PERGAMON

European Journal of Cancer 38 (2002) 2316–2324

European Journal of Cancer

www.ejconline.com

Downregulation of survivin expression by induction of the effector cell protease receptor-1 reduces tumor growth potential and results in an increased sensitivity to anticancer agents in human colon cancer

Tetsuhisa Yamamoto[a], Yoshinobu Manome[b], Motoyuki Nakamura[a], Nobuhiko Tanigawa[a,*]

[a]*Department of General and Gastroenterological Surgery, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki City, Osaka, 569-8686, Japan*
[b]*Department of Microbiology, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-Ku, Tokyo, 105-0003, Japan*

Received 11 December 2001; received in revised form 17 April 2002; accepted 3 July 2002

Abstract

Survivin, a novel inhibitor of apoptosis, is expressed in cancer cells and not in normal adult tissues, and is recognised as a potential target in anticancer therapy. The induction of a natural antisense of survivin, effector cell protease receptor-1 (EPR-1), in a human colon cancer cell line resulted in a downregulation of survivin expression, with a similar decrease in cell proliferation, an increase in apoptosis and an increase in the sensitivity to anticancer agents. In addition, subcutaneous (s.c.) tumours from *EPR-1* transfectants showed a significant reduction in size compared with parental cells, and this antitumour efficacy was further enhanced in combination with anticancer agents. These findings suggest that regulation of survivin by the induction of *EPR-1* cDNA may have significant potential as a therapy for human colon cancer.

Keywords: Survivin; Effector cell protease receptor-1; 5-Fluorouracil; Cisplatin; Colon carcinoma

1. Introduction

Aberrant inhibition of programmed cell death (apoptosis) plays an important role in the regulation of normal cellular homeostasis and in the promotion of tissue tumorigenesis [1,2]. Several proteins that inhibit apoptosis, including bcl-2 and the inhibitor of apoptosis (IAP) family, partly regulate these processes [3]. These members are thought to directly inhibit the terminal effector caspases through baculovirus IAP repeat (BIR)-dependent recognition [4]. Unlike other IAP proteins [5,6], survivin has recently been characterised by a unique structure with a single BIR and no zinc-binding domain [7]. Survivin expression is found during embryonic and fetal development [8], but is undetectable in terminally differentiated adult tissues [7]. It subsequently becomes re-expressed in most common human cancer cells [7–14]. Our previous studies demonstrated that survivin expression in cancer was strongly correlated with a poor prognosis in gastric [9], colorectal [11] and breast cancer [14]. Interestingly, the coding sequence of survivin is extensively complementary to effector cell protease receptor-1 (*EPR-1*) [7,15], and the interaction between *EPR-1* and *survivin* transcripts becomes a potential mechanism of gene regulation [16] that can be used therapeutically. Although survivin has been recognised as ''an ideal therapeutic target'' in cancer [7], and although there have been recent studies concerning the potential efficacy of antisense cDNA [13,16] or oligonucleotides [17,18] in various tumour cell lines, these studies were mainly conducted to elucidate the biological function of survivin, in addition to examining whether it augments the drug sensitivity of cancer cells *in vitro*. In addition, there was also a recent report of having used survivin mutant to experiment on melanoma cell lines *in vivo* [19].

* Corresponding author. Tel.: +81-726-84-6441; fax: +81-726-84-6541.
E-mail address: sur001@poh.osaka-med.ac.jp (N. Tanigawa).

0959-8049/02/$ - see front matter
PII: S0959-8049(02)00247-2

2. Materials and methods

2.1. Tumour cell line and animals

HT29 human colon adenocarcinoma cells (from Dr M. Fukushima, Taiho Pharmaceutical, Japan) were maintained in McCoy's 5A medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Four-week-old female BALB/c nu/nu mice were purchased from SLC Japan, Ltd. (Shizuoka, Japan). All animal procedures were carried out under guidelines approved by the Animal Care and Use Committee in Osaka Medical College.

2.2. Expression plasmid

The non-coding human *EPR-1* cDNA, a potential survivin antisense, was provided by Dr D.C. Altieri (Yale University School of Medicine, New Haven, CT, USA) [7,16]. The EPR-1 cDNA was ligated into the mammalian cell expression vector pMTN2 (from Dr M. Hirano, Dana-Farber Cancer Institute, Boston, MA, USA). In this pMTN2-EPR-1 vector, a metallothionein promoter, direction zinc-dependent, transcribes the *EPR-1* gene, and the simian virus 40 (SV40) promoter derives the neomycin 3′-phosphotransferase (*neo*) gene.

2.3. Transfection of EPR-1 cDNA into HT29 cells

HT29 parental (HT29-P) cells in six-well tissue culture plates (Falcon, Oxnard, CA, USA) were transfected with the control pMTN2 vector alone or the pMTN2-EPR-1 vector using LipofectAMINE (Life Technologies, Inc.). Cells were maintained for 4 weeks in medium supplemented with 800 μg/ml G418 sulphate (Life Technologies, Inc.). Stable HT29 subclones with survivin antisense (HT29-AS49 and HT29-AS33) and a positive control with *neo* gene (HT29-Neo) were selected, and detection of survivin expression in both control cultures and zinc-inducible transfectants was carried out by immunoblotting as described below.

2.4. Immunoblotting

HT29-P cells and subclones were grown in complete growth medium supplemented with or without 200 μM $ZnSO_4$. Cells were lysed and the protein concentration was determined with bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA). Then, 50 μg of total protein was separated by 15% sodium dedecyl sulphate (SDS)–polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore, Co., MA, USA). After incubation in phosphate-buffered saline (PBS) containing 5% non-fat milk, blots were incubated with anti-human survivin antibody (Alpha Diagnostic International, San Antonio, TX, USA), and reacted with anti-rabbit Ig (Amersham Life Science, Arlington Heights, IL, USA). After washing, blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham). To quantify survivin expression, densitometric analysis was performed using NIH image version 1.6 software. For control experiments, monoclonal anti-actin antibody (Actin Kit) (Calbiochem-Novabiochem, San Diego, CA, USA) was used as a positive control antibody.

2.5. In vitro cell proliferation assay

The cells (5000 cells/well) were seeded into 24-well tissue culture plates and 200 μM $ZnSO_4$ was introduced into growth medium. Cell proliferation was determined at 24 h intervals by the methylene blue staining procedure [20]. The percentage of cell proliferation was calculated by defining the value of the cells after zinc exposure for 12 h as 100%. For control experiments, the zinc induction step was omitted. All determinations were repeated six times.

2.6. Cell-cycle analysis

At 48 h after zinc induction in the presence or absence of chemotherapeutic drugs, the cells were collected by trypsinisation, pooled with non-attached cells, and fixed in cold ethanol. After 20 min incubation in RNase A, the cells were suspended in PBS containing propidium iodide. Samples were analysed by flow cytometry using EPICS ELITE ESP (Beckman Coulter, Tokyo, Japan).

2.7. Measurement of caspase-3 activity

Caspase-3 protease activity was measured by use of the CPP32/Caspase-3 colorimetric protease assay kit (MBL, Nagoya, Japan). Briefly, total protein and DEVD-para-nitroanilide substrate were mixed in a 96-well plate and incubated at 37 °C for 1 h in the presence or absence of the caspase inhibitor DEVD-CHO. The cleavage of substrate was monitored at 405 nm using a microplate reader (Model 450; Bio-Rad).

2.8. In vitro sensitivity to chemotherapeutic drugs

The cells (5000 cells/well) were seeded into 96-well microtitre plates. After 24 h, the cells were cultured with 200 μM $ZnSO_4$ supplemented in growth medium and treated with varying doses of 5-fluorouracil (5-FU) (provided by Kyowa Hakko Co., Tokyo, Japan) or CDDP (provided by Nippon Kayaku Co., Tokyo, Japan). At 5 days after exposure to drugs, cell proliferation was determined by the methylene blue staining procedure. For control experiments, the drug-treated step was omitted. All determinations were repeated six times.

2.9. Growth inhibition of human colon cancer xenografts by induction of EPR-1

The cells (1×10^6) were inoculated subcutaneously (s.c.) into the bilateral flanks of BALB/c nu/nu mice ($n=6$ mice in each group). All mice were given 25 mM zinc sulphate (acidified with hydrochloric acid to pH 2.5) through their drinking water to stimulate the induction of EPR-1 from the day following tumour inoculation [21]. Tumour size and body weight were measured every 3 days until the experiments were terminated. Tumour volume was estimated by using the following equation: $V=ab^2/2$, where a and b are tumour length and width, respectively.

2.10. Antitumour efficacy of the combination of EPR-1 induction and chemotherapeutic drugs on human colon cancer xenograft models

HT29-Neo and HT29-AS33 cells (1×10^6) were inoculated s.c. into the bilateral flanks of BALB/c nu/nu mice ($n=6$ mice in each group), respectively. At 14 days after tumour inoculation, the mice were given 25 mM zinc sulphate, together with intraperitoneal (i.p.) injections of PBS alone, 0.21 mmol/kg of 5-FU (daily for 5 days), or 3.5 mg/kg of CDDP (once at 14 days). The tumour volumes were measured at regular intervals as described up to day 42.

2.11. Histochemical detection of survivin expression and tumour cell apoptosis

The cells were cultured with 200 μM $ZnSO_4$ for 48 h and plated on coverslips treated with 1% paraformaldehyde. Tumour samples at the 60th day were obtained from animals maintained on drinking water containing zinc sulphate. Specimens were fixed in formalin and embedded in paraffin blocks, then sections 2–4 μm thick were cut and put on adhesive slides. For histochemical staining of survivin, anti-survivin antibody (Alpha Diagnostic International, San Antonio, TX, USA) was added to the slides after quenching in 3% hydrogen peroxide and blocking, then the slides were incubated overnight. After washing, the histofine avidin–biotin method (Nichirei, Tokyo, Japan) was applied. Colour was developed with 3,3′-diaminobenzidine and haematoxylin was used for counterstaining. Negative control slides in the absence of primary antibody were included for each staining. For histochemical detection of apoptotic cells, *in situ* DNA nick-end labelling (TUNEL) was carried out using the Apop Tag kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions [9,11,14].

2.12. Statistical analysis

Statistical significance between different groups was determined by the Student's *t*-test (two-tailed). P values of <0.05 were considered statistically significant.

3. Results

3.1. Effect of metallothionein induction of EPR-1 on survivin expression in HT29 cells in vitro

Expression vectors including *EPR-1* cDNA were constructed and stably transfected into HT29-P cells. Each clone was then screened for the expression of survivin. The results of two neo-preservative transfectants (HT29-AS49 and HT29-AS33) are shown in Fig. 1. EPR-1 induction by $ZnSO_4$ suppressed the expression of endogenous survivin, represented by a 16.5 kDa band in the figure. No significant change in survivin expression was observed in the absence of $ZnSO_4$ in each clone (Fig. 1a). In contrast, it was dramatically decreased in

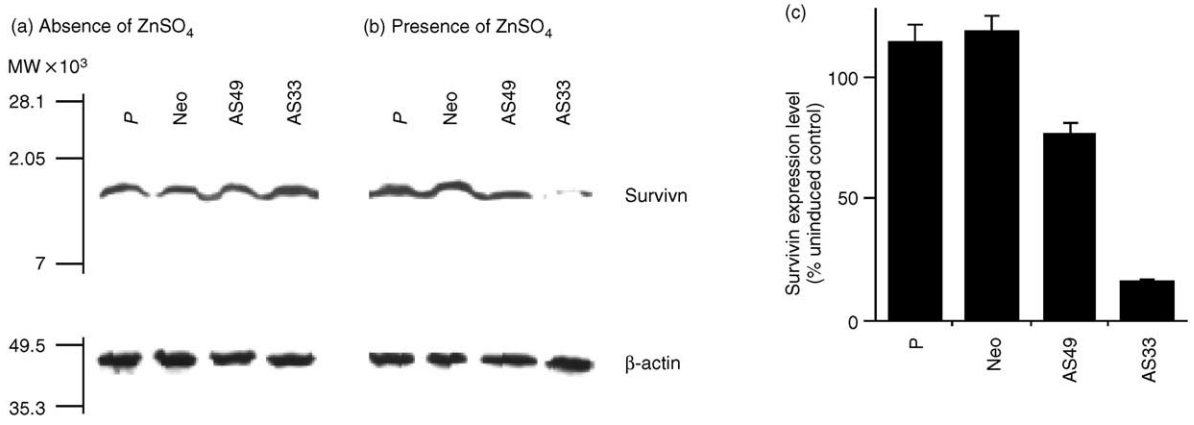

Fig. 1. Western blot analysis of survivin expression in HT29 cells. Cells were grown in medium in the absence (a) or presence (b) of $ZnSO_4$. (c) Intensity of the survivin expression as a % of the uninduced control. This experiment was repeated six times.

HT29-AS33 cells in culture medium mixed with $ZnSO_4$, but not in control cells (Fig. 1b). The survivin expression level in the presence of $ZnSO_4$ decreased to 76.8% for HT29-AS49 cells (compared with cells incubated without the corresponding figure $ZnSO_4$) and for HT29-AS33 cells was 15.9%. In contrast, an increase to 119% was seen for the control cells (Fig. 1c).

3.2. *Inhibition of cell proliferation by EPR-1* in vitro

The effect of EPR-1 induction on HT29 cell proliferation was also investigated. There were no significant differences in the cell proliferation rate for HT29-P, HT29-Neo and HT29-AS49 cells incubated in culture medium without $ZnSO_4$ (Fig. 2a). However, there were small, but significant, differences in cell proliferation between HT29-AS33 and the other cells when incubated in culture medium without $ZnSO_4$ ($P<0.05$). In contrast, in culture medium including $ZnSO_4$, a significant inhibition of cell proliferation in both HT29-AS49 and HT29-AS33 cells was observed when compared with the control cells ($P<0.01$ for each cell line; Fig. 2b). The cell proliferation rate on the sixth day following the addition of $ZnSO_4$ decreased to 67.2% for HT29-AS49 cells and 23.9% for HT29-AS33 cells compared with control cells. The proliferation rates of the control cells were increased approximately 1.4-fold following the addition of $ZnSO_4$ into the culture medium. When the proliferation of HT29-AS33 cells was examined with and without $ZnSO_4$, there was a 45.7% decrease in proliferative activity following the addition of $ZnSO_4$, while no significant decrease was seen in the HT29-AS49 cells.

3.3. *Induction of apoptosis by EPR-1* in vitro

To determine whether the inhibition of cell proliferation was due to the induction of apoptosis, we analysed tumour cell apoptosis, DNA content and enzymatic caspase-3 activity. HT29-AS49 and HT29-AS33 cells cultured with $ZnSO_4$ demonstrated a decreased expression of endogenous survivin by immunohistochemistry, and increased labelling for internucleosomal DNA fragmentation by the TUNEL method (Fig. 3a). As shown in Table 1, zinc induction of EPR-1 revealed an approximately 2.8-fold increase in HT29-AS49 cells and an approximately 5.4-fold increase in HT29-AS33 cells in the fraction of apoptotic cells (sub-G_1 peak) ($P<0.01$ in each cell line) with a decrease in the S phase population compared with HT29-P and HT29-Neo cells, demonstrated by an analysis of DNA content (Fig 4c, left). However, there were no significant differences in the G_2/M phase between the four cell lines. Furthermore, the caspase-3 protease activity in lysates of survivin antisense transfectants was significantly higher than that of vector control cells (2.1 times higher in HT29-AS49 cells and 5.7 times higher in HT29-AS33 cells) ($P<0.01$ for each cell line; Fig. 3b). In addition, it was entirely suppressed by the caspase-3 inhibitor, DEVD-CHO in the survivin antisense transfectants and vector control cells (Fig. 3b). There were no differences in the sub-G_1 phase and caspases-3 protease activity between the HT29-P and HT29-Neo cells.

Table 1
Effect of metallothionein induction of EPR-1 on the cell cycle in HT29 cells. Cells were cultured in complete growth medium supplemented with $ZnSO_4$ and subjected to flow cytometric analysis

Cells	DNA content (%)			
	Sub-G_1	G_1	S	G_2/M
HT29-P	2.1	45.2	34.1	18.6
HT29-Neo	2.4	43.5	35.9	18.2
HT29-AS49	5.9	44.3	30.7	19.1
HT29-AS33	11.4	39.1	29.7	19.8

Data represent the mean values from three separate experiments; Standard Deviation S.D., <5%.

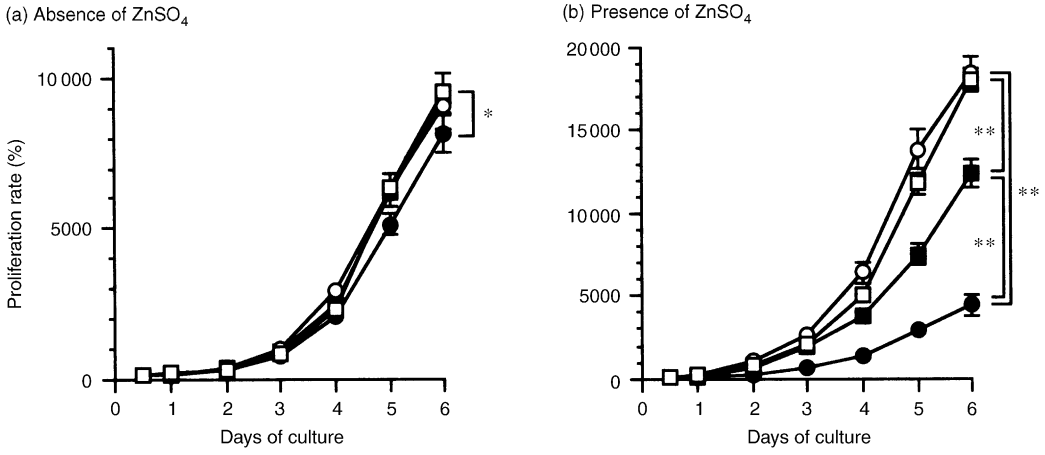

Fig. 2. Cells were cultured in growth medium in the absence (a) or presence (b) of $ZnSO_4$. Symbols: HT29-P (□); HT29-Neo (○); HT29-AS49 (■); HT29-AS33 (●). *, $P<0.05$; **, $P<0.01$.

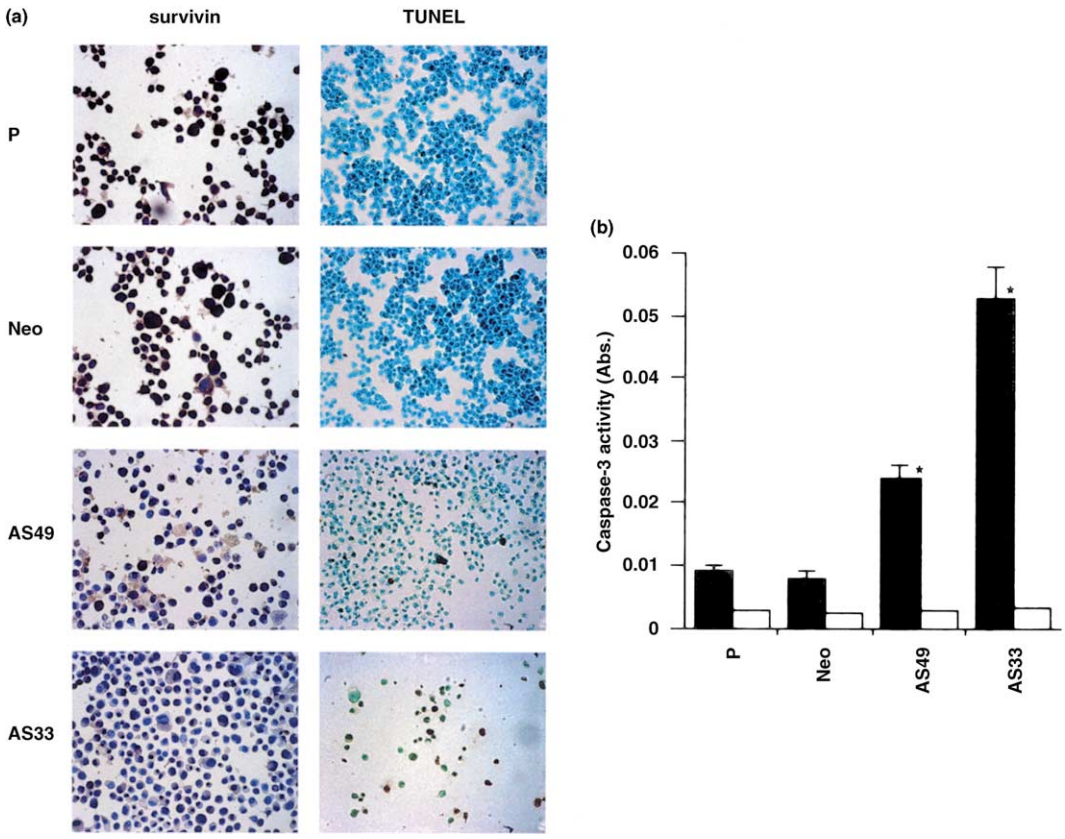

Fig. 3. (a) Immunohistochemical detection of survivin (left) and apoptosis (right) in HT29 cells cultured with $ZnSO_4$ (×400); (b) caspase-3 activity of HT29 cells cultured with $ZnSO_4$ in the presence (white-painted) or absence (black-painted) of the caspase-3 inhibitor, DEVD-CHO. *, $P<0.01$. Abs, absorbance.

Based on the morphological and biochemical differences that distinguish apoptotic from necrotic cell death that are reported elsewhere [22], we analysed *in situ* internucleosomal DNA fragmentation by TUNEL, DNA content by flow cytometry and enzymatic caspase-3 activity. These data are shown in Figs. 3a, 3b and 4c and Table 1 and indicate that the inhibition of cell proliferation was due to the induction of programmed cell death (apoptosis) and not to accidental cell death (necrosis).

3.4. *Drug sensitivity of HT29 cells* in vitro

The *in vitro* sensitivities to 5-FU and CDDP of the various HT29 cells cultured with $ZnSO_4$ were assessed. HT29-AS49 and HT29-AS33 cells showed increased sensitivities to 5-FU (Fig. 4a) and CDDP (Fig. 4b) showing corresponding increases in percentage of apoptotic cells increases that were not observed HT29-P and HT29-Neo cells (Fig. 4c). The 50% inhibitory concentration (IC_{50}) values for 5-FU treatment were 0.52 μM for HT29-AS49 cells and 0.31 μM for HT29-AS33 cells, while the IC_{50} for HT29-P and HT29-Neo cells were 1.21 and 1.19 μM, respectively. Similarly, the IC_{50} values for CDDP treatment were 1.72 μM for HT29-AS49 cells and 0.40 μM for HT29-AS33 cells, while the IC_{50} for HT29-P and Neo cells were 3.41 and 3.68 μM, respectively. Thus, HT29-AS49 cells were 2.3 and 2.1 times more susceptible to 5-FU and CDDP, respectively, than the control cells ($P<0.01$ for each drug). HT29-AS33 cells were 3.9 and 9.2 times more susceptible to 5-FU and CDDP, respectively, than the control cells ($P<0.01$ for each drug). In addition, there were no differences in the drug sensitivities between HT29-P and HT29-Neo cells. Also, there were no differences in the drug sensitivities between survivin antisense transfectants and control cells in the absence of $ZnSO_4$ (data not shown). The increase in drug sensitivities was proportional to the survivin expression level in these cell lines. Furthermore, the increase in drug sensitivity of the survivin antisense transfectants was associated with an increase in apoptotic cells evaluated by a DNA content analysis (Fig. 4c). HT29-AS49 cells treated with 5-FU or CDDP in the presence of $ZnSO_4$ demonstrated a 2.5- or 2.0-fold increase in apoptotic cells, respectively, compared with control cells. In HT29-AS33 cells, there was a 4.2- or 3.2-fold increase in apoptotic cells, respectively, compared with control cells.

3.5. *Efficacy of EPR-1 alone on HT29 tumours* in vivo

The efficacy of EPR-1 induced by $ZnSO_4$ on tumour growth *in vivo* was studied in human colon cancer

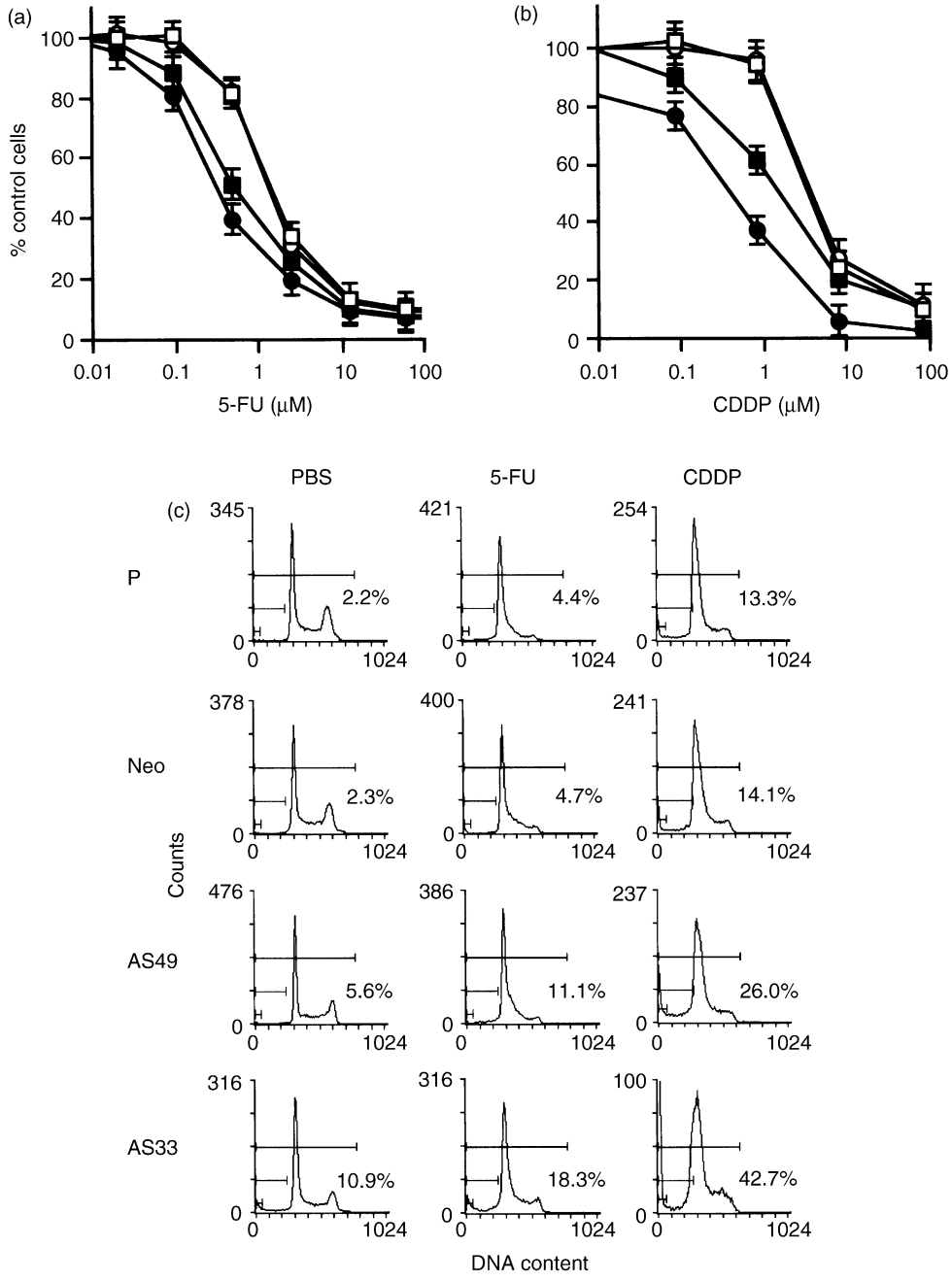

Fig. 4. Drug sensitivity *in vitro* to 5-FU (a) or CDDP (b) of HT29 cells cultured with $ZnSO_4$. Symbols: HT29-P (□); HT29-Neo (○); HT29-AS49 (■); HT29-AS33 (●); (c) analysis of DNA content by flow cytometry in HT29 cells treated with PBS (left), 5-FU (1 μM) (Middle), or CDDP (3 μM) (right) in the presence of $ZnSO_4$. Percentages shown are for the fraction of apoptotic cells (sub-G_1 peak).

xenografts. As shown in Fig. 5a, a significant growth suppression was observed in the HT29-AS49 and HT29-AS33 tumours ($P < 0.01$ for each tumour), while there was no evaluable change in the HT29-P and HT29-Neo tumours. The mean doubling time for each tumour model (from 100 to 200 mg) was 3 days for both the HT29-P and HT29-Neo tumours, 11 days for HT29-AS49 tumours, and 29 days for HT29-AS33 tumours. The mean tumour volume of the HT29-AS49 and HT29-AS33 tumours was markedly decreased to 26.2 and 9.5%, respectively, of the parental tumours at the 60th day after inoculation. In addition, we examined whether the growth suppression in the EPR-1-induced tumours was associated with a decreased survivin expression and increased spontaneous apoptosis (Fig. 5b). In the HT29-AS49 and HT29-AS33 tumours, there was decreased expression of endogenous survivin, and greatly increased labelling of internucleosomal DNA fragmentation using a TUNEL analysis compared with the HT29-P and HT29-Neo tumours.

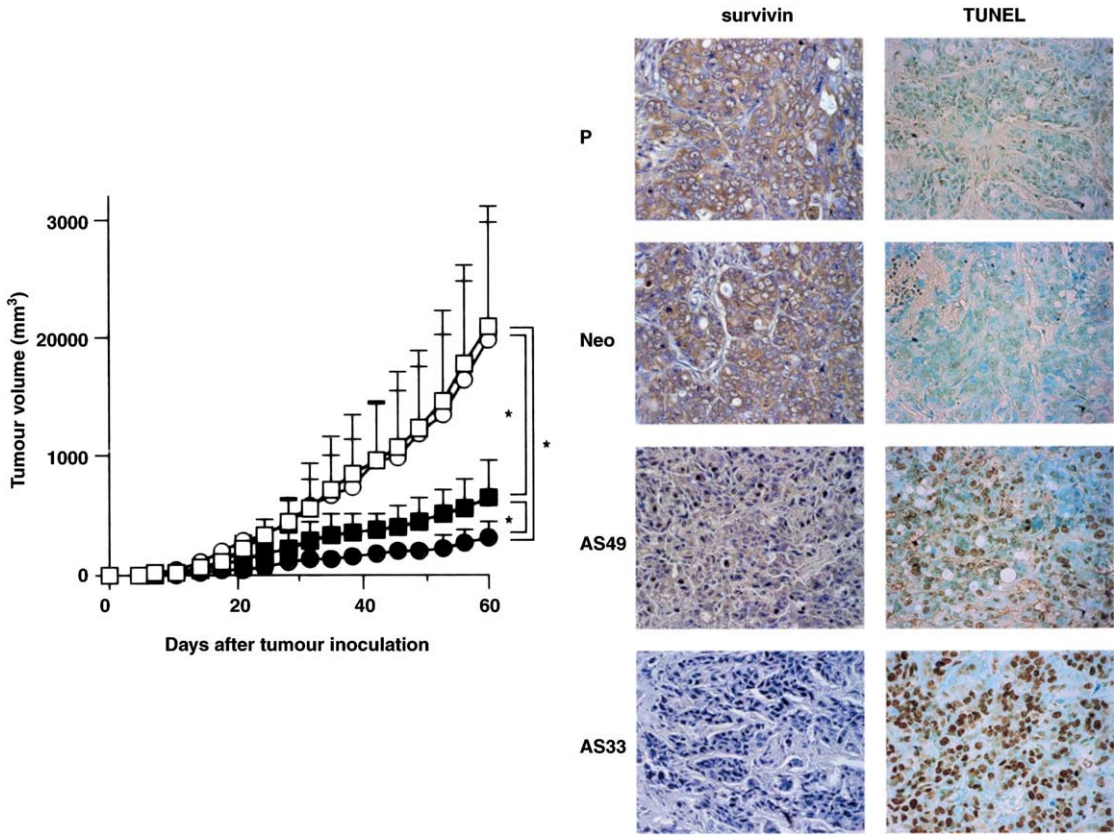

Fig. 5. (a) The efficacy of EPR-1 alone on HT29 tumours *in vivo*. Symbols: HT29-P (□); HT29-Neo (○); HT29-AS49 (■); HT29-AS33 (●). *, $P<0.01$; (b) immunohistochemical detection of survivin (left) and apoptosis (right) in HT29 tumours obtained at the 60th day from animals maintained on drinking water containing $ZnSO_4$ (×400).

3.6. *Efficacy of combined treatment with chemotherapeutic drugs and induction of EPR-1 in HT29 tumours* in vivo

The efficacy of the combination of EPR-1/5-FU or EPR-1/CDDP was also studied in an *in vivo* experiment using HT29-Neo and HT29-AS33 tumour models. As shown in Fig. 6a, there was a significant growth suppression in EPR-1-induced HT29-AS33 tumours ($P<0.01$ for each tumour); this suppression was not evident in PBS, 5-FU, or CDDP-treated HT29-Neo tumours. With the further addition of 5-FU or CDDP to the HT29-AS33 tumours, there was a significant decrease in tumour volume compared with EPR-1 alone ($P<0.05$ for each tumour). There were no differences in the tumour suppression between the EPR-1/5-FU and EPR-1/CDDP-treated HT29-AS33 tumours at the 42nd day (Fig. 6b). The tumour growth rate of EPR-1/5-FU and EPR-1/CDDP-treated HT29-tumours achieved an approximately 50% reduction compared with EPR-1 induction alone and an approximately 85% reduction compared with the HT29-Neo tumours. Throughout these experiments, there were no accidental deaths of the mice, and no additive effect in toxicity was observed in each group; however, the mice experienced cachexia with tumour growth in the HT29-Neo groups (data not shown). These findings suggest that enhanced EPR-1 expression in combination with 5-FU or CDDP treatment could lead to significant antitumour efficacy in the HT29 xenograft system.

4. Discussion

The recent discovery of survivin, a novel inhibitor of apoptosis, expressed in cancer cells alone and not in normal differentiated human cells has stimulated enthusiasm for its use as a target in cancer therapy [7–14,23–25]. In the present study, we successfully transfected HT29 cells with a vector incorporating an *EPR-1* cDNA fragment, which resulted in the downregulation of survivin expression and inhibition of cell proliferation activity *in vitro*. In these *EPR-1* cDNA transfectants, we were also able to witness increases in the sensitivity to anticancer agents and the induction of apoptosis. In addition, we demonstrated that treatment with EPR-1 alone or in combination with 5-FU or CDDP could lead to a significant antitumour effect in human colon cancer xenografts in mice.

The addition of $ZnSO_4$ to control cells resulted in an approximately 1.4-fold increase in cell proliferation, as

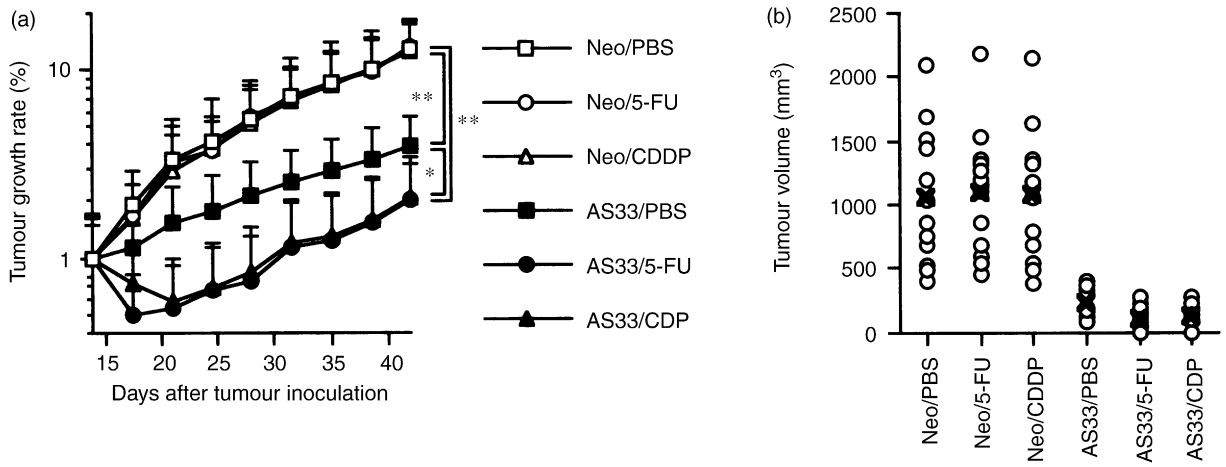

Fig. 6. (a) The efficacy of the combination of EPR-1/5-FU or EPR-1/CDDP in an *in vivo* experiment using HT29-Neo and HT29-AS33. *, $P<0.05$; **, $P<0.01$. (b) Each point represents tumour at day 42. Cross lines, median tumour volume.

well as a slight increase in survivin expression, suggesting that the presence of zinc itself might augment cell proliferation and exert an independent anti-apoptotic function [26]. However, in HT-29 cells transfected with *EPR-1* and induced by $ZnSO_4$, endogenous survivin expression was clearly downregulated, with a parallel inhibition seen in cell proliferative activity.

In several previous studies, it was postulated that survivin prevented apoptosis by targeting the terminal effectors caspase 3 and caspase 7, which act downstream in two major apoptotic pathways [27]. Survivin was thought to be expressed in the G_2/M phase of the cell cycle in a cell cycle-regulated manner and to be associated with microtubule formation of the mitotic spindle [23,27]. Our previous studies demonstrated that survivin expression was significantly associated with reduced apoptotic indices in various human cancers, including gastric, colorectal and breast cancers [9,11,14]. The current study also demonstrated a positive correlation between survivin expression and apoptosis of HT29 cells, assessed by TUNEL, flow cytometry and enzymatic caspase-3 activity. Downregulation of survivin expression was associated with increased rates of apoptotic cells. Taken together with the correlation between cell proliferation and survivin expression, it appears that survivin may have the potential to promote the cell cycle from the G_1 phase to the S phase [28]. Moreover, it has been suggested that the survivin antisense approach may be able to facilitate apoptosis through the two major apoptotic pathways [18,29].

In the present study, we also demonstrated that induction of EPR-1 resulted in an increased sensitivity of the tumour cells to the anticancer agents 5-FU and CDDP *in vitro*. In addition, the susceptibility to CDDP treatment, which acts in the G_2/M phase, was greater than the sensitivity to 5-FU treatment, which acts in the G_1 phase. An increase in chemosensitivity has been reported to be inversely correlated with survivin expression, as well as increased rates of apoptosis [19] and these findings correspond well with those of previous studies that reported survivin was expressed in the G_2/M phase of the cell cycle [17].

In the present study, we demonstrated for the first time a significant antitumour effect of either EPR-1 induction alone or in combination therapy with 5-FU or CDDP treatment against human colon cancer xenografts. As demonstrated by a decrease in survivin expression and an increase in apoptosis in survivin-targeted tumours, HT29-AS33 tumours in mice showed a significant reduction in size, with the tumour growth rate being greatly suppressed by the induction of EPR-1 alone. In addition, tumour growth was arrested by the combination of EPR-1/5-FU or EPR-1/CDDP in three of 12 tumours. These findings suggest that the EPR-1/5-FU or EPR-1/CDDP system could be a powerful gene therapy approach for colorectal cancer treatment. Moreover, further elucidation of a mechanism that would regulate the homeostatic balance between survivin and EPR-1 may lead to the development of a novel specific cancer therapy with no adverse effect on normal tissues.

References

1. Cross M, Dexter TM. Growth factors in development, transformation, and tumorigenesis. *Cell* 1991, **64**, 271–280.
2. Nagata S. Apoptosis by death factor. *Cell* 1997, **88**, 355–365.
3. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995, **267**, 1456–1462.
4. Takahashi R, Deveraux Q, Tamm I, *et al.* A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* 1998, **273**, 7787–7790.
5. Duckett CS, Nava VE, Gedrich RW, *et al.* A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J* 1996, **15**, 2685–2694.

6. Hay BA, Wassarman DA, Rubin GM. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* 1995, **83**, 1253–1262.
7. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997, **3**, 917–921.
8. Adida C, Crotty PL, McGrath J, *et al*. Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol* 1998, **152**, 43–49.
9. Lu CD, Altieri DC, Tanigawa N. Expression of a novel anti-apoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res* 1998, **58**, 1808–1812.
10. Adida C, Berrebi D, Peuchmaur M, *et al*. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998, **351**, 882–883.
11. Kawasaki H, Altieri DC, Lu CD, *et al*. Inhibition of apoptosis by survivin predict shorter survival rates in colorectal cancer. *Cancer Res* 1998, **58**, 5071–5074.
12. Swana HS, Grossman D, Anthony JN, *et al*. Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. *N Engl J Med* 1999, **34**, 452–453.
13. Grossman D, McNiff JM, Li F, Altieri DC. Expression of the apoptosis inhibitor, survivin, in nonmelanoma skin cancer and gene targeting in a keratinocyte cell line. *Lab Invest* 1999, **79**, 1121–1126.
14. Tanaka K, Iwamoto S, Gon G, *et al*. Expression of survivin ant its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res* 2000, **6**, 127–134.
15. Altieri DC. Xa receptor EPR-1. *FASEB J* 1995, **9**, 860–865.
16. Ambrosini G, Adida C, Sirugo G, Altieri DC. Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem* 1998, **273**, 11177–11182.
17. Li F, Ackermann EJ, Bennett CF, *et al*. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat Cell Biol* 1999, **1**, 461–466.
18. Olie RA, Simoes-Wust P, Baumann B, *et al*. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res* 2000, **60**, 2805–2809.
19. Grossman D, Kim PJ, Schechner JS, Altieri DC. Inhibition of melanoma tumor growth in vivo by survivin targeting. *Proc Natl Acad Sci* 2001, **98**, 635–640.
20. Manome Y, Wen PY, Chen L, *et al*. Gene therapy for malignant gliomas using replication incompetent retroviral and adenoviral vectors encoding the cytochrome P450 2B1 gene together with cyclophosphamide. *Gene Ther* 1996, **3**, 513–520.
21. Shibata M, Ward JM, Green JE, Merlino G. Enhanced sensitivity to tumor growth and development in multistage skin carcinogenesis by transforming growth factor-á-induced epidermal growth factor receptor activation but not p53 inactivation. *Mol Carcinog* 1997, **18**, 160–170.
22. Thompson HJ, Strange R, Schedin PJ. Apoptosis in the genesis and prevention of cancer. *Cancer Epidem Biomarkers Prev* 1992, **1**, 597–602.
23. Li F, Ambrosini G, Chu EY, *et al*. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998, **396**, 580–583.
24. Monzo M, Rosell R, Felip E, *et al*. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognostic marker in non-small cell lung cancers. *J Clin Oncol* 1999, **17**, 2100–2104.
25. Nakagawara A. Molecular basis of spontaneous regression of neuroblastoma: role of neurotrophic signals and genetic abnormalities. *Hum Cell* 1998, **11**, 115–124.
26. Newmeyer DD, Farschon DM, Reed JC. Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* 1994, **79**, 353–364.
27. Tamm I, Wang Y, Sausville E, *et al*. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res* 1998, **58**, 5315–5320.
28. Ito T, Shiraki K, Sugimoto K, *et al*. Survivin promotes cell proliferation in human hepatocellular carcinoma. *Hepatology* 2000, **31**, 1080–1085.
29. Asanuma K, Moriai R, Yajima T, *et al*. Survivin as a radioresistance factor in pancreatic cancer. *Jpn J Cancer Res* 2000, **91**, 1204–1209.