Cancer Chemother Pharmacol (2000) 46: 467–476

ORIGINAL ARTICLE

E. Pace · M. Melis · L. Siena · F. Bucchieri
A. M. Vignola · M. Profita · M. Gjomarkaj
G. Bonsignore

Effects of gemcitabine on cell proliferation and apoptosis in non-small-cell lung cancer (NSCLC) cell lines

Received: 24 March 1999 / Accepted: 25 July 2000

Abstract We evaluated the antiproliferative and the proapoptotic ability of gemcitabine in three non-small-cell lung cancer (NSCLC) cell lines. NCI-H292 (mucoepidermoid carcinoma), NCI-CorL23 (large-cell carcinoma) and NCI-Colo699 (adenocarcinoma) cells were cultured with and without 0.5, 0.05 and 0.005 μ*M* gemcitabine for 24, 48 and 72 h, respectively. Gemcitabine exerted a stronger and earlier antiproliferative and proapoptotic effect on H292 cells than on CorL23 or Colo699 cells. Fas receptor expression was increased in all three cell lines and was higher in Colo699 than in CorL23 cells. The incubation of NSCLC with anti-Fas agonistic monoclonal antibody (CH11) induced cell apoptosis in H292 cells, demonstrating that the Fas receptor was functionally active. Finally, gemcitabine and CH-11 exerted a synergistic effect on cell apoptosis in H292 cells. This study demonstrates that gemcitabine induces apoptosis in NSCLC and that this effect might be exerted by modulating functionally active Fas expression, and these effects of gemcitabine were stronger in H292 cells than in either CorL23 or Colo699 cells.

Key words Gemcitabine · Non-small-cell lung cancer · NSCLC · Apoptosis

Introduction

Non-small-cell lung cancer (NSCLC) is notorious for its resistance to chemotherapeutic agents. Most chemotherapeutic drugs, both alone and in combination, are not particularly active against this type of cancer and are at best strictly palliative in the clinical setting [1].

E. Pace (✉) · M. Melis · L. Siena · F. Bucchieri
A. M. Vignola · M. Profita · M. Gjomarkaj · G. Bonsignore
Italian National Research Council,
Institute of Respiratory Pathophysiology,
Via Trabucco 180, 90146 Palermo, Italy
e-mail: pace@ifr.pa.cnr.it
Tel.: +39-091-6882879; Fax: +39-091-6882165

Gemcitabine is a new nucleoside analogue with potent antitumour activity both in vitro and in vivo [2, 3, 4]. Clinical studies have shown that this drug has promising therapeutic effects in the treatment of certain solid tumours including NSCLC. In particular, gemcitabine exerts its cytotoxic effect primarily through its incorporation into DNA during cellular replication leading to the inhibition of cell proliferation [5] as well as to the induction of apoptosis in several tumour cell lines such as myeloma cell lines [6] and leukaemic cell lines [7].

Apoptosis is a distinct mode of cell death responsible for deletion of cells in normal tissue; it also occurs in specific pathological contexts [8]. Morphologically, apoptosis involves rapid nuclear alterations characterized by chromatin condensation and nuclear fragmentation [9]. A characteristic biochemical feature of the process is double-strand cleavage of the nuclear DNA at the linkage regions between nucleosomes, leading to the production of oligonucleosomal fragments [10]. Apoptosis occurs spontaneously in tumours, often markedly retarding their growth. Irradiation [11], heat, hormone ablation [12], and cytotoxic chemotherapy [13] enhance tumour cell susceptibility to apoptosis. In this regard, a variety of anticancer drugs have been shown to produce tumour regression by inducing extensive apoptosis in malignant cells [9].

Although the cytotoxic effect of gemcitabine has already been demonstrated in several tumours including lung cancer [1, 14], there are no data on its potential effect on cell proliferation and apoptosis in lung cancer, or on its capacity to modulate biological and molecular mechanisms involved in the pathogenesis of programmed cell death in this disease. In particular, it is unclear whether gemcitabine can induce cell apoptosis by activating the Fas receptor/Fas ligand system, a key regulator of apoptosis [15]. Fas-mediated apoptosis can be triggered following engagement of the target cell Fas receptor by its specific cognate ligand (i.e. FasL) or by the prototypic agonist, anti-Fas monoclonal antibody (mAb) CH11 [16].

The aims of the present study were to evaluate the ability of gemcitabine to affect cell proliferation as well as to induce apoptosis in three histologically different NSCLC cell lines and to determine whether this drug might promote Fas expression.

Materials and methods

Cell lines

The effects of gemcitabine were assessed in three NSCLC cell lines: NCI-H292 (mucoepidermoid carcinoma), NCI-CorL23 (large-cell carcinoma), and NCI-Colo699 (adenocarcinoma) (Interlab Cell Line Collection, Genova, Italy). Cells were cultured as adherent monolayers and maintained in RPMI-1640 complete medium (CM) supplemented with heat-inactivated (56 °C, 30 min) 5% fetal bovine serum, 1% penicillin-streptomycin solution, and 1 m*M* glutamine (all from GIBCO, Grand Island, N.Y.).

Incubation of tumour cell lines with gemcitabine

Cultured cells were harvested with a rubber policeman, washed twice with phosphate buffer solution (PBS) (GIBCO), counted using a haemocytometer, and resuspended in CM at a concentration of 10^5 cells/ml. Cell suspensions were seeded in 5-ml aliquots into 25-cm^2 tissue culture flasks 1 day before treatment with gemcitabine (Eli Lilly Co., Indianapolis, Ind.). To select the effective gemcitabine concentrations, dose-response and time-response curves were generated from tests of drug concentrations ranging from 0.5 to 0.0005 μ*M*, and from incubations of cells at 37 °C in an atmosphere containing 5% CO_2 for 1, 4, 24, 48 and 72 h. Cell viability was assessed by trypan blue dye exclusion.

Human tumour clonogenic assay

The colony growth of cells exposed to gemcitabine was evaluated following a previously described method [17] with minor modifications. Briefly, the lower layer was prepared in 35-mm Petri dishes (Falcon, Becton Dickinson, Franklin Lakes, N.J.) using CM supplemented with heat-inactivated (56 °C, 30 min) 10% fetal bovine serum in 0.5% agarose. Exponentially growing tumour cells were exposed to gemcitabine, as described above and, after each incubation time, cells were harvested and seeded (5×10^4) on the upper layer with 0.3% agarose prepared with the same medium as the lower layer, and finally incubated for 21 days at 37 °C in an atmosphere containing 5% CO_2. Each experiment was conducted in triplicate. At the end of incubation, colonies were counted under an inverted phase-contrast microscope (Leitz, Wetzlar, Germany). Colonies were defined as cell aggregates with at least 40 cells. The true number of colonies was calculated as the number of aggregates on the positive control subtracted from the number of colonies on the experimental plates. The results are expressed as percentages of the control value.

Determination of apoptosis by flow cytometry

Apoptosis was determined using a FACStar Plus (Becton Dickinson, Mountain View, Calif.) analyser equipped with an argon ion laser (Innova 70 Coherent) with Consort 32 computer support. After incubation with gemcitabine, adherent as well as detached cells were collected and evaluated for phosphatidylserine (PS) expression using a commercial kit (Bender MedSystem, Vienna, Austria) in accordance with the manufacturer's instructions. Briefly, cells were harvested, washed with PBS, resuspended in prediluted binding buffer at a concentration of 5×10^5 cells/ml, and incubated for 15 min at room temperature with annexin V labelled with fluorescein isothiocyanate. Cells were then washed in PBS and resuspended in 1 ml diluted binding buffer (5×10^5 cells/ml). Finally, 1 μg/ml propidium iodide (PI) was added to the cell suspensions and the percentages of positive and negative cells were determined using a fluorescence-activated cell sorter.

Determination of apoptosis by cell morphology

The effect of gemcitabine on cell morphology was evaluated by fluorescent triple staining with Hoechst 33258, acridine orange, and PI. After incubation with gemcitabine, cells were harvested, washed in PBS, resuspended in PBS (5×10^5 cells/ml), and incubated with Hoechst 33258 (10 m*M*) for 20 min in the dark. Cells were than washed again in PBS and incubated with acridine orange (1.5 ng/ml) and PI (1.5 μg/ml) for 5 min in the dark. The cells were examined using a Leitz Orthoplan microscope with Plum-Opak illuminator (Wetzlar, Germany). Apoptosis was identified according to the presence of characteristic morphological changes: cell shrinkage, nuclear condensation, fragmentation of the nuclei, and rupture of the apoptotic cells into debris.

Determination of apoptosis by DNA fragmentation assays

DNA fragmentation into nucleosomal bands was detected by agarose gel electrophoresis, as described previously, with minor modifications [7]. Briefly, the cells were lysed with 0.5 ml hypotonic lysis buffer (10 m*M* Tris, 1 m*M* EDTA, 0.2% TRITON X-100), and 0.5 mg/ml proteinase K (Sigma-Aldrich, Steinheim, Germany) was then added to the tubes. Lysates were incubated for 1 h at 50 °C. At the end of incubation, 10-μl volumes of DNase-free RNase A (0.5 mg/ml) were added to the lysates and the tubes were incubated (37 °C) for an additional hour. The samples were then precipitated overnight (−20 °C) with one-tenth volume of 3 *M* sodium acetate and three volumes of propanol. Pellets were air-dried and dissolved in TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.4). Loading buffer (Sigma) (3 μl) was added to the tubes, and the samples were heated at 65 °C for 15 min. Electrophoresis was performed in 1.8% agarose for 2 h at 80 V. DNA laddering was visualized under UV light by staining the agarose gel with ethidium bromide.

Determination of Fas expression

The expression of Fas was determined by direct immunofluorescence using a FACStar Plus (Becton Dickinson) analyser equipped with an argon ion laser (Innova 70 Coherent) with Consort 32 computer support. Cells were harvested from culture flasks and washed in PBS. Viability was assessed by trypan blue dye exclusion, and cells were incubated with gemcitabine as indicated above. At the end of incubation, the cells were harvested, washed in PBS and resuspended in PBS (10×10^6/ml). Aliquots of cell suspension (0.1 ml) were incubated in the dark (30 min, 4 °C) with mouse antihuman CD95 mAb (clone DX2; Dakopatts, Glostrup, Denmark) conjugated with phycoerythrin (PE). An irrelevant antibody (PE-conjugated X-928; Dakopatts) was used as a negative control. Results are expressed as both the means and mean percentages in relation to baseline log fluorescence intensity.

Effects of the agonistic anti-Fas mAb in NSCLC cell lines

To demonstrate whether or not Fas receptor expression in NSCLC cell lines induced by gemcitabine was associated with an increased susceptibility of the cells to Fas-mediated cytotoxicity, H292, Colo699 and CorL23 cell lines were incubated in the presence and absence of 0.5 and 0.05 μ*M* gemcitabine (0.005 μ*M* gemcitabine was not included because Fas expression was not induced at this concentration) for 24, 48 and 72 h, respectively. At the end of incubation, an agonistic anti-Fas antibody (CH11,

IgM; Genzyme, Cinisello Balsamo, Milan, Italy) was added to the cultures (200 ng/ml), and cells were incubated for an additional 3 h. Cell viability was assessed by trypan blue dye exclusion and the percentage of annexin-positive cells was evaluated by flow cytometry.

Statistical analysis

Conventional fluorescent microscopic observations were performed by an examiner who was blinded to the treatment. Data are expressed as mean counts ± SE. The Mann Whitney *U*-test was used for unpaired comparisons. Analysis of variance (ANOVA) was used for comparisons among different experimental conditions. For the Mann Whitney *U*-test and ANOVA, *P*-values <0.05 were taken as indicating statistical significance.

Results

Inhibition of colony growth of cells exposed to gemcitabine

We first determined whether gemcitabine was able to affect the viability of the NSCLC cell lines using trypan blue dye exclusion. Interestingly, gemcitabine (0.5 μ*M*) significantly decreased the viability of the H292 cells after 48 and 72 h of incubation, while the viability of CorL23 cells was decreased only after 72 h. Surprisingly, the viability of Colo699 cells was not affected by gemcitabine under any of the experimental conditions (Table 1). We next evaluated the long-term cytotoxic effects of gemcitabine on the self-renewal capacity of the NSCLC cell lines using a clonogenic assay. Interestingly, gemcitabine (0.05 μ*M*) was able to abrogate and to significantly reduce colony formation in H292 and Colo699 cells, respectively, and it exerted this effect in a dose-dependent fashion (Fig. 1A,B). Surprisingly, gemcitabine at 0.05 μ*M* was able to significantly reduce tumoral colony formation in CorL2323 cells after 48 and 72 h of incubation only. Moreover, gemcitabine at 0.005 μ*M* induced a significant inhibition of colony growth at all time-points in H292 cells while it was able to exert this inhibitory effect in CorL23 cells only after 72 h of incubation (Fig. 1A,C). No effect was observed in Colo699 cells at this drug concentration (Fig. 1B).

Induction of apoptosis in NSCLC cell lines by gemcitabine: evaluation by flow cytometry, morphology and DNA fragmentation

To determine whether gemcitabine was able to trigger apoptosis in the NSCLC cell lines, we evaluated the presence of PS on the cell surface using FITC-conjugated annexin V. Interestingly, although the time required for the optimum effect in each incubation was different among the three cell lines, gemcitabine induced apoptosis in the cell lines both in a dose-dependent and in a time-dependent fashion (Fig. 2A,B,C). The apoptotic effect of gemcitabine in the H292 cells occurred after 24 h of incubation, reached a maximum at 48 h ($P < 0.002$, ANOVA), and was still clearly evident after 72 h ($P < 0.003$, ANOVA) (Fig. 2A). In contrast, gemcitabine did not induce any apoptosis at any of the concentrations tested after 24 h of incubation in either the Colo699 or the CorL23 cells, but significantly induced apoptosis after 72 h in the Colo699 cells, and after 48 and 72 h in the CorL23 cells (Fig. 2B,C). In addition, under all experimental conditions, the proapoptotic effect of gemcitabine was significantly stronger in the H292 cells than in the other two cell lines ($P < 0.01$, Mann Whitney *U*-test), and was significantly stronger in the CorL23 cells than in the Colo699 cells ($P < 0.012$, Mann Whitney *U*-test) (Fig. 2A,B,C).

We next determined whether the gemcitabine-triggered apoptosis in the NSCLC cell lines was associated with specific alterations in cell morphology using fluorescent triple staining. Figure 3 shows H292, Colo699 and CorL23 cells incubated in the absence or presence of gemcitabine (0.5 μ*M*, 72 h) and costained with PI and Hoechst 33258 or acridine orange. The bright blue fluorescence of Hoechst 33258 allowed the visualization of the typical nuclear alterations of apoptosis (nuclear fragmentation and condensation of chromatin) in all the NSCLC cell lines incubated with gemcitabine. The red fluorescence of PI, indicating membrane barrier dysfunction, was not detected in any cell displaying the above-mentioned nuclear alterations using the same microscopic field. In addition, the green fluorescence of acridine orange, indicating a well-conserved metabolic

Table 1 Effect of gemcitabine on the viability of NSCL cancer cell lines (data are expressed as the percentage of viable cells ± SD from three experiments)

Cell lines	Drug concentration (μ*M*)	Cell viability		
		24 h	48 h	72 h
H292	Baseline	93.3 ± 3.1	93.6 ± 1.2	91 ± 1
	0.5	90 ± 3	56.3 ± 3.3	19.5 ± 0.5
	0.05	90.3 ± 2.4	72.3 ± 2.04	21 ± 1
	0.005	91 ± 2.7	78 ± 9	94.5 ± 0.5
Colo699	Baseline	89 ± 1.1	93 ± 1.2	93.5 ± 0.9
	0.5	85 ± 0.8	90.5 ± 3.7	68.2 ± 10.8
	0.05	91.5 ± 1.5	86 ± 3.5	83 ± 1.7
	0.005	90.5 ± 2.1	81 ± 3.4	92.2 ± 0.3
CorL23	Baseline	94.6 ± 0.2	93.6 ± 0.8	93.8 ± 0.7
	0.5	92.8 ± 0.3	90.8 ± 2.9	57.3 ± 2.1
	0.05	95.8 ± 0.5	75.1 ± 3.6	74.3 ± 1.7
	0.005	92.1 ± 0.5	89.5 ± 2.8	93.1 ± 0.6

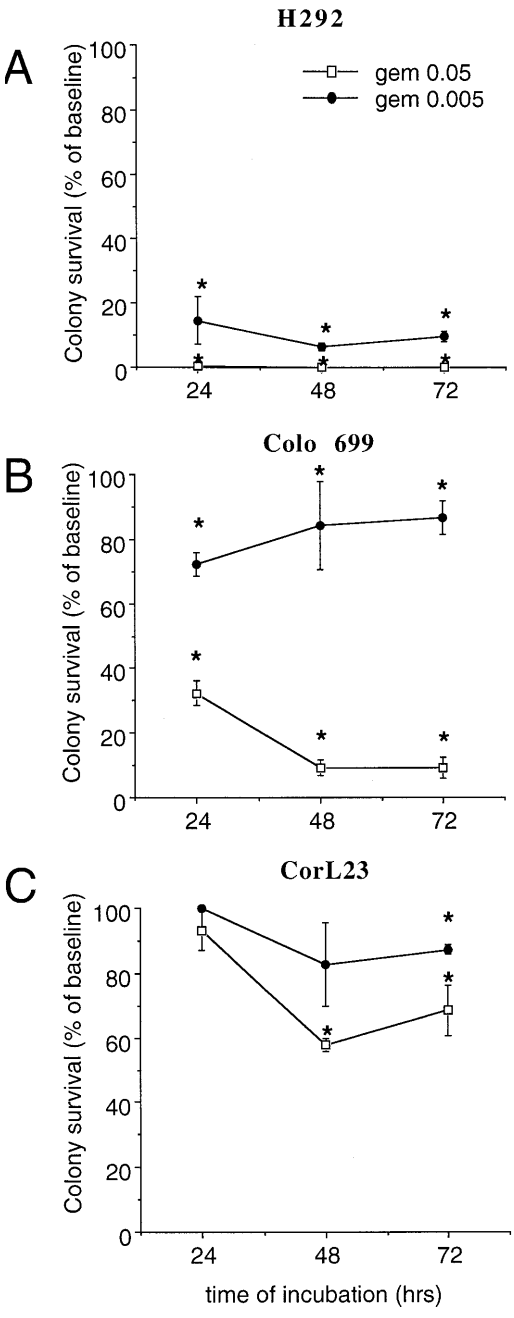

Fig. 1A–C Effects of gemcitabine on proliferation of H292 (**A**), Colo699 (**B**) and CorL23 (**C**) cells. Cells were incubated for 24, 48 and 72 h in the presence and in the absence of different concentrations of gemcitabine. Cell proliferation was evaluated in terms of colony formation by a clonogenic assay (see Materials and methods for details). The data are expressed as percent of baseline colony survival (means ± SD from three experiments). *$P < 0.0001$, ANOVA in comparison with baseline condition

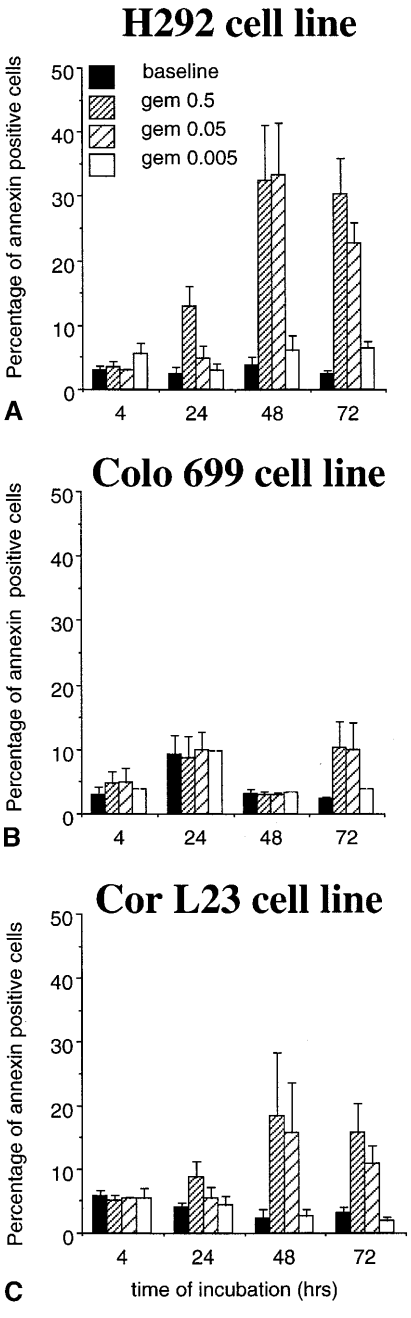

Fig. 2A–C Effects of gemcitabine on apoptosis in H292 (**A**), Colo699 (**B**) and CorL23 (**C**) cells. Cells were incubated for 4, 24, 48 and 72 h in the presence and in the absence of different concentrations of gemcitabine. Apoptosis was evaluated by cytometric analysis (see Materials and methods for details). The data are expressed as percent of annexin V-positive cells (means ± SD from four experiments)

activity, showed that the cells with the typical apoptotic nuclear alterations were viable.

Finally, we performed agarose gel electrophoresis of DNA to determine whether gemcitabine-induced apoptosis was also associated with the appearance of apoptosis-related DNA fragmentation (approximately 200 bp and multiples). We performed these experiments using H292 cells because gemcitabine exerted the strongest proapoptotic effect in this cell line. After gemcitabine exposure, we observed DNA damage (Fig. 4, lanes 3 and 6) but not typical DNA fragmentation probably due to the presence also of necrotic cells that obscured the evidence of DNA laddering.

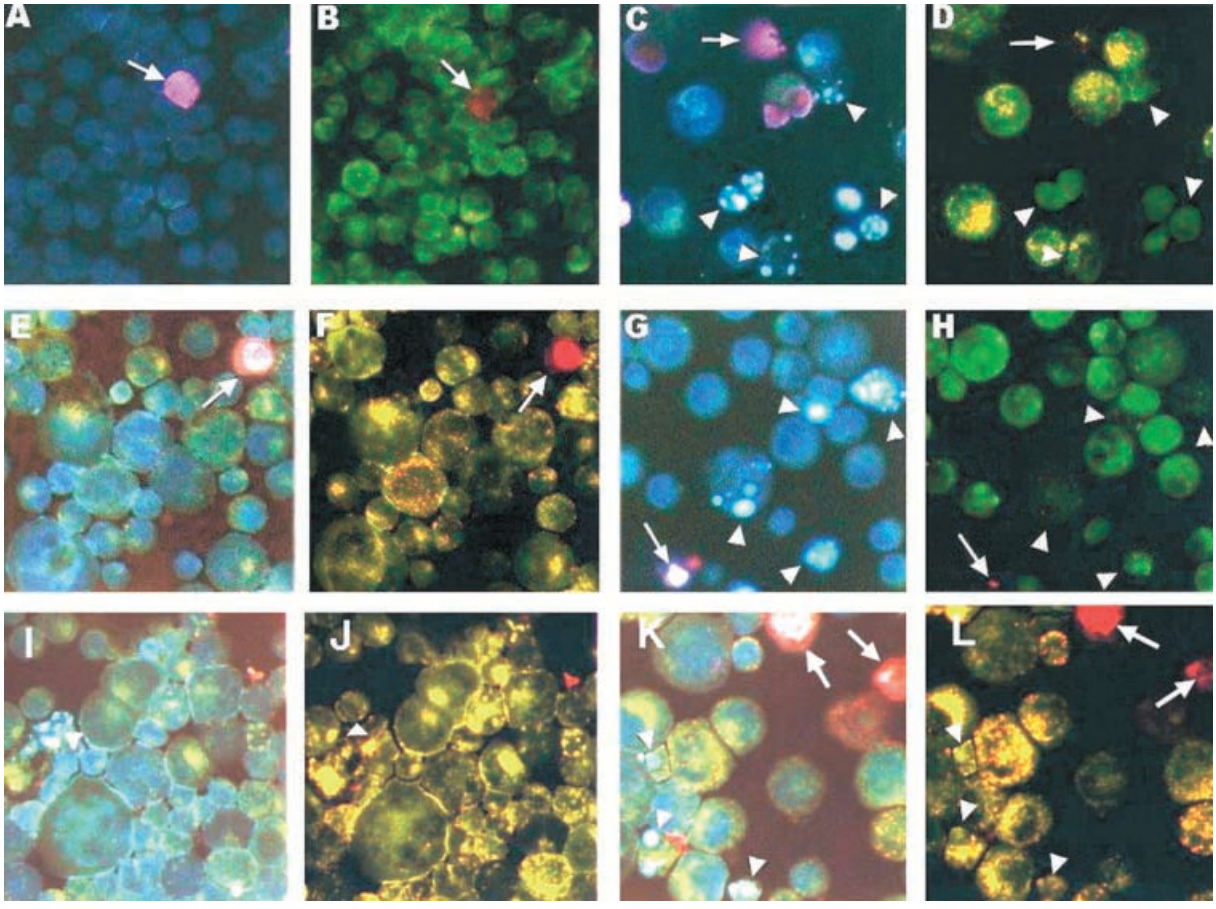

Fig. 3A–L Morphological alterations in H292, Colo699 and CorL23 cells incubated for 72 h in the presence and absence of gemcitabine (0.5 μ*M*). Cells were stained with PI and Hoechst or with PI and acridine orange (see Materials and methods for details). The pink-red fluorescence of PI identifies necrotic cells (white arrows). The bright blue fluorescence of Hoechst identifies typical nuclear alterations due to apoptosis. The green fluorescence of acridine orange identifies viable cells (Apoptotic cells are identified by white arrows). Cells cultured without gemcitabine: **A**,**B** H292; **E**,**F** CorL23; **I**,**J** Colo699. Cells cultured with gemcitabine: **C**,**D** H292; **G**,**H** CorL23; **K**,**L** Colo699. Final magnification ×400

Analysis of Fas expression in gemcitabine-treated NSCLC cell lines

We performed cytometric analysis using an anti-CD95 mAb to investigate the hypothesis that gemcitabine also exerts its effect by modulating Fas receptor expression in NSCLC cell lines. Interestingly, untreated mucoepidermoid H292 cells showed significantly higher spontaneous expression of the Fas molecule than either untreated Colo699 or untreated CorL23 cells ($P < 0.0001$, ANOVA) (Fig. 5A,B,C). Moreover, gemcitabine significantly increased the expression of Fas in H292 cells at all the incubation time points ($P < 0.0001$, ANOVA) (Fig. 5A) whereas the expression of this receptor was increased in the Colo699 and CorL23 cells after 48 and 72 h of incubation only (Fig. 5B,C). In addition, the maximum effect of gemcitabine in the H292 and Colo699 cells was observed after incubation with gemcitabine at 0.5 and 0.05 μ*M* ($P < 0.0001$, ANOVA). In the CorL23 cells, gemcitabine exerted its maximum effect only at 0.05 μ*M* ($P < 0.002$, ANOVA) (Fig. 5B,C).

Effects of the anti-Fas agonistic mAb on NSCLC cell lines

To determine whether Fas expression was associated with cell apoptosis, we first sought to determine whether there was a positive correlation between Fas expression and cell apoptosis in the NSCLC cell lines. Interestingly, a positive correlation was present after 48 and 72 h (Fig. 6) but not after 24 h (data not shown) of incubation under all the experimental conditions tested in H292 cells suggesting that, for this cell line, apoptosis increases when Fas expression increases. In contrast, no positive correlation was detected between Fas expression and cell apoptosis in Colo699 and for CorL23 cells at any time-point (data not shown). To further support the role played by Fas in cell apoptosis and to determine whether the Fas receptor present on the surface of the NSCLC cells was functionally active, we evaluated the cell apoptosis displayed by all three cell lines after incubation with the agonist mAb CH-11 (Fig. 7A,B,C). Interestingly, incubation with CH-11 induced a time-dependent increase in the percentage of apoptosis in

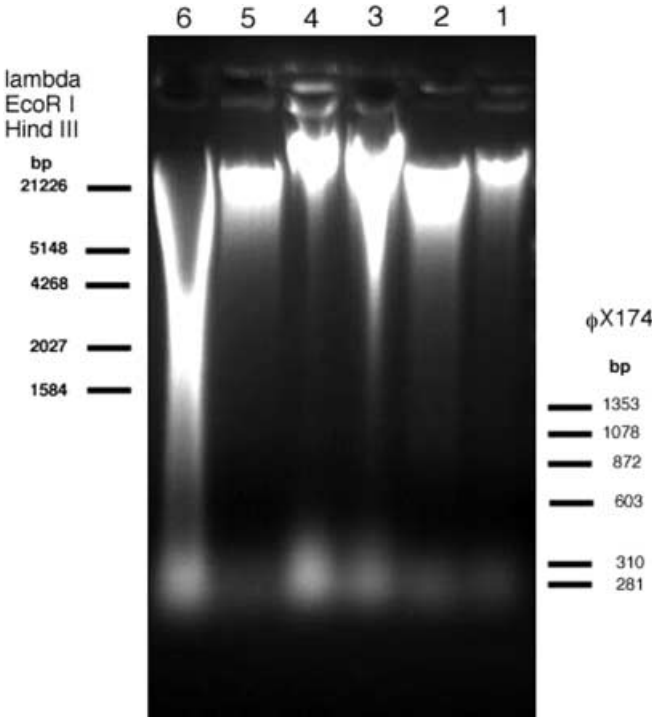

Fig. 4 Agarose gel electrophoresis of DNA extracted from H292 cells performed after incubation of cells with gemcitabine (0.5, 0.05 and 0.005 μ*M*) for 48 and 72 h (see Materials and methods for details). *Lane 1* DNA from H292 cells incubated for 48 h with gemcitabine 0.005 μ*M*; *lane 2* DNA isolated from H292 cells incubated for 48 h with gemcitabine 0.05 μ*M*; *lane 3* DNA isolated from H292 cells incubated for 48 h with gemcitabine 0.5 μ*M*; *lane 4* DNA isolated from H292 cells incubated for 72 h with gemcitabine 0.005 μ*M*; *lane 5* DNA isolated from H292 cells incubated for 72 h with gemcitabine 0.05 μ*M*; *lane 6* DNA isolated from H292 cells incubated for 72 h with gemcitabine 0.5 μ*M*

H292 cells which reached a maximum after 72 h (Fig. 7A). In contrast, incubation for any length of time with CH-11 did not affect apoptosis in Colo699 and CorL23 cells (Fig. 7B,C).

To establish whether incubation with gemcitabine affected the induction of cell apoptosis by CH-11, we compared the percentages of apoptotic cells in the three cell lines after incubation with CH-11 in the presence or absence of gemcitabine. Surprisingly, incubation with both CH-11 and gemcitabine dramatically increased apoptosis in H292 cells after 24 and 48 h compared to incubation with CH-11 or gemcitabine alone at both drug concentrations tested. No synergistic effect of gemcitabine and CH-11 on H292 cells was observed after 72 h of incubation (Fig. 7A). Incubation with gemcitabine and CH-11 did not exert a synergistic effect on cell apoptosis in CorL23 and Colo699 cells (Fig. 7B,C).

Finally, we determined whether activation of the Fas/Fas ligand system could affect colony formation in the NSCLC cells. Negligible effects on colony formation in the NSCLC cell lines were observed after incubation with CH11 alone or with both CH11 and gemcitabine (data not shown).

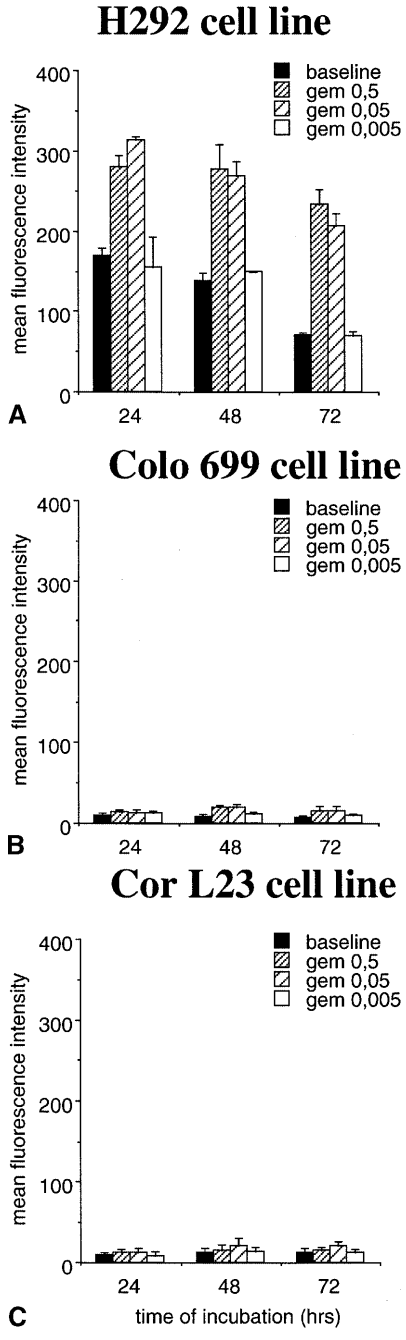

Fig. 5A–C Modulation of Fas receptor by gemcitabine in H292 (**A**), Colo699 (**B**) and CorL23 (**C**) cell lines. Cells were incubated for 24, 48 and 72 h in the presence and absence of different concentrations of gemcitabine. The expression of FAS receptor was evaluated by cytometric analysis (see Materials and methods for details). The data are expressed as mean fluorescent intensity (mean ± SD from three experiments)

Discussion

The ability of cytotoxic agents to limit tumour growth may be due to their capacity to inhibit cancer cell proliferation and/or to increase cancer cell death [5]. The major objectives of this study were to assess the

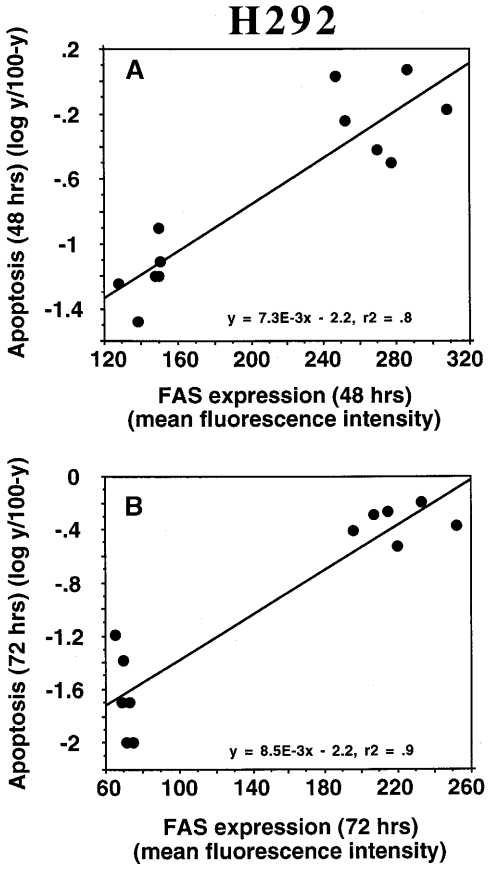

Fig. 6A,B Correlation between Fas receptor expression and apoptosis in H292 cells. Fas expression by H292 cells was evaluated after 24, 48 and 72 h in the presence and in the absence of gemcitabine at 0.5, 0.05 and 0.005 μM and apoptosis was evaluated under the same experimental conditions (see Materials and methods for details). Each point represents a single condition in the context of a single experiment ($n = 3$)

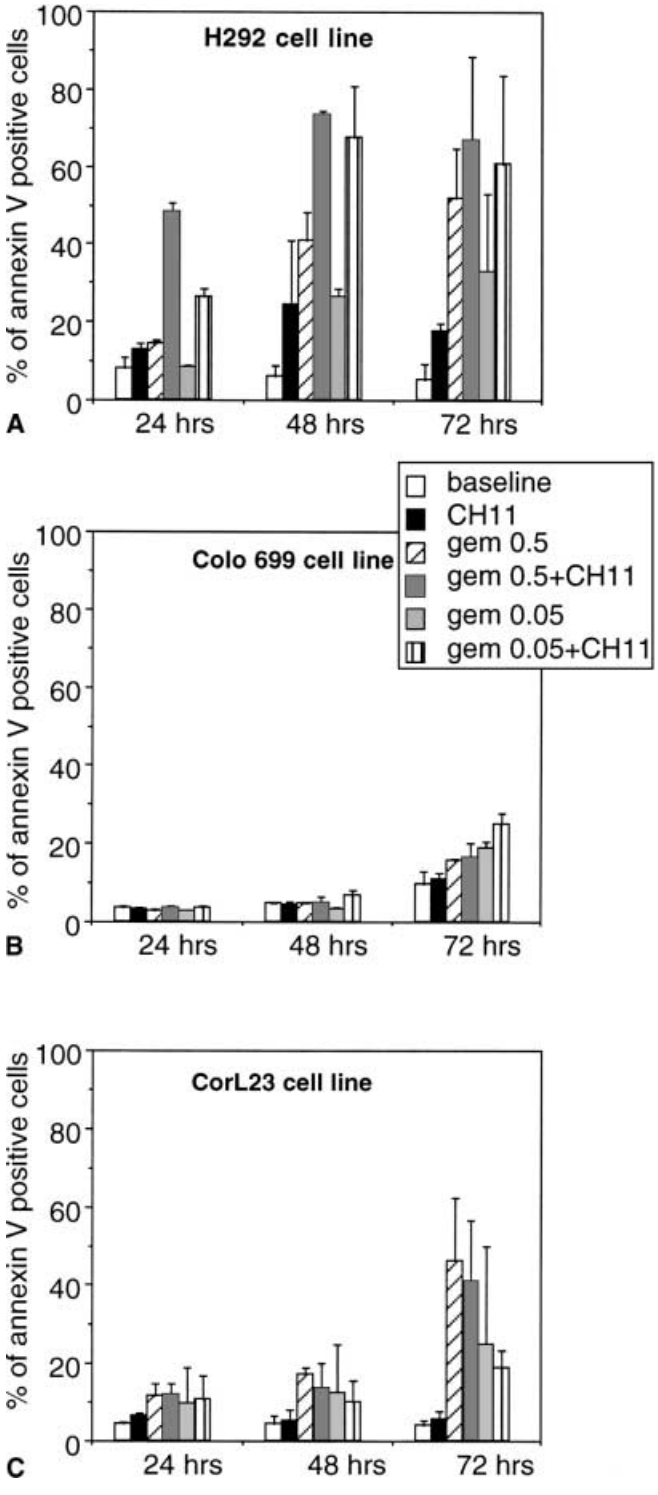

Fig. 7A–C Effects of CH-11 mAb on H292 (**A**), Colo 699 (**B**) and CorL23 (**C**) cell lines. Cells were incubated for 24, 48 and 72 h in the presence and absence of different concentrations of gemcitabine. Apoptosis was evaluated by cytometric analysis (see Materials and methods for details). The data are expressed as percent of annexin V-positive cells (means ± SD from three experiments)

possibility that gemcitabine, a promising anticancer drug in lung cancer, exerts its therapeutic effect by both inhibiting self-renewal capacity and triggering apoptosis in some NSCLC cell lines and to establish whether such a proapoptotic effect occurs in a direct or in an indirect fashion. Although several studies have shown the proapoptotic and the antiproliferative effects of gemcitabine in different tumours such as human ovarian cancer [18] and chronic myeloproliferative disorders [5], this is the first study in which this drug has been shown to exert such effects on lung cancer cells. This study showed that gemcitabine was able to inhibit colony growth as well as to induce apoptosis directly in NSCLC cell lines, and that these phenomena were stronger in H292 cells than in Colo699 or CorL23 cells. In addition, we demonstrated that gemcitabine may also promote cell apoptosis in an indirect fashion by increasing Fas expression in NSCLC cell lines and that this effect was stronger in H292 cells than in Colo699 or CorL23 cells.

In the present study, the antiproliferative and the proapoptotic effects of gemcitabine were evaluated by selecting NSCLC cell lines with different invasive behaviours. The mucoepidermoid lung carcinoma cell line (H292) is characterized by a low-grade malignancy, while the adenocarcinoma (Colo699) and the large-cell

carcinoma (CorL23) cell lines are characterized by high-grade malignancy [19]. The antiproliferative activity of gemcitabine was evaluated in the studied NSCLC cells by performing a clonogenic assay. We chose this method because gemcitabine, as do other deoxycytidine analogues, exerts delayed as well as short-term cytotoxic effects on cell viability which take longer than 72 h [5, 17]. The antiproliferative effect of gemcitabine was different in the three cell lines and, under all experimental conditions, this effect was significantly stronger in H292 cells than in cells of the other two cell lines. Moreover, gemcitabine inhibited tumoral colony growth in a dose- and time-dependent fashion, and this inhibition was stronger in Colo699 than in CorL23 cells.

With regard to the proapoptotic activity of gemcitabine, we chose to use several methods for evaluating cell apoptosis in vitro. It is widely accepted that there is no single method which, when used alone, provides irrefutable evidence in the evaluation of apoptosis. Even observations of morphological cell changes have several drawbacks [20]. Therefore, we used several techniques, including cytometry, evaluation of cell morphology and evaluation of DNA fragmentation, to detect apoptosis in the NSCLC cell lines. Cytometry and evaluation of cell morphology allowed us to differentiate between apoptotic cells, characterized by both the absence of membrane barrier dysfunction and the presence of an intact cellular metabolism, from necrotic cells. By combining the above-mentioned methodological approaches, we demonstrated the proapoptotic effects of gemcitabine in NSCLC cell lines.

The proapoptotic effect of gemcitabine in the mucoepidermoid H292 cell line was different from that in the Colo699 or CorL23 cell lines in terms of cell kinetics and number of cells undergoing apoptosis. In particular, although the proapoptotic effect of gemcitabine in the H292 cell line occurred after 24 h of incubation (reaching a maximum after 48 h and persisting after 72 h), a significant increase in apoptosis in the Colo699 and CorL23 metastatic cell lines was observed only after 72 and 48 h of incubation, respectively. Therefore, while gemcitabine was capable of exerting a direct apoptotic effect on all the tested NSCLC cell lines, this effect was more rapid and caused a significantly higher number of apoptotic cells in the less-invasive H292 cell line than in cell lines characterized by more aggressive and stronger metastatic potential.

We were also interested in studying the effect of gemcitabine on the expression of the Fas molecule. The transmembrane receptor Fas, together with its binding protein Fas ligand, play a critical role in cell apoptosis and in the regulation of tumour-host interactions. The expression of the Fas ligand on the surface of activated T lymphocytes is an important mechanism by which the host immune system may induce apoptosis in Fas-expressing target tumour cells [15]. Consequently, alteration of this pathway within tumour cells has been considered a tumour cell escape mechanism from apoptosis and immune surveillance. The loss of Fas expression or an alteration of its function has been described in cancer cells of several tissues and organs, including breast [21], colon [22], testis [23], liver [24, 25], and the haematopoietic compartment [26]. A recent study has also demonstrated the presence of reduced Fas protein expression in lung adenocarcinoma, and has shown that the protein is sequestered into the cytoplasmic portion of secondary lung adenocarcinoma cell lines [27]. This finding together with the evidence that tumour-infiltrating lymphocytes are less active in adenocarcinoma [28], indicate that lung adenocarcinoma may constitute a neoplastic microenvironment where the activation of tumour-infiltrating lymphocytes is less effective [28]. Accordingly, we showed that the constitutive expression of the Fas receptor was significantly higher in the mucoepidermoid (H292) lung cancer cell line than in the metastatic adenocarcinoma (Colo699) or large-cell carcinoma (CorL23) cell lines, supporting the concept that the loss of this molecule might be a mechanism of tumour progression. We also demonstrated that gemcitabine can modify Fas molecule expression of mucoepidermoid as well as of metastatic cell lines from adenocarcinoma and large-cell carcinoma. Since gemcitabine induced apoptosis as well as the expression of Fas molecules in all the tested cell lines, and since we demonstrated a positive correlation between apoptosis and Fas expression (Fig. 6), it is conceivable that one of the mechanisms by which gemcitabine controls tumour progression could be an increased sensitivity of tumour cells to Fas-dependent apoptosis. However, the spontaneous increase in Fas expression by H292 cells as well as the ability of gemcitabine to cause a more rapid Fas modulation on the surface of these cells, suggest that mucoepidermoid cells are more susceptible to Fas-dependent apoptosis than are adenocarcinoma cells.

To investigate this hypothesis, we determined whether the modulation of Fas by gemcitabine led to an increase in apoptosis in the presence of the anti-Fas agonistic mAb CH-11 which induces apoptosis in cell lines with a functionally active Fas [16]. Considering the inherently different Fas receptor expression among the three cell lines, we showed that the mucoepidermoid H292 cell line, when incubated with CH-11, undergoes a faster and greater apoptosis than that observed in both adenocarcinoma (Colo699) and large-cell carcinoma (CorL23) cell lines. Interestingly, although both the Colo699 and the CorL23 cell lines exhibited a similar constitutive Fas receptor expression, incubation with CH-11 induced apoptosis in the CorL23 cell line only, suggesting a loss of Fas function only in the Colo699 cell line. In addition, while coincubation of the H292 and CorL23 cell lines with CH-11 and gemcitabine caused a significant increase in the percentage of apoptotic cells compared to the effects of CH-11 alone, gemcitabine (0.05 μM) promoted a weak increase in the percentage of apoptotic Colo699 cells after 72 h of incubation.

These findings again highlight the possibility of different biological behaviours among the three cell lines in terms of response to gemcitabine treatment. There are

several possible explanations for the different patterns of response. The transition of a noninvasive tumour cell population into a more invasive population is indeed a hallmark of tumour progression [29], giving rise to a variety of phenotypes with distinct clinical and biological behaviours. Consequently, metastatic cells are subjected to selection pressures, and frequently have a higher predisposition to nonrandom allelic loss and chromosomal mutations [30, 31, 32]. These changes cause progressive genetic rearrangements which, allowing protection from external factors (including drugs) or intrinsic mechanisms of uncontrolled proliferation, are associated with more advanced and aggressive disease. Accordingly, it has been previously demonstrated that among different human lung cancer cell lines, only H292 is wild-type for both p53 and ras, suggesting that H292 cells, a low-invasive tumour cell population arising from a human carcinoma with a better prognosis, has fewer genetic alterations than the markedly aggressive adenocarcinoma and large-cell lung cancer cell lines. Thus, the cooperation of p53 and ras represents an important mechanism by which cell growth and neoplastic transformation is controlled [33]. It is conceivable that the H292 cell line is more susceptible to the effects of gemcitabine than the markedly aggressive adenocarcinoma and large-cell lung cancer cell lines because of the well-conserved integrity of the DNA.

In conclusion, the ability of gemcitabine to reduce tumour growth by inhibiting cell proliferation and inducing apoptosis, together with its ability to induce the expression of functionally active Fas molecules in some of the lines studied, highlight the therapeutic and the putative immunostimulatory effects of this drug and support its usefulness in the management of NSCLC.

Acknowledgements This work was supported by the Italian National Research Council and by Eli Lilly Co., Italy

References

1. Carney DN (1995) Management (chemotherapy/best supportive care) of advanced-stage non-small cell lung cancer. Semin Oncol 22: 58–62
2. Bergman AM, Ruiz-van-Haperen VW, Veerman G, Kuiper CM, Peters GJ (1994) Synergistic interaction between cisplatin and gemcitabine in ovarian and colon cancer cell lines. Adv Exp Med Biol 370: 139–143
3. Huang P, Plunkett W (1995) Induction of apoptosis by gemcitabine. Semin Oncol 22: 19–25
4. Huang P, Plunkett W (1995) Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother Pharmacol 36: 181–188
5. Santini V, Bernabei A, Gozzini A, et al (1997) Apoptotic and antiproliferative effects of gemcitabine and gemcitabine plus Ara-C on blast cells from patients with blast crisis chronic myeloproliferative disorders. Haematologica 82: 11–15
6. Gruber J, Geisen F, Sgonc R, et al (1996) 2′,2′-Difluorodeoxycytidine (gemcitabine) induces apoptosis in myeloma cell lines resistant to steroids and 2-chlorodeoxyadenosine (2-CdA). Stem Cells 14: 351–362
7. Bouffard DY, Momparler RL (1995) Comparison of the induction of apoptosis in human leukemic cell lines by 2′,2′-difluorodeoxycytidine (gemcitabine) and cytosine arabinoside. Leuk Res 19: 849–856
8. Arends MJ, Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 32: 223–254
9. Kerr J, Winterford C, Harmon B (1993) Apoptosis. Its significance in cancer and cancer therapy. Cancer 73: 2013–2026
10. McConkey DJ, Aguilar-Santelises M, Hartzell P, et al (1991) Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. J Immunol 146: 1072–1076
11. Stephens LC, Ang KK, Schultheiss TE, Milas L, Meyn RE (1991) Apoptosis in irradiated murine tumors. Radiat Res 127: 308–316
12. Redding TW, Schally AV, Radulovic S, Milovanovic S, Szepeshazi K, Isaacs JT (1992) Sustained release formulations of luteinizing hormone-releasing hormone antagonist SB-75 inhibit proliferation and enhance apoptotic cell death of human prostate carcinoma (PC-82) in male nude mice. Cancer Res 52: 2538–2544
13. Johnston JB, Lee K, Verburg L, et al (1992) Induction of apoptosis in $CD4^+$ prolymphocytic leukemia by deoxyadenosine and 2′-deoxycoformycin. Leuk Res 16: 781–788
14. Miller VA, Rigas JR, Grant SC, Pisters KM, Kris MG (1995) New chemotherapeutic agents for non-small cell lung cancer. Chest 107: S306–S311
15. Krammer PH, Dhein J, Walczak H, et al (1994) The role of APO-1-mediated apoptosis in the immune system. Immunol Rev 142: 175–191
16. Yonehara S, Ishii A, Yonehara M (1989) A cell-killing monoclonal antibody (anti-FAS) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 169: 1747–1756
17. Shameem IA, Kurisu H, Matsuyama H, Shimabukuro T, Naito K (1994) Direct and indirect effects of recombinant human granulocyte-colony stimulating factor on in vitro colony formation of human bladder cancer cells. Cancer Immunol Immunother 38: 353–357
18. Cartee L, Kucera GL (1998) Gemcitabine induces programmed cell death and activates protein kinase C in BG-1 human ovarian cancer cells. Cancer Chemother Pharmacol 41: 403–412
19. Mackay B, Lukeman JM, Ordonez NG (1991) Tumors of the lung. In: Mitchell J (ed) Major problems in pathology, vol 24. W.B. Saunders Company, Philadelphia, pp 285–322
20. Sgonc R, Wick G (1994) Methods for the detection of apoptosis. Int Arch Allergy Immunol 105: 327–332
21. Keane MM, Ettenberg SA, Lowrey GA, Russell EK, Lipkowitz S (1996) Fas expression and function in normal and malignant breast cell lines. Cancer Res 56: 4791–4798
22. Moller P, Koretz K, Leithauser F, et al (1994) Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer 57: 371–377
23. Lebel M, Bertrand R, Mes-Masson AM (1996) Decreased Fas antigen receptor expression in testicular tumor cell lines derived from polyomavirus large T-antigen transgenic mice. Oncogene 12: 1127–1135
24. Natoli G, Ianni A, Costanzo A, et al (1995) Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene 11: 1157–1164
25. Ni R, Tomita Y, Matsuda K, et al (1994) Fas-mediated apoptosis in primary cultured mouse hepatocytes. Exp Cell Res 215: 332–337
26. Robertson MJ, Manley TJ, Pichert G, et al (1995) Functional consequences of APO-1/Fas (CD95) antigen expression by normal and neoplastic hematopoietic cells. Leuk Lymphoma 17: 51–61
27. Nambu Y, Hughes SJ, Rehemtulla A, Hamstra D, Orringer MB, Beer DG (1998) Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinomas. J Clin Invest 101: 1102–1110

28. Vitolo D, Palmieri MB, Ruco E, Rendina E, Bonsignore G, Baroni CD (1994) Cytokine production and expression of adhesion molecules and integrins in tumor infiltrating lymphomononuclear cells of non-small cell carcinomas of the lung. Am J Pathol 145: 322–329
29. Nowell PC (1982) Genetic instability in cancer cells: relationship to tumor cell heterogeneity. In: Owens AH, Coffey DS, Baylin SB (eds) Tumor cell heterogeneity. Academic Press, New York, pp 351–365
30. Fong KM, Kida Y, Zimmerman PV, Ikenaga M, Smith PJ (1995) Loss of heterozygosity frequently affects chromosome 17q in non-small cell lung cancer. Cancer Res 55: 4268–4272
31. Fong KM, Zimmerman PV, Smith PJ (1995) Microsatellite instability and other molecular abnormalities in non-small cell lung cancer. Cancer Res 55: 28–30
32. Shiseki M, Kohno T, Nishikawa R, Sameshima Y, Mizoguchi H, Yokota J (1994) Frequent allelic losses on chromosomes 2q, 18q, and 22q in advanced non-small cell lung carcinoma. Cancer Res 54: 5643–5648
33. Lehman TA, Bennett WP, Metcalf RA, et al (1991) p53 mutations, ras mutations, and p53-heat shock 70 protein complexes in human lung carcinoma cell lines. Cancer Res 51: 4090–4096