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Preclinical report

A hollow fiber model for *in vitro* studies of cytotoxic compounds: activity of the cyanoguanidine CHS 828

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The hollow fiber assay is currently used as an *in vivo* model for anticancer drug screening in nude mice, but it can also be used as an *in vitro* model. In the current study, an *in vitro* hollow fiber model was used to study the effect and mode of induced cell death of a new cyanoguanidine, CHS 828. Human leukemia, adenocarcinoma and lymphoma cell lines as well as primary cultures of human tumor cells from patients with chronic lymphocytic leukemia (CLL) and ovarian cancer (OC) and normal human lymphocytes were cultured in semipermeable hollow fibers. The fibers were incubated for 3 or 14 days prior to CHS 828 exposure for 72 h, followed by determination of living cell density by MTT staining. For cell morphology, using harvested cultures on cytospin slides had technical advantages compared to using paraffin sections of the formalin-fixed fibers. CHS 828 showed higher antitumor activity on CLL and normal human lymphocyte cultures compared to OC cultures, and cell lines cultured 3 days were more sensitive than those cultured 14 days. Morphological examination of CHS 828-treated cultures revealed a mixture of apoptosis and necrosis. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, CHS 828, hollow fiber, *in vitro*, proliferation.

Introduction

Many of the *in vitro* models used in preclinical anticancer drug development are based on monolayer or suspension cultures of human tumor cell lines. These models are technically simple and useful in selecting potentially active compounds for further study. However, they do not mimic the complex microenvironment, heterogeneity and proliferative properties of solid tumors, and this may contribute to incorrect predictions of *in vivo* efficacy.[1] Antineoplastic drugs can be effective in solid tumors only if they can penetrate several cell layers and retain their activity in the tumor microenvironment.[2] To address this, three-dimensional *in vitro* solid tumor models have been developed such as collagen gel–support cultures,[3] multicellular tumor spheroids[4] and the *in vitro* hollow fiber models.[5] The hollow fiber model was developed by Hollingshead[6] for drug screening *in vivo*. The assay is based on implanting tumor cells cultured in polyvinylidine fluoride hollow fibers into athymic mice and using a modified MTT ([3-4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St Louis, MO) assay *in vitro* for the living cell density assessment. The hollow fiber model could potentially also be used to study angiogenesis.[7]

The use of primary human tumor cells (PHTC) from patients and normal human lymphocytes in drug development is less common than the use of established cell lines, although they may mimic the clinical situation better to some extent. PHTC from different tumor types might provide information on diagnosis-specific activity of new drugs.[8]

CHS 828 is a novel drug that chemically belongs to the cyanoguanidines. Its mechanism of action is still unclear, but it shows promising activity in many preclinical *in vitro* and *in vivo* systems,[9] and is now in phase II clinical trials.

In the present study we applied an *in vitro* hollow fiber model for the assessment of growth and drug sensitivity of different tumor cells. More specifically, the sensitivity to CHS 828 of 3-day-old was compared with that of 14-day-old cultures of human tumor cell lines. The CHS 828 sensitivity of hollow fiber cultures

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of primary tumor cells from patients with chronic lymphocytic leukemia (CLL) and ovarian cancer (OC), and of normal human lymphocytes was also investigated. The feasibility of examining tumor cell morphology was also assessed.

Materials and methods

Cell lines

The cell lines used were the leukemia cell lines CCRF-CEM, a kind gift from WT Beck (Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, TN), the renal adenocarcinoma cells line ACHN, obtained from ATCC (Rockville, MD), and the histocytic lymphoma cell lines U937 GTB, kindly provided by Professor K Nilsson (Department of Genetics and Pathology, Uppsala, Sweden). The cells were grown in culture medium RPMI 1640 (HyClone, Northumbria, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin. Growth and morphology were monitored weekly.

PHTC

Tumor cell samples were obtained from five patients with CLL and five patients with OC. Four patient samples with OC were used for drug concentration–response experiments and one sample was used for assessment of the growth of the tumor cells inside the hollow fiber. The patient samples were obtained from routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. Leukemic cells were isolated from the blood by density-gradient centrifugation on 1.077 g/ml Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation.[10] Tumor tissue from solid samples was minced into small pieces and tumor cells were then isolated by collagenase dispersion followed by Percoll (Pharmacia Biotech) density-gradient centrifugation.[11] OC cells from ascites fluid were collected by centrifugation, followed by purification of the cells by Ficoll-Paque and Percoll density-gradient centrifugation. Normal mononuclear cells from three healthy donors were collected and prepared in the same way as leukemic samples. All patients were previously treated with standard cytotoxic drugs except two OC patients. Cell viability of the tumor cells was determined with the Trypan blue dye exclusion test and the proportion of tumor cells had to be more than 70%, judged by microscopic examination of May–Grünwald–Giemsa (MGG)-stained cytospin preparations by a trained cytopathologist. Cells were cryopreserved in FCS containing 10% dimethylsulfoxide (DMSO; Sigma) by initial freezing for 24 h at -70°C followed by storage in liquid nitrogen, which does not appear to affect drug sensitivity.[12] On the fiber filling day, the frozen cells were rapidly thawed, washed twice with supplemented RPMI 1640 culture medium and suspended in it.

Hollow fiber cultures

The hollow fiber procedure was modified from that of Hollingshead.[6] Polyvinylidine fluoride (PVDF) hollow fibers (500 kDa molecular weight cut-off, 1 mm inner diameter; Spectrum, Laguna Hills, CA) were flushed with and incubated in 70% ethanol for 72 h and then autoclaved in deionized water. On the filling day, the fibers were washed with supplemented medium, flushed with air, and filled with cell suspension using a 0.9 mm needle and 1 ml syringe. The inoculation densities were 1×10^6 cells/ml for cell lines, 30×10^6 cells/ml for CLL cells and normal lymphocytes, and 10×10^6 cells/ml for OC cells. The fibers were sealed with a hot smooth-jawed needle holder at the two ends and at intervals of 2 cm, and were then incubated in Petri dishes with supplemented medium at 37°C in an incubator in a humidified atmosphere containing 95% air and 5% CO_2.

Linearity and growth assay

To test the linearity of the MTT assay, fibers were filled with each of the three cell lines at densities of 2, 4, 8, 16, 32 and 64×10^6 cells/ml. Triplicate fibers from each cell line at all densities were stained with MTT immediately.

For purposes of identifying the growth potential of a cell line inside the hollow fiber, tumor cells were seeded at a density of 1×10^6/ml and were incubated as above. After 3, 6, 14 and 17 day's incubation, three fibers from each cell line were MTT stained.

Drug concentration–response experiments

CHS 828 was provided by Leo Pharmaceutical Products (Ballerup, Denmark) dissolved in DMSO and was tested at five concentrations obtained by 10-fold serial dilution in culture medium, using 1 μM as a maximum concentration. Epirubicin, paclitaxel, cisplatin, topotecan, cytarabine and vincristine were obtained from the hospital pharmacy, and were dissolved according to the manufacturer's instructions and tested at three concentrations obtained by 5-fold serial dilution. CCRF-CEM and ACHN cell lines were

cultured inside the fibers for 3 or 14 days prior to 72 h drug exposure in six-well plates containing 5 ml drug solution. PHTC from individual patients with CLL, OC and normal mononuclear cells from healthy donors were cultured inside the fibers for 3 days prior to 72 h CHS 828 exposure.

Assessment of living cell density

The living cell density was determined for triplicate fibers and evaluated by staining with MTT, which is converted by metabolically active cells to insoluble blue formazan crystals. The fibers were incubated in six-well plates with 3 ml of supplemented medium and 200 μl MTT [stock 5 mg/ml in phosphate-buffered saline (PBS), kept frozen at −20°C and protected from light] at 37°C for 4 h. The staining medium was then replaced by PBS with 2.5% of protamin sulfate stock solution (1%; Sigma) and the plates were incubated at 4°C overnight. The fibers were cut into two pieces and put in 24-well plates to dry until the end of the experiment. The formazan was extracted with 250 μl DMSO for 4 h at room temperature. Then 150 μl of the extract was transferred to a flat-bottomed 96-well plate for reading the absorbance at 570 nm in a plate reader (Dynatech, UK). Blank values for DMSO was subtracted from each reading. Dilutions of all wells with DMSO (1+1) were made when any fiber analysis resulted in an absorbance too high to be measured

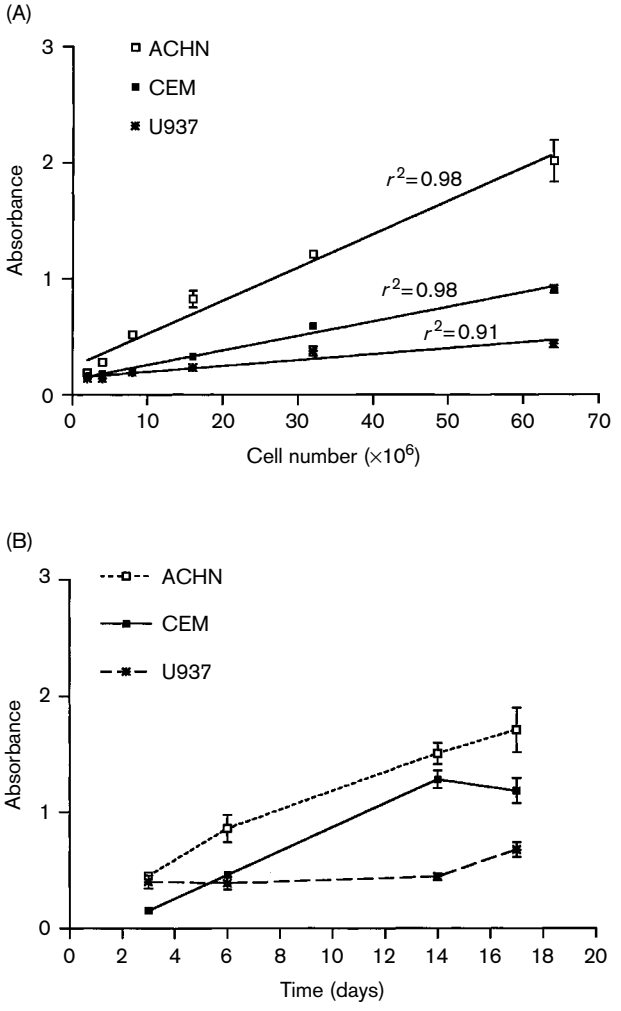

Figure 1. (A) Relationship between cell density and MTT absorbance for the cell lines CCRF-CEM, U937 GTB and ACHN in hollow fiber cultures. The results are presented as mean value of triplicate fibers ± SEM from one experiment. (B) The cell growth of CCRF-CEM, U937 GTB and ACHN inside hollow fibers as measured by MTT staining at different time points. Results are presented as mean ± SEM of three independent experiments.

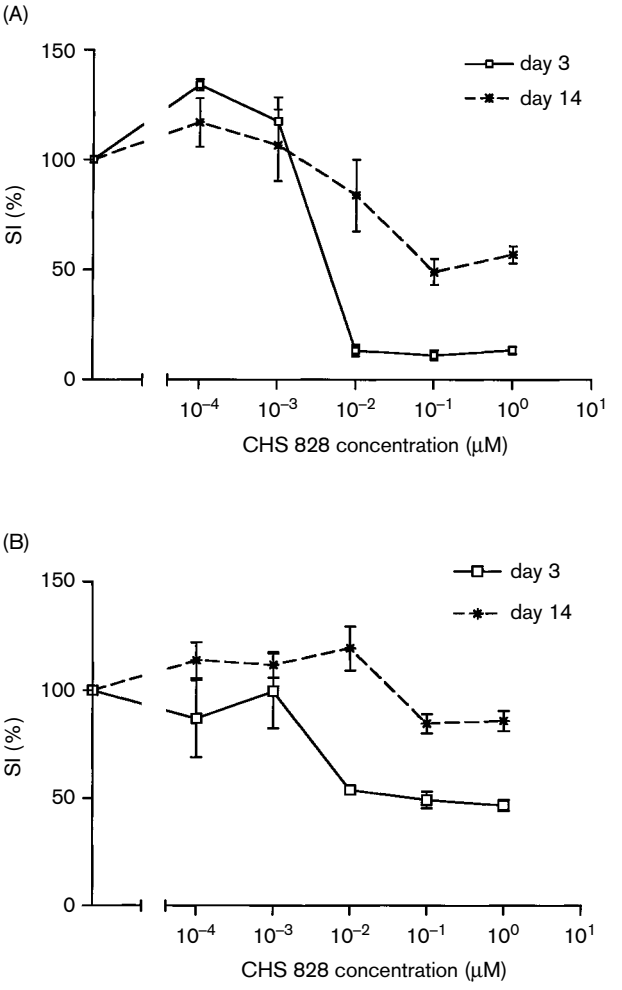

Figure 2. Concentration–response curves generated by CHS 828 on 3- and 14-day fiber cultures of CCRF-CEM (A) and ACHN (B) cells. The results are expressed as SI (cell survival in percent of control) and presented as mean ± SEM of three independent experiments.

(above 2.5). The cell densities in the treated fibers were expressed as survival index (SI%) defined as the absorbance of the treated fibers in percent of control fibers.

Morphology assessment

U937 GTB and ACHN cell lines were cultured in hollow fibers up to 28 days (seeding density 2×10^6 cells/ml), and triplicate fibers were investigated on day 3, 5, 14 and 28. One fiber was fixed in 10% buffered formalin, embedded in paraffin and sectioned at 5 μm intervals, and the sections were then stained in hematoxylin & eosin. The second fiber was used for cell harvest from inside the fiber by incubating the fiber with 2 ml trypsin for 5 min and then the sealed ends were cut off. The tumor cells were extruded in a flat-bottomed 96-well plate by flushing the fibers with 100 μl trypsin followed by 200 μl culture medium. This suspension was used to prepare MGG-stained cytospin slides. The third fiber as well as the empty fibers remaining after the cell harvest was also stained with MTT. Tumor cells from one patient with ovarian cancer were seeded at 10×10^6 cells/ml in hollow fibers which were incubated for 3, 14, 17 or 21 days. Fibers were fixed and sectioned as described above, and the tumor cell growth inside the fiber was assessed.

Three-day hollow fiber cultures of U937 GTB and ACHN were exposed to 1 μM CHS 828 for 24, 48 and 72 h or to 15 μg/ml etoposide for 4 h. Cells were harvested as described above and the cytospin slides were either MGG stained or TUNEL stained.

TUNEL assay

The TUNEL assay is used for detection and quantification of apoptosis (programmed cell death) at the single-cell level, and is based on labeling of DNA strand breaks. The assay was performed using the In Situ Cell Death Detection Kit, fluorescein (Boehringer Mannheim, Mannheim, Germany) on cytospin slides. The cytospin slides were mounted with Vectashield® antifade mounting solution (Vector, Burlingame, CA) and covered with a coverslip immediately after staining. The preparations were analyzed using a Leica microscope DMLB equipped for fluorescence with microphotoequipment MPS 60. The slides were examined using a $\times 40$ lens excitation at 480/40 nm with a bandpass emission filter of 527/30 nm. Cells were defined as apoptotic, TUNEL-positive, if their nuclei were fragmented with bright fluorescence. Apoptosis was judged morphologically by existence of intact cytoplasmic membrane and fragmented nuclei[13] in MGG-stained cells.

Statistical calculation

SI values at different drug concentrations for the various tumor cells tested were compared using an unpaired Student's *t*-test.

Results

Linear relationships were found between the absorbance signals and tumor cell numbers when the cells

Table 1. Activity of CHS 828 and other cytotoxic drugs on 3- and 14-day-old hollow fiber cultures of CCRF-CEM and ACHN

Drug	3-day culture SI (%)	14-day culture SI (%)	*p* value
CCRF-CEM			
CHS 828 (0.1 μM)	11	48	<0.01
cisplatin (2.5 μg/ml)	26	55	NS
epirubicin (2.5 μg/ml	39	68	$p<0.05$
paclitaxel (2.5 μg/ml)	12	60	$p<0.001$
topotecan (12.5 μg/ml)	17	32	NS
cytarabine (12.5 μg/ml)	22	48	NS
vincristine (12.5 μg/ml)	25	15	NS
ACHN			
CHS 828 (0.1 μM)	49	84	$p<0.01$
cisplatin (2.5 μg/ml)	30	61	$p<0.05$
epirubicin (2.5 μg/ml)	42	81	NS
paclitaxel (2.5 μg/ml)	45	79	NS
topotecan (12.5 μg/ml)	33	67	NS
cytarabine (12.5 μg/ml)	49	99	NS
vincristine (12.5 μg/ml)	69	58	NS

NS, not significant.
Activity expressed as surviving fraction (SI;%) after 72-h drug exposure with the indicated concentrations. Differences between drug activity in 3- and 14-day-old fiber cultures tested with Student's *t*-test, *p* values indicated.

were seeded at different densities (Figure 1A) The solid tumor cell line ACHN showed a higher absorbance signal per cell than the hematological CCRF-CEM and U937 GTB. This tendency was also evident when these tumor cells were cultured inside the hollow fiber for 2 weeks, although all three cell types had the same initial seeding densities (Figure 1B). There was gradual increase in the signals generated by the viable cells throughout the observation period under the growth condition evaluated.

The concentration response curves for CHS 828 in 3- and 14-day cultures of CCRF-CEM and ACHN cell lines are shown in Figure 2. A concentration-dependent decrease in SI followed by a plateau was observed for both cell lines. There was a tendency for the 3-day, high-proliferating cultures to be more sensitive than the 14-day low-proliferating cultures ($p < 0.05$ at 0.1 μM). This was observed also for the standard cytotoxic drugs studied (Table 1).

Figure 3 shows the activity of different CHS 828 concentrations on tumor cells from individual patients with CLL and OC. A concentration-dependent decrease in the SI followed by a plateau could be seen. The insets show the cell growth during the experimental period of the individual samples, as measured by the MTT assay. This pattern of CHS 828 effect was also noticed for the normal human lymphocytes (data not shown). The OC cells appeared to be less sensitive than the CLL and normal human lymphocytes.

Figure 4 shows longitudinal cross-sections and harvested cells of 14-day-old hollow fiber cultures of U937 GTB (Figure 4A and B) and ACHN (Figure 4C and D). U937 GTB grew mainly as single cells filling most of the fiber lumen (Figure 4A), while ACHN cells grew in clumps or aggregates near the fiber wall (Figure 4C). The harvested cells (Figure 4B and D) showed a microscopic picture similar to the cells originally seeded in the fiber, they appeared viable with their

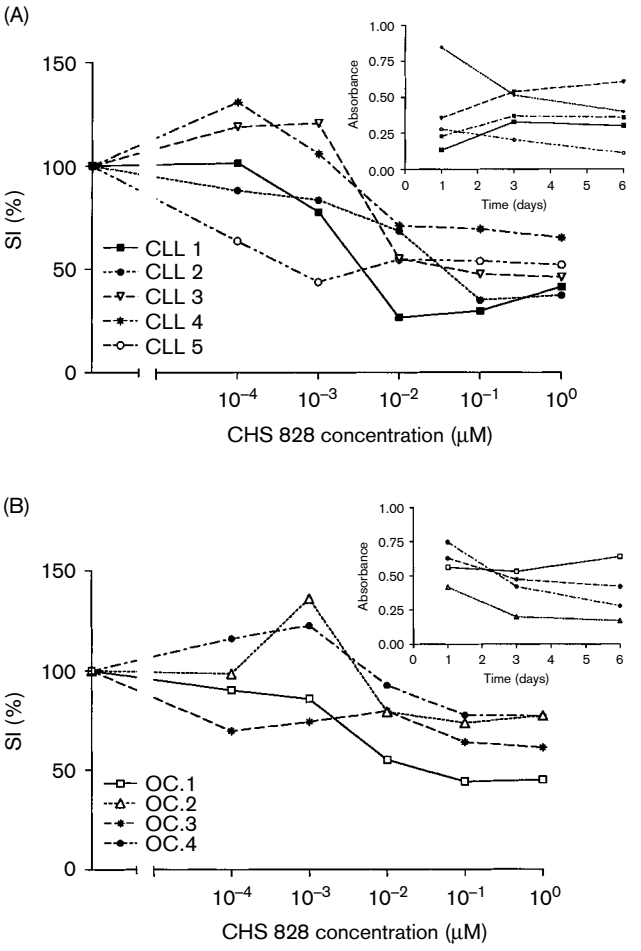

Figure 3. Concentration–response curves of CHS 828 on hollow fiber cultures of tumor cells from five individual patients with CLL (A) and four patients with OC (B). The results are expressed as mean SI (cell survival in percent of control) of triplicate fibers from one experiment. The growth curves of the individual samples are shown as insets.

nuclear and cytoplasmic morphology preserved, throughout the 28-day study period (not shown).

Figure 5 shows the successful harvest of U937 GTB and ACHN cells from the hollow fiber 14 days after seeding. The MTT signals of the emptied fibers were very low compared to the signals from the filled fibers. It was possible to harvest the tumor cells from the hollow fiber at any time point throughout the study period (data not shown).

Figure 6 shows longitudinal cross-sections of hollow fibers with malignant OC from one sample after 3 (Figure 6A), 14 (Figure 6B), 17 (Figure 6C and E) or 21 (Figure 6D and F) days incubation. Figure 6 demonstrates that the cells are growing along the fiber walls up to 14 days (Figure 6B). Later on, the cells show a tendency to coalesce forming bridges and solid groups towards the lumen of the fiber (Figure 6C and D).

When staining with TUNEL (Figure 7), unexposed hollow fiber cultures of U937 GTB showed positive staining in only a few isolated cells (Figure 7). In cultures exposed to etoposide 15 μg/ml for 4 h, the

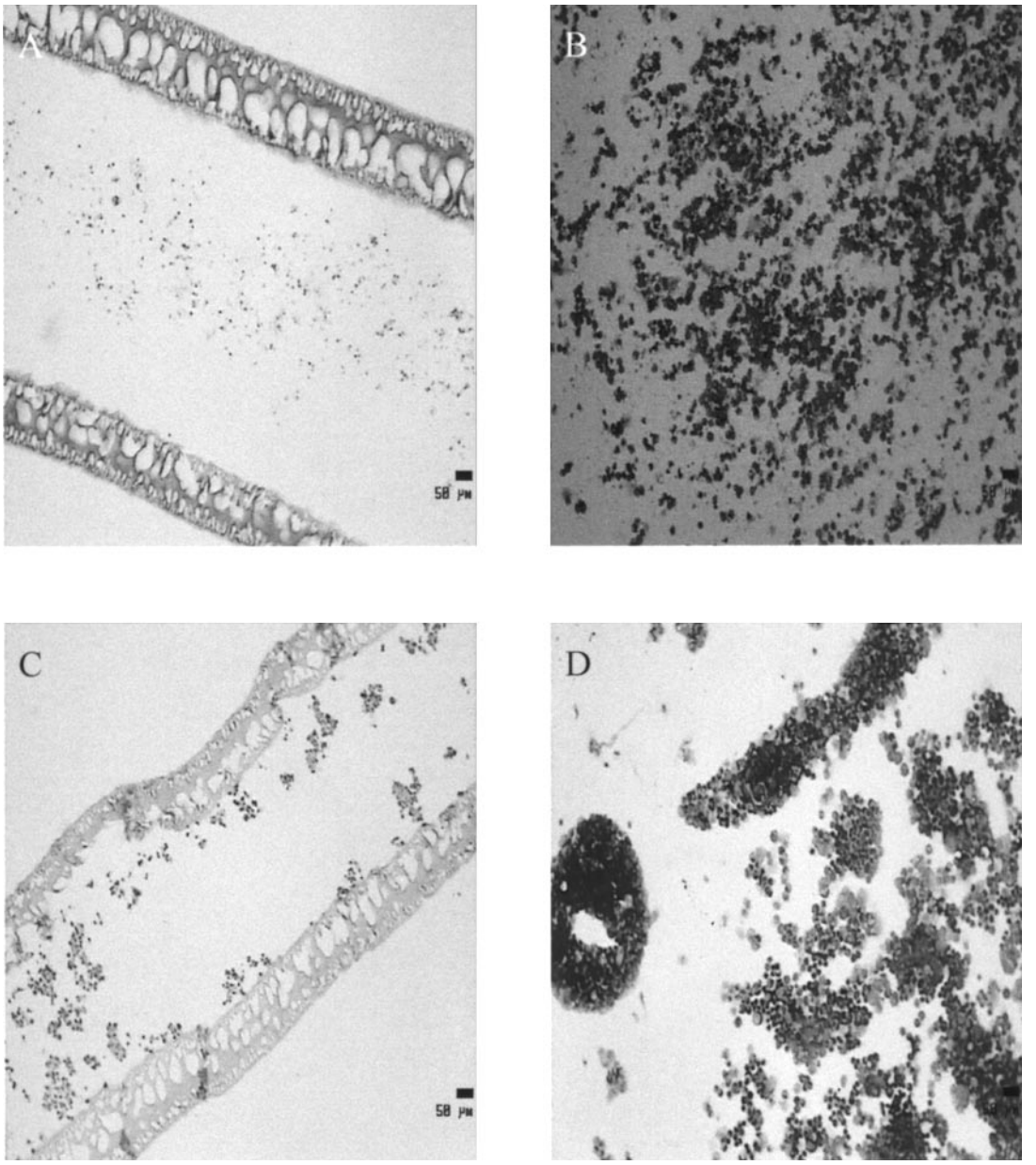

Figure 4. The 14-day-old hollow fiber cultures of cell line U937 GTB (A and B) and ACHN (C and D). Panels (A) and (C) show longitudinal cross-sections of the fiber, and panels (B) and (D) show cells retrieved from the fibers (10×0.025 MGG staining).

majority of the cells were positively stained (Figure 7C). In response to 1 μM CHS 828 for 48 h, there was an increase in the fraction of TUNEL-positive cells but less than after etoposide treatment (Figure 7E). There was no obvious difference in the staining pattern after 24 h CHS 828 exposure compared to control culture and the staining after 72 h exposure did not differ from that after 48 h exposure (not shown). In parallel, the MGG staining shows mainly viable cells in untreated culture (Figure 7B). Fragmented nuclei with an intact cytoplasmic membrane were clearly evident in etoposide-treated cells (Figure 7D). CHS 828 exposure for 48 h induced obvious apoptosis in a minority of the cells (Figure 7F). There were some cells showing vacuolization of the cytoplasm, degeneration and collapse. At this stage, it was impossible to distinguish morphologically between necrotic and apoptotic cells. After 72-h CHS 828 exposure, there were only a few isolated preserved viable cells. The majority of the cells appeared as cell debris or cell ghosts without nuclei, only cell contours were observed (not shown). No obvious changes in cell morphology could be observed after 24 h CHS 828 exposure (not shown).

Discussion

The results from the present study confirm the possibility of using primary human tumor cells from CLL and OC and normal human lymphocytes from healthy donors as well as tumor cells from established cell lines in the hollow fiber assay. Others have shown that hollow fibers are suitable for culturing a variety of tumor cell types and that the cells can grow inside the hollow fibers and form a heterogeneous *in vitro* solid tumor model.[5] The hollow fiber assay in nude mice is currently used routinely for drug screening *in vivo* at the NCI.[6] It has also been shown that immunocompetent rats can be used as hosts for hollow fibers containing human tumor cell lines[14] and primary culture of human tumor cells.[15,16]

Most human tumors have a large population of non-proliferating cells;[17] thus an old, low-proliferating system could be advantageous in resembling a clinical tumor. In the present study tumor cells from established cell lines CCRF-CEM and ACHN grew inside the hollow fibers for 2 weeks to form old, low-proliferating cultures which were used for drug sensitivity evaluation.

Our results show that the 3-day, high-proliferating *in vitro* cultures have higher sensitivity to CHS 828 than the 14-day, slow-proliferating cultures. This tendency could be seen for most of the cytotoxic drugs studied. Both 3 and 14-day cultures of the hematological tumor cell line CCRF-CEM showed higher sensitivity to CHS 828 than cultures of the solid tumor cell line ACHN. CHS 828 showed high *in vitro* activity against the hematological tumor CLL, while it showed less activity towards the solid tumor OC. This observation is similar to what was previously reported on the *in vitro* activity of CHS 828 in primary cultures of human tumors *in vitro* using the fluorometric microculture cytotoxicity assay (Åleskog *et al.*, manuscript in preparation). The possibility of culturing ovarian tumor cells from individual patients up to 3 weeks inside the hollow fiber is demonstrated in these results. More work is needed to assess the possibility of using these late *in vitro* cultures from individual patient's samples in cytotoxicity experiments. Such models might, for example, be used in selection and optimization of treatment of individual patients.

The stable end-point MTT dye conversion assay, for the determination of the living cell density, was used for its several advantages.[6] The present results show that the assay was simple, rapid and reproducible (linear relationship between viable cell number and signals generated).

Our results indicate the feasibility of harvesting tumor cells from inside the hollow fiber before and after drug treatment, and at any time of the cell growth period. The morphological assessment of the harvested tumor cells is easy, and has advantages over formalin fixation and sectioning. The harvested tumor cells retain their original size and shape. In contrast, the morphological changes were difficult to assess in the hollow fiber sections due to shrinkage of the tumor cell's cytoplasm during the fixation step and

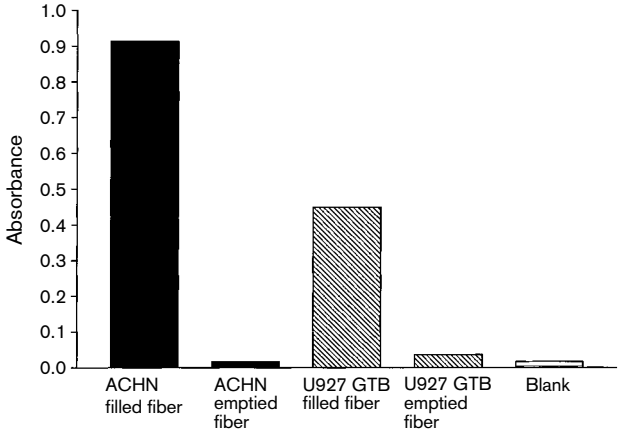

Figure 5. Successful harvest of 14-day-old hollow fiber cultures of cell lines U937 GTB and ACHN shown by low absorbance signals from emptied fibers after cell harvest.

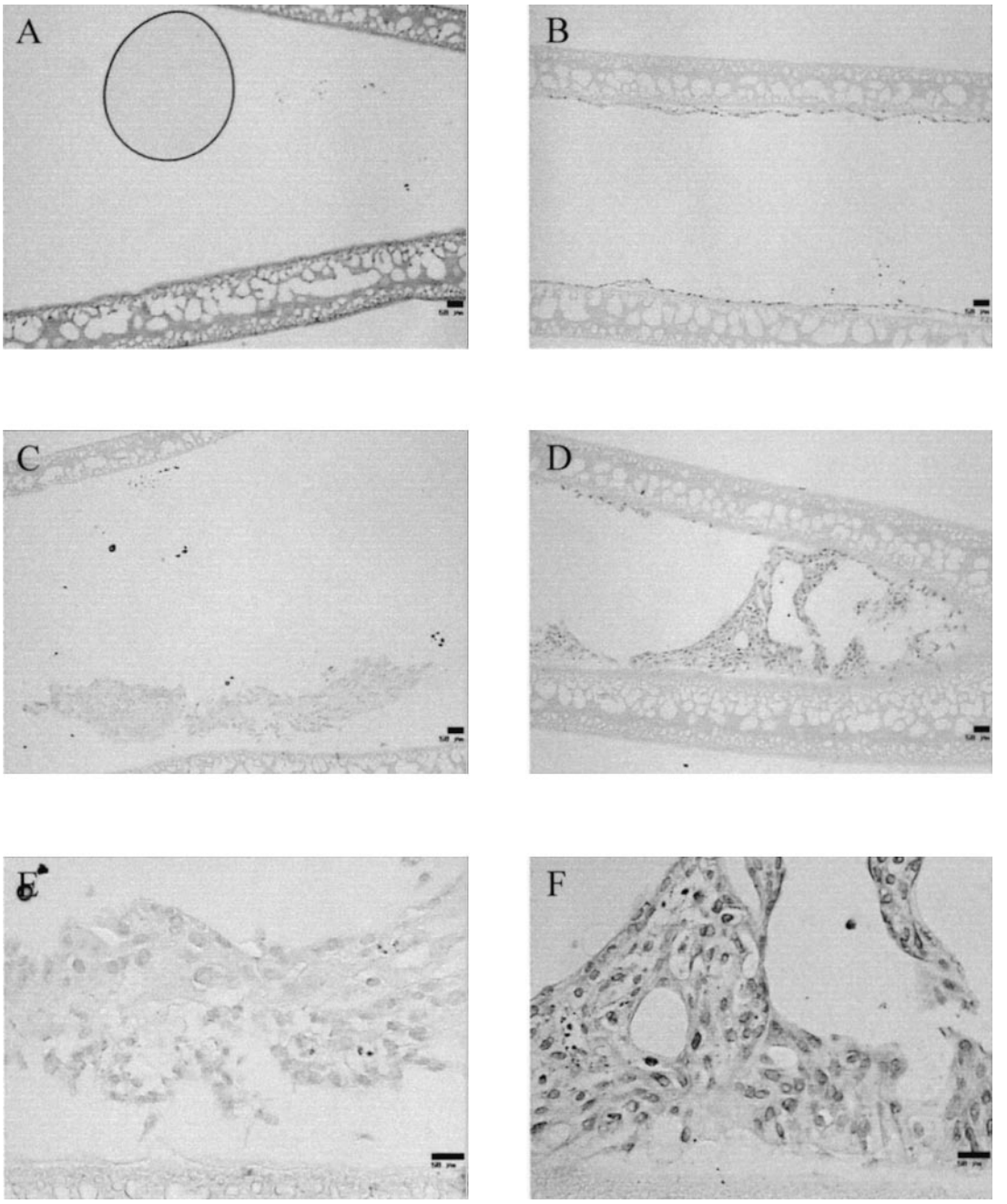

Figure 6. Longitudinal cross-section of hollow fibers filled with OC cells cultured *in vitro* for 3 (A), 14 (B), 17 (C and E) and 21 (D and F) days. The fiber walls are visible as parallel structures (A–D, 10×0.25) and the tumor cells are growing attached to them. Panels (E) and (F) (40×0.65) show the individual cells. The circle seen in (A) is an air bubble originating from the mounting procedure.

consequently squeezed nuclei. In the present study we have adopted the harvesting procedure to characterize the mode of cell death in CHS 828-treated hollow fiber cultures. No difference in cell morphology of U937 GTB was observed during the first 24 h exposure to 1 μM CHS 828 as compared to the control cultures. Our results show that the morphological changes after CHS 828 exposure occur after 24 h under these experimental conditions. Apoptotic features such as fragmented nuclei and intact cytoplasm could be seen

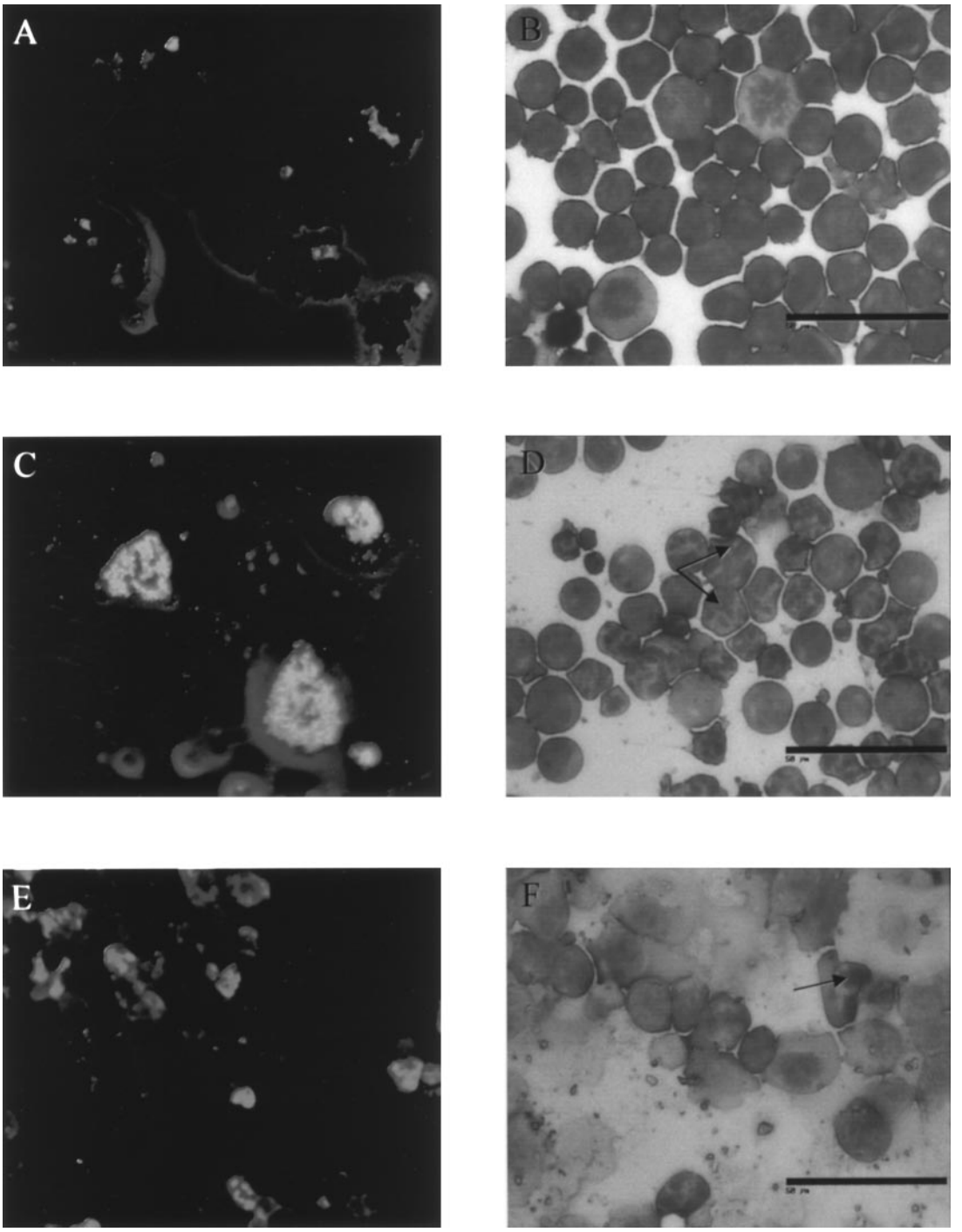

Figure 7. TUNEL (40 × 0.65) and MGG (100 × 1.25) staining of 3-day hollow fiber cultures of U937 GTB cells. Untreated control (A and B), 4-h etoposide (15 μg/ml) exposure (C and D) and 48-h CHS 828 (1 μM) exposure (E and F). Arrows indicate apoptotic cells.

in some of the cells, whereas some other dying cells lost their plasma membrane and cytoplasm. Similar morphological changes were observed with the other cell lines studied. These results suggest that CHS 828-induced cell death could be a mixture of necrosis and apoptosis, which is in accordance with previous findings.[18] However, the precise molecular mechanism of CHS 828 action needs to be investigated.

Conclusion

We have shown that the *in vitro* hollow fiber assay can be used to study the anticancer drug effects on cultures from several cell lines as well as primary human tumor cells. The tumor cells can be cultured inside the hollow fibers up to several weeks to form a solid tumor model. A high effect on hematological tumor cell lines and on primary tumor cells from patients with CLL was observed with the novel drug CHS 828. CHS 828 also showed substantial activity against hollow fiber cultures of cells originating from solid tumors. Morphological examination and characterization of the mode of cell death of the tumor cells can be performed on cytospin preparations of harvested tumor cultures.

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