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CHEMOSENSITIVITY OF SOLID TUMOR CELLS *IN VITRO* IS RELATED TO ACTIVATION OF THE CD95 SYSTEM

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We have identified the CD95 system as a key mediator of chemotherapy-induced apoptosis in leukemia and neuroblastoma cells. Here, we report that sensitivity of various solid tumor cell lines for drug-induced cell death corresponds to activation of the CD95 system. Upon drug treatment, strong induction of CD95 ligand (CD95-L) and caspase activity were found in chemosensitive tumor cells (Hodgkin, Ewing's sarcoma, colon carcinoma and small cell lung carcinoma) but not in tumor cells which responded poorly to drug treatment (breast carcinoma and renal cell carcinoma). Blockade of CD95 using F(ab′)$_2$ anti-CD95 antibody fragments markedly reduced drug-induced apoptosis, suggesting that drug-triggered apoptosis depended on CD95-L/receptor interaction. Moreover, drug treatment induced CD95 expression, thereby increasing sensitivity for CD95-induced apoptosis. Drug-induced apoptosis critically depended on activation of caspases (ICE/Ced-3-like proteases) since the broad-spectrum inhibitor of caspases zVAD-fmk strongly reduced drug-mediated apoptosis. The prototype substrate of caspases, poly(ADP-ribose) polymerase, was cleaved upon drug treatment, suggesting that CD95-L triggered autocrine/paracrine death *via* activation of caspases. Our data suggest that chemosensitivity of solid tumor cells depends on intact apoptosis pathways involving activation of the CD95 system and processing of caspases. Our findings may have important implications for new treatment approaches to increase sensitivity and to overcome resistance of solid tumors. *Int. J. Cancer* 76:105–114, 1998.
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Chemotherapeutic agents, irrespective of their intracellular target, act primarily through induction of apoptosis in susceptible cancer cells (Rowan and Fisher, 1997). However, the precise molecular requirements that characterize a chemosensitive phenotype in tumor cells are not well understood. Response to chemotherapy has been attributed to various mechanisms, including intact apoptosis pathways in target cells (Debatin, 1997; Rowan and Fisher, 1997).

Cell-surface-receptor molecules of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family, such as CD95, are involved in the regulation of apoptosis (Nagata, 1997; Oehm *et al.,* 1992; Trauth *et al.,* 1989). CD95 is a 45 kDa type I transmembrane receptor expressed on a variety of normal and neoplastic cells (Leithäuser *et al.,* 1993). Following cross-linking of CD95, a death signal is generated *in vitro* and *in vivo* in sensitive cells that catalyzes cleavage of the caspase (ICE/Ced-3) cascade of cysteine proteases, leading to proteolysis of substrates such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP) and, ultimately, to cell death (Peter *et al.,* 1996). The CD95 ligand (CD95-L) is a type II transmembrane molecule of 40 kDa and part of the TNF/NGF family of ligands (Suda *et al.,* 1993) which may occur in a soluble form released from the cell surface by proteolytic cleavage (Tanaka *et al.,* 1995).

Although the key role of the CD95 system in negative growth regulation has been studied mostly within the immune system (Debatin, 1996; Krammer *et al.,* 1994), there is mounting evidence that it is involved in negative growth regulation and drug-induced apoptosis of tumor cells. We have found that CD95-L was induced upon drug treatment in leukemia and neuroblastoma cells and mediated cell death in an autocrine/paracrine manner (Friesen *et al.,* 1996; Fulda *et al.,* 1997). Similarly, activation-induced autocrine suicide of T cells depends on CD95-L/receptor interaction following induction of CD95-L upon T-cell-receptor triggering (Dhein *et al.,* 1995). We therefore investigated whether or not activation of the CD95 system may contribute to chemosensitivity of solid tumor cells.

MATERIAL AND METHODS

Drugs

Doxorubicin (Farmitalia, Milan, Italy) and cisplatinum (Sigma, Deisenhofen, Germany) were provided as pure substances and dissolved in sterile water before each experiment (1 mg/ml).

Cell culture

Ewing's sarcoma (A17/95), Hodgkin (L540CY), breast carcinoma (MCF-7), colon carcinoma (HT-29), small cell lung carcinoma (H-146) and renal cell carcinoma (KTCTL-26) cells were cultured in RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 10 mM HEPES (pH 7.3) (Biochrom, Berlin, Germany), 100 U/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO) and 2 mM L-glutamine (Biochrom). For experiments, cells were seeded at a concentration of 5×10^4/cm^2 in 24-well plates or 75 cm^2 tissue culture flasks (Falcon, Heidelberg, Germany).

Determination of apoptosis

Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei (FACScan; Becton Dickinson, Heidelberg, Germany) as previously described (Nicoletti *et al.,* 1991). Cells were suspended in hypotonic lysis buffer (0.1% sodium citrate [Merck, Darmstadt, Germany], 0.1% Triton X [Serva, Heidelberg, Germany] and 50 µg/ml propidium iodide [Sigma]) and incubated at 4°C overnight. Cells were analyzed for DNA content by flow cytometry (FACScan) using CELLQuest software (Becton Dickinson).

Inhibition of apoptosis by zVAD-fmk or F(ab′)$_2$ anti-APO-1 (anti-CD95) fragments

The broad-range tripeptide inhibitor of caspases zVAD-fmk (Enzyme Systems Products, Dublin, CA) was used at a concentration of 60 µM. F(ab′)$_2$ anti-APO-1 (anti-CD95) antibody fragments and isotype-matched antibody FII23 (IgG$_3$) were prepared as previously described (Dhein *et al.,* 1995). Cells were incubated with 10 µg/ml F(ab′)$_2$ anti-APO-1 antibody fragments or 10 µg/ml F(ab′)$_2$ FII23 antibody fragments for 1 hr at 37°C prior to addition of drugs.

Determination of caspase activity

Caspase activity was measured by FACS analysis as previously described (Los *et al.,* 1995). Briefly, cells were loaded in hypotonic medium with the fluorogenic substrate VAD-MNA (50 µM; Bachem,

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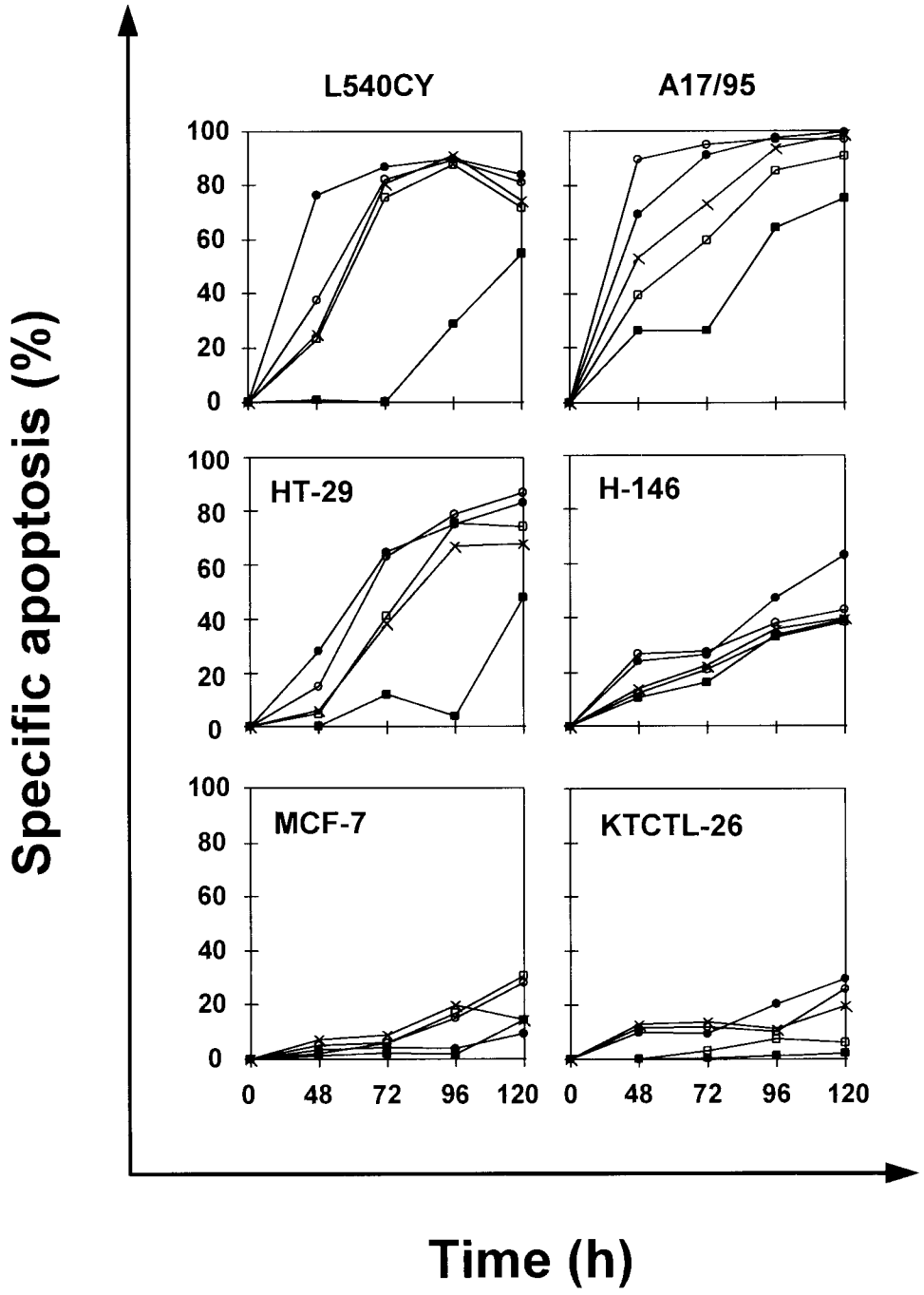

FIGURE 1 – Dose-response of doxorubicin-induced apoptosis. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated with 0.01 µg/ml (■), 0.05 µg/ml (□), 0.1 µg/ml (×), 0.5 µg/ml (○) or 1 µg/ml (●) doxorubicin at the time points indicated. Apoptosis was assessed by FACS analysis of propidium iodide-stained nuclei. Percentage of specific apoptosis was calculated as follows: 100 × [experimental apoptosis (%) − spontaneous apoptosis in medium (%)]/[100% − spontaneous apoptosis in medium (%)]. Time (abscissa) is plotted against cell number (ordinate). Data are the mean of triplicates with standard deviations of less than 10%. Similar results were obtained in 3 separate experiments.

FIGURE 2 – Activation of caspases. *(a)* Cleavage of PARP. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml (Hodgkin, Ewing's sarcoma, colon carcinoma, small cell lung carcinoma) or 1 µg/ml doxorubicin (breast carcinoma, renal cell carcinoma) at the indicated time points; 100 µg protein per lane isolated from cell lysates were separated by 12% SDS-PAGE. Immunodetection of PARP protein was performed by rabbit anti-PARP polyclonal antibody and ECL. *(b)* Analysis of caspase activity. Colon carcinoma (HT-29) and breast carcinoma (MCF-7) cells were incubated with 0.01 µg/ml (●), 0.05 µg/ml (♦) or 0.1 µg/ml (■) doxorubicin at the time points indicated. Cells were permeabilized by hypotonic shock, incubated with 50 µM of the fluorogenic substrate VAD-MNA and analyzed with a flow cytometer equipped with an argon laser. Data are given as mean of 3 independent experiments done in triplicate. Standard deviations were less than 10%. *(c)* Inhibition of doxorubicin-induced apoptosis by zVAD-fmk. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29) and small cell lung carcinoma (H-146) cells were treated with 0.5 µg/ml doxorubicin for 72 hr in the absence (black bars) or presence (white bars) of 60 µM zVAD-fmk. Specific apoptosis was determined and calculated as described in Figure 1. Data are given as means of 3 independent experiments done in triplicate. Standard deviations were less than 10%.

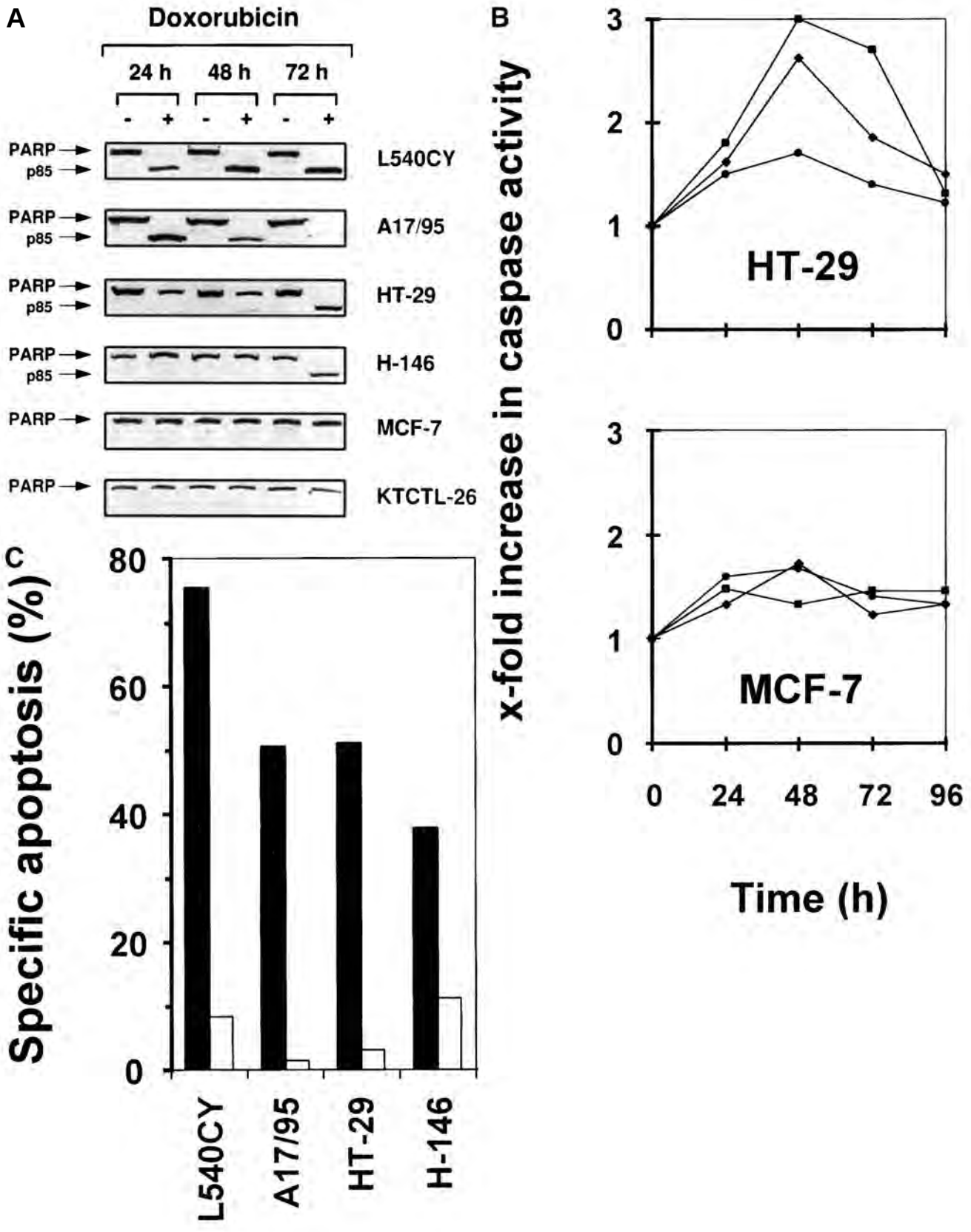
A
Doxorubicin
24 h
48 h
72 h
- + - + - +
PARP
p85
L540CY
A17/95
HT-29
H-146
MCF-7
KTCTL-26
B
x-fold increase in caspase activity
HT-29
MCF-7
0 24 48 72 96
Time (h)
C
Specific apoptosis (%)
0 20 40 60 80
L540CY
A17/95
HT-29
H-146

FIGURE 2

Heidelberg, Germany). Fluorescence was measured by a flow cytometer equipped with an argon laser (FACS Vantage, Becton Dickinson) using an excitation wavelength of 360 nm and an emission wavelength of 488 nm.

Determination of CD95 and CD95-L expression

For determination of CD95 expression, cells were stained with anti-APO-1 (anti-CD95) IgG_1 monoclonal antibody (MAb, 1 µg/ml) for 45 min at 4°C followed by goat anti-mouse IgG-phycoerythrin (Immunotech, Hamburg, Germany) or goat anti-mouse IgG-FITC (Calbiochem, Bad Soden, Germany) for 30 min at 4°C. FII23 IgG_3 antibody was used as isotype-matched antibody to control non-specific binding. For determination of CD95-L expression, cells were stained with FITC-conjugated rat anti-CD95-L MAb (H11; Alexis Corporation, Grünberg, Germany) for 45 min at 4°C. Rat IgG_{2a} antibody was used as isotype-matched control antibody (PharMingen, San Diego, CA). Cells were analyzed by a flow cytometer using CELLQuest software.

Determination of P-glycoprotein (MDR-1) expression

Cells were stained with mouse anti-MDR-1 MAb UIC2 (50 µg/ml; Immunotech, Marseilles, France) for 30 min at 4°C followed by goat $F(ab')_2$ anti-mouse IgG_{2a}-RPE (Southern Biotechnology Associates, Birmingham, AL) for 30 min at 4°C. Cells were analyzed by FACScan using CELLQuest software.

Determination of intracellular glutathione (GSH)

Cells were stained with 40 µM monochlorobimane (Molecular Probes, Eugene, OR) for 10 min at room temperature in the dark and immediately analyzed on a FACS Vantage flow cytometry system (Becton Dickinson) using an emission wavelength of 455 nm (Omega 450DF-65) and a 351 to 364 nm bandpass for excitation.

RT-PCR for CD95-L mRNA

Total RNA was prepared using the Qiagen (Hilden, Germany) total RNA kit. RNA was converted to cDNA by reverse transcription and amplified for 38 cycles by PCR in a thermocycler (Stratagene, Heidelberg, Germany) using the Gene Amplification RNA-PCR kit (Perkin-Elmer, Branchburg, NJ) following the manufacturer's instructions. A 500 bp fragment of CD95-L was amplified using primers 5′-ATGTTTCAGCTCTTCCACCTACAGA-3′ and 5′-CCAGAGAGAGCTCAGATACGTTGAC-3′ according to the sequence of human CD95-L (Suda *et al.,* 1993). Expression of β-actin (MWG-Biotech, Ebersberg, Germany) was used as a standard for RNA integrity and equal gel loading. PCR products were run at 60 V for 2 hr on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination.

Western blot analysis

Proteins for Western blot analysis were extracted from cells lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva) and 1 mM PMSF (Sigma) followed by high-speed centrifugation. Membrane proteins were eluted by buffer containing 0.1 M glycine, pH 3.0, and 1.5 M Tris, pH 8.8. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL); 40 µg protein per lane were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 hr in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection was done using the mouse anti-CD95-L MAb (1:2,500; Transduction Laboratories, Lexington, KY), mouse anti-CD95 MAb (1:500, Transduction Laboratories), rabbit anti-PARP polyclonal antibody (1:5,000, Enzyme Systems Products), rabbit anti-Bax polyclonal antibody (1:500, Calbiochem), rabbit anti-Bcl-x polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bcl-2 MAb (1:1,000, Santa Cruz Biotechnology) and mouse anti-p53 MAb (1:1,000, Transduction Laboratories) followed by horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Santa Cruz Biotechnology). ECL (Amersham) was used for detection.

RESULTS

Dose-response experiments of drug-induced apoptosis were performed by staining hypodiploid DNA with propidium iodide. Doxorubicin rapidly triggered apoptosis in Hodgkin (L540CY) and Ewing's sarcoma (A17/95) cells (Fig. 1). Colon carcinoma (HT-29) and small cell lung carcinoma (H-146) cells showed a delayed response, whereas breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells responded poorly to doxorubicin even after prolonged treatment (Fig. 1). A similar pattern of chemosensitivity among the different cell lines was found following treatment with other cytotoxic drugs, such as cisplatinum and VP-16 (data not shown). In addition to propidium iodide staining, apoptosis was assessed by annexin V staining, giving similar results (data not shown).

To gain insight into the molecular requirements of drug-induced apoptosis, we investigated whether caspases (ICE/Ced-3-like proteases), involved as effector molecules in various death-signaling pathways (Schulze-Osthoff *et al.,* 1996), were activated upon treatment. To assess activation of caspases, we monitored cleavage of the prototype caspase substrate PARP by Western blot analysis. PARP was processed proteolytically to its characteristic 85 kDa fragment in doxorubicin-sensitive cells, such as Hodgkin, Ewing's sarcoma, colon carcinoma and small cell lung carcinoma cells, but not in breast carcinoma and renal cell carcinoma cells, which responded poorly to doxorubicin (Fig. 2*a*). Kinetics of PARP cleavage corresponded to kinetics of apoptosis induction as PARP was processed rapidly in Hodgkin and Ewing's sarcoma cells, which also rapidly underwent apoptosis upon doxorubicin treatment (Fig. 2*a*). Colon and small cell lung carcinoma cells showed a more delayed kinetic of PARP cleavage corresponding to the delayed kinetics of apoptosis induction in these cells (Fig. 2*a*). Moreover, caspase activity, as measured by flow cytometry, markedly increased in cells which underwent apoptosis upon doxorubicin treatment, such as HT-29 cells, whereas little increase in caspase activity was observed in cells which showed a poor response to drug treatment, such as MCF-7 cells (Fig. 2*b*). To test whether apoptosis following doxorubicin treatment was the result of caspase activation, we used the broad-spectrum peptide inhibitor of caspases zVAD-fmk. Incubation with zVAD-fmk almost completely inhibited doxorubicin-triggered apoptosis, demonstrating that caspases are central to doxorubicin-mediated apoptosis (Fig. 2*c*).

Since caspases are an integral part of the CD95 signaling pathway (Los *et al.,* 1995), we asked whether stimulation of the CD95 system might be involved in doxorubicin-induced activation of caspases and cell death. CD95 was expressed constitutively at similar levels in all cell lines tested (Fig. 3*a*). Apoptosis was triggered by an agonistic anti-APO-1 antibody and cycloheximide, implicating that the CD95 pathway was intact in these cells (Fig. 3*b*). Following incubation with cisplatinum, expression of CD95 was up-regulated 3-fold (Fig. 3*a*), thereby increasing sensitivity to apoptosis triggered by an agonistic anti-APO-1 antibody (data not shown). The increase in CD95 expression was not specific to cisplatinum since a similar increase was found after exposure to doxorubicin or VP-16 (Fig. 3*c* and data not shown). However, CD95 expression or sensitivity to anti-APO-1-mediated apoptosis did not correspond to sensitivity to drug-induced cell death in all cell lines, suggesting that additional factors probably are involved in mediating drug-induced apoptosis. Treatment with doxorubicin strongly induced CD95-L mRNA and protein in Hodgkin, Ewing's sarcoma, colon carcinoma and small cell lung carcinoma cells, all of which exhibited sensitivity to doxorubicin, whereas weak induction of CD95-L mRNA and protein was observed in cells which responded poorly to drug treatment, such as breast carci-

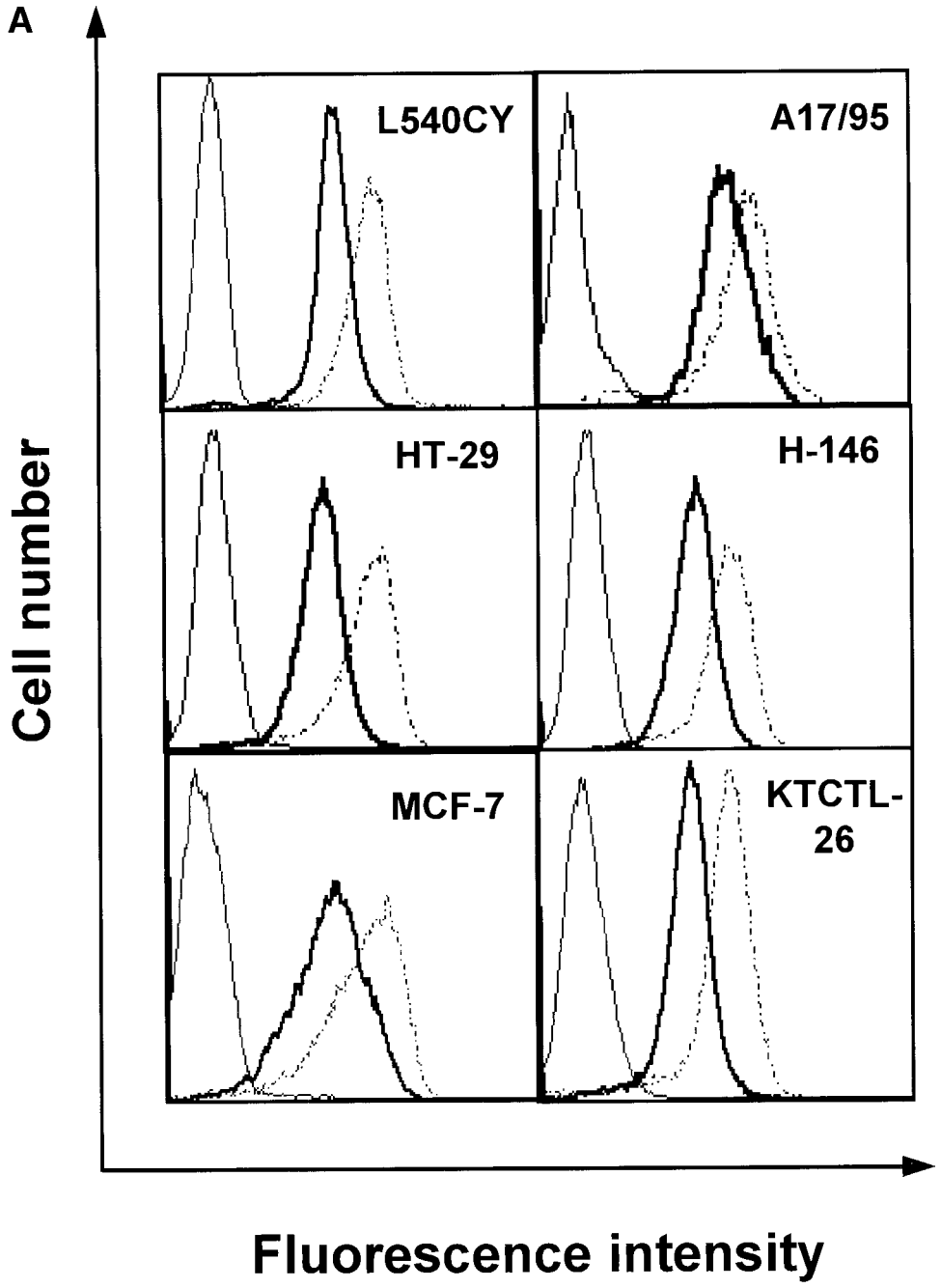

FIGURE 3 – Induction of CD95- and anti-CD95-induced apoptosis. *(a)* Induction of CD95 expression by cisplatinum. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated with 5 µg/ml cisplatinum for 24 hr, stained with mouse anti-APO-1 MAb followed by phycoerythrin-conjugated anti-mouse IgG antibody and analyzed by flow cytometry using CELLQuest software. Thick line, untreated cells stained with anti-APO-1 antibody; dotted line, treated cells stained with anti-APO-1 antibody; thin line, control cells stained with isotype-matched antibody. Fluorescence intensity (abscissa) is plotted against cell number (ordinate). All experiments were done in triplicate with standard deviations of less than 10%. Similar results were obtained in 3 separate experiments. *(b)* Induction of apoptosis by anti-APO-1 (anti-CD95). Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated for 72 hr with 1 µg/ml anti-APO-1 IgG_3 MAb and 1 µg/ml cycloheximide. Specific apoptosis was determined and calculated as described in Figure 1. Data are given as means of 3 independent experiments done in triplicate. Standard deviations were less than 10%. *(c)* Induction of CD95 expression by doxorubicin. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml doxorubicin for 24 hr. Protein of cell lysates, 40 µg per lane, was separated by 12% SDS-PAGE. Immunodetection of CD95 protein was performed by mouse anti-CD95 MAb and ECL. Panels *b* and *c* are continued on page 110.

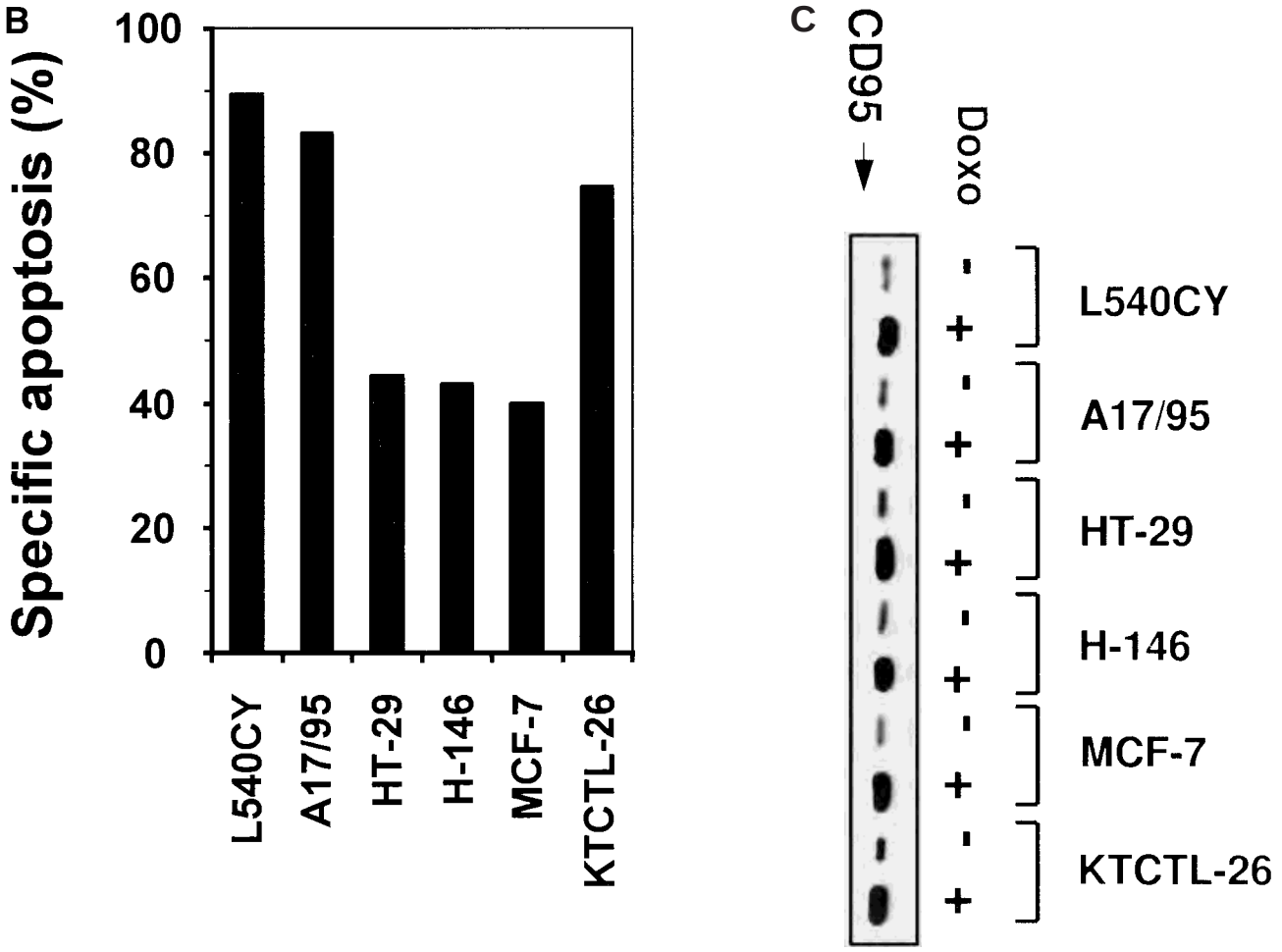

FIGURE 3 (*continued*). Legend appears on page 109.

noma and renal carcinoma cells (Fig. 4*a*,*b*). Moreover, kinetics of CD95-L induction corresponded to kinetics of cell death. CD95-L mRNA was up-regulated strongly after 24 hr incubation with doxorubicin in Hodgkin and Ewing's sarcoma cells, which rapidly underwent apoptosis upon drug treatment, whereas colon carcinoma and small cell lung carcinoma cells showed delayed induction of CD95-L mRNA expression corresponding to the delayed kinetics of apoptosis in these cells (Fig. 4*a*). Thus, CD95-L was induced upon drug incubation in chemosensitive tumor cells, whereas weak induction of CD95-L was seen in cell lines which responded poorly to doxorubicin. To see whether CD95-L was expressed at the cell surface, FACS analysis was performed. Upon treatment with doxorubicin, an increase in surface expression of CD95-L was detected (Fig. 4*c*).

To test whether CD95-L would mediate autocrine or paracrine cell death by cross-linking its cognate receptor, we blocked CD95-L/receptor interaction using $F(ab')_2$ anti-CD95 antibody fragments, which have been shown to inhibit CD95- and drug-triggered cell death (Dhein *et al.*, 1995; Friesen *et al.*, 1996; Fulda *et al.*, 1997). Incubation with $F(ab')_2$ anti-CD95 antibody fragments prior to addition of drugs markedly reduced cell death, whereas $F(ab')_2$ FII23 IgG_3 control antibody fragments had no effect (Fig. 4*d* and data not shown). This suggested that drug-triggered apoptosis depended, at least in part, on CD95-L/receptor interaction.

Response to cytotoxic drugs has been reported to be modulated by various mechanisms, including expression levels of Bcl-2-related proteins (Yang and Korsmeyer, 1996), over-expression of P-glycoprotein (MDR-1) (Bellamy, 1996) or the cellular redox state (Schroder *et al.*, 1996). However, the poor response to doxorubicin seen in breast carcinoma and renal cell carcinoma cells was not associated with over-expression of anti-apoptotic Bcl-2-related proteins such as Bcl-2 and Bcl-x_L or down-regulation of pro-apoptotic proteins such as Bax or Bcl-x_s before or after doxorubicin treatment (Fig. 5*a*). Furthermore, no over-expression of P-glycoprotein was detected in breast carcinoma or renal cell carcinoma cells or in any of the other cell lines before or after doxorubicin treatment (Fig. 5*b* and data not shown). Since functional studies revealed no difference in doxorubicin uptake or efflux among the cell lines (data not shown), altered drug accumulation probably did not account for the observed differences in drug sensitivity. In addition, no increase in intracellular GSH levels was found in cells which responded poorly to doxorubicin compared to chemosensitive cells (Fig. 5*c*). Wild-type p53 protein accumulated upon drug treatment in all cell lines except p53 mutant HT-29 cells (Fig. 5*d*).

DISCUSSION

Chemotherapeutic agents, irrespective of their putative intracellular target, activate programmed cell death in target cells (Rowan and Fisher, 1997). However, the molecular requirements which determine chemosensitivity of cancer cells have not been defined exactly. Here, we report that sensitivity of solid tumor cells to cytotoxic drugs is related to activation of the CD95 system. CD95-L was induced following drug treatment in chemosensitive

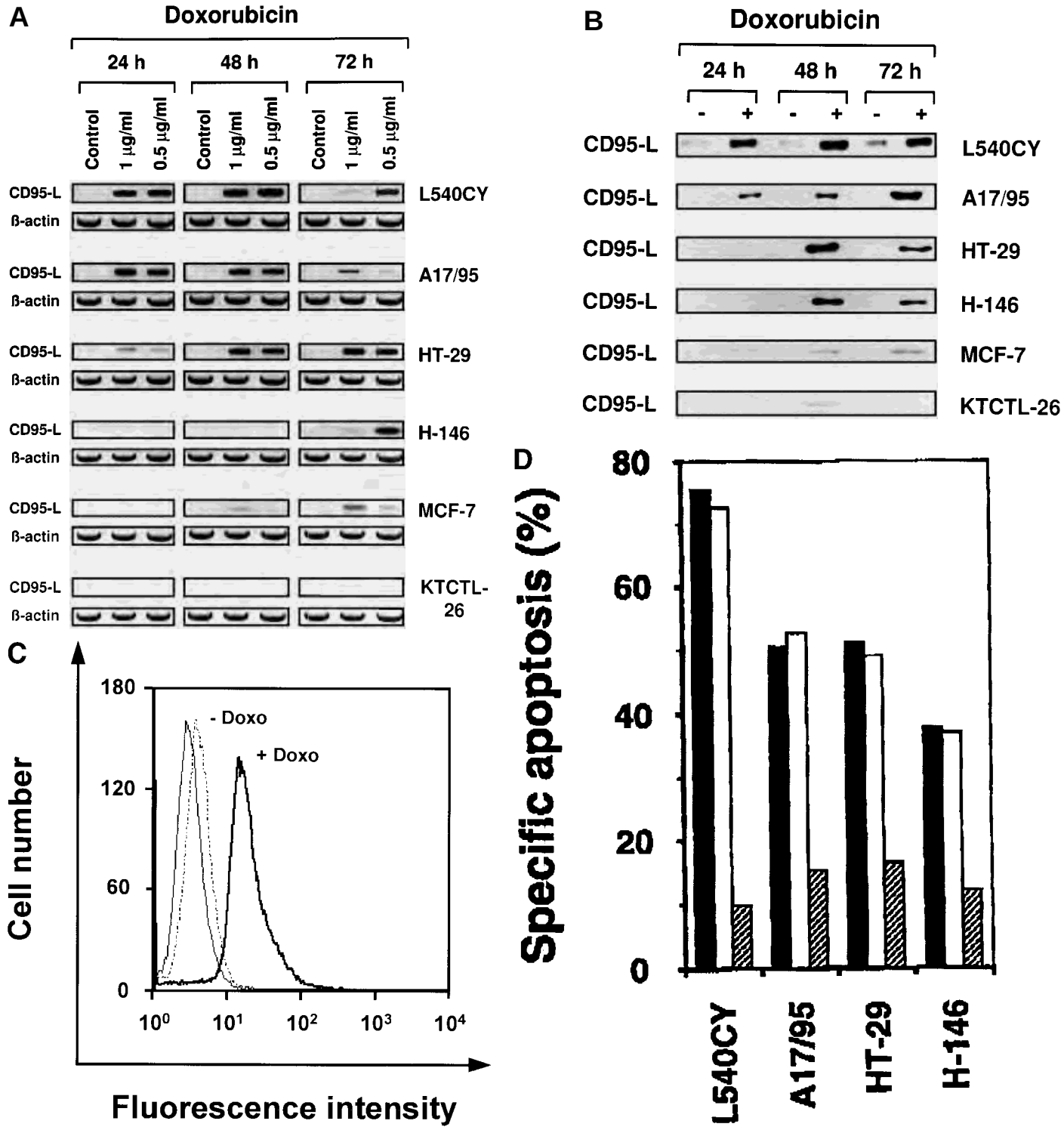

FIGURE 4 – Induction of CD95-L. *(a)* Analysis of CD95-L mRNA expression. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated with doxorubicin at indicated concentrations and time points. CD95-L mRNA expression was determined by RT-PCR. Expression of β-actin was used to control RNA integrity and equal gel loading. *(b)* Analysis of CD95-L protein by Western blot. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml (Hodgkin, Ewing's sarcoma, colon carcinoma, small cell lung carcinoma) or 1 µg/ml doxorubicin (breast carcinoma, renal cell carcinoma) at the indicated time points. Protein of cell lysates, 40 µg per lane, was separated by 12% SDS-PAGE. CD95-L protein was detected as a 37 kDa band by mouse anti-CD95-L MAb and ECL. *(c)* Analysis of CD95-L protein by flow cytometry. Ewing's sarcoma cells were treated with 0.1 µg/ml doxorubicin for 18 hr, stained with FITC-conjugated rat anti-CD95-L MAb and analyzed by flow cytometry using CELLQuest software. Thick line, treated cells stained with anti-CD95-L antibody; dotted line, untreated cells stained with anti-CD95-L antibody; thin line, control cells stained with isotype-matched antibody. Fluorescence intensity (abscissa) is plotted against cell number (ordinate). *(d)* Inhibition of doxorubicin-induced apoptosis by $F(ab')_2$ anti-CD95. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29) and small cell lung carcinoma (H-146) cells were treated with 0.5 µg/ml doxorubicin for 72 hr after pre-incubation for 1 hr with medium (black bars), 10 µg/ml $F(ab')_2$ FII23 (IgG_3 control antibody, white bars) or 10 µg/ml $F(ab')_2$ anti-CD95 (blocking antibody, hatched bars). Specific apoptosis was determined and calculated as described in Figure 1. Data are given as means of 3 independent experiments done in triplicate. Standard deviations were less than 10%.

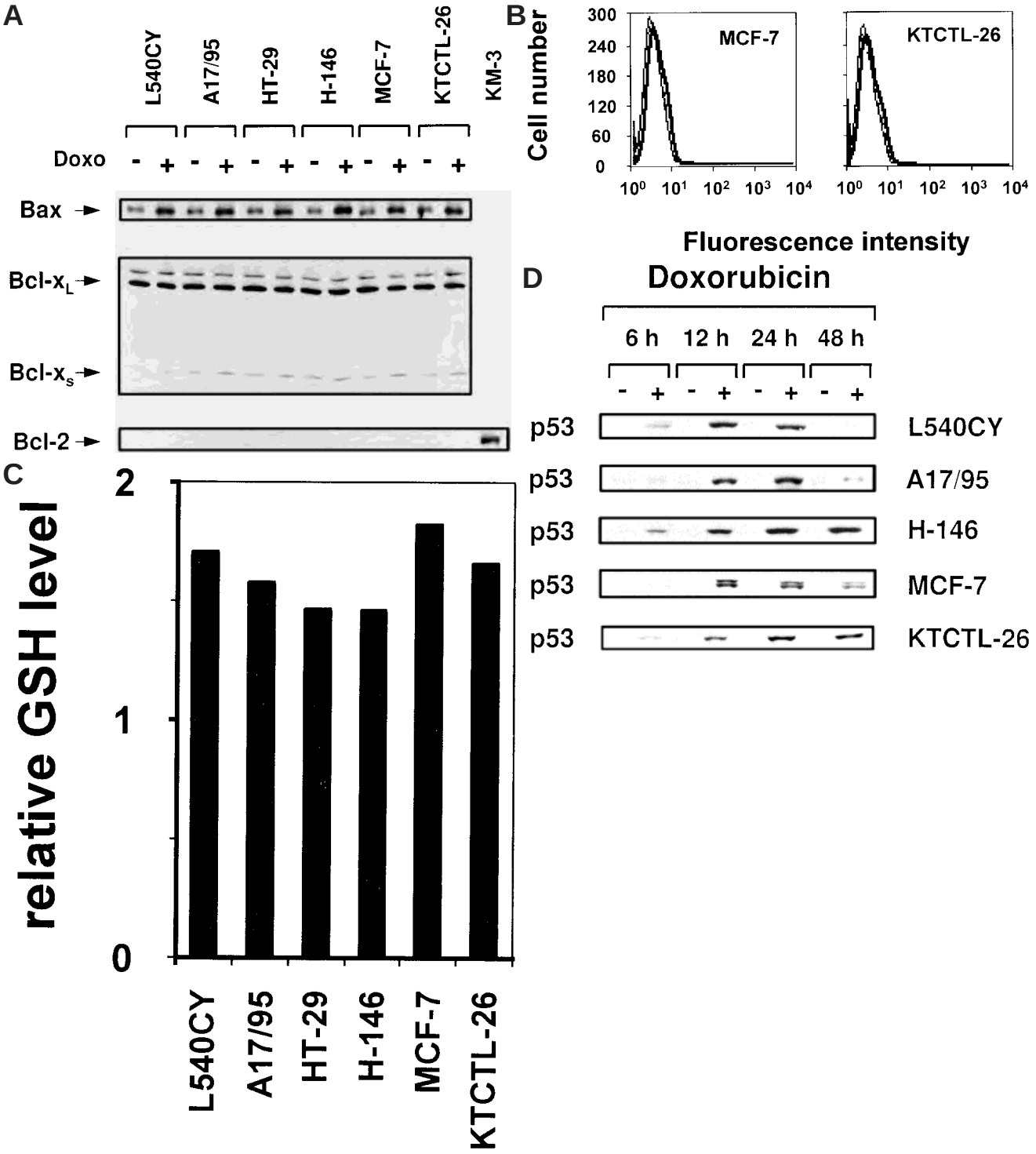

FIGURE 5 – Expression levels of Bcl-2-related proteins, P-glycoprotein (MDR-1), GSH and p53 protein. *(a)* Expression of Bcl-2-related proteins. Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml doxorubicin for 24 hr; 40 µg protein from cell lysates were separated by 12% SDS-PAGE. Immunodetection of Bcl-2, Bax and Bcl-x proteins was performed using mouse anti-Bcl-2 MAb, rabbit anti-Bax polyclonal and rabbit anti-Bcl-x polyclonal antibody and ECL. Untreated KM3 cells were used as positive control for Bcl-2 expression. *(b)* Expression of P-glycoprotein (MDR-1). P-glycoprotein expression of breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells was determined by FACS analysis using P-glycoprotein (MDR-1) MAb UIC2. Representative experiments out of 3 are shown. Fluorescence intensity (abscissa) is plotted against cell number (ordinate). *(c)* Analysis of GSH levels. GSH levels of Hodgkin (L540CY), Ewing's sarcoma (A17/95), colon carcinoma (HT-29), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were determined by flow cytometry, staining cells with 40 µM monochlorobimane. Data are shown as relative GSH levels compared to unstained control cells. *(d)* Expression of p53 protein. Hodgkin (L540CY), Ewing's sarcoma (A17/95), small cell lung carcinoma (H-146), breast carcinoma (MCF-7) and renal cell carcinoma (KTCTL-26) cells were treated (+) with 0.5 µg/ml (Hodgkin, Ewing's sarcoma, small cell lung carcinoma) or 1 µg/ml doxorubicin (breast carcinoma, renal cell carcinoma) at the indicated time points; 40 µg protein of cell lysates per lane were separated by 12% SDS-PAGE. Immunodetection of p53 protein was performed by mouse anti-p53 MAb and ECL.

tumor cells but not in tumor cells which showed a poor response to cytotoxic drugs. Binding of CD95-L to its receptor then triggered the apoptosis cascade since interfering with CD95-L/receptor interaction using $F(ab')_2$ anti-CD95 antibody fragments markedly reduced doxorubicin-induced apoptosis. Thus, production of CD95-L and cross-linking of its cognate receptor were probably key events in drug-mediated cell death. Moreover, CD95 expression was up-regulated upon treatment with cytotoxic drugs, thereby increasing sensitivity for physiological apoptotic signals. Caspases were centrally involved as death effector molecules during doxorubicin-induced apoptosis as the broad-spectrum tripeptide inhibitor of caspases zVAD-fmk almost completely inhibited doxorubicin-induced apoptosis. Activation of caspases resulted in cleavage of substrates, such as PARP, and in cell death. Sensitivity for drug-induced cell death has been reported to depend on activation of caspases (Los *et al.,* 1997). Taken together, our results suggest that chemosensitivity of solid tumor cells was determined, at least in part, by the presence of functional apoptosis pathways, such as the CD95 system involving activation of caspases. We have identified activation of the CD95 system as a key mechanism of drug-triggered apoptosis in leukemia and neuroblastoma cells (Friesen *et al.,* 1996; Fulda *et al.,* 1997). Thus, cytotoxicity mediated by the CD95 system might be central to cytotoxic drug action in a variety of sensitive tumor cells of different origin. Drug concentrations used in this study corresponded to plasma levels which can be achieved in patients following chemotherapy (Dominici *et al.,* 1989; Muller *et al.,* 1993), indicating that our findings may be of therapeutical relevance.

In addition to the CD95 system, various mechanisms have been implicated in regulation of drug-induced apoptosis. Members of the Bcl-2 family of proteins positively and negatively modulate apoptosis (Yang and Korsmeyer, 1996). However, imbalance in expression levels of anti-apoptotic to pro-apoptotic Bcl-2-related proteins did not appear to account for the differences in chemotherapy-induced apoptosis found among the cell lines. Moreover, the diminished response to chemotherapy seen in breast carcinoma and renal cell carcinoma cells was not related to an increase in intracellular GSH levels or over-expression of P-glycoprotein, which has been described to mediate resistance to certain chemotherapeutic agents, including doxorubicin, in some cell types (Bellamy, 1996). Furthermore, the cytotoxic response to chemotherapy was modulated variably by loss of wild-type p53 function as p53 mutant HT-29 cells were sensitive to doxorubicin, whereas wild-type p53 MCF-7 and KTCTL-26 cells responded only poorly to chemotherapy. Wild-type p53 is considered to mediate apoptosis in response to DNA damage in many tumor cells (Lowe *et al.,* 1994) and has been implicated in up-regulation of CD95 expression in hepatocellular carcinoma cells treated with cytotoxic drugs (Müller *et al.,* 1997). However, there have been conflicting reports on the relation of p53 status and the efficacy of cancer cells to treatment-induced cytotoxicity, suggesting that the response to cytotoxic agents might be influenced variably by loss of wild-type p53 function and might be tissue-specific (Cote *et al.,* 1997; Wahl *et al.,* 1996). In this respect, it is of interest that we found up-regulation of CD95 upon drug treatment in p53 mutant HT-29 cells, consistent with Micheau *et al.* (1997). Activation of the CD95 system with increased expression of CD95-L and CD95 may be mediated through several pathways. Thus, generation of oxygen radicals and ceramide followed by activation of cellular stress pathways may be involved in drug-induced apoptosis (data not shown).

Taken together, our findings suggest that sensitivity of solid tumor cells for cytotoxic drugs is related to activation of the CD95 system, including processing of caspases. Together with our previous findings that the CD95 system is centrally involved in drug-triggered apoptosis of leukemia and neuroblastoma cells, these results might have important implications for drug development and new therapeutic strategies to overcome drug resistance of tumor cells.

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