Profiling of angiogenic cytokines produced by hormone- and drug-refractory prostate cancer cell lines, PC-3 and DU-145 before and after treatment with gossypol

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ABSTRACT. In this study, we aimed to investigate the angiogenic cytokine profiles of hormone- and drug-refractory prostate carcinoma cell lines, PC-3 and DU-145. We also studied the effect of gossypol, a natural polyphenolic cotton-seed extract, on the angiogenic cytokine profile of these cell lines. XTT cell proliferation assay was used for the assessment of cytotoxicity. For apoptosis, both histone-DNA fragmentation by ELISA assay and caspase 3/7 activity measurement were used. Angiogenic cytokine profiles of supernatants from both cell lines, before and after treatment with gossypol, were investigated using the human angiogenesis antibody array I®. It was shown that the two different hormone- and drug-resistant prostate cancer cell lines, PC-3 and DU-145, constitutively express some important angiogenic cytokines, which are known to regulate tumorigenicity and angiogenesis in hormone-refractory prostate cancer. However, PC-3 and DU-145 cells have distinct angiogenic cytokine profiles. In addition, these two cells lines respond differently to gossypol treatment in terms of cytotoxicity and angiogenic cytokine secretion. After treatment with 10 μM of gossypol, there was a 1.5-fold decrease in angiogenin and IL-8 levels and a 1.7- and 1.8-fold decrease in ENA-78 and GRO-α levels respectively, in DU-145 cells. For PC-3 cells, there were 1.6- and 1.8-fold decreases in IL-8 and VEGF levels, respectively. We conclude that PC-3 and DU-145 cells secrete significant amounts of different angiogenic cytokines that may explain their aggressive nature and metastatic potential. Gossypol treatment affects angiogenic cytokine secretion from these two cell lines in a different manner. By expanding our knowledge of the heterogeneous biological behavior of these two cell lines, novel treatment approaches can be developed for the treatment of prostate cancer.

Keywords: gossypol, PC-3, DU-145, angiogenic cytokines

Prostate cancer is the most common malignancy and the second leading cause of cancer mortality in men [1]. Prostate cancer is a genetically and phenotypically heterogenous disease. This may be a consequence of mutations of different cell types resulting in different malignant maturation pathways. PC-3 and DU-145 are ideal cell models to study the effects and mechanisms of various anticancer agents since they represent very highly aggressive nature of metastatic human prostate cancer [2]. They are both derived from metastases of prostate cancer, either bone or brain, respectively. Both are also androgen receptor-negative cells. However, there are studies showing that they may differ genetically in some ways. For instance, PC-3 cells have higher levels of expression of the anti-apoptotic protein Bcl-2, leading to greater resistance to cytotoxic agents when compared to DU-145 [2, 3]. In addition, these two cell lines also have a distinct p53 gene status; DU-145 has a mutant p53 gene, but PC-3 lacks p53. Moreover, it has been proved that their metastatic potential and response to different cytotoxic agents also differ from each other [4].

Recent data concerning the growth and metastasis of prostate cancer strongly suggest that angiogenesis is a crucial prerequisite for progression to advanced disease. Although many autocrine and/or paracrine angiogenic factors lead tumor-associated angiogenesis, there are some specific angiogenic factors for prostate cancer shown to be linked with its angiogenic potency [5-7]. It is now well documented that tumor progression from its early stages to advanced metastatic state needs establishment of neovasculature. Recent interest in the therapeutic potential of using angiogenesis as a target mechanism for anticancer therapy has led to the identification of various antiangiogenic agents that interfere at various stages of angiogenesis [8].

Gossypol is a natural polyphenolic compound extracted from the cotton plant *(Gossypium species)* and the tropical tree, *Thepesia populnea.* Recently, gossypol was

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reported to have potent anticancer activities in many types of malignancies, including prostate cancer. Gossypol was shown to be a potent inhibitor of Bcl-2/Bcl-$X_{L,}$ however, the exact mechanisms responsible for inhibition of cell growth and stimulation of apoptosis have not been elucidated. There are also limited data in the literature on the antiangiogenic properties of gossypol [9-11].

In the present study, we aimed to investigate the angiogenic cytokine profiles of these two hormone- and drug-refractory prostate carcinoma cell lines, PC-3 and DU-145. We also studied the effect of gossypol on the angiogenic cytokine profile of these cell lines. Comparison of the angiogenic cytokine profiles of these two cell lines will allow a better understanding of the heterogeneous biology of prostate cancer. As a consequence, novel treatment approaches can be developed.

METHODS

Cell lines and reagents

Human hormone- and drug-resistant prostate cancer cell lines, PC-3 and DU145 were obtained from ICLC (Genova, Italy). These cells were grown as monolayers. Adherent cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin in 75 cm^2 polystyrene flasks (Corning Life Sciences, UK), and maintained at 37°C in a humidified atmosphere with 5% CO_2. Growth and morphology were monitored and cells were passaged when they had reached 90% confluence. Cell culture supplies were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Gossypol (> 98% purity) was purchased from Sigma Chemical Co. (USA). A stock solution of gossypol (10^{-2} M) was prepared in DMSO. The DMSO concentration in the assay did not exceed 0.1% and was not cytotoxic to the tumor cells. The final dilutions were made immediately before use, and new stock solutions were made for each experiment. All other chemicals, unless mentioned, were purchased from Sigma.

Viability assay

The cytotoxic effect of gossypol in PC-3 and DU-145 cells was determined by XTT cell proliferation assay (Roche Applied Science, Mannheim, Germany). In short, cells (1 x 10^4 cells/well) were seeded into 96-well flat-bottomed microtiter plates containing 200 µL of growth medium in the presence or absence of increasing concentrations of gossypol at 37°C in 5% CO_2 after verifying cell viability by trypan blue dye exclusion test using Cellometer automatic cell counter (Nexcelom Inc.,USA). After an incubation period of indicated time intervals, 100 µL of XTT (Roche Applied Science, Mannheim, Germany) was added to each well, and plates were incubated at 37°C for another four hours. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter). The mean of triplicate experiments for each dose was used to calculate the IC_{50} value.

Evaluation of apoptosis by histone-DNA fragmentation analysis

Apoptosis was evaluated by enzyme-linked immunosorbent assay (ELISA) using a Cell Death Detection ELISA-PLUS kit (Roche Applied Science, Mannheim, Germany) according to the instruction manual. The relative amounts of mono- and oligonucleosomes generated from the apoptotic cells were quantified using monoclonal antibodies directed against DNA and histones by ELISA. Briefly, cytoplasmic fractions of the untreated control and gossypol-treated cells were transferred onto a streptavidin-coated plate and incubated for two hours at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled antihistone. The plate was washed thoroughly, incubated with 2,29-Azino-di-[3-ethylbenzthiazolinesulfonate] diammonium salt (ABTS), then absorbance was measured at 405 nm with a reference wavelength of 490 nm (DTX 880 Multimode Reader, Beckman Coulter). All experiments were set up in triplicate.

Measurement of caspase 3/7 enzyme activity

Apoptosis was also confirmed by using the Caspase-Glo 3/7 assay (Promega, WI, USA) as described by the manufacturer. Briefly, PC-3 and DU-145 cells, at a concentration of 10^4 cells/well, were plated onto a 96-well plate in 100 µL of culture medium in the presence or absence of increasing concentrations of gossypol for the desired period of time. Then, 100 µL of Caspase-Glo 3/7 reagent was added to each well and the plates were incubated at room temperature for one more hour. Finally, the luminescence of each sample was measured with a luminometer (DTX 880 Multimode Reader, Beckman Coulter).

Cytokine profiling

To determine the profile of angiogenic cytokine production by PC-3 and DU-145 cells, a Human Angiogenesis Antibody Array I (Raybiotech, GA, USA) was used according to the manufacturer's instruction manual. The list of cytokines studied using this technique is presented in *table 1*. The supernatants collected from untreated controls and the cells that were exposed to 10 µM of gossypol for 72 hours were investigated for cytokine profiling. The principle of the method comprised a membrane that was coated with specific antibodies for each cytokine forming an array. After blocking the membrane, the sample was added and incubated at room temperature. Cytokine detection was completed by incubation with a biotinylated antibody followed by horseradish peroxidase-conjugated streptavidin. Chemiluminescence detection of signals was processed by the Kodak® Gel Logic 1500 imaging system. The spots were quantified using a computer-assisted system for image analysis (Koadaarray® 2.6 software); normalized intensities were calculated from each array by first subtracting the local background from each spot and then normalizing by the average intensity of the arrays. The data were then corrected for the cell protein content of each well. The relative expression level of each cytokine was calculated according to both spot pixel mean values ± standard devi-

Table 1
The list of human angiogenic cytokines studied by antibody array (RayBiotech®)

Cytokine	Name	Cytokine Group
ANGIOGENIN	Angiogenin	Cytokine
bFGF	Fibroblast Growth Factor	Growth Factors
EGF	Epidermal Growth Factor	Growth Factors
ENA-78	Epithelia Neutrophil Activating Peptide	Cytokine
IFN-γ	Interferon γ	Cytokine
IL-6	Interleukin 6	Cytokine
IL-8	Interleukin 8	Cytokine
IGF-1	Insulin Like Growth Factor	Growth Factors
GRO-α	Growth Related Gene α	Cytokine
LEPTIN	Leptin	Cytokine
MCP-1	Monocyte Chemoattractant Protein1	Cytokine
PDGF-BB	Platelet Derived Growth Factor	Growth Factors
PIGF	Placental Growth Factor	Growth Factors
RANTES	Regulated Upon Activated Normal T-Cell Expressed And Secreted	Cytokine
TGF-β1	Transforming Growth Factor β1	Cytokine
THROMBOPOIETIN	Thrombopoietin	Cytokine
TIMP-1	Tıssue Inhibitor of Metalloproteinases 1	Anticollagenase
TIMP-2	Tıssue Inhibitor of Metalloproteinases 2	Anticollagenase
VEGF	Vascular Endothelial Growth Factor	Growth Factors
VEGF-D	Vascular Endothelial Growth Factor	Growth Factors

ation (SD) and a confidence index from 0 to 100 assigned to the each spot by the Koadarray® algorithms. The spot pixel mean value represents the background-subtracted, total intensity of each spot. The spot intensity is then given by the total of all the background-substracted values within the spot area. Pixels determined by Koadarray® to be part of an artefact are excluded. The confidence index is based on several parameters, including spot shape, intensity and homogeneity. A value more than 50 ± (SD) indicates a reliable spot. Changes in cytokine expression after exposure to the drugs were expressed as a -fold decrease.

Statistical analysis

Data were analyzed by using GraphPad PRISM 5.0 software (San Diego, CA, USA). All experiments were set up in triplicate and the results were expressed as the mean ± SD. The final value was expressed as the ratio of expression (y axis) ± SD from three experiments. Data were analyzed using Student's *t* test to compare the results in two groups ($p < 0.05$ was considered significant).

RESULTS

PC-3 cells are more resistant than DU-145 cells to the cytotoxic effect of increasing concentrations of gossypol

In order to investigate the growth inhibitory effect of gossypol on prostate cancer cells, PC-3 cells were exposed to different concentrations of gossypol at 24, 48, and 72 h and XTT cell proliferation assay was performed. There were parallel decreases in the percentage cell proliferation in a time- and dose-dependent manner (data not shown). The highest cytotoxicity was observed at 72 h. As shown in *figure 1*, there was a 16, 42, and 70% decrease in proliferation of PC-3 cells exposed to 5, 10, and 20 μM of gossypol, respectively, as compared to the untreated control. The IC_{50} value of gossypol for PC-3 cells at 72 hours was calculated from the dose-response curve and it was found to be 13.6 μM. We performed the same set of experiments with DU-145 cells. There were 2, 52, and 76% decreases in cell proliferation in DU-145 cells exposed to 5-, 10-, and 20 μM gossypol, respectively, as compared to untreated controls *(figure 2)*. The IC_{50} value of gossypol for DU-145 cells, calculated from

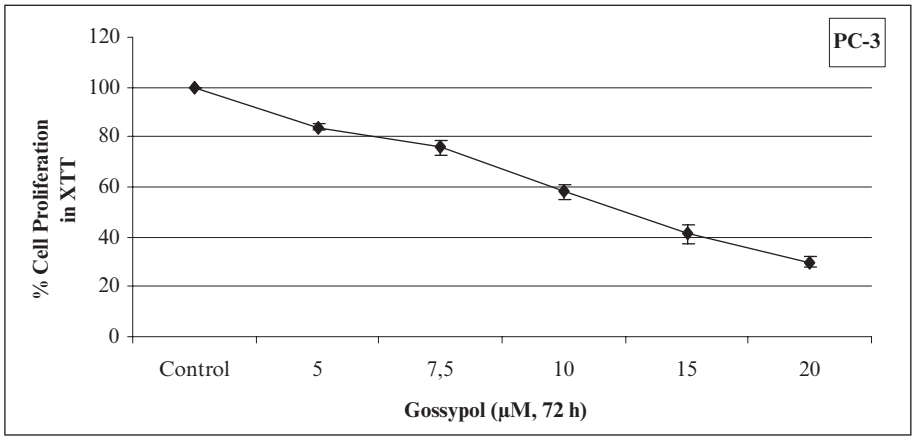

Figure 1
Effect of gossypol treatment on the growth of PC-3 cells. The data represent the mean of three independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant.

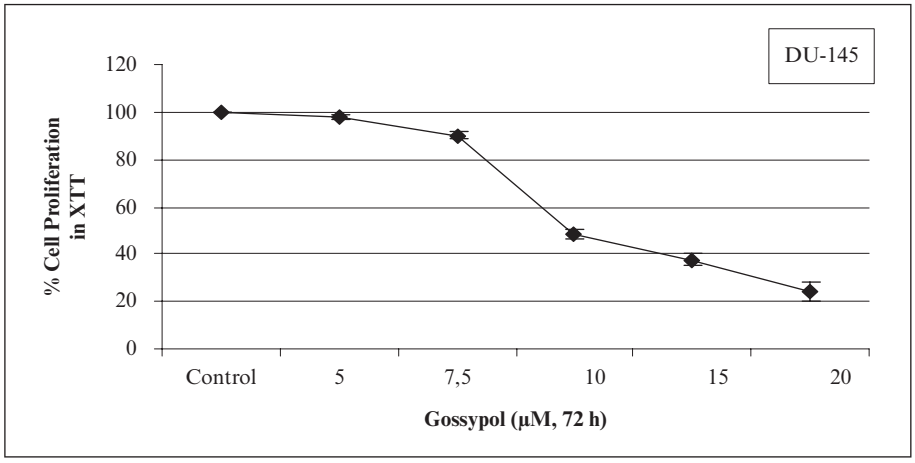

Figure 2
Effect of gossypol on the growth of DU-145 cells. The data represent the mean of three independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant.

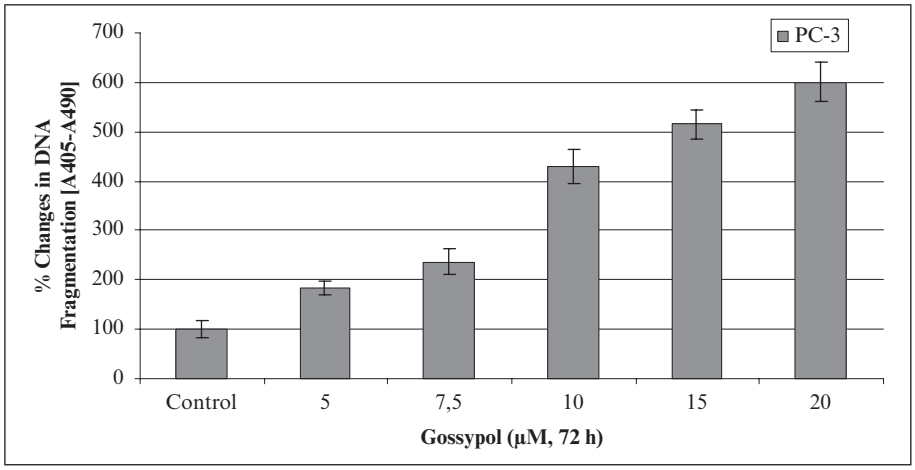

Figure 3
Analyses of apoptosis by DNA fragmentation in response to gossypol in PC-3 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant.

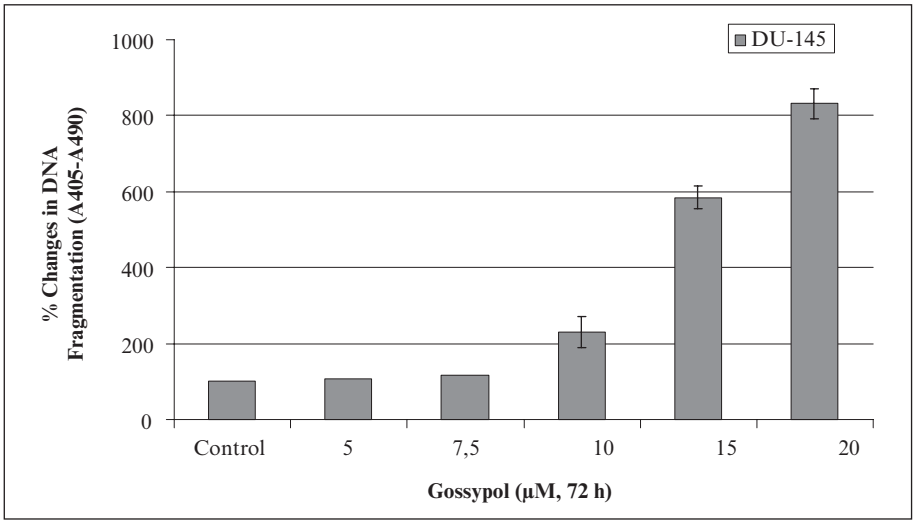

Figure 4
Analyses of apoptosis by DNA fragmentation in response to gossypol in DU-145 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. $p < 0.05$ was considered significant.

the dose-response curve was found to be 10 μM. Similar results were obtained using the trypan blue dye-exclusion assay. These results showed that PC-3 cells are more resistant to the cytotoxic effect of gossypol than DU-145 cells.

DU-145 cells have a higher sensitivity to the apoptotic effect of gossypol when compared to PC-3 cells

To examine the apoptotic effect of gossypol in PC-3 and DU-145 cells, increasing concentrations of gossypol (1.5 to 20 μM) were applied to the cells for 72 hours. The levels of mono-oligonucleosome fragments were quantified using a Cell Death Detection Kit. As shown in *figure 3*, there were 1.85-, 4.3-, and 6-fold increases in histone-DNA fragmentation in 5, 15, and 20 μM gossypol-treated PC-3 cells respectively, compared to the untreated control. Similar results were also observed with DU-145 cells, verifying that gossypol also induces apoptosis in DU-145 cells in a dose-dependent manner. As shown in *figure 4* there were 2.3-, 5.8- and 8.7-fold increases in 10, 15, and 20 μM gossypol-treated DU-145 cells, respectively, as compared to the untreated control *(figure 4)*.

Gossypol-induced apoptosis was also confirmed using the Caspase-Glo 3/7 assay in PC-3 and DU-145 cells. To this aim, different concentrations of gossypol were applied to PC-3 and DU-145 cells for 72 h before the addition of Caspase-Glo 3/7 reagent to each well. The luminescence of each sample was measured using a plate-reading luminometer (DTX 880 Multimode Reader, Beckman Coulter). As shown in *figure 5*, there were 1.7-, 2.8-, and 4.2-fold increases in caspase 3/7 enzyme activity in 5, 7.5, and 10 μM gossypol-treated PC-3 cells after 72 hours incubation respectively, as compared to the untreated control. On the other hand, caspase 3/7 enzyme activity was examined in DU-145 cells treated with increasing concentrations of gossypol. There were 2-, 4.9-, and 8.1-fold increases in caspase 3/7 enzyme activity in 5, 7.5, and 10 μM gossypol-treated DU-145 cells, respectively, as compared to the untreated controls *(figure 6)*.

Our data clearly showed that gossypol induces apoptosis significantly in both cell lines in a dose-dependent manner, but DU-145 cells are more prone to apoptosis than PC-3 cells with the same doses of gossypol.

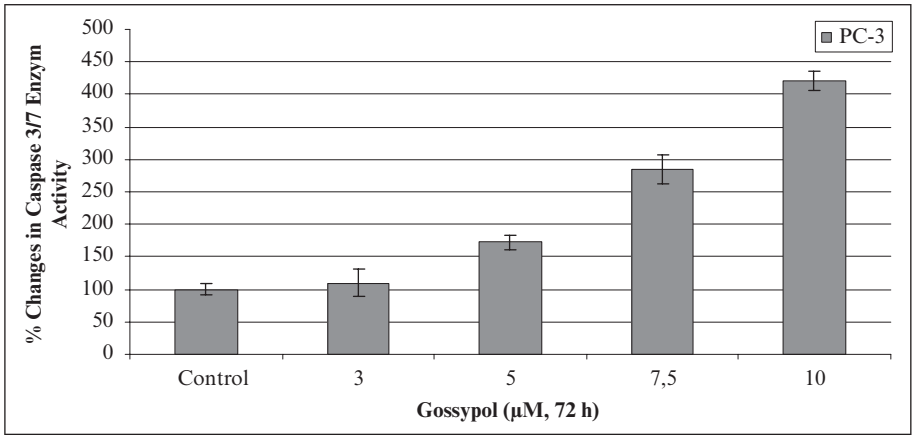

Figure 5

Analyses of apoptosis by caspase-3/7 enzyme activity in response to gossypol in PC-3 cells. The error bars represent the standard deviations. $p < 0.05$ was considered significant.

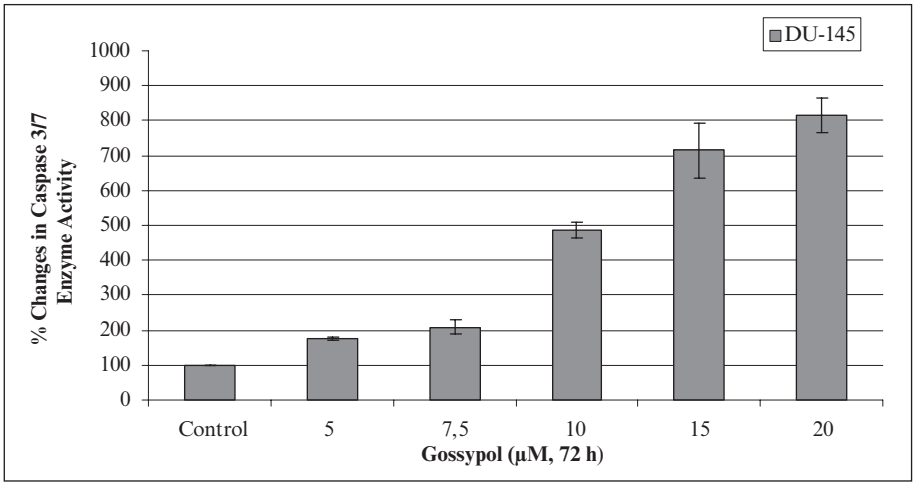

Figure 6

Analyses of apoptosis by caspase-3/7 enzyme activity in response to gossypol in DU-145 cells. The error bars represent the standard deviations. $p < 0.05$ was considered significant.

Angiogenic cytokine profiling of PC-3 and DU-145 cells

To expand our understanding of the heterogeneous biology of PC-3 and DU-145 cells, we profiled angiogenic cytokines secreted by these cell lines. Both cell lines constitutively secrete some important angiogenic factors as listed in *table 2* (PC-3 cells) and *table 3* (DU-145 cells). However, the angiogenic cytokine profile was different in the two cell lines. For example, ENA-78, TIMP-2, EGF, bFGF, and PIGF were cytokines secreted by DU-145 cells, but not by PC-3 cells. On the other hand, TIMP-1 and thrombopoietin were secreted by PC-3 cells, but not by DU-145 cells. Moreover, there was a -fold change difference in expression levels of some angiogenic cytokines secreted by both cell lines. For instance, secretions of GRO-α, MCP-1 and angiogenin were 1.6-, 1.7 and 1.5-fold higher respectively, in DU-145 cells compared to PC-3 cells ($p < 0.05$), but, the secretion levels for IL-8 and VEGF were 1.3- and 1.5-fold higher, respectively, in PC-3 cells ($p < 0.05$).

The effect of gossypol treatment on the secretion of angiogenic cytokines by PC-3 and DU-145 cells

As described above, gossypol has significant cytotoxic and apoptotic effect in PC-3 and DU-145 cells. However, PC-3 and DU-145 cells have different angiogenic cytokine profiles. We also investigated the effect of gossypol treatment on the angiogenic cytokine profiles of these cells. It was found that 10 μM of gossypol treatment, which was the optimal effective dose for both cell lines, resulted in different angiogenic cytokine secretion changes in PC-3 and DU-145 cells. There was a 1.5-fold decrease in angiogenin and IL-8 levels; a 1.7- and 1.8-fold decrease in ENA-78 and GRO-α levels respectively, in DU-145 cells, but there was no change in VEGF secretion. There was a significant decrease in IL-8 secretion (1.6-fold, $p < 0.05$) in PC-3 cells, as in DU-145 cells *(figure 7)*. However, unlike DU-145 cells, VEGF levels decreased significantly (1.8-fold, $p < 0.05$) in PC-3 cells after gossypol treatment. There were no changes in angiogenin and GRO-α secretion in PC-3 cells ($p > 0.05$). These results showed that gossypol inhibited secretion of angiogenic cytokines in PC-3 and DU-145 cells differently, and this is strongly related to the metastatic potency of these cells.

Table 2
The angiogenic cytokine profile of PC-3 before gossypol treatment. The relative expression level of each cytokine was calculated according to both spot pixel mean values ± standart deviation (SD) and a confidence index from 0 to 100 assigned to the each spot by the Koadarray® algorithms

Cytokine	Name	Spot Pixel Mean Value ± SD	Confidence Index ± SD
Angiogenin	Angiogenin	43.0 ± 2,3	52.5 ± 0.7
IGF-I	Insulin Like Growth Factor	16.0 ± 1.4	59.0 ± 0.7
PDGF-BB	Platelet Derived Growth Factor BB	16.5 ± 0.2	70.1 ± 2.1
VEGF	Vascular Endothelial Growth Factor	210.5 ± 2.1	86.5 ± 4.9
VEGF-D	Vascular Endothelial Growth Factor D	30.5 ± 2.4	70.0 ± 2.3
GRO-α	Growth Related Gene α	162.5 ± 3.8	60.5 ± 3.5
IFN-γ	Interferon γ	26.5 ± 5.7	68.5 ± 4.9
IL-6	Interleukin 6	20.5 ± 0.7	72.0 ± 2.3
IL-8	Interleukin 8	250.5 ± 4.9	87.0 ± 2.9
LEPTIN	Leptin	44.5 ± 0.7	60.5 ± 0.7
MCP-1	Monocyte Chemoattractant Protein 1	23.5 ± 2.5	51.5 ± 0.7
Thrombopoietin	Thrombopoietin	35.5 ± 2.3	65.5 ± 5.7
TIMP-1	Tıssue Inhibitor of Metalloproteinases 1	52.0 ± 4.2	61.5 ± 4.9

Table 3
The angiogenic cytokine profile of DU-145 before gossypol treatment. The relative expression level of each cytokine was calculated according to both spot pixel mean values ± standart deviation (SD) and a confidence index from 0 to 100 assigned to the each spot by the Koadarray® algorithms

Cytokine	Name	Spot Pixel Mean Value ± SD	Confidence Index ± SD
Angiogenin	Angiogenin	64.5 ± 2.1	78.7 ± 2.6
bFGF	Fibroblast Growth Factor	28.5 ± 3.5	71.5 ± 4.9
EGF	Epidermal Growth Factor	42.0 ± 1.4	71.5 ± 2.1
IGF-I	Insulin Like Growth Factor	16.5 ± 2.1	57.0 ± 1.4
PDGF-BB	Platelet Derived Growth Factor BB	34.7 ± 2.1	68.5 ± 0.7
PIGF	Placental Growth Factor	32.5 ± 2.1	57.5 ± 3.5
VEGF	Vascular Endothelial Growth Factor	140.3 ± 2.1	57.6 ± 5.6
VEGF-D	Vascular Endothelial Growth Factor D	39.5 ± 0.7	69.5 ± 2.1
GRO-α	Growth Related Gene α	260.0 ± 1.4	97.0 ± 4.2
ENA-78	Epithelia Neutrophil Activating Peptide	32.5 ± 2.1	79.5 ± 0.7
IFN-γ	Interferon γ	23.0 ± 0.7	68.5 ± 4.9
IL-6	Interleukin 6	35.0 ± 0.7	75.0 ± 0
IL-8	Interleukin 8	192.3 ± 5.6	66.9 ± 2.1
LEPTIN	Leptin	50.0 ± 2.1	59.0 ± 0
MCP-1	Monocyte Chemoattractant Protein 1	39.9 ± 3.5	87.5 ± 0.7
TIMP-2	Tıssue Inhibitor of Metalloproteinases 2	112.5 ± 0.7	57.5 ± 0.7

DISCUSSION

Tumors originating from the same organ may give different responses to chemo- and radiotherapy. For this reason, it is important to expand our knowledge of cancer cells to understand the tumor's pleiotropic, biological behavior. Metastasis is accepted as a complex process of tumors being influenced by many tumor-host reactions [12, 13]. The metastasis of the tumor is mainly associated with tumor-related angiogenic cytokines. The balance between stimulatory and inhibitory factors originating from both the tumor and microenvironment regulates angiogenesis [13]. Thus, it is important to determine the angiogenic factors leading to metastasis in prostate cancer.

Although PC-3 and DU-145 are both androgen receptor-negative and drug-resistant prostate carcinoma cell lines,

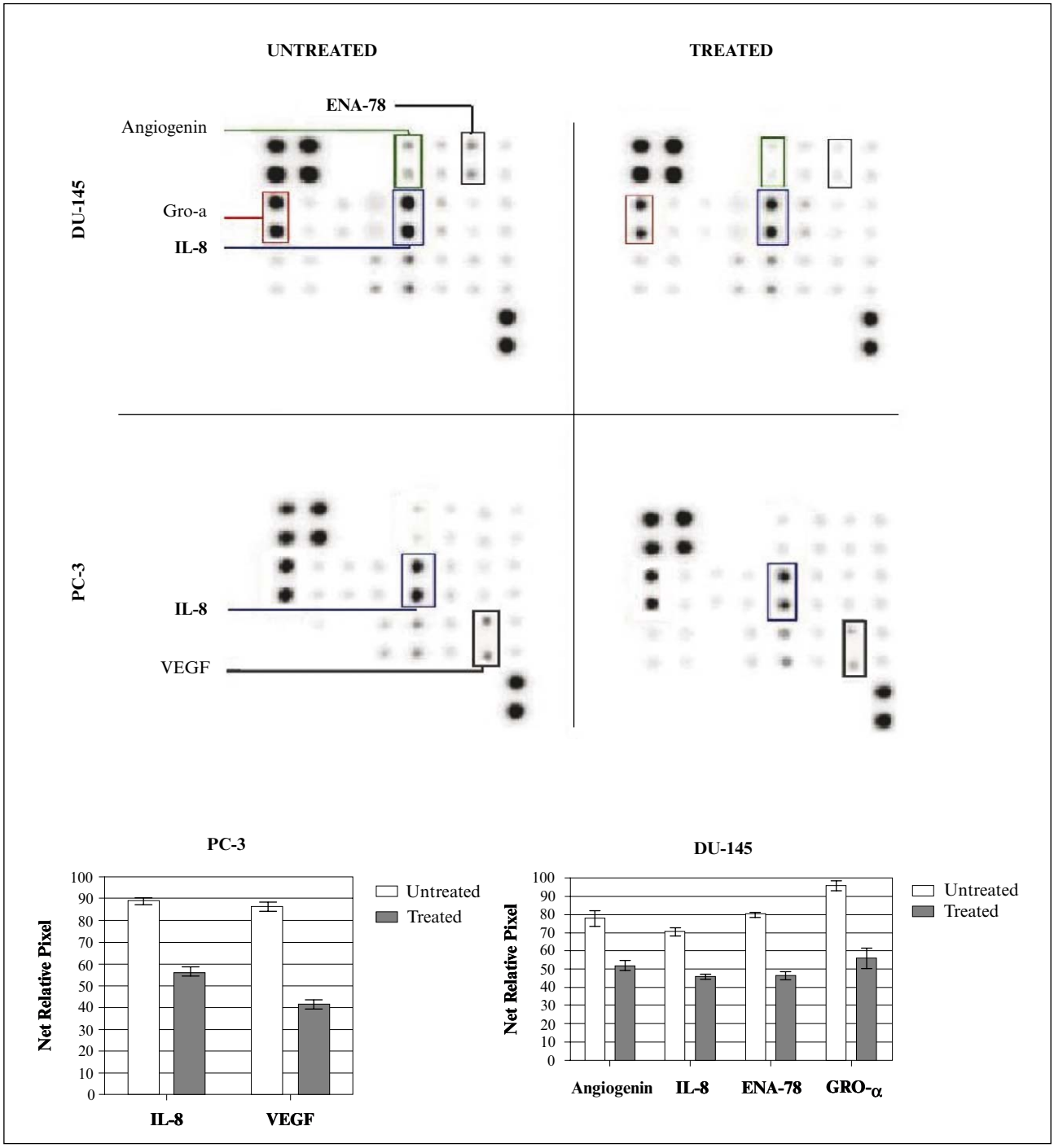

Figure 7
The cytokine expression profile of untreated and 10 μM gossypol-treated DU-145 (upper figure) and PC-3 (lower figure) cells. PC-3 and DU-145 cells responded differently to gossypol treatment in terms of angiogenic cytokine secretion. $p < 0.05$ was considered significant.

there is a body of evidence in the literature that indicates that they respond differently to chemotherapeutic agents. Skjoth and Issınger have shown that the DU-145 cell line was 20-fold more sensitive to cisplatin-induced apoptosis compared to the PC-3 cell line [2]. These results were also consistent with our study demonstrating that DU-145 cells were more prone to apoptosis with the same doses of gossypol compared to PC-3 cells.

We have shown that two different hormone- and drug-resistant prostate cancer cell lines, PC-3 and DU-145, constitutively express some important angiogenic cytokines which are known to regulate tumorigenicity and angiogenesis in hormone-refractory prostate cancer. However, as we show in *table 2 and 3*, the angiogenic cytokine profile of the two cell lines differed from each other, demonstrating their heterogeneous biological behavior.

CXC type cytokines are present in every kind of cell, and have been shown to be associated with angiogenesis. Depending on the presence or absence of the 'ELR' motif (Glu-Leu-Arg) preceding the first cysteine amino acid in the NH_2 terminal, there are two subclasses of these mole-

cules [14]. The first one containing the ELR motif has angiogenic properties. Growth-related genes (*GRO-α*, *GRO-β*, *GRO-γ*), IL-8 and ENA-78 are the members of this ELR-positive subclass [14, 15]. GRO-α has been shown to be a mitogenic and angiogenic factor for some types of human cancer including hormone-refractory prostate cancer [14]. Several studies in the literature have demonstrated that GRO-α, as well as IL-8 and ENA-78, play an important role in tumor progression and metastasis by inducing the angiogenic process in non-small cell lung carcinoma [16, 17], stomach cancer [18], melanoma [19] and prostate cancer [20]. Expression of IL-8 and ENA-78 in prostate cancer is associated with its metastatic potency [21, 22]. It has been shown that GRO-α is a main regulator of neovascularisation and tumorigenesis in the DU-145 cell line, however,the PC-3 cell line uses IL-8 as a major cytokine for angiogenesis [20]. Consistent with these results, we have also demonstrated that GRO-α expression was 1.6-fold higher in DU-145 cells compared to PC-3, and IL-8 expression was 1.3-fold higher in PC-3. On the other hand, ENA-78 was secreted by DU-145 cells, but not by PC-3 cells. Monocyte chemoattractant protein-1 (MCP-1) is a member of CCβ cytokine family and it strongly induces monocyte and macrophage chemotaxis to inflammatory sites [23, 24]. However, recent studies have shown that MCP-1 may also have a direct effect on the epithelial cells of many tumor types, leading migration of those cells, and resulting in a metastatic process. Loberg *et al.* showed that MCP-1 has a role in tumorigenesis and metastasis of prostate cancer by a direct inducer effect on cell proliferation and angiogenesis [25]. In parallel with these results, Lu and co-workers observed a correlation between expression of MCP-1 and the stage of prostate cancer patients [26]. These studies demonstrated that MCP-1 is not only an important cytokine acting in the inflammatory state in cells, but also a cytokine present in the tumor. Data from the present study show that it is secreted by both cell lines: however, its expression is 1.7-fold higher in DU-145 cells.

Angiogenin is another cytokine worthy of mention. This is secreted by both cell lines. It is a 14-kDa molecule, which was first identified in HT-29 colon adenocarcinoma cells [27]. Angiogenin has the particular feature of acting in a dual-role in proliferation and angiogenesis for prostate cancer. Human angiogenin is up-regulated in prostate epithelial cells, promoting them to invasive prostate carcinoma cells. In a study by Yoshioka *et al*., it was shown that knocking down angiogenin expression in PC-3 cells inhibited cell proliferation by 65%. In our study, angiogenin secretion was 1.5-fold higher in DU-145 cells compared to PC-3.

Among the cytokines mentioned here, VEGF is of unique importance in the process of angiogenesis. VEGF is hypothesized to be a critical regulator of angiogenesis during prostate carcinogenesis [28]. VEGF, originally described as vascular permeability factor, has been implicated as one of the most important proangiogenic growth factors during angiogenesis. Increases in VEGF levels are associated with the progression of prostate cancer to locally advanced and metastatic disease with the development of androgen resistance [29]. Accumulated evidence shows that both cell lines, PC-3 and DU-145, express significant levels of VEGF [30, 31]. In our study, VEGF expression was 1.5- fold higher in PC-3 compared to DU-145, which is consistent with the existing literature. This may be explained by the more aggressive nature of PC-3 cells.

Gossypol treatment does influence the secretion of some of the angiogenic cytokines mentioned above. However, both cell lines responded differently to gossypol treatment. This could be explained by genetic differences between two cell lines. Gossypol treatment decreased the secretion of angiogenin and the CXC cytokine family (GRO-α, IL-8 and ENA-78) in DU-145 cells. On the other hand, IL-8 and VEGF levels were decreased significantly in PC-3 cells, but angiogenin and GRO-α secretion were not affected. It is worth mentioning that both cell lines secrete VEGF, but a significant decrease was seen after gossypol treatment only in PC-3 cells. It is known that the overexpression of Bcl-X_L induces increased expression of proangiogenic cytokines [32]. It is also known that gossypol is a potent inhibitor of Bcl-2/Bcl-X_L in prostate cancer cells. Inhibition of Bcl-2/Bcl-X_L may be possibly be one of the routes for inhibition of angiogenesis by gossypol in prostate cancer.

In conclusion, data presented here provide evidence that PC-3 and DU-145 cells secrete significant amounts of different angiogenic cytokines. This may explain their aggressive nature and metastatic potency. It may also explain why they have distinct biological behaviors. Furthermore, they responded to gossypol treatment in a distinct manner in terms of cytotoxicity and angiogenic cytokine secretion. By expanding our knowledge of the heterogeneous, biological behavior of these two cell lines, novel treatment approaches can be developed for the treatment of prostate cancer.

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