Cyclin D1 Antisense Oligonucleotide Inhibits Cell Growth Stimulated by Epidermal Growth Factor and Induces Apoptosis of Gastric Cancer Cells

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The cyclin D1 protein is one of the cell cycle regulators required for cell cycle progression through G1 phase to S phase. The cyclin D1-cyclin-dependent kinase (CDK) system is thought to control the cell cycle through mediating extracellular signals from mitogens, such as epidermal growth factor (EGF). In this study, we attempted to examine the therapeutic effect of cyclin D1 antisense oligonucleotides (AS/D1) on cell proliferation and apoptosis of the gastric cancer cell line MKN-74, in the presence and absence of EGF-stimulation. Evaluation of cell survival and DNA synthesis revealed that enhanced cell growth following EGF-stimulation was completely inhibited by a 24 h pre-incubation with 100 nM AS/D1. This inhibition was down to 19.3% compared with maximal DNA synthesis after stimulation with 3 nM EGF alone. Western blotting demonstrated that while EGF-stimulation led to cyclin D1 over-expression, AS/D1 inhibited cyclin D1 protein expression. We also demonstrated the induction of apoptosis in MKN-74 cells by AS/D1. In conclusion, EGF-stimulated MKN-74 cell proliferation was inhibited by AS/D1, which could overcome EGF-induced cyclin D1 over-expression. AS/D1 also affected cell survival by inducing apoptosis through cell cycle arrest following cyclin D1 depletion. Thus, AS/D1 may be a candidate for use as a novel cancer therapy specifically targeted against the over-expression of cyclin D1 enhanced by EGF in malignant cells.

Key words: Gastric cancer cell lines — Antisense oligonucleotide — Epidermal growth factor — Cyclin D1 — Apoptosis

Cell cycle progression is regulated by the formation of specific protein kinase complexes, consisting of a cyclin-dependent kinase (CDK), proliferating cell nuclear antigen (PCNA), and regulatory cyclin molecules. Cyclin D1 is a 36 kDa protein recognized to be a cell cycle regulator required for cell cycle progression through G1 phase to S phase. Cyclin D1 activates specific CDKs by forming a complex, allowing cell cycle progression,[1, 2] and the cyclin D1-CDK system is a critical factor in mid-G1 to late G1 phase cell cycle progression.[3, 4] The cyclin D1-CDK system also allows the cell cycle to be modulated via extracellular signals, including mitogens.[5, 6]

Epidermal growth factor (EGF) is a 6 kDa protein that acts as a potent mitogen for both normal and malignant cells through interaction with specific cell surface receptors. EGF binding leads to phosphorylation of receptor tyrosine residues, resulting in intracellular signal transduction and enhancement of several regulatory cascades associated with mitogenic events.[7, 8] EGF is clinically recognized as a critical factor in over 30% of gastric cancers, especially in gastrointestinal cancers that show high EGF receptor expression levels.[9, 10] High levels of EGF receptor expression or EGF production have been correlated with poor prognosis in gastric cancer patients in several studies.[11] EGF is also known to up-regulate the expression of cyclin D1, which appears to be a major determinant of EGF-mediated CDK activation.[12–14]

In order to investigate gene targets for antitumor therapy, it is necessary to understand the fundamental differences in gene expression between normal and tumor cells.[15] Knowledge of these differences has allowed the development of antisense strategies that target genes expressed by cancer cells, but not by normal cells. Antisense oligonucleotides (ON) are short stretches of nucleic acids that bind to complementary target mRNA forming mRNA-ON hybrid molecules that inhibit mRNA translation and thereby reduce the activity of the targeted gene product.[16] Cyclin D1 antisense ON have been reported to show growth-inhibitory effects against ovarian cancer cells,[17] while another study proposed that cyclin D1 antisense ON were able to revert malignant cells as a method of cancer chemoprevention.[18]

In this study, we used the gastric cancer cell line MKN-74 (derived from a differentiated gastric carcinoma), which expresses cyclin D1 protein.[19, 20] The purpose of

this study was to investigate the inhibitory effects of antisense ON of cyclin D1 (AS/D1) on MKN-74 cell growth, both in normal proliferation and EGF-stimulated proliferation, and to clarify mechanisms of AS/D1 including cyclin D1 protein expression and induction of apoptosis.

MATERIALS AND METHODS

Cell lines and culture Both MKN-74, a moderately differentiated gastric adenocarcinoma cell line, and MKN-45, a poorly differentiated gastric adenocarcinoma cell line, were established at Niigata University.[19] TMK-1, a poorly differentiated gastric adenocarcinoma was established at Hiroshima University.[21] Human umbilical vein endothelial cells (HUVEC), which do not show strong growth enhancement with EGF, but do so with vascular endothelial cell growth factor (VEGF), was used as non-cancerous cells.[22, 23] Cancer cells were suspended in control culture medium containing RPMI 1640 with 20% fetal bovine serum (FBS) F4010 (Sigma, St. Louis, MO), or HUVEC was suspended in endothelial basic medium (EBM, Sigma) with EBM supplement (Sigma). One hundred microliters aliquots containing 10^4 cells were plated into 96-well tissue culture plates (35-3072 Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). After 24 h preincubation in control medium with FBS and another 24 h incubation in FBS-starved or EBM supplement-starved control medium, 100 μl of medium containing the required concentrations of AS/D1 or control vehicle was added. Plates were incubated for 24 h, then EGF was added at various concentrations and incubation was continued for the required periods. All incubations were carried out at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Drugs and oligonucleotides EGF was purchased from Research Plus, Inc., Bayonne, NJ, anti-cyclin D1 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) for cell suspension assay was purchased from Sigma. AS/D1, corresponding to cyclin D1 DNA coding sequence, were designed, synthesized, and modified by insertion of a phosphothionate bond at the 3′-end to increase the resistance to endogenous nucleases (Yale Medical School Keck Oligonucleotide Synthesis Facility). Nucleotides of the same length with randomized sequences were prepared as control nucleotides (nonsense ON of cyclin D1, NS/D1). Each lyophilized product was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and stored at −20°C. ON sequences used were: AS/D1, 5′-ATGGAACACCA-s-G-s-C-s-T-s-C-3′; NS/D1, 5′-GCAATGACCAG-s-C-s-C-s-A-s-T-3′ (where -s- shows the site of a phosphothionate bond).

Cell survival assay The viability and survival of cancer cells *in vitro* were evaluated using the MTT assay devel-oped by Mosmann, with some modifications.[24] This method evaluates the number of viable cells by measuring the activity of mitochondrial succinate dehydrogenase. After each treatment, 20 μl of MTT (4 mg/ml) and sodium succinate (0.1 M) in phosphate-buffered saline (PBS) filtered through 0.45 μm membrane filter (Millipore Co., Bedford, MA) was added to each well, and plates were incubated for a further 4 h at 37°C. Dimethylsulfoxide (150 μl/well) was then added to dissolve the formazan salt, the plates were shaken for a few minutes, and the absorbance of each well was read on a Bio-Kinetics Reader (Model EL312e, BIO-TEK Instruments, Inc., Winooski, VT) at 540–630 nm. Survival rates were calculated as: (absorbance of treated group − absorbance of blanks)/(absorbance of control − absorbance of blanks)× 100, as a percentage. Cells in control medium without MTT and sodium succinate were measured as blanks. Each experiment was performed in triplicate.

[^{3}H]Thymidine incorporation DNA synthesis was measured in terms of [^{3}H]methylthymidine incorporation. After 0–48 h treatment as described above, [^{3}H]thymidine at a final concentration of 1 μCi/ml was added to each well, and the cells were incubated for an additional 6 h. Medium was removed gently, replaced with ice-cold 10% trichloroacetic acid (TCA), and incubated for 10 min at 4°C. TCA was removed twice, followed by washing with 100% ethanol. Precipitated material was solubilized in 0.2 N NaOH/0.1% sodium dodecyl sulfate (SDS), and incorporated radioactivity was assessed by liquid scintillation counting. Experiments were performed in triplicate.

Western blotting MKN-74 cells (1×10^5) were plated into flasks and incubated for 24 h in control medium with FBS followed by another 24 h in FBS-starved control medium. Cells were then treated in 100 nM AS/D1, 100 nM NS/D1 or control medium for 24 h, EGF (1 nM final concentration) or control vehicle was added, and incubation was continued for another 24 h. Cells were harvested and lysed with lysis buffer {50 mM Tris HCl pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1.5 mM MgCl$_2$ and protease inhibitor cocktail tablets (Boehringer Mannhein GmbH, Mannheim, Germany) according to the manufacturer's instructions}. Protein was quantified by bicinchoninic acid (BCA) protein assay and 40 μg aliquots of protein were electrophoresed on 8% SDS-polyacrylamide gels, then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Co.). After incubation with 5% non-fat skim milk in Tris-buffered saline (TBS) for 1 h, the membranes were probed with anti-cyclin D1 rabbit polyclonal antibody overnight at 4°C, and reactivity was detected using the BM chemiluminescent Western Blotting Kit (Boehringer Mannhein GmbH) according to the manufacturer's instructions. Experiments were performed in triplicate, and

quantification of the detected bands was performed using the NIH image 1.6.2 system, in order to evaluate differences of cyclin D1 expression among the groups.

Apoptosis AS/D1-induced apoptosis was assessed morphologically by TUNEL assay using the In Situ Cell Death

Fig. 1. A. Survival rates of MKN-74 cells were determined using the MTT assay. Cells were incubated with control vehicle (○), 100 nM NS/D1 (□) or 100 nM AS/D1 (●) for 24 h, followed by incubation with various concentrations of EGF (0, 1, 5, 10 or 50 nM) for 48 h. Data are representative of three experiments. Values are given as means±SE (bars) of triplicate samples. Where bars are not shown, the SE is smaller than the symbol. ∗ Significant difference from the control ($P<0.05$). B. DNA synthesis in MKN-74 cells was evaluated by [^{3}H]thymidine uptake incorporation assay. Cells were incubated with control vehicle (○), 100 nM NS/D1 (□) or 100 nM AS/D1 (●) for 24 h. After incubation for a further 24 h with the indicated concentrations of EGF, [^{3}H]thymidine was added and incubation was continued for an additional 6 h. Data are representative of three experiments. Values are given as means±SE (bars) of triplicate samples. Where bars are not shown, the SE is smaller than the symbol. ∗ Significant difference from the control ($P<0.05$).

Detection Kit (Cat. No. 1 684 817, Boehringer Mannheim GmbH). Cells were incubated in chamber-slides as described above, and after fixation with 4% formaldehyde, they were stained according to the manufacturer's instructions. Apoptosis levels were also quantified using the Cell Death Detection ELISA (Cat. No. 1 544 675, Boehringer Mannheim GmbH) which detects the small nucleotide fragments produced as a result of cell apoptosis. The apoptotic index was calculated as an enrichment factor, that is, the ratio of the result compared with the control set arbitrarily at 1.0. Each experiment was performed in triplicate, and means and standard deviations were calculated.

RESULTS

Inhibitory effect on survival rate by AS/D1 The survival rate of MKN-74 cells increased after EGF stimulation, with up to 125.1±4.4% increase at 1 nM EGF (Fig. 1A). AS/D1 at 100 nM showed no inhibitory effect on survival rate in the absence of EGF stimulation, while 100 nM AS/D1 demonstrated a significant inhibitory effect on EGF-stimulated cells (at 1, 5, 10 and 50 nM EGF) with a maximal inhibition down to 20% observed (Fig. 1A). In contrast, NS/D1-treated cells showed no differences in cell proliferation compared with control cells.

Inhibitory effect on DNA synthesis by AS/D1 Measurement of DNA synthesis was used as a sensitive assay for changes in cell proliferation. Fig. 1B shows the DNA synthesis curves for EGF concentrations of 0.01, 0.1, 1, 3 and 10 nM. EGF at concentrations of 1, 3 and 10 nM significantly increased DNA synthesis in cells incubated without AS/D1 or with NS/D1, with a maximal increase of 182±21% compared to controls at 3 nM EGF. The addition of NS/D1 caused no inhibition of DNA synthesis at any EGF concentration examined. However, DNA synthesis in the absence of EGF-stimulation was inhibited 72.6% compared to controls by 100 nM AS/D1, while EGF-stimulated DNA synthesis was strongly suppressed down to 19.3%.

The concentration dependency of AS/D1 was confirmed using the [^{3}H]thymidine incorporation assay on cells stimulated with 1 nM EGF for 6 h. While 1 nM AS/D1 showed no significant inhibition of cells stimulated with 1 nM EGF (221.7±30.0% compared to controls) or of cells incubated in medium only (223.0±7.9% compared to controls), 10 nM and 100 nM AS/D1 inhibited EGF-enhanced DNA synthesis to 83.2±10.6% and 31.4±4.6%, respectively (Fig. 2). Thus, stimulation of DNA synthesis was inhibited by AS/D1 in a concentration-dependent manner.

The time-course of changes in DNA synthesis levels was evaluated using MKN-74 cells incubated with 100 nM AS/D1 or in media alone for 24 h and then stimulated with 1 nM EGF (Fig. 3). AS/D1-mediated inhibition of
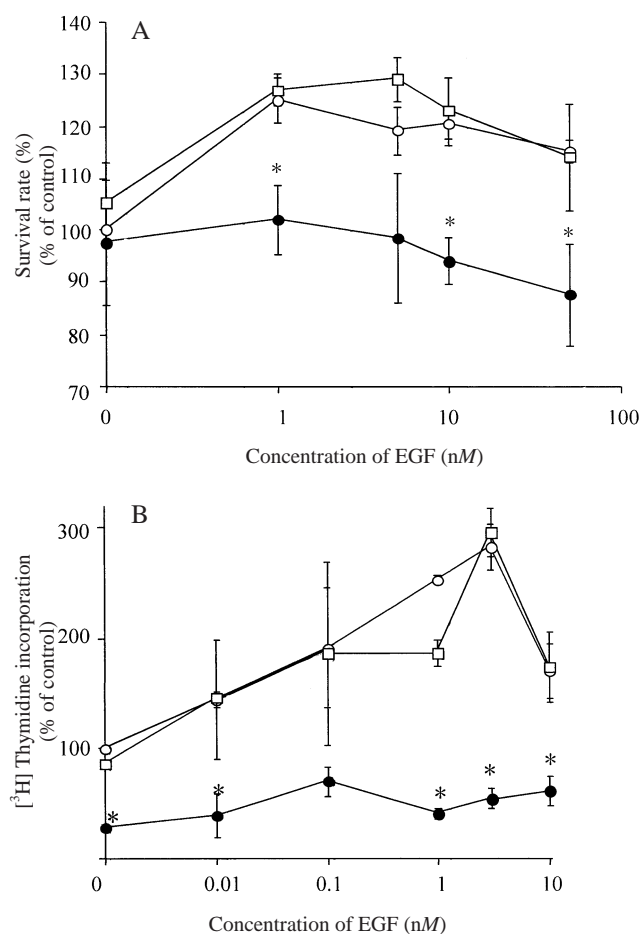
DNA synthesis was observed after the initial 24 h incubation (68.7±17.8%), prior to EGF-stimulation, and this inhibitory effect was maintained for 24 h following EGF-stimulation. However, the EGF-stimulatory and AS/D1-inhibitory effects on DNA synthesis diminished 48 h after EGF-stimulation.

Cyclin D1 expression decreased by AS/D1 (Fig. 4) While MKN-74 cells in the absence of EGF-stimulation showed moderate cyclin D1 expression, the cells stimulated by 1 nM EGF over-expressed cyclin D1 protein compared with the cells without EGF-stimulation ($P<0.05$). Exposure to 100 nM AS/D1 led to marked suppression of cyclin D1 protein levels with significant differences in the cells without EGF (25.2% of the control, $P=0.0085$) and in the cells with EGF (44.4% of the cells with EGF alone, $P=0.0274$) (Fig. 4). Thus, the AS/D1 suppression of EGF-stimulated cell proliferation appears to be mediated via inhibition of over-expressed cyclin D1. NS/D1 treatment had no significant effect on cyclin D1 expression compared with controls, indicating that the inhibition was AS/D1-specific.

Apoptotic induction by AS/D1 Apoptosis was determined morphologically using the TUNEL assay. After 24 h culture of MKN-74 cells in medium alone or with 100 nM AS/D1, marked induction of apoptosis was observed in AS/D1-treated cells, while few apoptotic cells were found in cells cultured without AS/D1 (Fig. 5). We also observed that AS/D1-induced apoptotic cells tended to be located at the outer portions of cell clusters. Quantification of apoptosis was then performed using a commercially available apoptosis detection kit. The apoptosis enrichment factors of cells cultured with AS/D1 alone and AS/D1 followed by 1 nM EGF were 1.75±0.15 and 1.5±0.09, respectively, with both groups exhibiting a significant
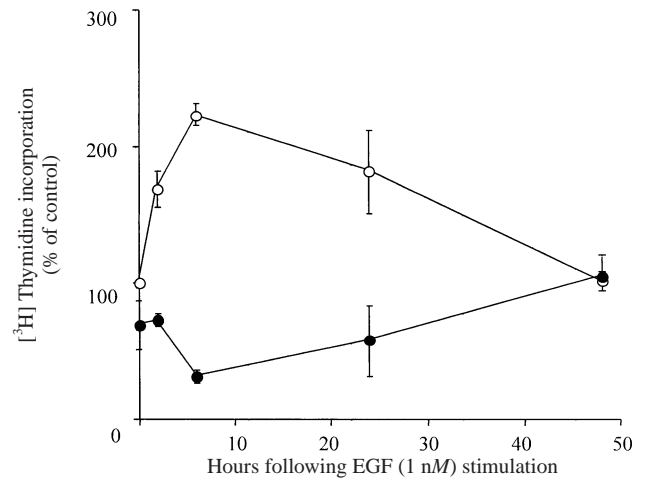

Fig. 3. Time-courses of change in DNA synthesis after stimulation with 1 nM EGF were compared between control cells (no AS/D1; ○) and cells cultured in 100 nM AS/D1 (●). Cells were treated as described in Fig. 1B. Data are representative of three experiments. Values are given as means±SE (bars) of triplicate samples.
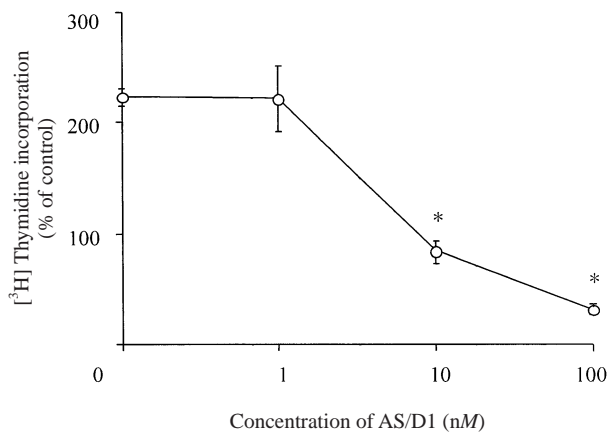

Fig. 2. Dose-dependency of AS/D1 was evaluated using the [³H]thymidine incorporation assay. Cells were treated as described in Fig. 1B except for the EGF incubation, which was reduced to 6 h. Final AS/D1 concentrations were 0, 1, 10 or 100 nM, and the final concentration of EGF was 1 nM. Data are representative of three experiments. Values are given as means±SE (bars) of triplicate samples. Where bars are not shown, the SE is smaller than the symbol. * Significant difference from 0 nM AS/D1 ($P<0.05$).

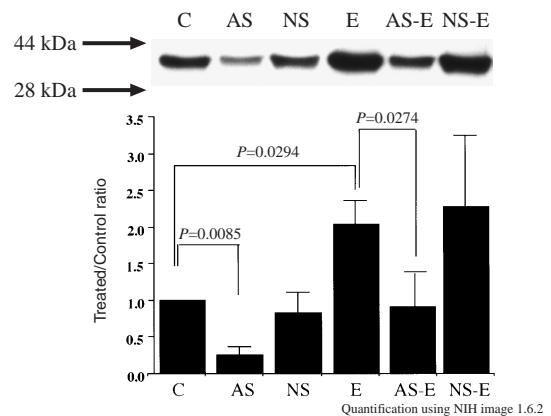Fig. 4. Cyclin D1 protein expression was determined by western blotting in MKN-74 cells treated with control vehicle (C), AS/D1 (AS), NS/D1 (NS), 1 nM EGF (E), AS/D1 followed by 1 nM EGF (AS-E), or NS/D1 followed by 1 nM EGF (NS-E). Treatments and evaluation were performed as described in "Materials and Methods." Ps show P values determined by paired Student's t test with triplicate results quantified by the NIH image 1.6.2 system.
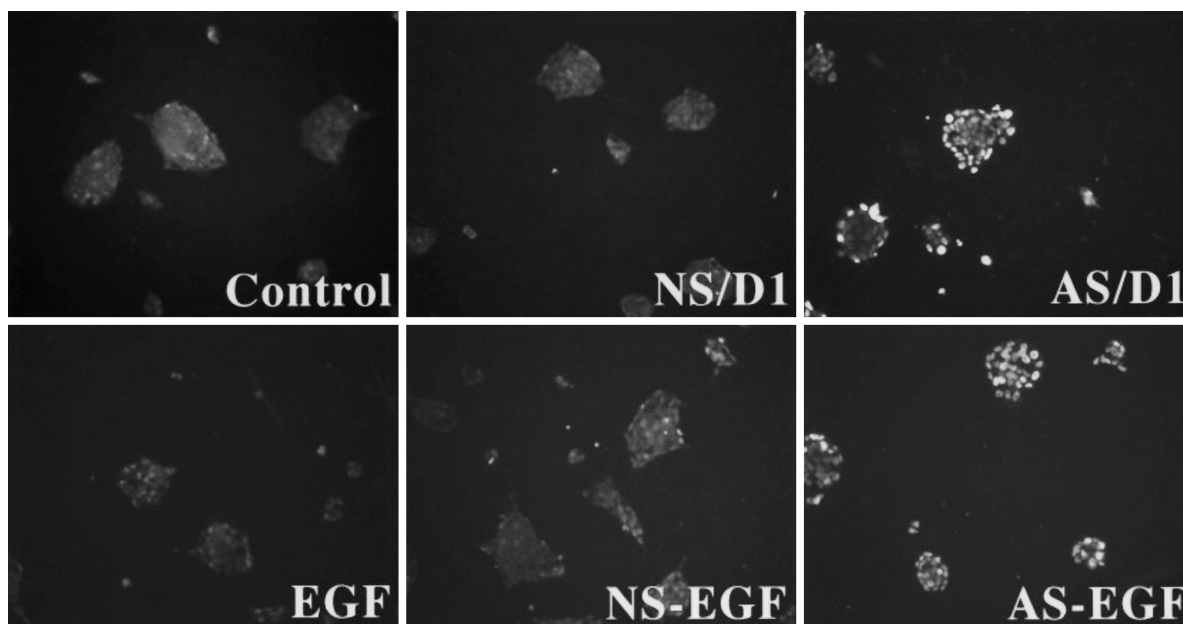
Fig. 5. TUNEL assay was used for the morphological detection of apoptotic changes. Cells were treated as described in Fig. 4. Cells were stained according to the manufacturer's instructions after fixation with 4% formaldehyde. Results were evaluated by light microscopy. Untreated (Control), NS/D1-alone treated (NS/D1), AS/D1-alone treated (AS/D1), EGF treated (EGF), EGF after NS/D1 treated (NS-EGF), and EGF after AS/D1 treated (AS-EGF) are shown.
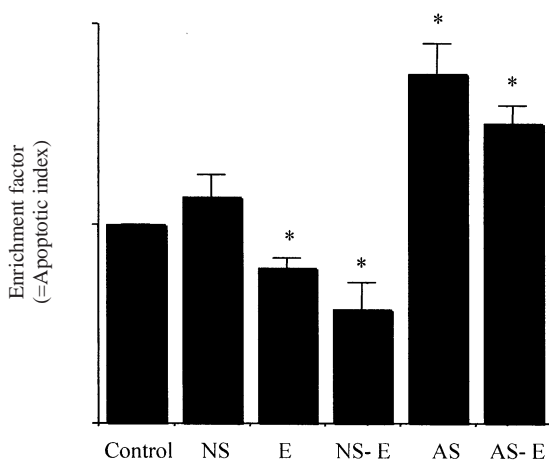

Fig. 6. Apoptosis was quantified using a commercially available apoptosis detection kit that uses an ELISA method to detect the small nucleotide fragments produced as a result of cell apoptosis. Enrichment factor is shown as an apoptotic index that correlates with the number of apoptotic cells. Untreated (Control), NS/D1-alone treated (NS), EGF treated (E), EGF after NS/D1 treated (NS-E), AS/D1-alone treated (AS), EGF after AS/D1 treated (AS-E) are shown. Data are representative of three independent experiments. Values are described as means±SE (bars) of triplicate samples. * Significant difference from the control ($P<0.05$).

increase in apoptotic index compared with controls (Fig. 6). These results confirmed that treatment with 100 nM AS/D1 for 24 h led to the induction of apoptosis.

AS/D1 generality in gastric cancer cells and AS/D1 specificity for cancer cells Under stimulation with 5 nM EGF, cell proliferation rates of MKN-74, MKN-45 and TMK-1 cells were suppressed by AS/D1 up to 81.2±4.2%, 73.7±9.9%, 76.5±10.3% of the respective control, as determined by MTT assay. On the other hand, in HUVEC cells as a non-cancerous cell line, proliferation in the presence of 5 nM EGF was not suppressed by AS/D1, as HUVEC cells did not show enhanced proliferation when treated with EGF.[22, 23]

DISCUSSION

In this study, we attempted to clarify the inhibitory effects of AS/D1, especially against EGF-stimulated proliferation, using a gastric cancer cell line. This inhibition represents a new strategy in cancer therapy as it targets cyclin D1, a protein that appears to be strongly expressed in cancer cells (especially when stimulated by mitogens such as EGF), while relatively weakly expressed in normal cells.

In order to prove the specificity of AS/D1, we demonstrated the dose-dependency of the effect of AS/D1 on
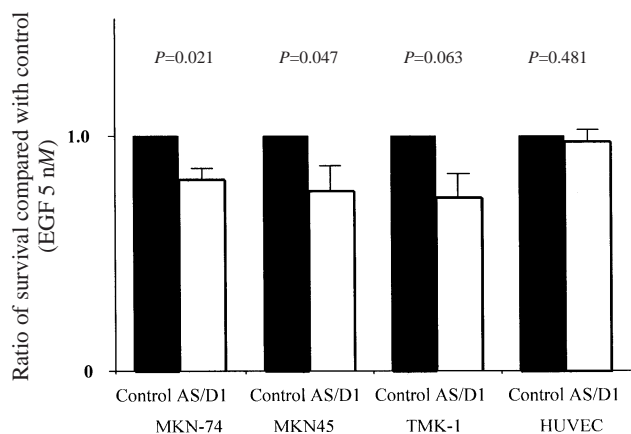
Fig. 7. Growth-inhibitory effect of AS/D1 on cells stimulated with 5 nM EGF was evaluated in MKN-74, MKN-45 and TMK-1 cells by means of MTT assay as described in "Materials and Methods." AS/D1 efficacy was confirmed in these three gastric cancer cells. Proliferation of HUVEC cells in the presence of 5 nM EGF was not suppressed by AS/D1.

cell proliferation stimulated with 1 nM EGF (Fig. 2), and we also showed the ineffectiveness of NS/D1, which was designed as an ON with randomized sequences, but composed of the same bases as AS/D1 (Fig. 1, A and B).

Our study demonstrated that 24 h incubation with 100 nM AS/D1 in media alone inhibited basal DNA synthesis in MKN-74 cells down to 27.4% of the controls (Fig. 1B). This is in accord with a previous study that showed several cyclin D1 antisense RNA constructs reduced DNA synthesis to 40% in an ovarian cancer cell line.[17] We also demonstrated that AS/D1 had a strong inhibitory effect on cells stimulated with 1 nM EGF, decreasing DNA synthesis to 19.3% of that in cells cultured without AS/D1, while 0.1 to 3 nM EGF strongly stimulated DNA synthesis by over 2.5-fold (Fig. 1B). These results suggested that the use of AS/D1 in cancers such as gastric carcinoma, in which a poor prognosis is correlated with EGF or EGF receptor expression, may be an attractive new strategy. This method may regulate EGF-stimulated cell proliferation without inhibiting the normal physiological roles of EGF necessary to maintain normal cellular metabolism and survival.

AS/D1 generality and specificity for cancer cells were supported by the results in Fig. 7. Inhibitory effects against EGF-stimulating growth by AS/D1 were confirmed in all three gastric cancer cell lines, indicating generality of the AS/D1 effect in cancer cells (Fig. 7). Although growth of HUVEC cells stimulated by EGF was not suppressed by AS/D1, AS/D1 specificity for cancer cells is still unclear, because AS/D1 may affect EGF-stimulated growth in all cells, even normal cells, if they show enhanced growth through cyclin D1 over-expression induced by EGF.

Other approaches using antisense ON targeted against oncogenes like c-*myc* have been designed, and some protocols have been successfully applied to *in vitro* and *in vivo* models in human leukemia or melanoma cells.[25, 26] Several approaches to antisense therapy against cyclin D1 have been reported,[17, 27–33] most of which utilize a drug delivery system, such as liposomes or vector transfection, to allow the antisense ON molecules to gain access to the cells. In this study, we showed the effectiveness of drug delivery simply by means of spontaneous uptake of antisense ON composed of 15 nucleotides with phosphothionate bonds at the 3′-end to increase resistance to endogenous nucleases.

Several clinical trials using antisense therapies against cancer have targeted protein kinase C, c-raf, Ha-ras, and bcl-2, and phase I trials have been completed.[34–37] While improvements in antisense technology have overcome problems such as instability, drug delivery, and toxicity, there remain unanswered questions concerning clinical application, including efficacy, host resistance, and toxicity in the clinical setting.[38] As with these other studies, our investigation into the clinical application of AS/D1 also requires further research into areas such as pharmacokinetics, efficacy and *in vivo* toxicity.

Our study demonstrated that AS/D1 specifically inhibited cancer cell proliferation in a gastric cancer cell line, MKN-74. We also demonstrated that AS/D1 decreased both EGF-stimulated cell proliferation and EGF-enhanced cyclin D1 expression, and induced apoptosis in these cells. Thus, this AS/D1 strategy seems to be a promising approach to suppress enhanced cancer growth evoked by EGF.

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