**PI3K inhibitor reduced the expression level of pAkt and induced apoptosis in MKN1 cells**

Through SN-38 treatment, pAkt expression in MKN45 cells was reduced but not in MKN1 cells while MKN45 cells have a higher sensitivity to SN-38 than MKN1 cells. To elucidate that the PI3K-Akt pathway is involved in SN-38-induced apoptosis, we examined whether LY294002 could affect SN-38-induced apoptosis in MKN1 cells. LY294002 is a specific PI3K inhibitor that has been widely used to study the role of PI3K in various biological responses.

The expression level of pAkt in MKN1 cells reduced in an SN-38 dose-dependent manner after

**Fig. 3.** Influence of SN-38 to MKN1 cells.
A: Effect of 20 nM or 250 nM SN-38 on the cell cycle progression of MKN1 cells after 48 h incubation determined by flow cytometry.
B: Fluorescence microscopy dyeing, Hoechst 33342 after 250 nM SN-38 treatment for 48 h.

**Fig. 4.** Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 for 48 h. *P < 0.05 using Student’s *t* test.
pretreatment with 50 µM LY294002 (Fig. 6). We also examined PTEN expression using Western blotting, but LY294002 had no influence on PTEN expression (Fig. 6).

Moreover, by cell cycle analysis, the apoptotic sub-G1 fraction appeared by flow cytometry (Fig. 7A). By morphological examination, apoptotic cells were increased by treatment with SN-38 and LY294002 (Fig. 7B). The apoptotic sub-G1 fraction of the population of MKN1 cells was 35.9% 48 h after treatment with 250 nM SN-38 and 50 µM LY294002 (Fig. 8). Exposure to 50 µM LY294002 increased SN-38-induced apoptosis in MKN1 cells to as much as that in MKN45 cells after treatment with only SN-38 (Fig. 8). These results indicated that the PI3K-Akt pathway plays a significant role in SN-38-induced apoptosis in human gastric cancer cells.

Discussion

The MKN45 cells were more sensitive to SN-38 than the MKN1 cells. The difference of sensitivity mainly comes from the percentage of apoptotic cells. The pattern of tumor response after in vivo topotecan treatment was reported to correlate with the ability of the drug to induce apoptosis but not with its in vitro antiproliferative activity (Caserini et al., 1997). Apoptosis is a major mode of cell death in response to chemotherapeutic drug treatment (Hickman, 1992; Kerr et al., 1994), and resistance to apoptosis induction has been proposed as a critical mechanism in drug resistance (Kataoka et al., 1994; Chen et al., 1996; Seimiya et al., 1997). However, the mechanisms by which chemotherapeutic agents induce apoptosis are not fully understood. The sensitivity of cells to chemotherapeutic drug-induced apoptosis appears to depend on the balance between proapoptotic and antiapoptotic signals. Therefore, it is possible that a chemotherapeutic drug may induce apoptosis not only by increasing the proapoptotic signals but also by decreasing the antiapoptotic ones.

The PI3K-Akt signaling pathway is known to transmit survival signals and to protect a variety of cells from apoptosis. Akt plays a critical role in controlling the balance between cell survival and apoptosis (Balendran et al., 1999). Phosphorylation of Akt is promoted by phosphatidylinositides converted by PI3K products. Previous reports have
shown that Akt delivers antiapoptotic survival signals by phosphorylating Bad and activating caspase-9 (Cardone et al., 1998; Brunet et al., 1999). In addition, inhibition of Akt signaling can induce apoptosis in some human cancer cell lines (Page et al., 2000; Yuan et al., 2000). It has also been reported that Akt plays an important role in cell survival when cells are exposed to different apoptotic stimuli, such as growth factor withdrawal, ultraviolet radiation, matrix detachment, cell cycle discordance and DNA damage (Hemmings, 1997; Kulik et al., 1997; Kulik and Weber, 1998; Ng et al., 2000; Brognard et al., 2001). Moreover, inactivation of PI3K using a specific inhibitor has led to dephosphorylation of Akt at Ser473, consequently causing translocation of Akt to nuclei,

Fig. 7. Influence of LY294002 to MKN1 cells.

A: Cell cycle analysis by flow cytometry.
B: Morphological detection of apoptosis by Hoechst 33342 staining.

Fig. 8. Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 in the presence or absence of LY294002 for 48 h. *P < 0.05 using Student’s t-test.
where it is believed to regulate the transcription of genes mediating cell survival or apoptosis (Hemmings, 1997). In a previous study, TRAIL and Fas induced apoptosis by inhibition of the PI3K-Akt pathway in human gastric cancer cells (Nam et al., 2003; Osaki et al., 2004). Our study showed that apoptosis was induced in cells where pAkt expression was reduced (MKN45), but not in cells where pAkt expression showed no change (MKN1) by SN-38. It has been reported that exposure to camptothecin induced a dose-dependent trapping in the G2/M phase and dose-dependent delay in the S phase. Treatment of MKN1 cells with 48 h 20 nM SN-38 induced G2/M phase arrest, and 48 h 250 nM SN-38 treatment induced S phase delay. Meanwhile, in MKN45 cells, 48 h 20 nM SN-38 treatment induced G2/M phase arrest tendency and 48 h 250 nM SN-38 treatment induced sub-G1 peak. We speculate that low dose SN-38 treatment below 20 nM induces the G2/M arrest in MKN45 cells and 20 nM SN-38 treatment begins to induce S phase cells. Moreover, in MKN45 cells, 250 nM SN-38 treatment induces apoptosis by reducing pAkt expression but MKN1 cells showed resistance for apoptosis by no change in pAkt expression.

Therefore, inactivation of pAkt using the PI3K inhibitor, LY294002, finally induced apoptosis in MKN1 cells as well as in only SN-38-treated MKN45 cells. It was also reported (Nakashio et al., 2000) that constitutively active Akt results in a reduction of the cytotoxic effect of topotecan in lung carcinoma cells. The PI3K-Akt pathway might thus play a significant role in SN-38-induced apoptosis in human gastric cancer cells.

The tumor suppression gene PTEN is a natural biological inhibitor of Akt cell survival signals. PTEN expression sensitizes cells to death receptor-mediated apoptosis induced by a variety of stimuli, such as tumor necrosis factor (Mayo et al., 2002), TRAIL (Thakkar et al., 2001) and chemotherapeutic agents (Tanaka et al., 2000). Akt is abnormally activated in several human tumors by loss of PTEN (Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001). Recent studies have documented that PTEN appears to negatively regulate PI3K-Akt survival signaling by removing the D-3 phosphate from PIP3, which is a primary activator of Akt (Maehata and Dixon, 1998). Loss of PTEN results in high basal activity of Akt in a variety of tumors, while introduction of PTEN suppresses the activity. It is reported (Saga et al., 2002) that overexpression of PTEN increased sensitivity to SN-38 in ovarian cancer cells. However, in our study, the expression of PTEN was not correlated with SN-38-induced apoptosis; in human gastric cancer cells, SN-38 might suppress the PI3K-Akt signaling via a PTEN independent pathway. Rokudai et al. (2002) reported that suppression of PDK1, but not of PI3K, was one of the mechanisms of chemotherapeutic drug-mediated Akt inactivation, because VP-16 indirectly suppressed PDK1 kinase activity in 293T cells. However, the mechanisms of SN-38-mediated Akt inactivation remain unclear. Overexpression of constitutive active Akt using a transfected active form of akt cDNA overcame chemotherapeutic drug-induced apoptosis. Akt inactivation might be a sign of chemotherapeutic drug sensitivity of cancer cells, because it was found only in chemosensitive cells (MKN45).

Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor involved in a wide spectrum of cellular functions like cell cycle control, stress adaptation, inflammation and control of apoptosis (Pahl, 1999). NF-κB is constitutively activated in various types of tumors, including gastric cancer (Sasaki et al., 2001). Several investigators have reported that inhibition of the NF-κB pathway by adenoviral delivery of an IκBα superrepressor, small interfering RNA, against the p65 subunit of NF-κB or TNFα increased SN-38-induced apoptosis (Camp et al., 2004; Guo et al., 2004; Valente et al., 2003). Besides the PI3K-Akt pathway, the NF-κB pathway might also facilitate an antiapoptotic signal to inhibit SN-38-induced apoptosis.

In conclusion, we found that SN-38 induced apoptosis by decreasing the PI3K-Akt survival signaling, the antiapoptotic signals in human gastric cancer cells. A selective small molecule inhibitor of Akt, the Akt/protein kinase B signaling inhibitor-2 (API-2) was recently discovered by screening the National Cancer Institute Diversity Set (Yang et al., 2004). Such an Akt inhibitor might be a useful
anti-tumor agent in combination with a chemotherapeutic agent, such as CPT-11.

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