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BIOLOGY CONTRIBUTION

SPONTANEOUS AND RADIATION-INDUCED APOPTOSIS IN COLORECTAL CARCINOMA CELLS WITH DIFFERENT INTRINSIC RADIOSENSITIVITIES: SURVIVIN AS A RADIORESISTANCE FACTOR

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Background: Spontaneous apoptosis has been shown to predict tumor response to radiochemotherapy in rectal cancer *in vivo*. It remains to be elucidated, however, which genetic profile determines whether a tumor is more or less prone to apoptosis. Recently, a novel member of the inhibitor of apoptosis protein family, designated survivin, was identified. We investigated the impact of surviving expression on tumor cell apoptosis in three colorectal cell lines of different intrinsic radiosensitivities.

Methods and Materials: Survivin protein expression was measured by Western blot analysis, and survivin mRNA expression by quantitative TaqMan reverse transcription polymerase chain reaction, both in untreated cell and after irradiation with 2 and 8 Gy. The expression profile was then correlated to spontaneous and radiation-induced apoptosis (Tunel-Assay, DAPI-staining) in three colorectal cell lines of low (SW 480), intermediate (HCT-15), and high radiosensitivity (SW 48), as determined by the colony-forming assay.

Results: *In vitro* analysis revealed higher spontaneous and higher radiation-induced apoptosis rates in the radiosensitive line (SW 48), as compared with the more resistant line (SW 480). In Western blot analysis and in TaqMan analysis, SW 480 was characterized by a higher spontaneous expression and a pronounced induction of survivin 48 h after irradiation, whereas survivin expression was low when untreated and not increased after irradiation in the most radiosensitive line SW 48. HCT-15 was intermediate, both with respect to the level of survivin mRNA and protein expression.

Conclusion: The inverse correlation of survivin-expression with spontaneous and radiation-induced apoptosis suggests that survivin is an important inhibitor of apoptosis in colorectal cancer cell lines. Analysis of survivin mRNA or protein expression may therefore provide predictive information on radio- and chemoresistance of individual colorectal tumors. **© 2003 Elsevier Science Inc.**

Survivin, Apoptosis, Radiosensitivity, Colorectal cancer.

INTRODUCTION

Both radiation and chemotherapy will induce apoptosis in neoplastic cells. Studies in irradiated murine tumors of different histologies suggest that the extent of radiation-induced apoptosis correlates closely with radiosensitivity, and that the spontaneous apoptotic rate of untreated tumor cells lines may also correlate with the induced apoptotic rate and radiosensitivity (1,2). In the clinical setting, we previously demonstrated in a series of patients with rectal cancer treated uniformly by neoadjuvant radiochemotherapy (RCT) that the immunohistochemically determined levels of apoptosis varied considerably among individual tumors, ranging from 0.3% to 3% of all malignant cells within a biopsy specimen (3). In this series, a high level of spontaneous apoptosis emerged as a significant predictor for tumor response to neoadjuvant RCT and for improved disease-free survival.

Cell inactivation by apoptosis is regulated by a complex balance in signal transduction pathways between apoptosis-activating factors, such as p53 and bax, and antiapoptotic factors, such as the bcl-2 family and the inhibitor of apoptosis protein (IAP) family. Recently, a novel and structurally unique member of the IAP gene family, designated survivin, was identified (4). Unlike other IAP proteins, survivin is found during fetal development, but is completely downregulated in normal adult tissues. Interestingly, this protein was found to be prominently reexpressed in a variety of human tumors, including cancers of the lung (5), pancreas (6), stomach (7), breast (8), esophagus (9), soft tissue (10), and colon (11, 12).

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In a more recent clinical study comprising pretreatment biopsies of 54 patients with rectal cancer who treated uniformly by neoadjuvant RCT within a prospective protocol (13), we could demonstrate that the expression of survivin correlated inversely with the spontaneous apoptotic rate (14). Moreover, a high survivin expression turned out to be associated with a significantly higher risk of tumor recurrence after preoperative RCT, indicating that overexpression of survivin may confer a certain degree of radioresistance to rectal cancer cells *in vivo*. In the present *in vitro* study, using three colorectal cell lines with different intrinsic radiosensitivities, we attempted to find a relationship between spontaneous and radiation-induced apoptosis and radiosensitivity, as determined by the clonogenic survival. Furthermore, we analyzed the expression levels of survivin messenger RNA and protein both untreated and after irradiation and compared them with the occurrence of apoptosis and overall cell survival.

METHODS AND MATERIALS

Cell lines

The human colorectal cancer cell lines SW 48, SW 480, and HCT-15 were obtained from the American Type Culture Collection (LGC-Promochem, Wesel, Germany). SW 48 and SW 480 were isolated by A. Leibovitz (15) between 1971 and 1976 as human colorectal adenocarcinoma cell lines. SW 480 is described to harbor a G → A mutation in codon 273 of the p53 gene and a C → T mutation in codon 309. SW 48 is wild-type p53 (16). HCT-15 was established from a colorectal adenocarcinoma (17) and is characterized by a heterozygous wild-type and C → G mutation at codon 153 of the p53 gene (18). The cells were cultured in Dulbecco's Modified Eagle's Medium (Biochrom, Germany) supplemented with 10% heat inactivated fetal calf serum, 1% sodium pyruvate, and 2 mM Glutamine (all supplements from Biochrom, Berlin, Germany) at 37 °C, 5% CO_2, and 95% humidity.

Irradiation procedure

Colorectal cells were irradiated at room temperature in culture flasks (Nunc, Wiesbaden, Germany) using orthovoltage-irradiation (Stabilipan, Siemens, Erlangen, Germany) at 250 kV/15 mA 40 cm focus–surface distance at a dose rate of 1.15 Gy/min with single doses of 2 and 8 Gy. Sham-irradiated cultures were kept at room temperature in the X-ray control room while the other samples were irradiated. After irradiation, the cells were kept in culture medium up to 48 h.

Determination of cell survival

The clonogenic assay was performed on single-cell suspension of exponentially growing cells. Cells were counted using a Coulter Counter (Casy1, Schärfe System, Reutlingen, Germany), plated in growth-medium into Petri dishes and were irradiated 12 h after plating. After 10–14 days, cells were stained with methylene-blue for 30 min, colonies greater than 50 cells were counted using an Automatic Colony Analyzing Machine, and survival data were fitted according to the linear quadratic equation using TechPlot software (Techplot 3, Braunschweig, Germany). All experiments were repeated at least three times.

Detection and quantification of apoptosis

Apoptotic carcinoma cells were either identified using the terminal-deoxynucleotidyl-transferase–mediated dUTP nick-end-labeling (TUNEL) technique (*in situ* cell death Detection Kit, Boehringer Mannheim, Germany) or DAPI (4′,6′-diamino-2 phenylindolehydrochlorid)-staining procedure. Briefly, cells were washed with phosphate-buffered saline (PBS) and then incubated with TUNEL-reaction-mixture in a humidified chamber for 60 min at 37°C in the dark according to the manufacturer's recommendation. For DAPI-staining, cells were fixed in 3,7% formaldehyde at room temperature and stained with a 1:10.000 DAPI-solution (Sigma, Germany) for 15 min, rinsed with PBS and analyzed using a fluorescence microscope (Leitz, Germany). The apoptotic index was obtained by dividing the number of apoptotic tumor cells by the total number of tumor cells, multiplied by 100. A minimum of 1000 tumor cells were counted using an Image-System (Optimas 6.2, Stemmer PC Systeme, Puchheim, Germany). All sections were scored by two independent observers (FR, AG).

Automated Survivin and GAPDH transcript analysis by quantitative fluorescence polymerase chain reaction

Total mRNA from the cell lines was isolated using RNeasy-Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Contaminating chromosomal DNA was digested with DNase I on the column before preparation of RNA. Survivin mRNA subsequences (77 bp), which do not overlap with the EPR-1 mRNA, and that are specific for all three known survivin transcripts, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts (63 bp) were amplified from cDNA in duplicate experiments by ready-to-use quantitative polymerase chain reaction (PCR) assays (Roboscreen Gesellschaft für molekulare Biotechnologie mbH, Leipzig, Germany). Briefly, conventional 96-well bases were loaded with an 8-well ready-to-use standard DNA strip either coated with eight different amounts of reference-DNA for quantitation survivin or GAPDH transcripts. Mastermixes contained forward and reverse primer and the TaqMan probe. The double-labeled probes were either 5′-labeled with the fluorescent reporter dye 6-carboxyfluorescein for detection of survivin or VIC for GAPDH, and the common 3′-fluorescent quencher dye 6-carboxytetra-methylrhodamine to generate the respective survivin or GAPDH standard reference curves for each run. The remaining free base positions were loaded with the required number of sample tubes containing just the respective TaqMan oligonucleotide sets. Reaction premixes containing PCR buffer, which was supplemented with the passive fluorescence dye 6-carboxy-tetramethyl-rhodamin, dNTPs, and 1.25 U of AmpliTaq

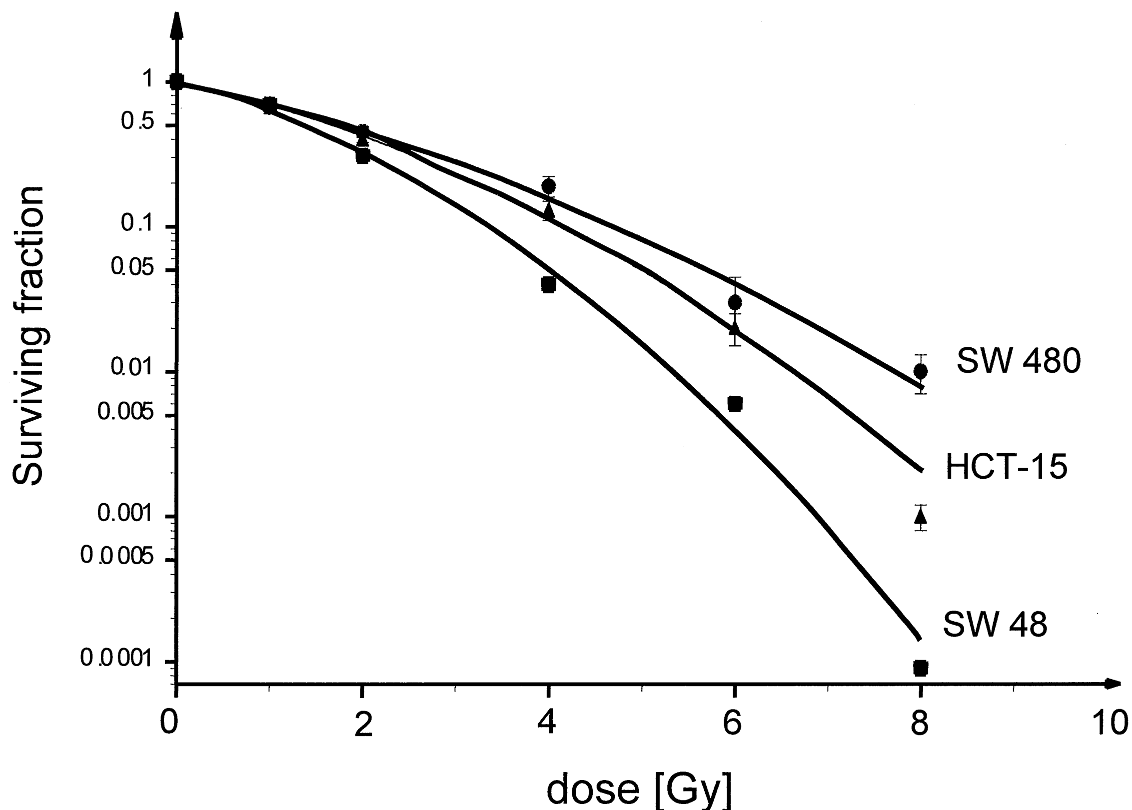

Fig. 1. Cell survival of three colorectal cell lines as determined by the clonogenic assay.

"GOLD" (Applied Biosystems, Darmstadt, Germany) were assembled according to the manufacturer's instructions. Aliquots of the mixes were added to each reaction tube by using a BIOMEK 2000 laboratory automation workstation (Beckman Instruments, Fullerton, CA). Sample and standard reactions (final volume of 25 μL) differed only by the addition of 2-μL aliquots of the analyzed cDNA sample. PCR amplification and detection was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Target cDNA amounts were calculated from the simultaneously processed reference DNA strips. Survivin data were correlated to GAPDH cDNA and are given as zeptomoles (10^{-21}) survivin mRNA per attomole (10^{-18}) of GAPDH mRNA, which was calculated from the same cDNA sample.

Immunoblotting

Briefly, subconfluent cells were harvested and lysed in RIPA buffer (50 mM Tris, ph 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate) supplemented with protease inhibitors (1 mM phenylmethylsulfonylfluoride, 10 μg/ml pepstatin, 10 μg/mL aprotinin, and 5 μg/mL leupeptin). Protein concentrations were determined using BCA-protein assay (Pierce Biotechnology, Rockford, IL). Equal amounts of protein (15 μg) were run on a 12.5% SDS polyacrylamide-gel and transferred to a nitrocellulose membrane (Hybond C, Amersham) using a Protean-system (BioRad Laboratories, Munich, Germany). Blots were then washed (PBS/0.1% Tween 20), blocked for 30 min in PBS containing 5% skim milk powder/0.1% Tween and probed with antibodies against human survivin (ab469, dilution: 1:1000, Abcam, UK) overnight at 4 °C. Blots were next incubated in horseradish peroxidase–linked secondary antibodies (Dako, Hamburg, Germany) and developed by a chemoluminescence detection system (ECL, Amersham Pharmacia, Freiburg, Germany). To confirm equal protein loading per lane, membranes were subsequently reprobed with a 1:5000 dilution of an anti–α-tubulin antibody (Biozol, Germany) and developed as described previously.

RESULTS

Clonogenic survival

The intrinsic radiosensitivity of the three colorectal cell lines was first determined by the colony-forming assay. As shown in Fig. 1, SW 48 was the most sensitive line with a survival fraction at 2 Gy (SF_2) of 31% ($\pm$3%). SW 480 was most radioresistant (SF_2: 45% $\pm$ 4%), and HCT-15 was of intermediate radiosensitivity (SF 2: 40% $\pm$ 4%). These results are in line with previous findings in the literature, reporting a SF_2 of SW 48, HCT-15 and SW 480 of 21%, 41% and 51%, respectively (19).

Spontaneous and radiation-induced apoptosis

A high spontaneous TUNEL-positive fraction (2.5%) was detected in the most radiosensitive SW 48 cells (Fig. 2).

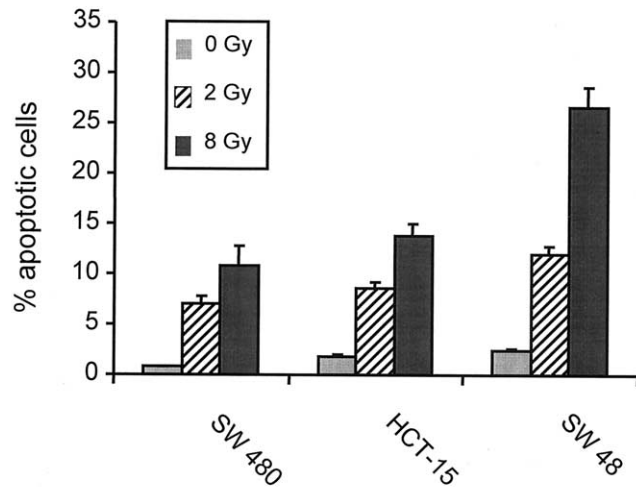

Fig. 2. Spontaneous and radiation-induced apoptosis in three colorectal cell lines of different intrinsic radiosensitivity. The apoptotic fractions were determined by terminal-deoxynucleotidyl-transferase–mediated dUTP nick-end-labeling staining in unirradiated cells (0 Gy) and 48 h after irradiation with 2 and 8 Gy, respectively.

Irradiation with 2 and 8 Gy induced a 4-fold and 10-fold increase of the TUNEL-positive fraction at 48 h postirradiation. Conversely, the spontaneous and radiation-induced TUNEL-positive fractions were considerable lower in the most radioresistant SW 480 line and turned out to be intermediate in the HCT-15 colorectal cell line (Fig. 2). Comparable results were obtained using DAPI-staining as a second method to determine the apoptotic fraction (data not shown).

Survivin protein and mRNA expression levels

Almost no detectable levels of survivin protein was found by Western blot in untreated and irradiated SW 48 cells after 48 h (Fig. 3). Survivin expression in untreated cells was highest in the radioresistant cell line SW 480 and intermediate in HCT-15 cells. Moreover, in these both cell lines the expression levels of survivin increased 48 h after irradiation with 2 and 8 Gy, respectively (Fig. 3).

Table 1. Relationship of cell survival (SF_2) and survivin mRNA expression in 3 colorectal cell lines*

	Relative survivin mRNA expression	(SF_2) mean ± SD
SW 480	1.000	45% ± 4%
HCT-15	0.850	40% ± 4%
SW 48	0.715	31% ± 3%

* Survivin mRNA expression was measured by a quantitative TagMan reverse transcription-polymerase chain reaction and is indicated as values to SW 480 expression.

Expression of survivin mRNA was examined during the logarithmic phase of cell growth in untreated cells and after irradiation with 2 and 8 Gy, respectively. Survivin mRNA was expressed at a high level in untreated SW 480 cells, a low level in SW 48, and an intermediate level in HCT-15 cells. Table 1shows the relative survivin mRNA expression of the three colorectal cell lines together with their survival fraction at 2 Gy. To determine whether survivin mRNA is induced after irradiation, the mRNA expression was measured by a quantitative reverse transcriptase–PCR assay 24 and 48 h postirradiation (Fig. 4). In SW 480 cells, expression of survivin mRNA markedly increased, whereas in the most radiosensitive SW 48 cell line no or only a slight increase occurred. The expression of survivin mRNA in HCT-15 cells increased gradually, ranging between both other lines.

DISCUSSION

Apoptosis occurs spontaneously in tumors, and the degree to which it occurs varies between experimental tumors even of the same histopathologic origin (1). In murine tumors, Meyn *et al.* have reported on a significant correlation between spontaneous apoptosis, radiation-induced apoptosis and tumor growth delay and the TCD_{50} (the radiation dose at which 50% of the tumors are locally controlled) (2). In clinical series of preoperative RCT/radiotherapy in rectal cancer, several groups, including our own, have dem-

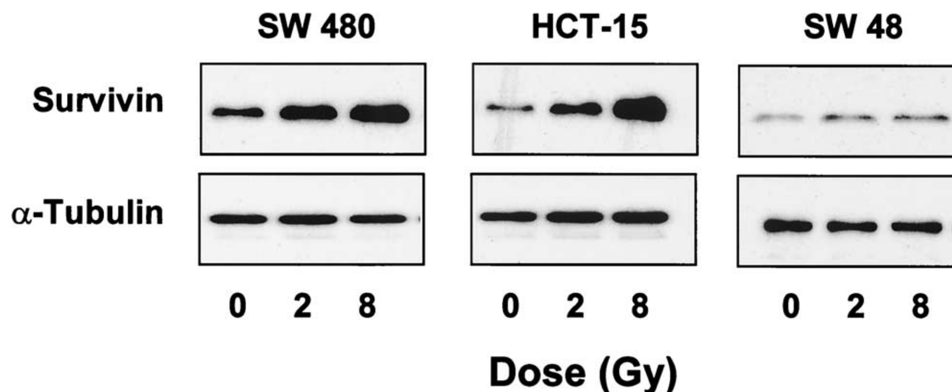

Fig. 3. Western blot analysis of expression levels of survivin protein in unirradiated cells (0 Gy) and 48 h after irradiation with 2 and 8 Gy, respectively.

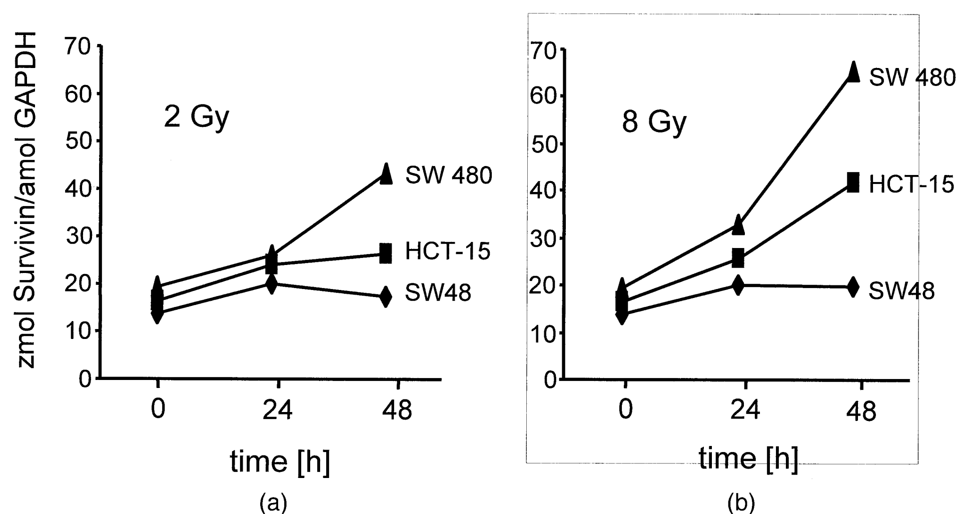

Fig. 4. Survivin mRNA expression in three colorectal cell lines, untreated (0 h), 24 h, and 48 h postirradiation with 2 Gy (a) and 8 Gy (b). Expression of survivin mRNA was measured by quantitative TaqMan reverse transcription-polymerase chain reaction.

onstrated a significant predictive value of pretreatment levels of apoptosis for tumor response and relapse-free survival (3, 20–23). Comparative studies on pretreatment levels of apoptosis in rectal cancer biopsies and post-RCT levels in the resected specimen have demonstrated increased rates of the apoptotic cell death after RCT (24) and have shown higher rates of induced apoptosis in responsive as compared with more radioresistant rectal cancer specimen (25).

The first question of this article was if these clinical findings can be confirmed *in vitro* using three colorectal cell lines with different intrinsic radiosensitivities. Higher level of spontaneous and radiation-induced apoptosis were detected in the most sensitive cell line SW 48, compared with HCT-15 cells, and the most radioresistant SW 480 cells showed the lowest amount of spontaneous and radiation-induced apoptosis, suggesting an association between the ability of colorectal cancer cells to undergo apoptosis and their radiosensitivity. In three lung carcinoma cells with different intrinsic radiosensitivities, Sirzen *et al.* have also found higher levels of spontaneous apoptosis to be predictive for radiosensitivity *in vitro* (26).

Based on these *in vivo* and *in vitro* results, the question that needs further investigation is, What determines whether a colorectal tumor is more or less prone to apoptosis? Several apoptosis inhibitors related to the baculovirus *iap* gene were recently identified in *Drosophila*, mouse, and humans (27). Survivin, a member of the IAP family, is involved in both inhibition of apoptosis and control of cell division. Its antiapoptotic function seems to be related to its ability to directly or indirectly inhibit caspases (28). Genome-wide searches revealed that survivin constituted the fourth top "transcriptome" in colorectal, lung, brain, and breast cancers, among others (29). In a immunohistochemical study comprising pretreatment biopsies of 54 patients with rectal cancer, we could recently demonstrate that the percentage of survivin-positive cells within a tumor specimen is variable, ranging from 0% to more than 75% (14). Moreover, survivin expression inversely correlated with the apoptotic index and was a highly significant predictor of relapse-free survival after preoperative RCT and surgical resection. A significant inverse correlation between survivin expression and tumor cell apoptosis *in vivo* was also found in colon (11, 12), breast (8), gastric cancer (7), and pancreatic duct cell tumors (6), and its expression in these cancers was associated with an unfavorable prognosis.

In our present *in vitro* study, these clinical results could be confirmed: among the colorectal cancer cells examined, SW 480 cells, which had the highest levels of survivin mRNA and protein, had the lowest rate of spontaneous and radiation-induced apoptosis and were the most resistant to irradiation as determined using the clonogenic assay. Conversely, SW 48 cells, which had the lowest level of survivin protein and mRNA, showed the highest level of apoptosis and were most sensitive to irradiation, suggesting that survivin may act as a *constitutive* radioresistant factor in colorectal cancer cells. These results are in line with studies on pancreatic cancer cells *in vitro*, where an inverse relationship between survivin mRNA expression and radiosensitivity also was found (30). Recent studies also revealed that survivin correlated with the chemoresistance of cancer cells: in patients with advanced esophageal cancer who underwent chemotherapy, survivin mRNA expression was significantly lower in patients with response to treatment as compared with patients with no change or progressive disease (9). Tamm *et al.* reported that survivin-transfected cells inhibited etoposide-induced apoptosis (31). Thus, there is now compelling evidence that survivin plays an important role in chemo-radio–resistance of malignant cells.

In the present study, we also analyzed the expression pattern of survivin mRNA and protein in colorectal cancer cells *after* irradiation. We found a dose-dependent increase both of survivin mRNA and protein expression, at least in

the more radio-resistant cell lines SW 480 and HCT-15. The same results were reported by Asanuma *et al.*, who found that survivin mRNA expression in pancreatic cell lines increased gradually after irradiation, and that caspase-3 activity was significantly suppressed in these cells (30). Interestingly, this group also demonstrated that cell survival after exposure to high doses (5 and 10 Gy) was significantly increased by pretreatment of cells with sublethal doses (1–2 Gy), suggesting that the increase of survivin protein expression after irradiation may also act as an inducible radioresistance factor. In this respect, it is notable that we found no increase in survivin protein and mRNA expression in the most sensitive SW 48 cell line, which—in contrast to the both other lines tested—is known to be p53 wild-type. Recently, Hoffman *et al.* (32) and Mirza *et al.* (33) reported that wild-type p53 represses survivin expression at both mRNA and protein levels.

In conclusion, we have found survivin expression both *in vivo* and *in vitro* to be closely related to the ability of colorectal cells to undergo apoptotis. Analysis of survivin mRNA or protein expression may therefore provide important predictive information on the radio- and chemo-resistance of individual colorectal tumors. Moreover, strategies to inhibit survivin mRNA (e.g., by antisense oligonucleotides) (34) may finally emerge as a new gene therapeutic treatment option in a variety of tumor entities.

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