The effect of the catalytic topoisomerase II inhibitor dexrazoxane (ICRF-187) on CC9C10 hybridoma viability and productivity

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Abstract

The effect of dexrazoxane on monoclonal antibody (Mab) production by CC9C10 hybridoma cells was investigated. Dexrazoxane is a catalytic inhibitor of DNA topoisomerase II. DNA topoisomerase II has a critical role in DNA metabolism and its inhibition by dexrazoxane can prevent completion of cytokinesis. Incubation of hybridomas with dexrazoxane was found to increase specific monoclonal antibody production by up to four-fold. However, due to the growth inhibitory effects of dexrazoxane the total Mab yield decreased by 40%. Under high density culture conditions (defined here as 10^6 cells ml^{-1}) specific monoclonal antibody production increased by up to 37%, which was, however, accompanied by up to a 48% decrease in Mab yield. Hybridomas that were incubated with dexrazoxane significantly increased in size due to the inhibition of cytokinesis. Dexrazoxane was also observed to induce a delayed apoptosis in the hybridomas. The caspase inhibitor Z-VAD-fmk slightly decreased the apoptotic effects of dexrazoxane. Preincubation with the caspase inhibitor Z-Asp-CH₂-DCB had no effect on dexrazoxane-treated hybridomas, but it did have antiapoptotic effects on the untreated hybridomas which normally undergo a significant basal level of apoptosis. In conclusion, dexrazoxane-induced growth inhibition (which results in higher specific antibody production) and apoptosis inhibition (which results in prolonged viability) has the potential to significantly enhance the productivity of hybridoma cell cultures.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; Mab, monoclonal antibody; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate-buffered saline (145 mM NaCl, 10.7 mM Na₂HPO₄, 2.5 mM NaH₂PO₄, pH 7.2); q_{Mab} , specific monoclonal antibody production (μg antibody $(10^6$ cells) $^{-1}$ d $^{-1}$); SFM, serum-free medium; T_i , total cell index, integral of total cell density and time; TE, 10 mM Tris-HCl, 1 mM disodium EDTA buffer, pH 8.0; TBE, 100 mM Tris-base, 100 mM boric acid, 20 mM disodium EDTA, 0.2 μg ml^{-1} ethidium bromide buffer, pH 8.3; V_i , viability index, integral of viable cell density and time; Z-Asp, benzyloxycarbonyl-Asp-2,6-dichlorobenzoyloxymethylketone or Z-Asp-CH₂-DCB; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone or Z-VAD-fmk.

Introduction

One of the goals of monoclonal antibody (Mab) production technology is the development of processes aimed at improving cell culture efficiency. To this end, conditions that favor increased specific Mab pro-

ductivity may be beneficial and cost effective. Various reports have indicated that high antibody production can occur under conditions that are not optimal for hybridoma cell growth. Culture conditions of hyperosmolarity, high temperature or addition of growth inhibitors have been shown to increase the specific

Mab production rates (Barnabé and Butler, 1994; Ozturk and Palsson, 1991; Whiteside et al., 1992). This suggests the possibility of dissociating the conditions optimal for cell growth from the conditions optimal for monoclonal antibody production.

Hybridoma cells are, however, prone to apoptosis which is also a critical factor limiting the productivity of cultures. This is due to the relation between the number of viable cells and Mab production (Renard et al., 1988). Apoptosis or programmed cell death is characterized by distinct biochemical and morphological changes such as activation of caspases and nucleosomal DNA fragmentation (Nicholson and Thornberry, 1997; Wyllie, 1980). Apoptosis in hybridomas may be induced by nutrient depletion or by the addition of growth inhibitors (Cotter et al., 1992; Mercille and Massie, 1994). An ideal growth inhibitor would arrest cell growth without inducing apoptosis.

Dextrazoxane (Figure 1A) is used clinically to reduce doxorubicin-induced cardiotoxicity (Hasinoff, 1998; Hasinoff et al., 1998a). Under physiological conditions dextrazoxane undergoes a slow ring-opening hydrolysis to ADR-925 (Figure 1A) (Hasinoff, 1998; Hasinoff et al., 1998a), an analog of EDTA. Dextrazoxane likely exerts its cardioprotective effects through its rings-opened hydrolysis product ADR-925 by virtue of its ability to strongly chelate free iron, or to quickly and efficiently remove iron from its complex with doxorubicin (Hasinoff, 1998; Hasinoff et al., 1998a), thus reducing doxorubicin-induced iron-based oxygen free radical damage.

The bisdioxopiperazine dextrazoxane (ICRF-187, Zinecard®) is also a strong catalytic inhibitor of mammalian DNA topoisomerase II (EC 5.99.1.3) (Fortune and Osheroff, 2000; Hasinoff et al., 1995; Ishida et al., 1991). Dextrazoxane has been shown not to induce DNA-topoisomerase II covalent complexes, unlike the topoisomerase II poisons, etoposide and doxorubicin (Fortune and Osheroff, 2000; Ishida et al., 1991). Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix (Fortune and Osheroff, 2000). Topoisomerase II has a critical role in DNA metabolism including replication, transcription and recombination and is required for the separation of chromosomes during mitosis. The bisdioxopiperazines have been proposed to act by trapping the enzyme in the form of a closed ATP-modulated protein clamp (Roca et al., 1994), thus preventing the formation or stabilization of cleavable complexes. Dextrazoxane may be

promoting an energy-dependent inappropriate binding of topoisomerase II to DNA after the resealing step.

We previously showed (Hasinoff et al., 2000) that CHO cells that were continuously exposed to dextrazoxane did not complete cytokinesis, yet continued to increase in size (up to 150-fold greater volume) and ploidy (up to 32N DNA). We also showed that human leukemia K562 cells continuously exposed to dextrazoxane also dramatically increased in size and ploidy and also induced erythroid differentiation and a slow induction of apoptosis (Hasinoff et al., 2001). Given the fact that dextrazoxane was able to inhibit cell division, yet allowed the cells to continue to grow, we hypothesized that dextrazoxane might be able maximize antibody production in a monoclonal hybridoma cell line. In this study, we report how the growth inhibitory effects of dextrazoxane may alter the specific monoclonal antibody productivity of a well characterized mouse hybridoma cell line and how apoptotic induction may be prevented by caspase inhibitors.

Materials and methods

Materials

Dextrazoxane (Zinecard®, ICRF-187) was a gift from Pharmacia and Upjohn (Columbus, OH). Ultrapure agarose, DMEM, Ham's F12 and Nunc Maxisorp ELISA plates were obtained from Gibco BRL (Burlington, ON). Cell Titer 96® Aqueous One Solution Cell Proliferation Assay was from Promega (Madison, WI). Z-VAD-fmk was from Alexis Biochemicals (San Diego, CA), and Z-Asp from Bachem (Torrance, CA). Chemicals not listed above were obtained from the Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Cells, media and incubation conditions

CC9C10 hybridoma cells were obtained from the American Type Culture Collection (Rockville, MD). CC9C10 is a murine hybridoma cell which produces an antibody (IgG₁) directed against bovine insulin. Cells were grown in a humidified atmosphere of 10% CO₂ at 37 °C in a defined SFM buffered with 20 mM HEPES, pH 7.1 (Barnabé and Butler, 1994). Cells were counted with a hemocytometer and viability was determined by the trypan blue exclusion method.

Figure 1. (A) Dexrazoxane and its hydrolysis product ADR-925. (B) The effect of dexrazoxane on CC9C10 hybridoma cell density (○), Mab concentration (●), and q_{Mab} (■). Hybridomas were incubated in 24-well plates with dexrazoxane for 4 d ($n = 3$). An IC_{50} value of $0.96 \pm 0.03 \mu\text{M}$ for growth inhibition was calculated from a non-linear least squares fit of the hybridoma cell density-dexrazoxane concentration data to a logistic equation. The amount of monoclonal antibody produced was determined by ELISA from cell culture supernatant collected after 4 d. Specific monoclonal antibody production (q_{Mab}) was determined from the final Mab yield divided by the total cell index (T_i).

Growth inhibition assay

Cells in exponential growth were inoculated into 24-well plates ($1 \times 10^5 \text{ cells well}^{-1}$). Freshly prepared dexrazoxane was dissolved in HEPES-buffered SFM and added to the wells to give a final volume of 1 ml. Three replicates were measured at each drug concentration. Total cell counts were determined with a model Z_F Coulter Counter (Coulter Electronics, Hialeah, FL) after 4 d of incubation. The IC_{50} value for growth inhibition was obtained from a non-linear

least squares fit of the cell density-drug concentration data to a 4-parameter logistic equation (SigmaPlot, San Rafael, CA).

Dexrazoxane treatment of high density cultures

Because dexrazoxane is growth inhibitory (Hasinoff et al., 2001; Hasinoff et al., 2000; Hasinoff et al., 1995), a series of experiments were done at high cell density in order to evaluate the effect of dexrazoxane on cell viability. CC9C10 hybridoma cells from exponentially growing cultures were centrifuged at $200 \times g$ for 5 min

and resuspended in HEPES-buffered SFM. Duplicate T-flasks (75 cm²) were inoculated at 1×10^6 cells ml⁻¹ in the presence of 0, 2, 5, or 100 μ M dextrazoxane. A low density control culture (1×10^5 cells ml⁻¹) was also included in this study. Cell density and Mab levels were monitored for 10 d.

Determination of monoclonal antibody concentration and productivity

Monoclonal antibody concentration was determined by double antibody sandwich ELISA (Harlow and Lane 1988). Briefly, ELISA plates were coated overnight at 4 °C with goat-antimouse IgG (1:1000) made up in 0.1 M sodium carbonate buffer (pH 9.5). Wells were then washed 4 times with 0.1% (v/v) Tween 20/PBS. Wells were blocked with 1% BSA (w/v) in PBS for 1 h at 37 °C and washed again. Dilutions of standards and samples were prepared in 0.1% BSA (w/v) in PBS and added to wells for 1 h at 37 °C. Standards were obtained from purified CC9C10 monoclonal antibody (Barnabé and Butler, 1998). After another washing cycle, goat antimouse IgG alkaline phosphatase conjugate (1:50,000) was added to the wells and incubated at 37 °C for 1 h. After a final wash, SIGMA FASTTM *p*-nitrophenyl phosphate was added to the wells and the absorbance at 405 nm was read on a Molecular Devices (Menlo Park, CA) plate reader after 30 min incubation at room temperature.

The specific Mab production rate (q_{Mab}) was calculated from the slope of a plot of the V_i vs. Mab concentration. The viability index, V_i , was determined from the integral of the growth curve (viable cells vs. time) and is a measure of the number of viable cells during the cell culture period (Renard et al., 1988). In cases where growth curves were not available, the total cell index (T_i), the integral of the total cells vs. time plot, was used.

Apoptosis determination and DNA fragmentation

CC9C10 hybridomas from exponentially growing cultures were centrifuged and resuspended in HEPES-buffered SFM with either 0 or 100 μ M dextrazoxane at an initial cell density of 1×10^5 cells ml⁻¹. After various times of dextrazoxane exposure, cell samples were harvested and the degree of apoptosis and DNA fragmentation was determined. The degree of apoptosis was determined morphologically (Mercille and Massie, 1994). A dye mix (4 μ l) working solution of ethidium bromide and acridine orange (100 μ g ml⁻¹

each in PBS, Molecular Probes, Eugene, OR) was added to 100 μ l of cell suspension. The slide was examined under epifluorescence with a suitable filter combination. Hybridoma cells with uniformly stained, highly condensed chromatin appearing as spherical beads or crescents around the periphery of the nucleus were scored as apoptotic. Hybridoma cells with diffuse fluorescent nuclei were scored as non-apoptotic.

DNA fragmentation was analyzed by electrophoresis on 2% (w/v) agarose gel. Cells (6×10^6) were harvested, washed twice with ice-cold PBS and lysed in 250 μ l lysis buffer (50 mM Tris-base, 10 mM disodium EDTA, 0.5% w/v sodium N-laurylsarcosine) and 125 μ l DNase-free RNase (0.5 mg ml⁻¹). After a 1 h incubation at 50 °C, 225 μ l Proteinase K (1.3 mg ml⁻¹) was added and the solution was incubated for 1 h at 50 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v). One volume of 3 M sodium acetate (pH 5.2) and 25 volumes of ice-cold ethanol were added to 10 volumes of the DNA extract to precipitate the DNA. The pellet was collected by centrifugation at $11,000 \times g$ for 20 min (4 °C) and washed once with 70% (v/v) ice-cold ethanol. The pellet was air-dried and resuspended in 100 μ l TE buffer. DNA was quantified by UV absorbance spectroscopy at 260 nm. One volume of sample (3 μ g DNA) was mixed with 6 volumes of gel loading dye and run on the agarose gel in TBE buffer for 90 min at 50 V. A Lambda DNA Bst II digest standard was used as a molecular weight marker.

MTS assay

The spectrophotometric 96-well MTS assay, which measures the ability of the cells to reduce MTS, was used as a measure of viable cell number. MTS, a tetrazolium compound, is reduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. MTS reagent (20 μ l) was added to 100 μ l of cell culture on the last day of incubation. The absorbance was read at 490 nm on a plate reader after 4 h of incubation at 37 °C. The absorbance values ($\pm$ SD) plotted were from an average of 6 wells.

Caspase inhibitor experiments

Caspase inhibitors were dissolved in DMSO and added to cultures so that the final DMSO concentration was 0.5% (v/v). CC9C10 hybridoma cells from exponentially growing cultures were inoculated into 96-

well plates at 5000 cells well⁻¹ with a total volume of 100 μ l. Z-VAD (0–200 μ M) or Z-Asp (0–200 μ M) were added to the wells. After 1 h of pre-treatment with the caspase inhibitors, dexrazoxane was added. An MTS assay was performed 72 h after dexrazoxane addition. An increase in MTS absorbance was interpreted as inhibition of apoptosis.

In a second series of experiments, CC9C10 hybridomas from exponentially growing cultures were collected by centrifugation and resuspended in HEPES-buffered SFM at a cell density of 1×10^5 cells ml⁻¹. Cells were treated with 5 or 10 μ M Z-Asp for 0, 1, 12, 24, 48, or 72 h. After treatment, cells were centrifuged and resuspended at 5×10^4 cells ml⁻¹ in fresh medium containing the original amount of Z-Asp and 100 μ l of each culture was added to microtiter wells at a cell density of 5000 cells well⁻¹ ($n = 6$). Dexrazoxane (0 or 100 μ M) was then added to the wells and an MTS assay was performed 72 h later. Unless otherwise indicated, all errors quoted are standard errors (SE) from least squares analyses.

Results

Growth inhibitory effects of dexrazoxane and its effect on monoclonal antibody production

The structures of dexrazoxane its hydrolysis product, ADR-925, are shown in Figure 1A. The growth inhibitory effects of 4 d of continuous exposure of dexrazoxane on the CC9C10 hybridoma cells are shown in Figure 1B. The monophasic survival curve gave an IC_{50} value of 0.96 ± 0.03 μ M. It was also observed that in the presence of dexrazoxane the hybridoma cell size increased as was previously seen for CHO cells (Hasinoff et al., 2000). Estimates of cell size on hemacytometer counting grids showed that CC9C10 hybridomas treated with greater than 5 μ M dexrazoxane increased to 25–33 μ m in diameter compared to untreated controls, which were 17–20 μ m. Cellular debris from apoptosis was also observed.

The monoclonal antibody content of the cell culture supernatant (Figure 1B) was determined to see if dexrazoxane-induced reduced growth or growth inhibition would lead to enhanced Mab production. A 44% decrease in total Mab (18 μ g vs. 32 μ g over 4 d) was observed in cells treated in the range of 5–500 μ M dexrazoxane compared to untreated cells, whereas less than 5 μ M dexrazoxane exposure had no effect on Mab yield. Large decreases in Mab

Figure 2. The effect of dexrazoxane on CC9C10 hybridoma growth at high cell density. Hybridomas were inoculated at 1×10^6 cells ml⁻¹ and treated with 0 μ M (●, control A), 2 μ M (▼), 5 μ M (■) or 100 μ M (◆) dexrazoxane. Control B (○) cultures were inoculated at 1×10^5 cells ml⁻¹ and were not treated with dexrazoxane.

concentration were, however, observed in 1000 μ M and 5000 μ M dexrazoxane-treated cells. However, at these high dexrazoxane concentrations we previously showed (Hasinoff et al., 1997) that the metal chelating hydrolysis product ADR-925 that would form over the 4 d of this experiment (Hasinoff et al., 1998b) inhibits growth due to the depletion of calcium and magnesium from the medium. Assuming constant Mab production over the analysis period, the q_{Mab} was determined as a function of dexrazoxane concentration (Figure 1B). The q_{Mab} production curve peaked at 5–500 μ M dexrazoxane. At the highest point in the curve, q_{Mab} was 4-fold higher than controls (0 μ M dexrazoxane). Thus, dexrazoxane appeared to reduce cell growth and enhance specific Mab productivity.

Dexrazoxane treatment of high density cell cultures

Because of the growth inhibitory effect of dexrazoxane, further experiments were carried out in cell cultures inoculated at high cell densities prior to dexrazoxane treatment as it was hypothesized that a higher starting cell density would increase Mab yield in dexrazoxane-treated cultures in comparison to control cultures.

As shown in Figure 2, all cultures, including the controls, inoculated at 1×10^6 cells ml⁻¹ decreased in

viable cell density as expected from high cell density cultures. The rate of decline was, however, dependent on the concentration of dexrazoxane. Exposure of hybridomas to 5 and 100 μM dexrazoxane had similar effects on viability, such that approximately 50% of the cell population remained viable after 2 d of incubation. At 2 μM dexrazoxane, viable cell density decreased to 50% of the inoculated density after 3 d of incubation, whereas control A cultures that were not exposed to dexrazoxane were 50% viable after 8 d. Hybridomas inoculated at a 10-fold lower cell concentration (control B) were also included in this study. The purpose of this control was to distinguish the effects of high cell density and dexrazoxane on Mab productivity. In the control B cultures, a typical 4 d exponential phase was followed by a brief stationary phase and then a death phase as has been reported elsewhere (Barnabé and Butler, 1994).

The Mab production in these experiments is shown in Figure 3A. The highest Mab yield ($150 \mu\text{g ml}^{-1}$) was attained in control A cultures. Mab yields of 102, 91, and $78 \mu\text{g ml}^{-1}$ after 8 d were found in cultures grown in the presence of 2, 5, and 100 μM dexrazoxane, respectively, which was 32–48% lower than the corresponding control A cultures. The q_{Mab} calculated from the linear least squares fit of the data in Figure 3B were determined for each culture condition. Cultures treated with 5 and 100 μM dexrazoxane had q_{Mab} values of 31 ± 2 and $32 \pm 2 \mu\text{g (10}^6 \text{ cell)}^{-1} \text{ d}^{-1}$ respectively. This represented a 34 and 37% increase, respectively, compared to the corresponding control A cultures ($23 \pm 1 \mu\text{g (10}^6 \text{ cell)}^{-1} \text{ d}^{-1}$). A smaller q_{Mab} increase (17%) was observed in cultures treated with 2 μM dexrazoxane.

When comparing the effects of cell density on q_{Mab} , it can be seen that in the absence of dexrazoxane, inoculation density had a significant effect. Culturing cells at high cell density (control A, q_{Mab} of $23 \pm 1 \mu\text{g (10}^6 \text{ cells)}^{-1} \text{ d}^{-1}$) resulted in a 56% higher q_{Mab} than cultures inoculated at low cell density (control B, q_{Mab} of $15 \pm 1 \mu\text{g (10}^6 \text{ cells)}^{-1} \text{ d}^{-1}$). This effect was probably related to the previously established correlation between a high specific productivity and a low growth rate (Figure 2). The fact that dexrazoxane-treated cells also have reduced growth rates compared to untreated cells (Figure 2) may also explain why dexrazoxane-treated cultures show a higher q_{Mab} .

Determination of dexrazoxane-induced apoptosis

As seen in Figure 4A, 100 μM dexrazoxane totally inhibited cell growth after 2 d and caused a decrease in viable cell number. The degree of apoptosis (Figure 4B) was determined by morphological changes in the nucleus. As shown in Figure 4A, control cultures (0 μM dexrazoxane) that grew exponentially for 3 d had basal levels of apoptosis that were 2–5% of the total cell population (Figure 4B). However, apoptosis in the controls increased to 10% on the fourth day of culture which coincided with the end of exponential growth (Figure 4A). In contrast, significant apoptosis (15%) was observed in dexrazoxane-treated cultures after only 2 d of incubation. Thereafter, the percentage of apoptosis increased approximately linearly from day 1 to day 4 with maximal apoptosis reaching 35% on day 4.

The degree of apoptosis was also determined by the extent of DNA laddering caused by internucleosomal DNA fragmentation using gel electrophoresis (Figure 5). Little laddering was observed in control cells during the exponential phase of growth (days 1–3) and only on day 4, when exponential growth ceased, did a typical DNA ladder pattern emerge in control cells (Wyllie, 1980). In contrast, in the dexrazoxane-treated cultures, DNA laddering was apparent from day 2 with more extensive fragmentation occurring on days 3 and 4. However, the distinct ladder pattern was lost on these last two days, probably due to extensive late apoptotic non-specific DNA fragmentation.

Inhibition of dexrazoxane-induced apoptosis by caspase inhibitors

As shown in Figure 3B higher q_{Mab} levels were induced in CC9C10 cultures treated with dexrazoxane. However, due to dexrazoxane-induced apoptosis the viable cell density and total Mab yield decreased (Figures 2 and 3). Thus, we hypothesized that the caspase inhibitors, Z-VAD and Z-Asp, might be able to inhibit apoptosis. It was previously shown (Gaber-net et al., 1999) that Z-VAD inhibited apoptosis in hybridomas. Likewise, Z-Asp was shown to inhibit dexrazoxane-induced apoptosis in human leukemic CEM cells (Khelifa and Beck, 1999).

As shown in Figure 6A a 1 h pre-treatment of cells with 200 μM Z-VAD partially antagonized the effect of 100 μM dexrazoxane cytotoxicity (1.8-fold increase in MTS absorbance) compared to controls

Figure 3. (A) The effect of dexrazoxane on CC9C10 monoclonal antibody production as a function of time. (B) The specific monoclonal antibody production as a function of the viability index. Hybridomas were inoculated at 1×10^6 cells ml⁻¹ and treated with 0 μM (○, control A), 2 μM (□), 5 μM (△) or 100 μM (◇) dexrazoxane. Control B (●) cultures were inoculated at 1×10^5 cells ml⁻¹ and were not treated with dexrazoxane.

without the caspase inhibitor. Z-VAD did not, however, totally inhibit apoptosis as cultures never reached the same MTS absorbance as in the absence of dexrazoxane (Figure 6A). In the absence of dexrazoxane Z-VAD had little effect on MTS reduction.

Z-Asp used at concentrations recommended by the supplier (50–200 μM) was found to be cytotoxic to CC9C10 hybridomas (data not shown). In order to circumvent this cytotoxicity hybridomas were pre-incubated at lower Z-Asp concentrations for extended periods, treated with dexrazoxane, and then cultured for a further 72 h. The effects on MTS absorbance on Z-Asp pre-incubation are shown in Figure 6B. Pre-incubation of Z-Asp (5–10 μM) for 1–72 h had no effect on dexrazoxane-induced apoptosis. However, pre-incubation with Z-Asp (5 or 10 μM) for 24 to 72 h did have a significant antiapoptotic effect on untreated control cells (Figure 6B). For example, cells pre-incubated for 72 h with 10 μM Z-Asp gave a 21% higher MTS absorbance than cells not exposed to the caspase inhibitor.

Discussion

The CC9C10 hybridoma cell line was used to study the effect of dexrazoxane on growth and monoclonal antibody productivity because of its well characterized metabolism and productivity profiles (Barnabé and Butler, 1998; Butler et al., 1997; Petch and Butler,

1994). The CC9C10 hybridoma is of mouse origin and produces an antibody (IgG₁) directed against bovine insulin (Schroer et al., 1983). A dexrazoxane *IC*₅₀ value of 0.96 μM for growth inhibition was determined for the CC9C10 cells which is in the same range as that determined for Chinese Hamster Ovary cells (Hasinoff et al., 1997). Dexrazoxane treatment of hybridomas resulted in an increase in cell size (data not shown). This effect was similar to that seen in CHO cells, which upon treatment with dexrazoxane continued through multiple rounds of cell cycling without completion of cytokinesis due to inhibition of topoisomerase II (Hasinoff et al., 2000).

The growth inhibitory properties of dexrazoxane were exploited for the purpose of enhancing the specific Mab production rate (q_{Mab}). The dexrazoxane-induced enhancement of q_{Mab} is consistent with the hypothesis that energy resources normally destined for cell growth have been made available for secondary metabolism (i.e. non-essential product formation). Specific antibody production rates can be significantly increased in a number of hybridoma cell lines in response to lower specific growth rates due to growth inhibitors, temperature shifts, nutrient limitations, and dilution rates (Barnabé and Butler, 1994; Hayter et al., 1992; Jang and Barford, 2000; Martens et al., 1993; Miller et al., 1998). However, the dexrazoxane-induced increase in hybridoma cell size may, in part, also account for the observed increase in q_{Mab} . Evid-

Figure 4. Effect of dexrazoxane (100 μM) on cell viability and apoptosis in CC9C10 cultures. Hybridomas were inoculated at 1×10^5 cells ml^{-1} and incubated for 4 d in 0 or 100 μM dexrazoxane. (A) Total (open symbols) and viable (closed symbols) cell density of control cultures ($\circ$, $\bullet$), and cultures treated with 100 μM dexrazoxane (∇ , $\blacktriangledown$). (B) Degree of apoptosis of control cultures ($\bullet$), and 100 μM dexrazoxane-treated cultures ($\blacktriangledown$).

ence of the effect of growth rate on Mab production was also shown in untreated CC9C10 control cultures inoculated at different densities (Figure 3A). Exponentially growing cells in low inoculum cultures had a significantly lower q_{Mab} than high inoculum cultures which had little exponential growth.

We previously showed that dexrazoxane induced apoptosis and differentiation in human leukemia K562 cells (Hasinoff et al., 2001). Dexrazoxane was shown to slowly induce apoptosis in CC9C10 cells, both as evidenced by the morphological changes in the nucleus characteristic of apoptosis, and by the appearance

Figure 5. DNA fragmentation of CC9C10 hybridoma cells visualized by ethidium bromide-stained agarose gel electrophoresis. The hybridomas were inoculated at 1×10^5 cells ml^{-1} and incubated for 4 d in 0 or 100 μM dexrazoxane. From the left, lane 1: cells at inoculum (day 0); lanes 2–5: controls on days 1–4, respectively; lanes 6–9: 100 μM dexrazoxane-treated cells on days 1–4, respectively. Rightmost lane: Lambda Bst II digest molecular weight marker. The progressive appearance of a DNA ladder pattern with increasing time of dexrazoxane exposure was indicative of apoptosis.

Figure 6. The effect of caspase inhibitors on dexrazoxane-mediated growth inhibition of CC9C10 hybridoma cells measured in a MTS assay. (A) The effect of Z-VAD (0–200 μM) on dexrazoxane-mediated cell growth. The cells were pre-treated with Z-VAD (or not) for one h after which 100 μM dexrazoxane was added (or not). The cells were then grown for 72 h in the presence of 100 μM dexrazoxane (■), or without dexrazoxane (●) and Z-VAD (or not). The increase in MTS absorbance of the cells grown in the presence of dexrazoxane indicate that Z-VAD partially inhibits the apoptotic effect of dexrazoxane. (B) The effect of Z-Asp on dexrazoxane-mediated cell growth. The control cultures are denoted by closed symbols and the 100 μM dexrazoxane-treated cells are denoted by open symbols. Cells from both groups were preincubated for 1 h (●), 12 h (■), 24 h (▲), 48 h (▼), or 72 h (◆) with 0–10 μM Z-Asp prior to the addition of dexrazoxane. The increase in MTS absorbance for the control cultures in (B) indicates that Z-Asp reduces basal apoptosis levels in hybridomas.

of internucleosomal DNA fragmentation (Figure 5). Dexrazoxane has also been reported to induce apoptosis in other mammalian cell lines (Khelifa and Beck, 1999; Kizaki and Onishi, 1997), though it did not do so in CHO cells (Hasinoff et al., 2000). The low levels of apoptosis after the end of exponential growth in control cells, as evidenced by both methods of detection, was likely due to nutrient limitation (*i.e.* glucose or glutamine) in the culture medium as we have previously shown in the CC9C10 cell line (Barnabé, 1998), and has been shown in cells of lymphoid origin (Gabernet et al., 1999; Mercille and Massie, 1994; Petronini et al., 1996).

Caspases mediate highly specific proteolytic cleavage events in cellular apoptosis. Z-VAD, a highly cell permeable and irreversible broad-spectrum caspase inhibitor (Lemaire et al., 1998), was shown to only slightly antagonize dexrazoxane-induced apoptosis in CC9C10 hybridomas (Figure 6A). This result is consistent with the antiapoptotic effects of Z-VAD in a hybridoma cell line deprived of glutamine (Gabernet et al., 1999). Z-Asp, another broad spectrum caspase inhibitor (Mashima et al., 1995), did not antagonize dexrazoxane-induced apoptosis in CC9C10 cells (Figure 6B). Z-Asp did, however, have an antiapoptotic effect on untreated control cells (Figure 6B). Z-Asp had previously been shown to inhibit apoptosis in dexrazoxane-treated CEM cells through inhibition of caspase-3 and -7 like activities (Khelifa and Beck, 1999). The result that Z-Asp inhibited basal levels of apoptosis in untreated CC9C10 cells was probably due to inhibition of apoptosis that occurs even under normal culture conditions (Solis-Recendez et al., 1995).

Based on the studies presented here, we have shown that it was possible to use the catalytic topoisomerase II inhibitor dexrazoxane to dissociate cell growth from monoclonal antibody production. Exposure of CC9C10 cells to dexrazoxane resulted in hybridoma cell growth inhibition and inhibition of cytokinesis which may have led to the increase in specific monoclonal antibody production that was observed. Exposure of CC9C10 cells to dexrazoxane also induced apoptosis that was partially inhibitable by the caspase inhibitor Z-VAD. This work indicates the potential for the use of dexrazoxane in combination with Z-VAD in the commercial production of therapeutic or diagnostic antibodies from cell culture processes. These molecules are relatively small and unlikely to affect the purification of an antibody by

the standard techniques of affinity or ion exchange chromatography.

Future studies are required to fully evaluate the potential of culture supplements of topoisomerase and caspase inhibitors to contribute to improvements in the productivity of antibody-producing hybridomas. Such studies may establish whether cell growth can be inhibited without inducing apoptosis or if apoptosis is induced by growth inhibitors whether or not caspase inhibitors can prevent induction. The combination of growth inhibition (which results in higher q_{Mab}) and apoptosis inhibition (which results in prolonged viability) may have the potential to significantly enhance the productivity of hybridoma cell cultures.

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