinositol 3′-Kinase, in Human Colon Cancer Cells

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ABSTRACT

Purpose: Phosphatidylinositol 3′-kinase (PI3K) and Akt/protein kinase B (PKB) allow for escape from apoptosis in various human cancer cells. We postulated that 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a PI3K inhibitor, should inactivate Akt/PKB, consequently inhibiting cell proliferation and inducing apoptosis in vitro and in vivo.

Experimental Design: Human colon cancer cell lines (DLD-1, LoVo, HCT15, and Colo205) and their mouse xenografts (DLD-1 and LoVo) were used in this study. The expression of phosphorylated Akt (Ser473) and apoptosis in cancer cells were determined by immunoblotting and immunohistochemistry. To evaluate the activity of caspase-3 in culturing cells, the caspase colorimetric assay was also performed.

Results: LY294002 demonstrated a remarkable growth-inhibitory and apoptosis-inducing effect in these colon cancer cell lines, with decreased expression of phosphorylated Akt (Ser473). However, there was a great discrepancy between the sensitivity for LY294002 and the level of expression of phosphorylated Akt. Although the LoVo and Colo205 cells exhibited high sensitivity to LY294002 with increased apoptosis, the DLD-1 and HCT15 cells did not show rapid induction of apoptosis. The caspase-3 activity was significantly high in the LoVo cells but not in the DLD-1 cells. In the experiments using mouse xenografts, we found that LY294002 administration in vivo also resulted in suppression of tumor growth and induction of apoptosis, especially in the LoVo tumors, and therefore showed remarkable effectiveness in the mouse peritonitis carcinomatosa model.

Conclusions: PI3K-Akt/PKB plays an important role in colon cancer development and progression by helping to promote cell growth and allowing cells to escape apoptosis. These results propose the usefulness of LY294002 as an antitumoral agent for patients with colorectal cancer.

INTRODUCTION

Although genetic alterations on oncogenes and tumor suppressor genes have been identified in human colorectal cancer (1, 2), the contribution of altered extracellular communication to tumor growth and apoptotic potential has not been well studied. The fact that multiple growth factors may contribute to the development or progression of colorectal cancer highlights the importance of understanding how growth factor signals are transduced in colon cancer cells and identifying which pathways are important for cellular survival and therapeutic resistance. Among kinases identified to be downstream of growth factor receptor activation, a PI3K pathway is probably the best-characterized pathway known to promote cellular growth or survival and suppress apoptosis in cancer cells (3–5).

PI3K regulates a vast array of fundamental cellular responses and plays a critical role in controlling the balance between cell survival and apoptosis (4, 5). Akt/PKB, a key effector of PI3K in signaling cell survival, is phosphorylated in response to activation by many different growth factors and interleukins through phosphatidylinositides converted by PI3K (6, 7). Four phosphorylation sites have been identified at the NH2-terminal pleckstrin homology domain of Akt: (a) Ser124; (b) Thr308; (c) Thr450; and (d) Ser473 (8, 9). Thr308 and Ser473 are inducibly phosphorylated after treatment of cells using extracellular stimuli, whereas Ser124 and Thr450 appear to be basally phosphorylated. Mutagenesis studies have also demonstrated that phosphorylation of Thr308 and Ser473 is required for Akt activation and that mimicking phosphorylation partially activates Akt (8).

Because Akt delivers antiapoptotic survival signals by phosphorylating BAD (10), glycogen synthase kinase 3 (11, 12), and caspase-9 (13), inhibition of PI3K-Akt/PKB signaling using 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of PI3K (14), can cause apoptosis in various human cancer cells in vitro (15–18). Additionally, LY294002 effec-

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3 The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; FBS, fetal bovine serum; PKB, protein kinase B; Ac-DEVd-pNA, N-acetyl-Asp-Glu-Val-Asp-pNA; Ac-DEVd-CHO, N-acetyl-Asp-Glu-Val-Asp-CHO; TNF-α, tumor necrosis factor α; SCID, severe combined immunodeficient; ssDNA, single-stranded DNA; ABI, apoptotic body index.
tively induced apoptosis in pancreatic cancer cell lines under nutrient starvation conditions (17) and greatly potentiated chemotherapy-induced apoptosis in lung cancer cells with high levels of Akt activity (18). However, the growth-inhibitory and apoptosis-inducing effect of LY294002 has not been studied in any tumor system in vivo.

In the present study, we evaluated the effects of LY294002 in vitro, especially targeting for the suppression of cell proliferation and induction of apoptosis, as well as altered expression of phosphorylated Akt. The biological effects of this agent in vivo were also evaluated with mouse xenografts, testing the possibility that LY294002 might be useful as an antitumoral drug for human colorectal cancer. These data show that targeting a specific kinase that promotes survival, such as Akt, can change the proliferative activity or the apoptotic potential of colon cancer cells, suggesting the possibility that LY294002 might be a therapeutic agent for colorectal cancers.

**MATERIALS AND METHODS**

**Cell Lines and LY294002 Treatment.** Colon cancer cell lines DLD-1, LoVo, HCT15, and Colo205 were used in this study. No genetic alterations in the Akt gene were detected in these cells (data not shown). The cells were routinely maintained in RPMI 1640 supplemented with 1 mM l-glutamine, 10% (v/v) FBS (Life Technologies, Inc., Rockville, MD), and 100 units/ml penicillin G. Cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. LY294002 (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO at a stock concentration of 10 mM and added to cell cultures at a final concentration of 0–50 μM. We found that the final concentration of DMSO used in our study did not affect cell survival or protein phosphorylation.

**WST-1 Cell Proliferation Assay.** Cell growth was determined using the Premix WST-1 Cell Proliferation Assay System (Takara Bio-chemicals Co., Tokyo, Japan); a new tetrazolium salt WST-1 was evaluated for use in a colorimetric System (Takara Bio-chemicals Co., Tokyo, Japan); a new tetraphosphorylation.

**Western Blotting.** The cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor (Sigma Chemical Co.) and 1% phosphatase inhibitor mixture II (Sigma Chemical Co.). Cell lysates (40 μg of each) were separated by SDS-PAGE on a polycrylamide gel followed by electrotransfer onto a sequi-blot polyvinylidene difluoride membrane (Bio-rad, Richmond, CA). Phosphorylation-specific rabbit polyclonal antibodies for Akt (Ser473) and Akt were purchased from New England Biolabs (Beverly, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Horseradish peroxidase-conjugated goat antirabbit or antimouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA) was used as a secondary antibody for enhanced chemiluminescence (NEF Life Science Products, Inc., Boston, MA).

**Apoptosis and Caspase-3 Activity.** After treatment with LY294002, apoptotic cells in DLD-1 and LoVo cell lines were counted using propidium iodide staining. To detect DNA fragmentation, genomic DNA extracted from cells treated with 20 μM LY294002 was electrophoresed in 1% agarose gels as described elsewhere (20).

The activity of caspase-3 was evaluated by the caspase colorimetric substrate/inhibitor (BioMed Research Laboratories, Inc., Plymouth Meeting, PA). Briefly, 5 × 10⁵ cells treated with 20 μM LY294002 under serum-free conditions were suspended in cell lysis buffer [50 mM HEPES, (pH 7.4), 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 1 mM DTT, and 1 mM EDTA] and then centrifuged at 10,000 × g at 4°C for 10 min. A total of 50 μl of assay buffer [100 mM HEPES (pH 7.4), 200 mM NaCl, 0.8% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 20 mM DTT, 2 mM EDTA, and 20% glycerol] and 10 μl of Ac-DEVD-pNA substrate were added to triplicate culture wells in 96-microwell plates. Ac-DEVDD-CHO, an aldehyde caspase-3-specific inhibitor, was also used to test the specificity of caspase-3. The plates were then incubated at 37°C for 6 h, and caspase-3 activity in the lystate was determined by absorbance at 405 nm on a microwell plate reader. As a control, TNF-α (100 ng/ml; Upstate Biotechnology, Lake Placid, NY) was also examined.

**Animal Models.** SCID 6-week-old female mice derived from C.B-17/Icr (Clea, Tokyo, Japan) were used in this study. All mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Yamagata University School of Medicine (Yamagata, Japan), in accordance with the Guide for Animal Experimentation, Yamagata University School of Medicine, and Japanese Governmental Law 105. Both DLD-1 and LoVo cancer cells (1.0 × 10⁶ cells) in PBS were inoculated either s.c. (group A, n = 10) or into the peritoneal cavity (group B, n = 10), respectively (day 0). The test mice showed s.c. tumor (>5 mm in the greatest dimension) or peritoneal dissemination (macroscopically/microscopically) at day 20.

From days 20–40, LY294002 (2.0 ng/ml/kg) was administered, via an i.v. or i.p. injection, to half (n = 5) of the mice in group A and B, every day, respectively. The control mice (n = 5) in this study were treated with vehicle (DMSO) via an i.v. or i.p. injection, respectively. At day 40, all mice were sacrificed to evaluate the effect of LY294002 on tumor growth, size, and so forth. In group A, tumor growth was assessed by measuring two perpendicular diameters with a caliper. Tumor volume (V) was calculated as V = A × B², where A was the width of the tumor in millimeters, and B was the length of the tumor, as described previously (21). In group B, the efficacy of this drug on peritoneal carcinoma nodules was confirmed macroscopically and microscopically, s.c. tumors and intra-abdominal organs were fixed with 10% buffered formalin and embedded in paraffin for subsequent immunohistochemical analyses. We found that the final concentration of DMSO and LY294002 used in these experiments did not affect any organs or life span.

**Immunohistochemistry.** The expression of Akt (Ser473) and the number of apoptotic bodies in the xenografts were examined immunohistochemically. A rabbit polyclonal anti-
body for Akt (Ser^{473}) used in immunoblotting was also available for immunohistochemistry. Apoptosis induced in the xenografts was detected by using ssDNA rabbit polyclonal antibody purchased from DAKO (Glutrup, Denmark). Modifications of the immunoglobulin enzyme bridge technique (avidin-biotin complex method) were used, as described elsewhere (22). Briefly, deparaffinized tissue sections were immersed in methanol containing 0.03% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in citrate buffer was performed to retrieve antigenicity. After incubation with a blocking buffer, the sections were treated at room temperature with each antibody for 6 h. Avidin DH-biotinylated horseradish peroxidase complex (Nichirei, Tokyo, Japan) was used. Peroxide staining was performed for 2–5 min using a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with 0.1% methylgreen or hematoxylin. The ABI was determined by counting the number of ssDNA-positive cells in a total of 10 high power fields (×400).

Statistical Analyses. The efficacies of LY294002 in vivo (the tumor volume and ABI) were investigated by a Mann-Whitney t test and a χ^{2} test. *P < 0.05 was considered statistically significant.

RESULTS

The in Vitro Effects of LY294002: Suppression of Cell Survival, Induction of Apoptosis, and Inactivation of Akt in Colon Cancer Cell Lines. To determine the role of PI3K in colon cancer cell lines, we first tested the ability of LY294002 to inhibit cell proliferation and Akt phosphorylation by culturing cells. When the cells were incubated in medium containing 20 μM LY294002 for 48 h, cell proliferation was remarkably decreased (>80%) in LoVo and Colo205 cells (Fig. 1A). Even for relatively tolerant cell lines such as DLD-1 and HCT15, >30% of cells died after 48 h. However, LY294002 inhibited phosphorylation of Akt (Ser^{473}) in the four colon cancer cell lines (Fig. 1B).

Next, we analyzed a dose-response effect of LY294002 using DLD-1 and LoVo cells. LY294002 (10–50 μM), when added for 24 h, significantly decreased the population of DLD-1 cells in a concentration-dependent manner. When the cells were treated with 10, 20, and 50 μM LY294002 in the presence (absence) of FBS, the mean absorbance decreased to 88% (44%), 61% (40%), and 52% (38%), respectively (Fig. 2A). In contrast, a marked growth-inhibitory effect (<30%) was observed in LoVo cells regardless of FBS or LY294002 concentrations (Fig. 2B). LY294002 (10–50 μM) significantly reduced the levels of phosphorylated Akt in both DLD-1 and LoVo cells in a concentration-dependent fashion, but the levels of total Akt were not changed (Fig. 2B).

To elucidate the apoptosis-inducing effect of LY294002, we counted apoptotic bodies and observed DNA fragmentation in DLD-1 and LoVo cells. The percentage of apoptotic bodies in DLD-1 cells was low (<10%) when cells were placed in medium containing 10% FBS, but cells underwent apoptosis in a concentration-dependent manner when kept under serum-free conditions and treated with the PI3K inhibitor LY294002 (Fig. 3A, left). LoVo cells rapidly underwent apoptosis when treated with LY294002 in the presence or absence of FBS (Fig. 3A, right). DNA fragmentation was also detected in LoVo cells but not in DLD-1 cells (Fig. 3B).

With regard to the induction of apoptosis, we investigated caspase-3 activity in cells treated with the PI3K inhibitor. The caspase-3 activity in DLD-1 cells was up-regulated by LY294002 and was greater than that induced by TNF-α (Fig. 4, left). However, the level of caspase-3 activity was significantly high in LoVo cells even when the cells were maintained under normal conditions (Fig. 4, right). When treated with 100 ng/ml TNF-α, only 10% of the cells underwent apoptosis in each cell line (data not shown).

The in Vivo Effect of LY294002: Antitumoral Effects for Human Colon Cancer Xenografts. We compared these findings with in vivo examinations using mouse models. We transplanted cancer cells into SCID mice to prevent host rejection. Human tumor cell line-SCID mouse chimeric models were proper to exclude the effect of the host immune system because PI3K also controls thymocyte development and T-cell activation (23). The effects of LY294002 on the volume of the tumors and ABI are summarized in Table 1. The mean tumor volumes of
DLD-1 and LoVo xenografts at day 0 were 1.230 ± 0.333 × 10³ and 0.822 ± 0.101 × 10³ mm³, respectively. The i.v. administration of LY294002 to mice in group A significantly suppressed the growth of s.c. tumors derived from both DLD-1 and LoVo cells, with a mean volume of 72% (range, 48–82%) and 55% (range, 40–61%), respectively. There were also statistical differences in the mean volume of each DLD-1 (P = 0.022) and LoVo (P = 0.011) xenograft according to the Mann-Whitney test. Intra-abdominal dissemination nodules were significantly suppressed in mice treated with LY294002 in group B, whereas multiple mesenteric dissemination was detected in the controls (Fig. 5). The i.p. injection of LY294002 was not effective in inducing any macroscopic changes in tumors of DLD-1 cell origin (data not shown).

Finally, we performed a histological examination and used immunohistochemistry to identify the biological influences of this drug on tumor morphology, induction of apoptosis, and expression of phosphorylated Akt. After LY294002 treatment, DLD-1 xenografts demonstrated necrotic change in the central region of the tumors, but no histological findings were detected by H&E staining (data not shown). Although distinct tubular formation and well-arranged nuclei were observed in the LoVo xenografts, these tubules converted into solid/agglutinated structures, and a simultaneous loss of nuclear polarity also occurred after LY294002 treatment (Fig. 6, A and B). The ABI in each s.c. tumor after LY294002 treatment was detected using anti-ssDNA antibody (Table 1). The LoVo xenografts in particular exhibited a marked 7.3-fold (P < 0.001) increase in ABI, indicating that the in vitro apoptosis-inducible effect of LY294002 also occurs in vivo (Fig. 6, C and D). Cytoplasmic/intranuclear phosphorylated Akt (Ser473) was remarkably decreased in LoVo tumors after treatment with the PI3K inhibitor (Fig. 6, E and F). No alterations of the level of expression of phosphorylated Akt (Ser473) and ABI were detected in DLD-1 xenografts (Table 1).

DISCUSSION

The uncontrolled proliferation of cancer cells is up-regulated mainly by growth factors via the activation of various intracellular signaling pathways associated with cell cycle entry and inhibition of apoptosis. PI3K is one of the core intracellular signaling molecules in the stimulation of growth factors, subsequently phosphorylating and activating a serine/threonine kinase, Akt/PKB (3–6). As reported previously, the PI3K-Akt/PKB pathway is an essential survival signaling pathway for...
The activity of caspase-3 was evaluated by the caspase colorimetric substrate/inhibitor as described in “Materials and Methods.” Briefly, 5 × 10^6 cells were suspended in cell lysis buffer and then centrifuged at 10,000 g at 4°C for 10 min. A total of 50 μl of assay buffer and 10 μl of Ac-DEVD-pNA substrate were added to triplicate culture wells in 96-microwell plates. Ac-DEVD-CHO, an aldehyde caspase-3–specific inhibitor, was also used to test the specificity of caspase-3. The plates were then incubated at 37°C for 6 h, and caspase-3 activity in the lysate was determined by absorbance at 405 nm on a microwell plate reader. Absorbance (405 nm) of cleaved Ac-DEVD-pNA substrate (●), Ac-DEVD-CHO substrate (□), and control (△) are illustrated. The cells were treated with 20 μM LY294002 or 100 ng/ml TNF-α.

**Fig. 4** Activity of caspase-3 in DLD-1 and LoVo cells. The activity of caspase-3 was evaluated by the caspase colorimetric substrate/inhibitor as described in “Materials and Methods.” Briefly, 5 × 10^6 cells were suspended in cell lysis buffer and then centrifuged at 10,000 g at 4°C for 10 min. A total of 50 μl of assay buffer and 10 μl of Ac-DEVD-pNA substrate were added to triplicate culture wells in 96-microwell plates. Ac-DEVD-CHO, an aldehyde caspase-3–specific inhibitor, was also used to test the specificity of caspase-3. The plates were then incubated at 37°C for 6 h, and caspase-3 activity in the lysate was determined by absorbance at 405 nm on a microwell plate reader. Absorbance (405 nm) of cleaved Ac-DEVD-pNA substrate (●), Ac-DEVD-CHO substrate (□), and control (△) are illustrated. The cells were treated with 20 μM LY294002 or 100 ng/ml TNF-α.

**Table 1** Growth inhibition and induction of apoptosis by the PI3K inhibitor LY294002 in human colon cancer xenografts.

<table>
<thead>
<tr>
<th>Tumor volume (×10^3 mm^3)</th>
<th>DLD-1</th>
<th>LoVo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean SD</td>
<td>Mean SD</td>
<td></td>
</tr>
<tr>
<td>Control 2.330 0.544</td>
<td>1.433 0.451</td>
<td>0.011</td>
</tr>
<tr>
<td>LY294002 1.591 0.120</td>
<td>0.646 0.176</td>
<td></td>
</tr>
<tr>
<td>ABI 11.4 3.97</td>
<td>19.0 6.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LY294002 12.4 4.66</td>
<td>139.4 16.41</td>
<td></td>
</tr>
</tbody>
</table>

*Ps for tumor volume of control and treatment groups were estimated by the Mann-Whitney U test and the χ² test, respectively.

**Fig. 5** Effects of the i.p. injection of LY294002 for intra-abdominal dissemination mouse models. A, control group; B, LY294002-treated group (2.0 ng/ml/kg). No disseminated nodules were detected in LoVo xenograft treated with 2.0 ng/ml/kg LY294002.

In the current study, we used immunoblotting with phosphorylation-specific antibody to demonstrate Akt activity in cultured cells and then confirmed the ability of the LY294002 PI3K inhibitor to decrease Akt phosphorylation and cell viability in these colon cancer cell lines. However, there was a great discrepancy between the sensitivity to LY294002 and the level of expression of phosphorylated Akt. The proliferation of DLD-1 cells was suppressed by LY294002 in a dose-dependent manner, but growth inhibition and apoptosis were immediately induced in LoVo cells regardless of LY294002 concentration. What causes these differences in cell sensitivity to LY294002? Possible explanations include mutations of Akt itself or other downstream kinases rather than activation of various growth factor receptors, mutations of PI3K, or other upstream regulatory molecules, such as PDK-1 and PTEN (6), because the phosphorylation level of Akt (Ser^473) in vitro was PI3K dependent in these colon cancer cell lines. Therefore, these results indicate not only the possibility that cell growth signals from growth factors might pass mainly through the PI3K-Akt/PKB pathway in LoVo cells but also the possibility that another growth/survival pathway may exist in DLD-1 cells, such as extracellular signal-regulated kinases (25, 26). However, because these examinations using LY294002 are unable to assay direct effects of PI3K in these cells, clarification of the intracellular signaling network of cells awaits additional investigations. Possible mechanisms for regulation of Akt include activation of upstream kinases such as PI3K or PDK-1, inhibition of lipid or protein phosphatases that normally regulate Akt function, and/or other downstream signaling molecules such as glycogen synthase kinase 3, BAD, and caspase-9 (10–13).

Additionally, caspase-3 works downstream of Akt in cell signaling, subsequently leading cells to undergo apoptosis (6, 15). In this study, caspase-3 in LoVo cells was significantly active in both the presence and absence of LY294002 or TNF-α, whereas the ratio of apoptotic bodies of LoVo cells was higher when cells were treated with LY294002 than when cells were treated with TNF-α. We considered the possibility that the apoptosis-blocking molecule(s) might be down-regulated by LY294002 and then lead LoVo cells into apoptosis. In contrast, DLD-1 cells exhibit an increase in caspase-3 activity after LY294002 treatment, which is relatively effective compared to various human cancers, including carcinomas of the prostate, ovary, breast, pancreas, and lung (15–18). Additionally, we previously investigated the expression of phosphorylated Akt (Ser^473) in colon cancer tissue samples immunohistochemically and found a close association with cell growth, resistance to apoptosis, and clinicopathological parameters (24). Therefore, in the current study, we evaluated the effects of LY294002 in vitro and in vivo, especially targeting altered expression of phosphorylated Akt (Ser^473) and the possibility that LY294002 might be a therapeutic agent for colorectal cancers.
with the effectiveness of TNF-α. It would be of great interest to see how the PI3K-Akt pathway controls apoptosis and regulates caspase-3 activity in colon carcinoma cells.

To the best of our knowledge, the present study is the first report that refers to the \textit{in vivo} effectiveness of LY294002. The \textit{in vivo} efficiency of LY294002 was significantly reduced in each tumor derived from DLD-1 and LoVo cells, indicating the possibility that LY294002 may be a useful therapy in inhibiting colon cancer. Moreover, a remarkable induction of apoptosis and suppression of tumor formation in the mesenterium of LoVo xenografts support the idea of using the LY294002 PI3K inhibitor as an antitumoral therapy for patients with colon cancer and peritonitis carcinoma. How xenograft growth will change after cessation of treatment with LY294002 is of great interest.

Although accumulation of genetic alterations in oncogenes and tumor suppressor genes has been reported in studies of multistep colon carcinogenesis (1, 2), response to stimuli from growth factors is considered to be a common characteristic of all carcinoma cells. Therefore, these results indicate that LY294002 may block tumor progression despite a patient’s genetic background. Brognard \textit{et al.} (18) have demonstrated previously that Akt activity might promote therapeutic resistance in human non-small cell lung cancer cells and that LY294002 greatly potentiated chemotherapy-induced apoptosis in cells with high Akt levels, but not in cells with low Akt levels. Moreover, LY294002 additively increased apoptosis and inhibited clonogenic growth when combined with radiation in cells with active Akt (18). In addition to showing the usefulness of LY294002 under a nutrient-deprived condition in pancreatic cancer cell lines, Izuishi \textit{et al.} (17) proposed that LY294002 administration with tumor embolization may be a new therapeutic strategy designed to prevent metabolic adaptation of cancer cells. Thus, these findings suggest the possibility of treating human malignancies using the PI3K inhibitor LY294002. The Akt phosphorylation status in each carcinoma case can monitor aspects of malignancies such as cell proliferation rate, resistance to chemotherapy and irradiation, invasion and metastasis, and patient prognosis (18, 24). Evaluation of Akt as a predictive or even a prognostic factor depends on the development of reliable assays to measure Akt activity. The fact that immunoblotting with phosphorylation-specific antibody correlated with \textit{in vitro} Akt kinase assays suggests that the use of this antibody in standard pathological techniques such as immunohistochemistry and/or immunoblotting might allow valid surrogate measurements of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6.png}
\caption{Representative fields of immunohistochemical examination of LoVo mouse s.c. tumors after treatment with the PI3K inhibitor LY294002.\textit{A} and \textit{B}, LoVo xenografts microscopically exhibited a tubular pattern and well-arranged nuclei, but the tubular structure disappeared, whereas apoptotic bodies increased significantly (\textit{inset}) after treatment with LY294002.\textit{C} and \textit{D}, apoptosis was confirmed by immunohistochemical staining of ssDNA. An increase in apoptosis was observed in tumors treated with LY294002.\textit{E} and \textit{F}, cytoplasmic and nuclear expression of Akt (Ser\textsuperscript{473}) decreased after LY294002 treatment.}
\end{figure}
Akt activity in vivo and would facilitate assessment of Akt’s clinical importance. It would be of great interest to see whether these observations can be generalized to other cancer-derived cell lines and tumor samples.

s.c. LoVo tumors without LY294002 injection showed a relatively distinct tubular formation, whereas cancer cells showed a solid pattern after administration of this PI3K inhibitor. Akt may not only mediate prevention of apoptosis and cell proliferation but may also be involved in the maintenance of morphological gastrulation, especially in a tubular pattern. Chung et al. (27) reported that addition of the PI3K inhibitor LY294002 results in a rapid loss of cell polarity in MGP1 cells, indicating that inactivation of PI3K may possibly block colon cancer cells from forming a glandular structure.

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