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Cell Biology International 28 (2004) 237–242

Cell Biology International

www.elsevier.com/locate/cellbi

Apoptosis and nitric oxide release induced by thalidomide, gossypol and dexamethasone in cultured human chronic myelogenous leukemic K-562 cells

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Received 23 October 2003; revised 20 November 2003; accepted 8 December 2003

Abstract

The effects of different dosages of thalidomide, gossypol and dexamethasone on the levels of apoptosis and nitric oxide (NO) production were studied in a human chronic myelogenous leukemic cell line, K-562. Increases in the levels of apoptosis were induced by both 15 and 30 μM of thalidomide but only 15 μM significantly increased NO production. All dosages of gossypol used increased the production of NO and all dosages except 200 μM raised the level of apoptosis. After dexamethasone treatment the level of NO either decreased or stayed constant. Thus, some dosages of thalidomide and gossypol concomitantly raise the levels of apoptosis and NO production, but dexamethasone, though it induced apoptosis, had no significant effect on NO production.
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Keywords: Apoptosis; Nitric oxide; Thalidomide; Gossypol; Dexamethasone; K-562

1. Introduction

Both drug resistance in leukemic cells, and the side effects of the drugs used, can cause problems in the treatment of leukemia (Law et al., 1993). The K-562 leukemia cell line used in this study has been deployed as a model system in other laboratories, mostly for studies of apoptosis (Durrieu et al., 1998; Konac et al., 2003).

Free radicals can cause cell death by apoptosis; however, the superoxide anions, H_2O_2 and NO, can affect both cancer suppression and cancer progression (Halliwel, 2000). NO, synthesized by nitric oxide synthase from L-arginine, has both pro-apoptotic and anti-apoptotic effects, and is an important regulator of apoptosis and cell signalling pathways (Zhang et al., 2000).

Thalidomide was used in 1950 as a sedative hypnotic but was later found to cause various congenital disorders during pregnancy (Bröker and Giaccone, 2002). Today it is used to treat leprosy and acquired immunodeficiency syndrome (AIDS). It has anti-inflammatory, anti-angiogenic and anti-tumor properties, and is an inhibitor of tumor necrosis factor (TNF-α) (Vescovo et al., 2002).

Gossypol, a polyphenolic aldehyde, is used as an oral contraceptive for males and also to treat a variety of cancers (Gilbert et al., 1995). It is a potent inhibitor of phospholipid-sensitive Ca^{++} dependent protein kinase (Kimura et al., 1985) and is capable of forming free radicals (Dao et al., 2000).

Dexamethasone is a corticosteroid used in the treatment of autoimmune system diseases such as systemic lupus erythematosus. It blocks interleukin-6 (Ho et al., 2001). It is also used, synergistically with thalidomide, for treating advanced melanoma (Eisen et al., 2000).

Genetic changes that impair apoptosis during tumor development may result in resistance to therapies that kill tumor cells by apoptosis. Therefore, further insight into the regulation of apoptosis could lead to new approaches in the treatment of cancer. We have measured the levels of apoptosis and NO production in

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1065-6995/04/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2003.12.005

K-562 cells after treatment with different dosages of thalidomide, gossypol and dexamethasone.

2. Materials and methods

2.1. Cell culture and treatment

K-562 erythroleukemic cells were obtained from the Department of Immunology, Faculty of Medicine, Gazi University, Ankara, Turkey. They were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO_2 atmosphere in air at 37 °C. The effects of the following dosages of drugs on the cultures were tested by comparison with controls: 15, 30, 50 and 100 μM thalidomide; 15, 50, 100 and 200 μM gossypol; and 1, 10, 50 and 100 μM dexamethasone (Sigma, Germany). All dose levels of each compound were tested on six different cultures. The cultures were treated for 6 h with thalidomide and gossypol, and 12 h with dexamethasone.

2.2. Nitrate and nitrite measurements

NO_2^- and NO_3^- levels were measured by the Griess Method (Green et al., 1982). Aliquots (0.5 ml) from each treated culture were mixed with 1 ml of 97% ethanol and incubated at 4 °C for 30 min. After centrifugation at 14,000 rpm for 5 min at 4 °C, 0.5 ml of each supernatant was transferred to a microfuge tube. To measure the level of nitrate, 1 unit of supernatant, 1 unit of $VaCl_2$, 0.5 unit of sulphanilamide and 0.5 unit of *N*-(1-Naphtyl)ethylenediamine (NEDD) mixture were incubated together at 37 °C for 30 min, followed by measurement of the E_{540} in a Bioelisa reader (Elx-800, Biokit, Spain). To measure nitrite, 1 unit of supernatant, 0.5 unit of sulphanilamide and 0.5 unit of NEDD were incubated at 37 °C for 30 min before reading the E_{540}.

2.3. Apoptosis

Treated cell suspensions were dropped on to slides and fixed sequentially with 70 and 97% ethanol. The slides were stained in 10% giemsa solution (Merck, Germany) and examined under a light microscope to count the apoptotic cells. One thousand cells were counted for each concentration of each test compound. Cells displaying membrane blebbing, cell shrinkage and nuclear condensation were considered apoptotic (Fig. 1A–D).

2.4. Statistics

Z-tests (difference between binomial sample proportions) were conducted to evaluate the significance of changes in the percentages of apoptotic cells. Student's *t*-tests were conducted to evaluate the NO levels statistically.

3. Results

Thalidomide at 15, 30, 50 and 100 μM caused the release from K-562 cells of 19.99 ± 1.76, 15.12 ± 2.35, 18.98 ± 2.37 and 19.92 ± 3.62 μmol NO, respectively, compared to the control value of 16.16 ± 1.65 μmol (Fig. 2A). Although increases in the release of NO were observed for 15, 50 and 100 μM thalidomide treatments, only with 15 μM was the increase statistically significant ($P<0.05$). The corresponding percentages of apoptotic cells were 26, 30, 12 and 11%, respectively. The two lowest dosages, 15 and 30 μM, significantly elevated the level of apoptosis above the control value (16%; $P<0.05$) (Table 1).

Gossypol at 15, 50, 100 and 200 μM caused the release of 19.76 ± 2.69, 19.20 ± 1.03, 22.28 ± 3.85 and 20.40 ± 2.07 μmol NO, respectively. The value for the control cells was 17.86 ± 3.75 μmol (Fig. 2B). Only 100 and 200 μM gossypol caused significant increases above this control value ($P<0.05$). The corresponding percentages of apoptotic cells were 40, 54, 50 and 18%, respectively; the first three of these values were significantly higher than the control (16%; $P<0.01$, see Table 2).

Finally, the addition of 1, 10, 50 and 100 μM dexamethasone to the cells caused the release of 13.85 ± 2.42, 14.20 ± 2.64, 17.79 ± 3.30 and 16.18 ± 4.56 μmol NO, respectively, all of which were lower than the control value of 18.48 ± 1.48 μmol (Fig. 2C), but none significantly. The corresponding percentages of apoptotic cells were 16, 40, 34 and 32%, respectively, and of these the last three were significantly higher than the control value (12%; $P<0.01$; see Table 3).

4. Discussion

Free radicals induce mutagenesis or carcinogenesis in normal cells by causing DNA damage (Dreher and Junod, 1996). Greater quantities of free radicals may lead to elimination of tumor cells by inducing apoptosis. NO has been recognized to induce cell death via apoptosis. NO also stimulates apoptosis in different human neoplastic lymphoid cell lines (Ushmorov et al., 1999). Nitric oxide synthase (NOS) activities have been shown to mediate inhibition of K-562 survival or growth. Also, TNF-related apoptosis-inducing ligand (TRAIL) is investigated to induce apoptosis by activating caspases and stimulating NO production (Secchiero et al., 2001).

Apoptosis can be regulated by either p53-dependent or p53-independent pathways (Clarke et al., 1993; Lowe et al., 1993); p53 is not normally expressed in K-562 cells

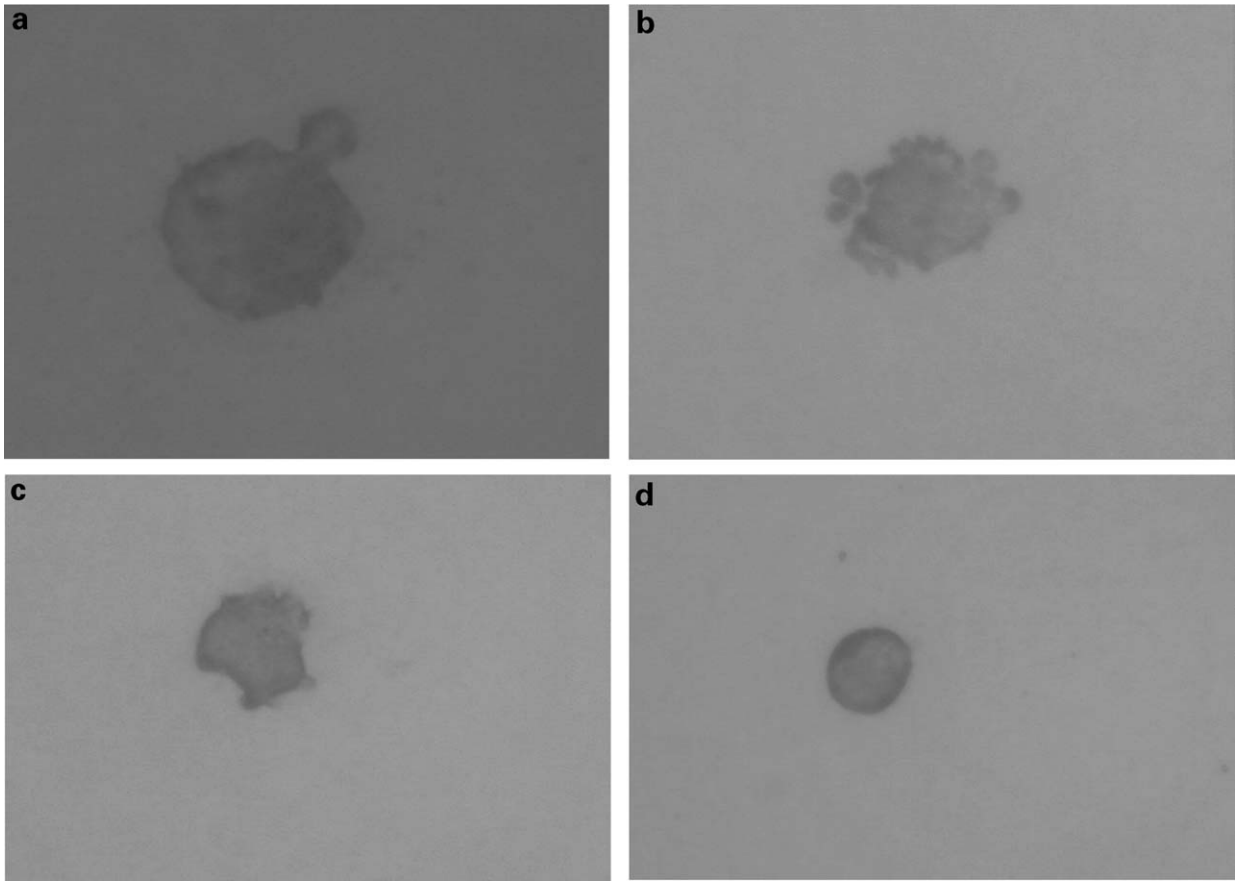


Fig. 1. Morphological evaluation of K-562 cell lines after treatment of different concentration of (A) thalidomide at 15 μM, (B) thalidomide at 30 μM, (C) gossypol at 50 μM, and normal cell morphology (D).

(Law et al., 1993). The present study and others have shown that apoptosis can be induced in K-562 cells without involvement of p53 (Ritke et al., 1994).

Although the exact mechanism of action of thalidomide remains unclear, oxidative DNA damage has been implicated (Parman et al., 1999). Thalidomide can directly inhibit the growth and survival of drug-resistant myeloma cells (Hideshima et al., 2000; Parman et al., 1999). Its activity, mediated through a liver metabolite, involves inhibition of (1) TNFα and (2) the angiogenic effects of basic fibroblast growth factor and vascular endothelial growth factor (Bauer et al., 1998; Richardson et al., 2002). Thalidomide is indicated to inhibit the growth of myeloma cells due to apoptosis (Yata et al., 2003). It also has an anti-angiogenic activity in human multiple myeloma (MM) cell lines and patient cells, including those resistant to conventional therapy. In addition, it is thought to mediate an anti-MM effect by modulating natural killer cell number and function (Davies et al., 2001).

While 15 and 30 μM thalidomide caused statistically significant increases in apoptosis in K-562 cells, only 15 μM caused a statistically significant increase in the release of NO. Induction of apoptosis by 15–30 μM thalidomide concentrations has previously been reported (Hansen et al., 1999); 20, 50 and 250 μg/ml thalidomide also exert a dose-dependent effect on TNFα level (Gallily et al., 1999). Intake of 100 mg/day thalidomide and 40 mg/day dexamethasone shows synergistic effects in the treatment of advanced melanoma (Eisen et al., 2000).

Gossypol, a male contraceptive drug, has been demonstrated to have anti-proliferative and anti-metastatic effects on many kinds of cancer cells, especially in melanoma and colon carcinoma cells in vitro (Zhang et al., 2003). Gossypol inhibits the colonization capability of the carcinoma cell lines HT29 and LoVo. Gossypol induced apoptosis may not be involved in the regulation of p53 (Wang et al., 2000). This study shows that gossypol increases the release of NO with the 100–200 μM gossypol concentrations and leads to a rise in the percentage of apoptotic cells between 15–100 μM gossypol concentrations. Others have also shown that apoptosis is induced by 50 and 100 μM gossypol (Quintana et al., 2000). The gossypol concentrations used in this study revealed that apoptosis was induced in a cell line, K-562, which does not express p53. Therefore, it could be used in the treatment of human leukemia as

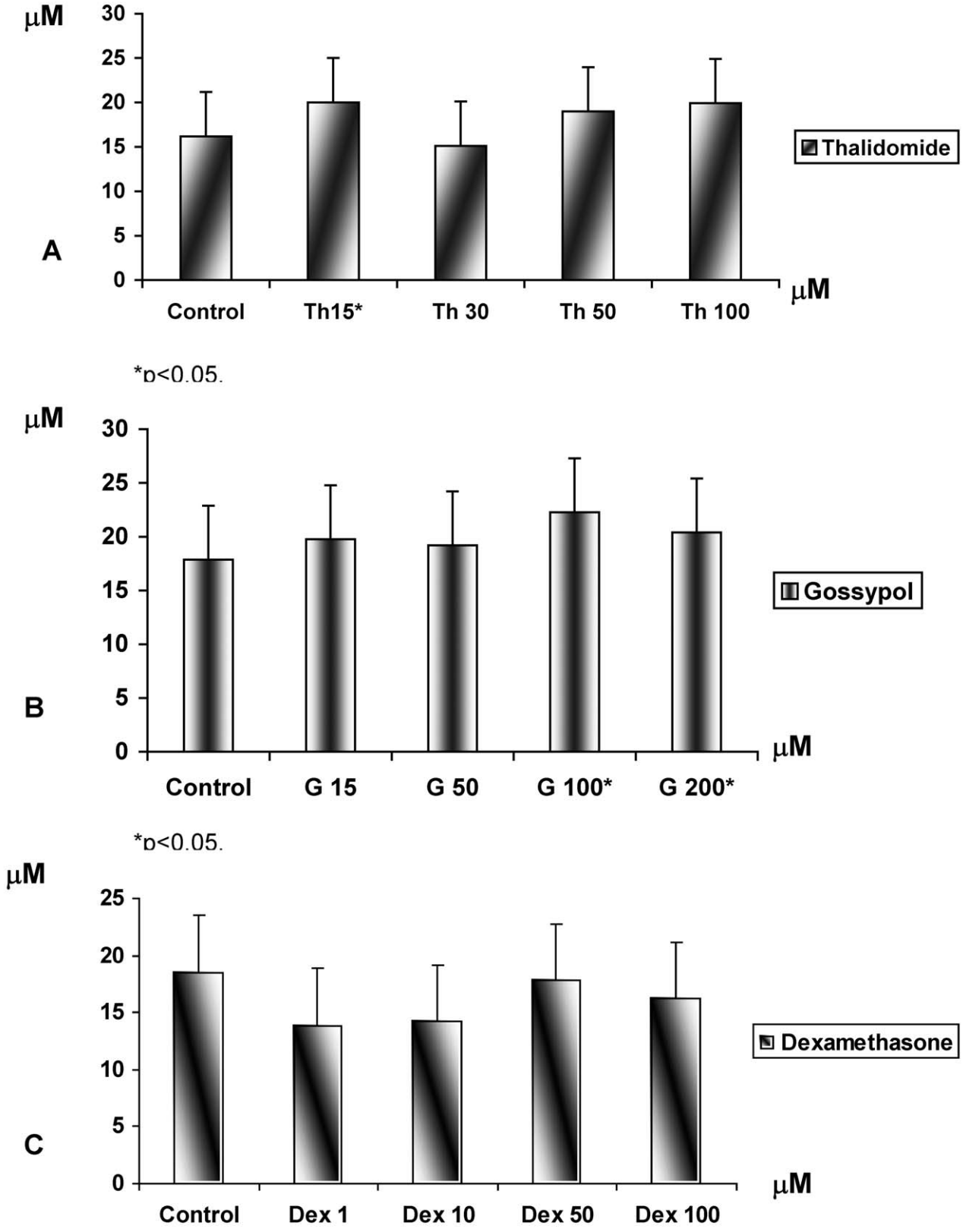


Fig. 2. Comparison of release of NO due to different concentrations of thalidomide (A), gossypol (B) and dexamethasone (C).

Table 1
The relation between concentration of thalidomide and percentage of apoptotic cells

Concentration	Apoptosis (%)
Control	16
Thalidomide 15 μM	26*
Thalidomide 30 μM	30*
Thalidomide 50 μM	12
Thalidomide 100 μM	11

* $P<0.05$.

Table 2
The relation between concentration of gossypol and percentage of apoptotic cells

Concentration	Apoptosis (%)
Control	16
Gossypol 15 μM	40*
Gossypol 50 μM	54*
Gossypol 100 μM	50*
Gossypol 200 μM	18

* $P<0.01$.

Table 3
The relation between concentration of dexamethasone and percentage of apoptotic cells

Concentration	Apoptosis (%)
Control	12
Dexamethasone 1 μM	16
Dexamethasone 10 μM	40*
Dexamethasone 50 μM	34*
Dexamethasone 100 μM	32*

* $P<0.01$.

well as in that of some other types of cancer (Balci et al., 1999).

Leukemia cells have been investigated to be arrested in the G1- and S-phase by exposure to the glucocorticoid dexamethasone (Renner et al., 2003). Also, 1–100 μM dexamethasone reportedly inhibits the release of NO (Li et al., 2001). In our study, 10–100 μM dexamethasone concentrations revealed apoptotic induction. After dexamethasone treatment, an increase in apoptosis and a decrease in the release of NO were observed, suggesting a different pathway of apoptosis induction from that involving thalidomide.

In most tumors, p53 expression is either the minimum required for essential functions, or zero, so these types of tumors could be resistant to p53-dependent apoptosis. Gossypol-induced apoptosis may be independent of p53 but associated with the regulation of Bcl-2 and Bax expression (Wang et al., 2000).

In conclusion, our findings reveal a correlation between NO release and apoptosis with different dosages of thalidomide and gossypol on K-562 cell line. Further, the common apoptotic effects of these drugs in this p53 deficient cell line suggests us a combination of the drugs will help us to overcome the therapy strategies in resistant cancers that are p53-deficient.

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