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Chemotherapeutic drugs and human tumor cells cytokine network

Vera Levina¹, Yunyun Su^{2,4}, Brian Nolen^{1,4}, Xiaoning Liu^{2,4}, Yuri Gordin⁴, Melissa Lee⁴, Anna Lokshin^{1,4}, and Elieser Gorelik^{2,3,4}

¹Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA

²Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, PA

³Department of Immunology, School of Medicine, University of Pittsburgh, Pittsburgh, PA

⁴University of Pittsburgh Cancer Institute, Pittsburgh, PA

Abstract

The ability of human tumor cell lines to produce various cytokines, chemokines, angiogenic and growth factors was investigated using Luminex multiplex technology. Media conditioned by tumor cells protected tumor cells from drug-induced apoptosis and stimulated tumor cell proliferation. Antibodies neutralizing IL-6, CXCL8, CCL2 and CCL5 blocked this stimulation. Treatment of tumor cells with doxorubicin and cisplatin resulted in a substantial increase in the production of IL-6, CXCL8, CCL2, CCL5, BFGF, G-CSF, and VEGF. This stimulation was associated with drug-induced activation of NF- κ B, AP-1, AP-2, CREB, HIF-1, STAT-1, STAT-3, STAT-5 and ATF-2 transcription factors and up-regulation of *IL-6*, *CXCL8*, *FGF-2*, *CSF-3* and *CCL5* gene expression. Treatment of tumor cells with doxorubicin and antibodies neutralizing G-CSF, CCL2 or CCL5 had higher inhibitory effects than each modality used alone. These results indicate that chemokines and growth factors produced by tumor by binding to the cognate receptors on tumor and stroma cells could provide proliferative and anti-apoptotic signals helping tumor to escape drug-mediated destruction. Clinical studies showed that antibodies neutralizing VEGF (Avastin/ Bevacizumab) or blocking HER2/neu signaling (Herceptin/ Trastuzumab) could increase the efficacy of chemotherapy although these beneficial effects have been limited. It is possible that drug-stimulated production of growth and pro-angiogenic factors could counterbalance the effects of antibody therapy. In addition, numerous growth factors and chemokines share angiogenic and growth-stimulating properties, and thus reduction of a single factor is insufficient to completely block tumor growth. Thus, a broad disruption of tumor cytokine network is needed to further increase the efficacy of cancer therapy.

Keywords

tumor cells; drugs; cytokines; chemokines; angiogenic factors

Introduction

The ability of tumor cells to produce various cytokines, chemokines, angiogenic and growth factors is crucial for tumor cell proliferation and the formation of stroma and blood vessel networks to provide oxygen and nutrients and support progressive tumor growth. Tumor-

produced VEGF is the most potent angiogenic factor, which stimulates migration and proliferation of endothelial cells and formation of blood vessels 2-3. Malignant transformation is often associated with overexpression of various growth factors including FGF, EGF, and HGF, all of which stimulate proliferation of tumor cells as well as stromal cells and manifest potent angiogenic effects 4-8.

Tumor cells also produce cytokines and chemokines such as IL-6, IL-8, IL-10, CCL2 (MCP-1), and CCL5 (RANTES) that have complex autocrine and paracrine effects in tumors. IL-6 is a pleiotropic pro-inflammatory cytokine that affects B- and T-cell differentiation, induces acute phase reactant production, and stimulates hematopoiesis. It has been shown that IL-6 could directly stimulate proliferation of tumor cells and manifests a potent angiogenic effect 9-11. Some reports indicate that IL-6 levels correlate with disease bulk and inversely correlate with response to treatment and survival 12. IL-8 belongs to the superfamily of CXC chemokines and has a wide range of pro-inflammatory effects. It stimulates migration of neutrophils, monocytes, and lymphocytes, and promotes tumor cell proliferation and metastasis 13-15. In addition, IL-8 exhibits strong angiogenic activity 13-16-17. Chemokines of the CC superfamily such as CCL5 (RANTES) and CCL2 (MCP-1) also are able to stimulate migration of normal and malignant cells, as well as promote tumor angiogenesis 18-22. Cytokines IL-10 and TGF- β are potent immunosuppressive factors that play an important role in protecting tumors from immune-mediated destruction 23-24.

The production of cytokines, growth and angiogenic factors is not an exclusive property of tumor cells. Stroma cells such as endothelial cells, fibroblasts, lymphocytes, and macrophages also produce various cytokines, chemokines, growth and angiogenic factors that could affect survival and proliferation of stroma cells as well as tumor cells. Thus, there exists perpetual cross-talk between tumor and stromal cells. Tumor- and stroma-produced soluble factors represent a tumor cytokine network that plays an important role in tumor growth and tumor protection from endogenous (hypoxia, oxygen free radicals) and exogenous (drugs, x-irradiation) damage. Overproduction of these factors by growing tumors could lead to the observed increase in blood levels of cytokines, chemokines, angiogenic and growth factors often associated with resistance to therapy and overall poor prognosis 12-13-25-27.

Thus, different types of tumor and stroma producing factors (cytokines, chemokines, angiogenic and growth factors) have overlapping functions in promoting tumor growth and thus, blocking of a single factor may not be sufficient to inhibit tumor growth as other factors are able to compensate for lost function. However, the breadth and magnitude of the complete network of cytokine and tumor-produced factors remains largely uninvestigated, and a comprehensive analysis of the cytokine network signature of various human tumor cells is therefore well warranted.

The importance of targeting angiogenic and growth factor signaling is well established. Humanized monoclonal antibodies (Avastin/Bevacizumab) that neutralize the angiogenic factor VEGF used in combination with chemotherapy have demonstrated a significant clinical benefit. Similarly, blocking HER2/neu signaling with a monoclonal antibody (Herceptin/Trastuzumab) may improve the therapeutic effects of chemotherapy in patients with metastatic breast cancer.

It remains unclear what effects chemotherapy has on tumor-produced cytokines. It is conceivable that killing tumor cells and/or inhibiting their DNA, RNA, and protein synthesis by chemotherapeutic drugs could reduce intratumor production of cytokines, chemokines, angiogenic and growth factors, however direct confirmation of this possibility remains elusive. In fact, the few studies in which the effect of chemotherapeutic drugs on cytokine

production by tumor cells was investigated suggested the opposite. Namely, dacarbazine treatment of melanoma cell lines stimulated IL-8 and VEGF production 30-31. Augmentation of IL-8 and TNF- α production was observed when human epithelial carcinoma cell lines were treated with etoposide and mitomycin C 32 and doxorubicin increased production of IL-8 and MCP-1 in human small cell lung cancer cell lines 33.

It is possible that drug treatment induces a cellular stress response resulting in the production of cytokines, chemokines, angiogenic and growth factors that, by binding to their receptors on tumor and stroma cells, provide pro-survival signaling that protects these cells from drug-induced apoptosis and stimulates their proliferation.

The broad investigation of tumor cell cytokine networks and analysis of the effects of drug on cytokine production is largely limited by the low throughput of conventional ELISA, which typically allows testing of only one cytokine at a time. In order to overcome this problem, we applied Fluorokine® MultiAnalyte Profiling (xMAP™) technology developed by Lumindex, Inc. (Austin, TX) to analyze simultaneously the production of 30 different cytokines, chemokines, angiogenic and growth factors by human H460 and A549 non-small cell lung cancer (NSCLC) lines, as well as MCF-7 and CAMA-1 breast tumor cell lines. The ability of chemotherapeutic drugs to affect production of these factors and mechanisms responsible for the drug-induced effect on cytokine production were investigated.

Materials and Methods

Cell Lines

Human H460 and A549 non-small cell lung cancer (NSCLC) cell lines, MCF-7 and CAMA-1 breast tumor, K562 erythroleukemia, LOX and FEMx melanoma and OVCAR-3 ovarian cancer cells tumor cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 supplemented with 10% FBS and antibiotics (Millipore Inc., Billerica, MA).

Reagents

Doxorubicin, cisplatin, bleomycin, etoposide, and monensin were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Neutralizing monoclonal antibodies (mAbs) against IL-10, G-CSF and CCL5 (RANTES) were obtained from BioVision Inc. (BioVision, Mountain View, CA). Neutralizing mAbs against TGF- β 1 and TGF- β 2 were obtained from R&D Systems (R&D Systems Inc., Minneapolis, MN). MAb against human MCP-1 (CCL2), VEGF, bFGF, IL-6 were purchased from Calbiochem (EMD Biosciences, Inc., San Diego, CA). Anti-IL-8 mAb was obtained from Abcam Inc. (Abcam, Cambridge, MA).

Drug treatment and proliferation assays

Tumor cells were seeded into 96-well or 24-well plates. The next day, drugs were added at indicated concentrations. Three days later, plates were centrifuged and supernatants from 4-6 wells were collected into 2-3 pools and frozen. Cell counts were estimated using the One Solution Cell Proliferation Kit (Promega, Madison, WI). In some experiments, cells harvested from T-25 flasks or 24-well plates were counted using a hemacytometer, whereas cells growing in 96-well plates were fixed with 2% paraformaldehyde (PFA), stained with Hoechst 33342 (2 μ g/mL), and counted using Cellomics Array Scan VTI (Cellomics/ThermoFisher, Pittsburgh, PA), as described.

Multiplex analysis of cytokine production by tumor cells

Concentrations of various tumor cell-produced factors (TPFs) in media conditioned by tumor cells were analyzed using multiplex bead-based sandwich immunoassay technology

(xMAP™, Luminex Corp., Austin, TX) using a 30-plex kit from Invitrogen/Biosource (Camarillo, CA) for testing the following cytokines: IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-13, IL-15, IL-17, GM-CSF, IFN- α , IFN- γ , TNF α ; chemokines: CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CCL11 (EOTAXIN), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10); growth factors: bFGF, EGF, G-CSF, HGF and VEGF. Analyses of tumor supernatants were performed in a 96-well microplate format according to the protocol by Invitrogen, as previously described 35, 36.

The analyte concentrations (pg/ml) were calculated as pg/1 \times 10⁶ tumor cells as follows:

$$\frac{\text{concentration of analyte (pg/ml)} \times \text{volume of media (ml/well)} \times 1 \times 10^6 \text{ cells}}{\text{number of tumor cells per well}}$$

Multiplex analysis of transcription factors

H460 cells were treated with doxorubicin for 0, 15, 30 or 60 min, nuclear extracts were prepared and levels of nine transcription factors (AP-1, AP-2, ATF-2, CREB, HIF-1, NF κ B, STAT 1, STAT 3, and STAT-5) were analyzed using the multiplex Procarta Transcription Factor Assay Kit (Panomics, Fremont, CA; P/N PC5101). Briefly, nuclear extracts were normalized for protein content and incubated with a mixture of biotin-labeled double-stranded oligonucleotide probes to form protein/DNA complexes. Unbound probes were then removed by filtration followed by elution of the complexes from the filter matrix. Bound probes were denatured and hybridized with transcription factor-specific antisense oligonucleotide-conjugated microbeads. Probe-bound microbeads were detected with streptavidin-conjugated R-phycoerythrin and analyzed using the Bio-Plex Luminex 100 system (Bio-Rad, Hercules, CA).

Quantative real-time PCR of cytokines gene expression

H460 tumor cells were treated with doxorubicin (0.125 μ g/ml) for 6h. RNA was extracted and cDNA was produced using the SuperArray ReactionReady First Strand cDNA Synthesis kit (SuperArray Bioscience). Real-time PCR was carried out using the SuperArray RT² Real-Time™ SYBR Green PCR Master Mix (SuperArray Bioscience) and was performed on the ABI Prism™ 7700 sequence detector real-time PCR system (AB Applied Biosystems). PCR conditions were as follows: denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Primers for human *IL-6*, *IL-8*, *VEGF*, *CCL5 (RANTES)*, *FGF2*, *CSF3 (G-CSF)* and *GAPDH* genes were from SuperArray RT2 PCR Primer Sets. Standard curves were generated from five 10-fold serial dilutions of tumor cells' cDNA, and no product could be seen in the no-template control. Differences in gene expression in doxorubicin-treated and untreated H460 tumor cells were calculated using the 2(- $\Delta\Delta$ Ct) method according to the manual from SuperArray Bioscience.

Analysis of the intracellular cytokines in tumor cells

H460 tumor cells (1 \times 10⁴/well) grown in 96-well plates were incubated overnight with doxorubicin (0.125 μ g/ml) then monensin (2 μ M) was added for 3h. Cells were fixed in 2% PFA, washed, permeabilized with 0.1% Triton X-100, washed with FACS buffer and then incubated with antibodies against various cytokines for 1h and after washing with secondary anti-mouse antibodies conjugated with Alexa 488, 546, or 680 fluorochromes (Molecular Probes/Invitrogen) for 1 h. Cell nuclei were stained with Hoechst 33342 at 2 μ g/ml for 20 min to identify individual cells.

Cell images were acquired using the Cellomics ArrayScan HCS Reader (Cellomics/ ThermoFisher, Pittsburgh, PA) and analyzed using the Target Activation BioApplication Software Module. The Cellomics ArrayScan HCS Reader collects information concerning distribution of fluorescently labeled components in stained cells. The scanner is equipped with emission and excitation filters (XF93, Omega Optical, Brattleboro, VT, USA) for selectively imaging fluorescent signals emitted by Hoechst 33342, Alexa 488, and Alexa 680. Data were captured, extracted and analyzed with ArrayScan II Data Acquisition and Data Viewer version 3.0 (Cellomics), Quattro Pro version 10.0.0 (Corel, Ottawa, Ontario, Canada), and MS Excel 2002 (Microsoft, Redmond, WA).

Effect of tumor-conditioned media (TCM) on tumor cell proliferation and drug-induced apoptosis

H460 tumor cells were cultured for 3 days in RPMI 1640 supplemented with 10% or 1% FBS until 80% confluence was achieved. TCM was collected and stored at -80°C . H460 cells were plated into 96 wells (1×10^4 cells/well) in fresh medium (FM) or TCM. The next day, doxorubicin ($0.125 \mu\text{g/ml}$) was added. The numbers of cells was determined after 3 days in culture using the One Solution Cell Proliferation Kit (Promega) or the Cellomics ArrayScan HCS Reader.

To assess the ability of TCM to protect tumor cells from doxorubicin-induced apoptosis, H460 tumor cells were cultured in FM or TCM in the presence of doxorubicin ($0.125 \mu\text{g/ml}$) for 6 or 20 h. Cells were stained with Annexin V-FITC and propidium iodine (PI) and by flow cytometry.

Effect of neutralizing antibodies (Abs) on tumor cells proliferation and sensitivity to doxorubicin

H460 cells were plated in 96-well plates (1.0×10^4 cells/ml) in culture medium with 10% FBS. Next day culture medium was replaced by FM supplemented with 1% FBS or TCM with 1% FBS. Cells were pre-treated with mAbs neutralizing different cytokines (100 ng/ml) for 1 h, and then doxorubicin ($0.125 \mu\text{g/ml}$) was added. After 72h of cultivation, samples of conditioned media were collected and cells were fixed in PFA and stained with Hoechst 33342. Cells were counted using Cellomics Array Scan VTI.

Statistical analysis

Data presented as mean \pm SD. Comparisons between the values were performed using a two-tailed Student's t-test. For the comparison of multiple groups, a one- or two-way ANOVA test was applied. For all statistical analyses, the level of significance was set at a probability of $P < 0.05$. All experiments were repeated 2-3 times.

Results

Analysis of soluble factors produced by human tumor cell lines

Human H460 and A549 non-small cell lung cancer, MCF-7 breast tumor, K562 erythroleukemia, LOX and FEMx melanoma, and OVCAR-3 ovarian cancer line were cultured for 3 days in T-25 flasks, supernatants were collected, tumor cells were counted and concentrations of 30 different cytokines, chemokines, angiogenic and growth factors in the supernatants were analyzed using Multiplex xMAP™ technology. In comparison to other cell lines, H460 lung tumor and OVCAR-3 ovarian cancer cell lines produced high levels of proangiogenic IL-6 and CXCL8 (IL-8). Most of tumor cell lines produced high levels of angiogenic factor VEGF (Table1). The highest level of VEGF was found in the supernatant of OVCAR-3 cells, whereas LOX melanoma was the lowest producer of VEGF. All tested lines, except MCF-7 and OVCAR-3, produced high levels of G-CSF. FEMx melanoma line

was a high producer of chemokine CCL5 (RANTES). Slow growing MCF-7 cells produced lower levels of growth and angiogenic factors compared with other cell lines (Table1).

Thus, tumor cells of different histological origin vary in their ability to produce various molecules essential for tumor cell proliferation, angiogenesis and migration of tumor and inflammatory cells. However, tumor cell lines of the same histological origin also showed different capacity in production of some factors. Some tested factors, such as IL-1 β , IL-2, IL-4, IL-5, IL-12, IFN- γ , TNF- α , GM-CSF, Eotaxin, IL-13, IP-10, MIG were undetectable or produced in very low quantities by tested cell lines.

Effect of anticancer drugs on the production of TPFs

The level of tumor cells produced factors (TPFs) could be a function of tumor cell number. To test this, H460 cells were seeded at 10×10^4 to 1.25×10^4 cells/well onto 96-well plate and supernatants were collected after 24 h of culture. Production of VEGF, IL-6 and IL-8 by H460 tumor cells was found to be proportional to the number of tumor cells. A reduction in cell number by only 2.5×10^4 tumor cells resulted in a proportional reduction in VEGF, IL-6 and IL-8 levels (Fig 1). Based on the correlation between tumor cell number and levels of produced factors, it is reasonable to expect that drugs that cause a reduction in tumor cell number might likewise reduce concentrations of the secreted TPFs. To investigate this possibility, H460 cells were treated for 3 days with the following drugs: doxorubicin (1.0, 0.5 and 0.25 $\mu\text{g/ml}$), cisplatin (1.5, 0.75, 0.38 $\mu\text{g/ml}$), bleomycin (4, 2, and 1 $\mu\text{g/ml}$) and etoposide (1.0, 0.5, and 0.25 $\mu\text{g/ml}$). Bleomycin is not a drug of choice for NSCLC and was used in our experiments to test whether any chemotherapeutic drugs are able to affect cytokine production and whether the patterns of changes are similar or different for different drugs. Bleomycin in comparison to doxorubicin, cisplatin and etoposide was less efficient in reduction of H460 cell numbers (Fig. 2). All drugs were able to significantly reduce the overall concentration of secreted VEGF, although bleomycin and etoposide were less effective than doxorubicin and cisplatin (Fig 1). IL-6 production was more resistant to drug treatment and was inhibited only at the two highest concentrations of doxorubicin and cisplatin. No inhibition of IL-6 was observed in the groups treated with bleomycin and etoposide at any tested concentration (Fig. 2). Similar results were obtained when CXCL8 (IL-8) levels were analyzed (data not shown). Although treatment of H460 cells with doxorubicin and cisplatin substantially reduced total cell numbers, the surviving cells showed a significant ($p < 0.05$) increase in production of bFGF (Fig. 2). In contrast, treatment of H460 cells with bleomycin and etoposide did not affect production of bFGF (Fig 2). All tested drugs increased production of G-CSF (Fig 2). High doses of doxorubicin (1 $\mu\text{g/ml}$) and cisplatin (1.5 $\mu\text{g/ml}$) decreased production of the chemokine CCL5 (RANTES), while lower doses (0.25 and 0.38 $\mu\text{g/ml}$, respectively) increased the production CCL5. Bleomycin and etoposide stimulated production of CCL5 at all tested concentrations (Fig 2).

Post-treatment effects on TPF production

We next tested whether drug-induced stimulation of TPFs is sustained after drug withdrawal by surviving tumor cells. H460 cells were cultured in 24-well plates (2×10^5 cells/well) with doxorubicin (0.125 $\mu\text{g/ml}$) or cisplatin (0.3 $\mu\text{g/ml}$) for 3 days. Both drugs exerted substantial cytotoxicity, resulting in about a 90% reduction in cell number, which was two-fold below the initial seeded level (Table 1S, Experiment A1, supplemented data). In spite of this, levels of IL-6, CXCL8 and bFGF were higher than those found in non-treated cells. Production of G-CSF and CCL5 in non-treated cells was below detectable levels, but was stimulated by drug treatment (Table 1S, supplemental data). To normalize cytokine production according to cell number, we calculated cytokine production per 1×10^6 cells. The data obtained revealed that doxorubicin and cisplatin induced dramatic per cell increases in IL-6, CXCL8, VEGF, bFGF, G-CSF and CCL5 (Table 2, Experiment A1).

After 3 days, tumor cells that survived drug treatment were harvested and viable cells were seeded into a 96-well plate (1×10^4 cells/well) in fresh media without drugs. Growth of tumor cells previously treated with doxorubicin or cisplatin was still impaired even in the absence of drugs, but these cells continued to produce significantly high levels of IL-6, CXCL8, VEGF, bFGF, G-CSF and CCL5 on a per cell basis (Table 2, Experiment A2, for total levels of cytokine see Table 1S, supplemental data). However, the production of these factors 3 days following cisplatin withdrawal significantly reduced, whereas in doxorubicin pre-treated cells no reduction of IL-6 and CXCL8 was found (Table 2, compare A1 and A2). Thus, these data indicate that drug treatment stimulates cytokine production by H460 NSCLC cells.

Effect of doxorubicin on cytokine production by tumor cells of different origin

We next sought to determine whether drug treatment stimulated cytokine production in other tumor cell lines. Another non-small cell lung cancer line A549 and breast tumor cell lines MCF-7 and CAMA-1 were treated with doxorubicin (0.125 μ g/ml) for 3 days that resulted in a substantial increase in the production of IL-6, CXCL8, VEGF, bFGF, CCL-2 and CCL-5 (Fig 3). Thus, drug treatment of tumor cells of different origin resulted in stimulation of cytokine, chemokine, growth and angiogenic factor production.

It is possible that increased levels of TPFs in the culture medium following drug treatment resulted from their massive release from dead cells. To test this, we analyzed media conditioned by H460 cells or by the lysates of these cells. The high levels of cytokines and chemokines were found in media conditioned by tumor cells (TCM) that were even higher in media conditioned by doxorubicin-treated tumor cells (TCMD) (Table 3). In fresh media (FM) conditioned by the lysates of tumor cells very low levels of cytokines were found (Table 3). It is important to note that a similarly low levels of tested cytokines and chemokines were found in lysates of doxorubicin-treated or untreated tumor cells probably due to the fact that these factors are not stored in cells but are quickly secreted. Thus, the increased levels of cytokines, chemokines and growth factors found in culture media were due to higher levels of production and secretion by surviving cells rather than the release from drug-killed tumor cells

Effect of doxorubicin on the intracellular cytokine production

To further verify the effects of drug treatment on cytokine production, we analyzed the levels of various cytokines in cells pretreated with monensin to block cytokine secretion. Intracellular staining of multiple cytokines revealed that doxorubicin treatment of H460 cells increased production of CXCL8, CCL2, G-CSF, CCL5 and bFGF (Fig. 4A). Analysis of VEGF production showed that populations of H460 tumor cells consist of low and high producers (Fig 4B). Doxorubicin treatment increased VEGF production in both subpopulations that still remained distinct (Fig 4B).

To test whether the same cells produce different cytokines we applied double fluorescent staining of doxorubicin-treated and untreated H460 cells (Fig 4C). Double staining with CXCL8 and CCL5 showed that a majority of tumor cells produced both CCL5 and CXCL8, but some cells preferentially produce CCL5 or CXCL8. After doxorubicin treatment, production of both CCL5 and CXCL8 substantially increased. Furthermore, cells that preferentially produced CXCL8 started to produce CCL5 (Fig 4C). Similarly, double staining showed that a vast majority of cells produce both CXCL8 and bFGF or CCL2 and doxorubicin treatment increased intracellular production of these factors (Fig 4B). These data brought additional confirmation that drug treatment stimulates cytokine production by tumor cells.

Effect of doxorubicin on transcription factor activation

Cytokine and chemokine production is regulated via activation of transcription factors 38-41. We analyzed the ability of doxorubicin to activate nine different transcription factors using the Procarta Transcription Factor Assay Kit (Panomics). H460 tumor cells were treated with doxorubicin (0.125 µg/ml). After 15 min of doxorubicin treatment, a significant ($p < 0.05$) increase in the activity of NF-κB, AP1, AP-2, CREB, HIF-1, STAT-1, STAT-3, STAT5 and ATF-2 was observed (Fig 5A). Drug-induced activation of NF-κB, AP-1, AP-2, CREB, HIF-1 and STAT-3 was maintained even after 30 min of doxorubicin treatment (Fig 5A). These transcription factors play an important role in the expression of various genes that encode cytokines, chemokines, angiogenic and growth factors 38-42.

Effect of doxorubicin on cytokine gene expression

To assess whether drug-induced activation of transcription factors is associated with up-regulation of cytokine gene expression, a real-time PCR analysis was performed. H460 cells were treated with doxorubicin (0.125 µg/ml) for 6h. Doxorubicin treatment increased expression of *IL-6*, *CXCL8* and *FGF-2* genes by more than two-fold, whereas *CSF-3* (*G-CSF*) and *CCL5* gene expression increased by 37- and 14-fold, respectively (Fig. 5B). No significant change in expression of the *VEGF* gene in H460 cells was found, suggesting that a doxorubicin-induced increase in VEGF production in H460 cells might be due to posttranslational mechanisms.

Drug-protective effects of TPFs

Production of cytokines and growth factors by tumor cells could be beneficial for their proliferation and survival. To test this, H460 tumor cells grown in FM or TCM were treated with doxorubicin (0.125 µg/ml) for 6h and then incubated in drug-free FM or TCM for the next 20h. After 6h of treatment, a high proportion (59%) of tumor cells cultured in a FM entered into apoptosis, whereas only 39% of cells were found to be apoptotic when they were cultured in the TCM (Fig 5C). After 20h of treatment, about 65% of cells cultured in FM entered into the necrosis phase as evidenced by propidium iodine (PI) staining, and only 26 % of these cells remained viable. In contrast, only 19% of H460 cells cultured in TCM were necrotic and 71% of these cells were viable (data not shown).

Next we tested whether TPFs could stimulate tumor cell proliferation. Indeed, H460 tumor cells cultured in TCM, in comparison with those cultured in FM, showed a higher rate of proliferation (Fig 5D). Doxorubicin (0.125 µg/ml) significantly ($p < 0.01$) reduced the number of surviving cells. However, the number of doxorubicin-surviving cells was higher when H460 cells were cultured in TCM than in FM (Fig 5D).

Effects of cytokine neutralizing mAbs on H460 tumor cell proliferation and sensitivity to doxorubicin

To determine which TPFs are responsible for stimulation of tumor cell proliferation and survival, we tested various neutralizing mAbs for their ability to abrogate proliferation of H460 tumor cells and increase their sensitivity to doxorubicin. H460 tumor cells were cultured in RPMI 1640 media supplemented with 1% of FBS to reduce the exogenous source of growth factors. Anti-bFGF mAb showed no effect on H460 cell proliferation, whereas neutralization of IL-6, CXCL8 or CCL2 inhibited tumor cell proliferation (Fig 6A). The highest inhibition of cell proliferation was found with anti-CCL5 mAb (Fig 6A).

Treatment of H460 cells with doxorubicin (0.125 µg/ml) substantially reduced the number of tumor cells (see control in Fig 6A and doxorubicin-treated group in Fig 6B). Anti-IL-6, anti-CXCL8, and anti-bFGF mAbs had no effect on the number of surviving tumor cells treated with doxorubicin (Fig 6B). A slight but significant ($p < 0.05$) reduction of the number

of tumor cells was observed with anti-CCL2 mAb. Anti-G-CSF Ab had a similar inhibitory effect (data not shown). Anti-CCL5 Ab in combination with doxorubicin showed the most efficient reduction in number of surviving tumor cells (Fig 6). Anti-TGF β 1 and TGF β 2 Abs did not affect H460 cell proliferation or their doxorubicin survival (data not shown).

DISCUSSION

In this report we demonstrated that human tumor cell lines of different histological origin are able to produce numerous cytokines, chemokines, angiogenic and growth factors. These TPFs represent a cytokine network of tumor cells that play an important role in tumor cell proliferation and formation of supporting stroma in vivo. We found that treatment of H460 and A549 human NSCLC lines, MCF-7 and CAMA-1 breast carcinoma cell lines with anticancer drugs killed tumor cells or inhibited their proliferation. However, in surviving cells this treatment substantially stimulates production of IL-6, VEGF, bFGF, G-CSF, CXCL8, CCL2 and CCL5.

The observed drug-mediated stimulation of TPFs could be a result of an adaptive stress response through which tumor cells protect themselves from drug-mediated toxicity. Doxorubicin treatment induced multifactorial signaling with activation of various transcription factors, such as NF- κ B, AP-1, AP-2, ATF-2, CREB, HIF-1, STAT-1, STAT-3, and STAT-5 that play an important role in the up-regulation of genes encoding various cytokine, chemokine and growth factors 38-40-42. Indeed, activation of these transcription factors paralleled drug-induced up-regulation of *IL-6*, *IL-8*, *bFGF*, *G-CSF*, *CCL2* and *CCL5* gene expression. Thus, increased extracellular levels of cytokines and chemokines were not a result of their release from drug-killed tumor cells but due to drug-induced stimulation production of these factors.

Media conditioned by tumor cells stimulated tumor cell proliferation and protected them from drug-induced apoptosis. We found that blocking IL-6, CXCL8, CCL2 and CCL5 by specific antibodies reduced H460 tumor cell proliferation, indicating that these factors are involved in stimulation proliferation of these cells. However, blocking of IL-6 and CXCL8 did not affect survival of doxorubicin-treated tumor cells whereas neutralization of G-CSF, CCL2 and especially CCL5 increased the lethal effects of doxorubicin, suggesting that these factors are involved in protection of tumor cells from doxorubicin-mediated killing. Our recent experiments with knockdown of the *CCL5* gene using specific siRNA transfection in H460 and CAMA-1 tumor cells also support the importance of CCL5 for tumor cell proliferation and sensitivity to doxorubicin (unpublished observations).

Initially, chemokine were identified for its ability to stimulate migration of proinflammatory cells. Recent studies demonstrated that they have broader biological effects both in normal and malignant cells. Chemokines, such as CXCL8, CCL2 and CCL5 are able to stimulate proliferation, migration and invasion of various tumor cells 18-43-44. They also manifest a potent angiogenic activity and could work in concert with other angiogenic factors to stimulate tumor angiogenesis 15-18-21. Thus, different types of tumor producing factors (cytokines, chemokines, angiogenic and growth factors) have overlapping functions in promoting tumor growth. Blocking a single factor could be compensated by other factors with a similar functional activity and thus might be insufficient to block tumor growth.

Cellular stress response could be induced not only by drugs but also by various apoptosis-induced molecules that results in augmentation of cytokine production. FasL-Fas signaling stimulates production of IL-6 in tumor cells. We found that TRAIL by binding to cognate death receptors stimulates production of CXCL8, CCL2, CCL5 and bFGF in MCF-7, H460 and OVCAR-3 tumor cells. This stimulation was associated with activation of caspases 1

and 8 and various transcription factors. Media conditioned by tumor cells protects tumor cells from the apoptotic effect of TRAIL and this protected effect was blocked by antibodies neutralizing IL-8, CCL5 and bFGF. Our studies with doxorubicin and TRAIL showed that antibodies against TGF β 1 and TGF β 2 did not abrogate the protective effects of media conditioned by H460 lung cancer cells. However, TGF β 2 was a major factor in media conditioned by prostate cancer cells that protected fibroblasts from TNF-induced apoptosis. Media conditioned by thyroid cancer cells protected cancer cells from apoptosis induced by anticancer drugs (doxorubicin, cisplatin or taxol) or FasL and this protection was abrogated by antibodies against IL-4 and IL-10 48. These data suggest that in different histological types different factors could be involved in protection of tumor cells.

The mechanisms by which cytokines and growth factors block the apoptotic effects of drugs and other apoptotic molecules have been intensively investigated. Growth factors by binding to their respective receptors could activate PI-3 kinase/Akt, Ras/MAP kinase, and Jak/signal transducers and activators of transcription. This signaling could change the balance between proapoptotic and antiapoptotic molecules, leading to increase in tumor cell surviving 49- 50.

Although all these studies are limited to *in vitro* cultured tumor cells we believe that drugs could induce similar effects *in vivo*. The tumor cytokine network of *in vivo* growing tumors is considerably more robust and contains factors produced by tumor cells as well as stroma cells (endothelial cells, pericytes, fibroblasts, lymphocytes, macrophages and other inflammatory cells). There is persistent cross-talk between tumor and stroma cells, producing factors that have autocrine and paracrine effects. These TPFs stimulate proliferation of tumor cells as well as endothelial cells, and drive the formation of new blood vessels to facilitate nutrient and oxygen supply and promote tumor survival. In addition, by binding to the receptors on tumor cells, cytokines, chemokines and growth factors provide proliferative signals that antagonize the harmful microenvironment (hypoxia, hypoglycemia, etc) or apoptotic effects of drugs. Thus, inhibition of such pro-survival responses might represent an important strategy for anticancer therapy.

Increased blood concentrations of cytokines, chemokines, angiogenic and growth factors were found in cancer patients, an observation often associated with resistance to therapy and a poor prognosis 12, 25, 26. It is possible that local, intra-tumor concentrations of these factors are higher than in circulation, and thus, TPFs could play an important role in tumor growth and resistance to drug therapy.

Chemotherapy is usually applied in several cycles, at three-week intervals in order to allow the body to restore hematopoietic and other normal cells damaged by drugs. However, some reports indicate that during this resting period tumor cells can aggressively repopulate the tumor and restore pre-treatment tumor size. Based on our *in vitro* findings, we believe that drug treatment may stimulate *in vivo* the production of various angiogenic and growth factors that stimulate tumor cell proliferation during resting periods, resulting in a rapid tumor repopulation. It remains undocumented whether chemotherapy results in the stimulation of cytokine production *in vivo*. It is unknown whether this stimulation is limited to the intra-tumor microenvironment or also results in a systemic increase of these factors in the circulation. However, some studies have shown that drug therapy stimulates the migration of endothelial precursors from the bone marrow into tumors, which is considered to be a result of drug stimulation of the production of pro-angiogenic factors. Therefore, targeting tumor-producing cytokines, chemokines, angiogenic and growth factors during drug-resting periods could reduce tumor repopulation by surviving tumor cells.

It was shown that conventional chemotherapy could be improved by combining it with a VEGF-neutralizing antibody (Avastin/ Bevacizumab) or blocking HER2/neu signaling using a mAb (Herceptin/ Trastuzumab). However, the beneficial effects of these strategies have been limited and manifested in slight increase in patient overall survival 28, 29. It is possible that drug-stimulated production of growth and pro-angiogenic factors could counterbalance the effects of antibody therapy. In addition, numerous growth factors and chemokines exhibit shared angiogenic and growth-stimulating properties, and thus reduction of a single factor is insufficient to completely block tumor growth. The efficacy of chemotherapy could be improved by modalities capable of broad inhibition or inactivation of multiple growth and proangiogenic factors involved in autocrine and paracrine regulation of tumor growth. Thus, it is necessary to develop a new approach for disrupting tumor cytokine networks in an effort to increase the efficacy of cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

TPFs	Tumor Produced Factors
FM	Fresh Medium
TCM	Tumor conditioned medium

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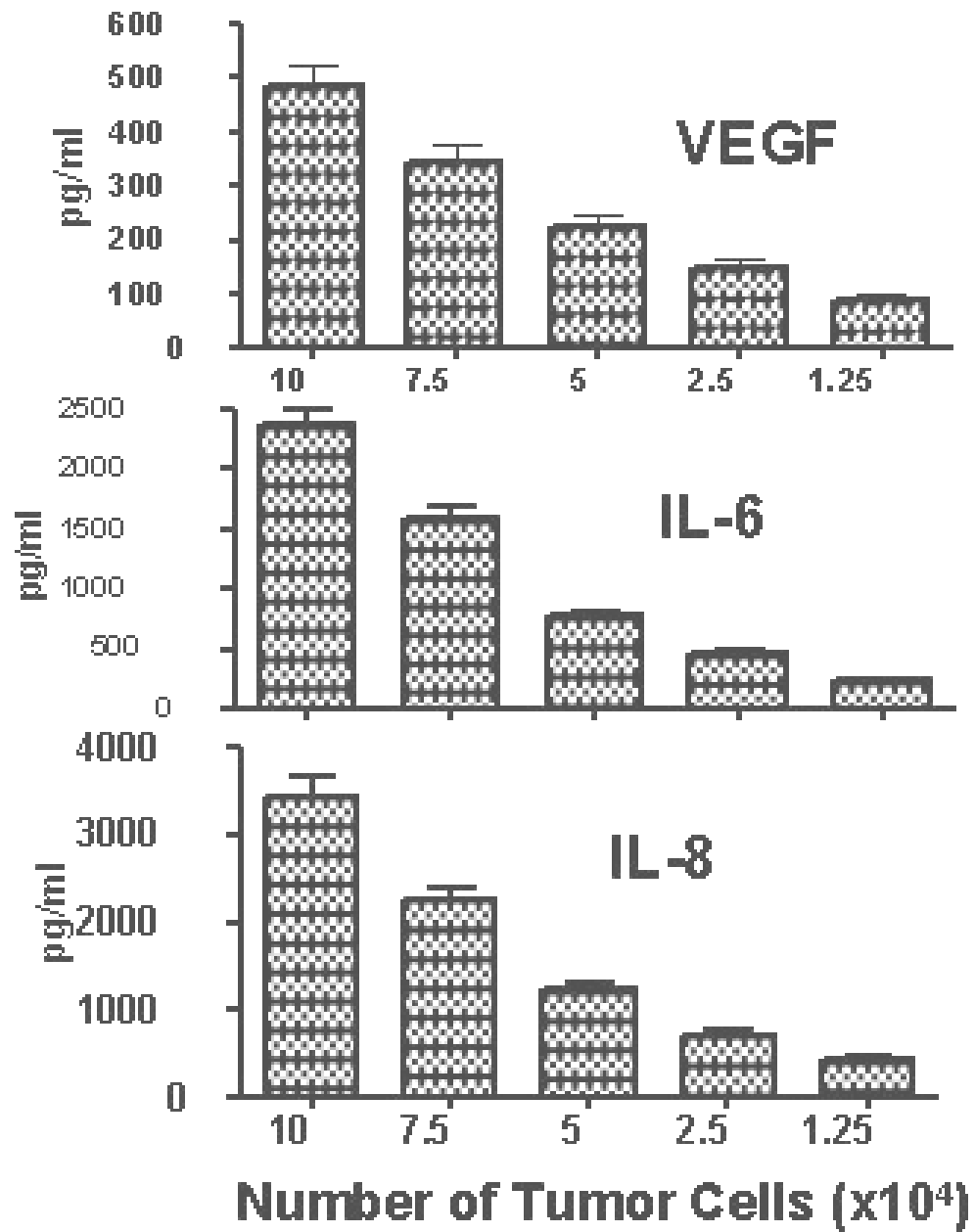


Fig. 1. Production of VEGF, IL-6 and IL-8 as a function of tumor cell number
H460 tumor cells were plated onto 96-well plate at 10×10^4 , 7.5×10^4 , 5×10^4 , 2.5×10^4 and 1.25×10^4 cells/well. After 24 h the supernatants were collected and concentrations of VEGF, IL-6, and IL-8 were determined using multiplexed immunobeads technology. Data presented as mean concentrations (pg/ml) \pm SD.

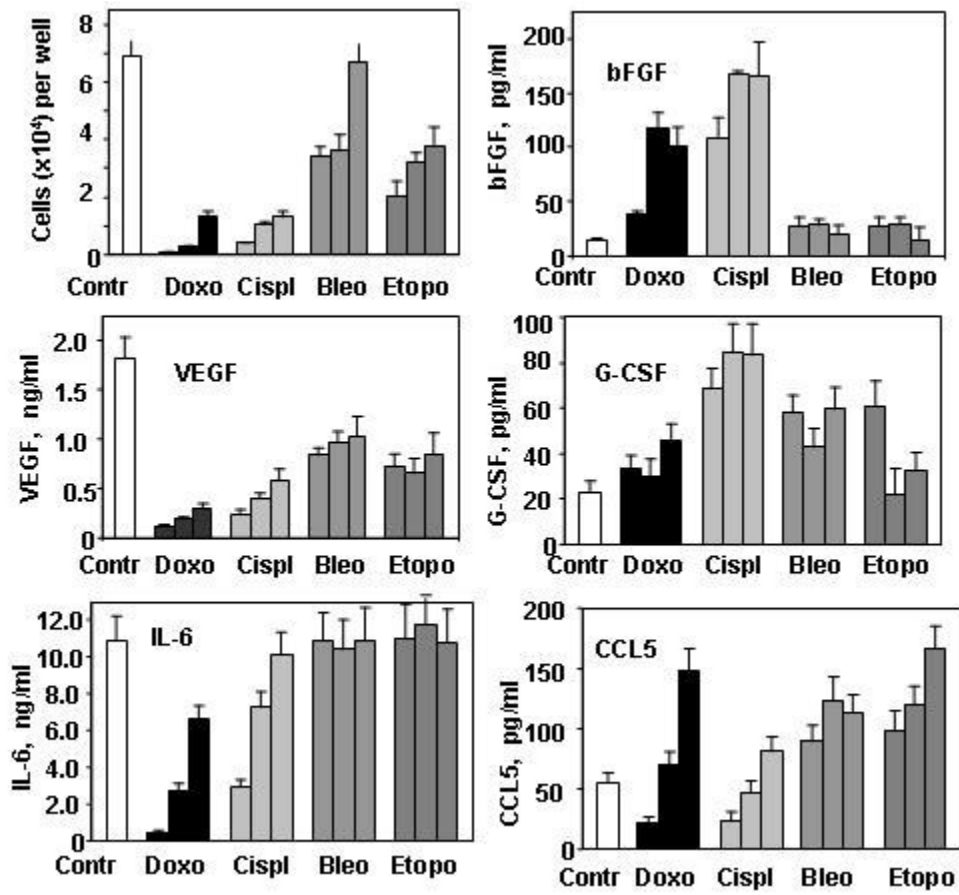


Fig 2. Effect of anticancer drugs on cytokine production by H460 cell line

H460 cells were seeded into a 96-well plate (1×10^4 cells/ well) and treated with doxorubicin (1, 0.5 and 0.25 $\mu\text{g/ml}$), cisplatin (1.5, 0.75, 0.38 $\mu\text{g/ml}$), bleomycin (4, 2, and 1 $\mu\text{g/ml}$) and etoposide (1, 0.5, and 0.25 $\mu\text{g/ml}$). After three days, conditioned media were collected, cell numbers were determined using the One Solution Cell Proliferation Kit (Promega) and concentrations of cytokines and growth factors were measured using a multiplex cytokine assay. * Significantly ($p < 0.05$) differed from the control group.

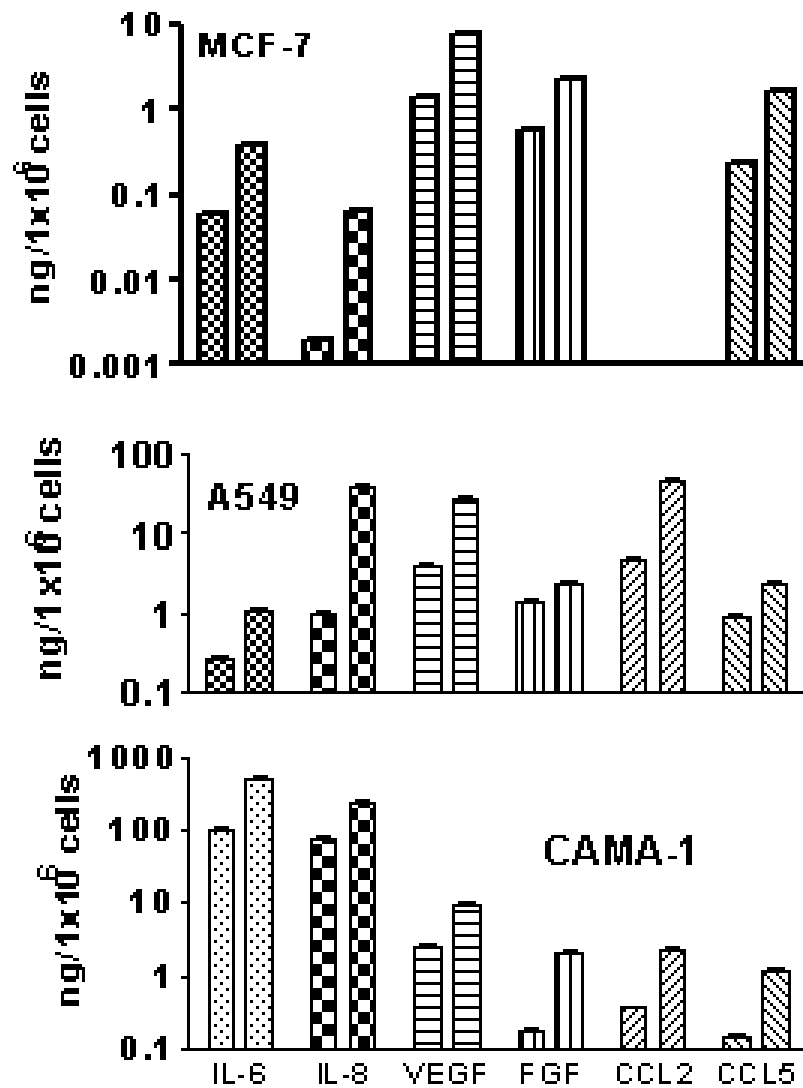


Fig 3. Cytokine production by human lung and breast tumor cells treated with doxorubicin MCF7 and CAMA-1 breast tumor cells, and A549, non-small cell lung cancer cells were seeded into 96-well plates, six wells per group (5×10^3 cells/well). After 24h, media was changed and doxorubicin ($0.125 \mu\text{g/ml}$) was added. After three days, conditioned media were collected; cells were stained with Hoechst 33342 and counted using Cellomics Array Scan VTI. Concentrations of various TPFs in the supernatants were determined using multiplex immunobeads technology. Data presented as mean \pm SE $\text{pg}/1 \times 10^6$ cells. Only cytokines with a significant ($p < 0.05$) drug-induced increased production are presented. Left columns for each cytokine show untreated control cells; right columns for each cytokine - doxorubicin-treated tumor cells.

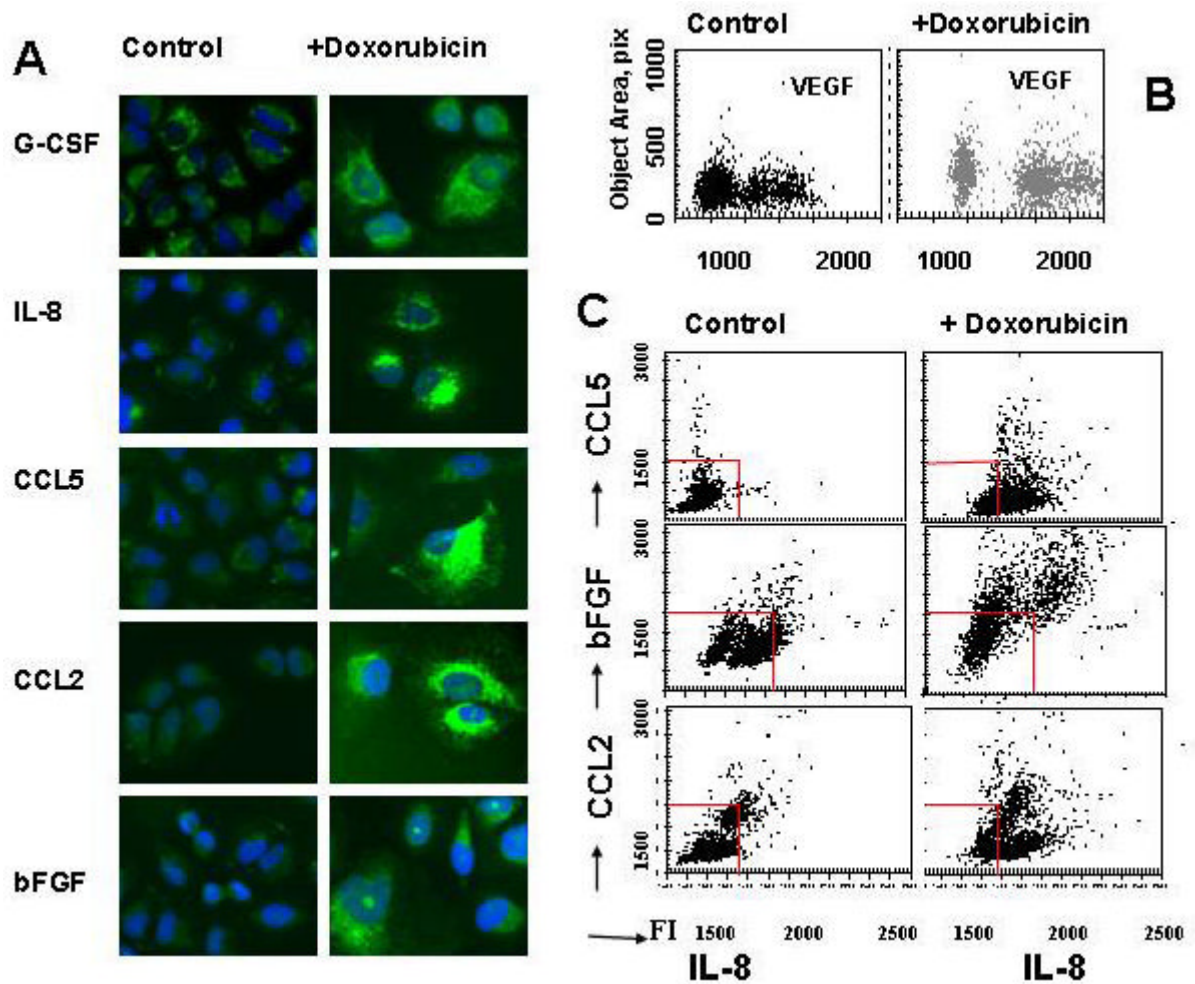


Fig 4. Intracellular levels of cytokines in doxorubicin-treated and untreated H460 tumor cells
H460 cells growing in 96-well plates were treated with doxorubicin (0.125 μ g/ml) for 16 h and then were incubated with monensin (2 μ M) for 3h. Cells were fixed, permeabilized and incubated with primary Abs against CXCL8, CCL2, CCL5, G-CSF, bFGF or VEGF and then with secondary Alexa Fluor 488 or Alexa 680-conjugated Abs. Cell nuclei were stained with Hoechst 33342 and cell images were acquired using the Cellomics ArrayScan HCS Reader (20X objective) and analyzed using the Target Activation BioApplication Software Module. **A.** Images of immunofluorescently stained H460 cells for CXCL8, CCL2, CCL5, G-CSF, or bFGF (Alexa488). **B** Fluorescence intensity of VEGF (Alexa488) is plotted against object area, pix. Each point represents a single cell. **C.** Doxorubicin-treated and untreated cells were incubated with monensin and double-stained for CCL5, bFGF or CCL2 (Alexa-488) and CXCL8 (Alexa-680). Fluorescence intensities of CCL5, bFGF or CCL2 were plotted against fluorescence intensity of CXCL8. Each point represents a single cell. The red lines show the boundaries of the fluorescence intensity of untreated cells.

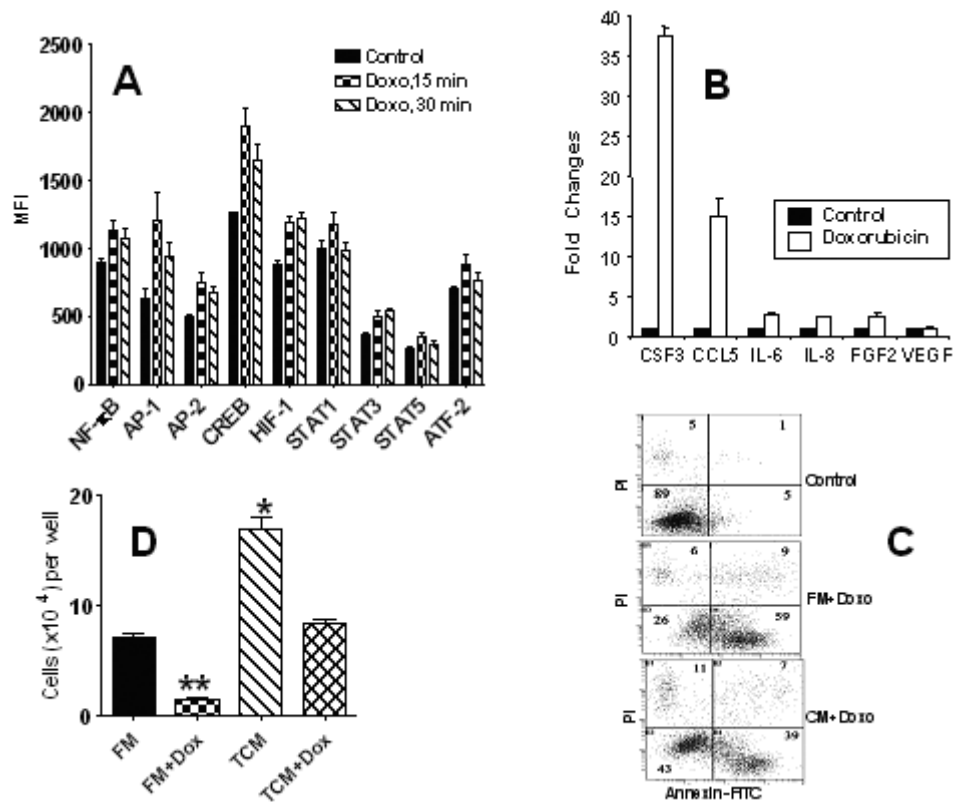


Fig 5.

A. Doxorubicin activation of transcription factors in human H460 tumor cells. H460 tumor cells were treated with doxorubicin (0.125 μ g/ml) for 0, 15 and 30 min. Nuclear extracts were prepared and an analysis of transcription factors was performed using the multiplex Procarta Transcription Factor Assay Kit. Only transcription factors with significant differences ($p < 0.05$) after the doxorubicin treatment are presented.

B. Real-time PCR of cytokine gene expression in doxorubicin-treated and untreated H460 tumor cells. H460 tumor cells were treated with doxorubicin (0.125 μ g/ml) for 6h. RNA from drug-treated and untreated tumor cells was extracted, and cDNA was produced from 2 μ g of RNA using the SuperArray ReactionReady First Strand cDNA Synthesis kit (SuperArray Bioscience). Real-time PCR was carried out using the SuperArray RT² Real-Time™ SYBR Green PCR Master Mix (SuperArray Bioscience) and was performed on the ABI Prism™ 7700 sequence detector real-time PCR system (AB Applied Biosystems). Fold changes in gene expression in doxorubicin-treated and untreated H460 tumor cells were calculated using the $\Delta \Delta C_t$ method. Control level was set at 1.0.

C. Tumor cell-conditioned medium (TCM) protects tumor cells from doxorubicin-induced apoptosis. H460 cells were incubated with doxorubicin (0.125 μ g/ml) in fresh medium (FM) or TCM. After 6 h, cells were harvested, stained with Annexin V-FITC and PI and analyzed by flow cytometry.

D. Effect of TCM on tumor cell proliferation and drug survival. H460 cells (1×10^4 /well) were cultured in FM or TCM for 3 days in a 96-well plate with and without doxorubicin (0.125 μ g/ml). Cell count was determined using the One Solution Cell Proliferation Kit (Promega, Madison, WI). * Differences between FM and TCM groups are significant ($p < 0.01$); ** Differences between doxorubicin-treated groups are significant ($p < 0.05$).

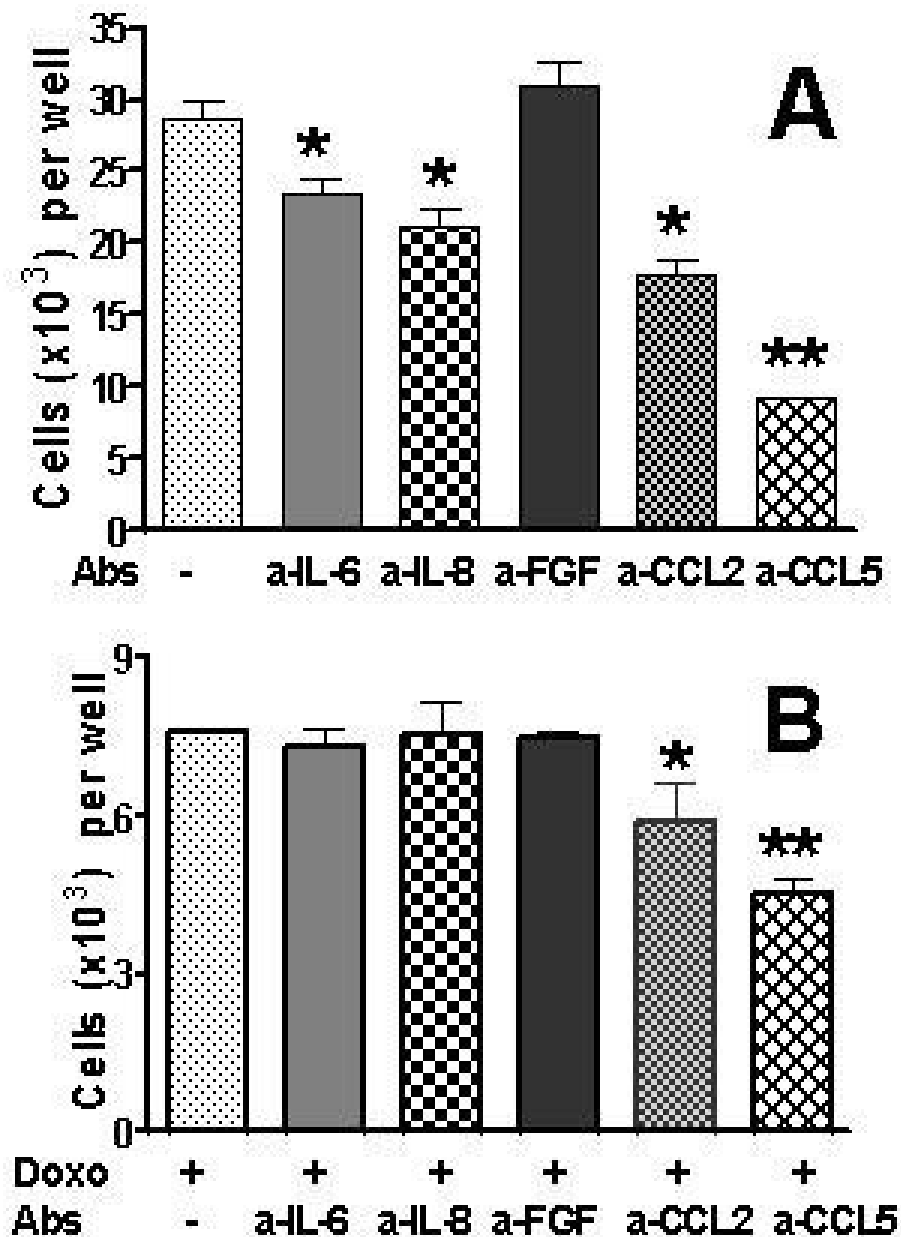


Fig 6. Effects of mAbs neutralizing growth factors and chemokines on tumor cell proliferation and drug sensitivity

H460 cells were plated in 96-well plates at 1×10^4 cells/well in FM with 10% FCS (six wells per group). The following day, cells were washed and FM with 1% FBS was added. Cells were preincubated with neutralizing mAbs (100 ng/ml) for 1 h before doxorubicin (0.125 μ g/ml) was added. Cells were cultured for 48 h. After removal of the media, cells were fixed, stained with Hoechst 33342 (2 μ g/mL), and counted using the Cellomics ArrayScan HCS Reader

A. Number of H460 tumor cells cultured in the presence of neutralizing mAbs. **B.** Number of H460 tumor cells cultured in the presence of neutralizing Abs and doxorubicin.

*Significantly differs ($p < 0.05$) from control groups.

Table 1

Multiplex Analysis of Soluble Factors Produced by Human Tumor Cell Lines

Tumor cells produced factors *	Cytokines (pg/1×10 ⁶ cells)									
	H460	A549	FEMX	LOX	MCF7	OVCAR-3	K562			
IL-6	12,385	205	11	18	0.8	6,646	51			
IL-8	13,600	444	217	354	3	12,281	2			
IL-10	10	20	86	33	2	23	26			
G-CSF	1,746	2,772	2,162	2,039	516	306	1,416			
HGF	31	131	161	98	12	70	70			
EGF	140	259	332	274	49	96	146			
VEGF	2,049	1,450	1,504	81	615	13,331	1,074			
bFGF	667	918	830	656	222	193	533			
MIP-1a	13	41	51	54	3.	0	24			
MIP-1b	119	280	0	163	28	0	133			
CCL2	17	675	0	0	2	0	30			
CCL5	416	0	1,259	780	93	188	209			
IFN-a	164	417	696	610	54	193	305			

* Human tumor cell lines H460 and A549 non-small cell lung cancer, LOX and FEMx melanoma, MCF7 breast, OVCAR-3 ovarian and K562 erythroleukemia cancer cells were cultured 3 days. Supernatants were collected and cells were counted. The concentrations of various tumor cell produced factors were analyzed using MAFx multiplex technology. Production of cytokines (pg/1×10⁶tumor cells) was calculated. Inter-assay coefficients of variation for individual cytokines in the multiplex assay were in the range of 3.5-9.8% and intra-assay coefficients of variation were in the range of 3.6-12.6%.

Table 2

Effect of drugs on cell proliferation and cytokine production by H460 cells

Ex p#	Groups	No. cells ($\times 10^3$)	Cytokines, (pg/ 1×10^6 cells)									
			IL-6	IL-8	VEGF	bFGF	G-CSF	CCL2	CCL5			
A1	Control	1,000 \pm 212	5,118	7,594	596	24	0	0	0	0	0	0
	Doxo	90 \pm 23*	116,955*	94,484*	4,108*	518*	514*	139*	276*			
	Cispl	100 \pm 31*	213,068*	107,964*	6,664	742*	1,104*	356*	920*			
A2	Control	47 \pm 8	5,970	15,349	638	34	0	11	0			
	PostDoxo	9 \pm 1*	137,689*	103,911*	3,177*	267*	623*	59*	111*			
	PostCispl	20 \pm 3*	121,120*	57,590*	3,530*	280*	60*	178*	80*			

Experiment A1: H460 tumor cells were seeded into a 24-well plate (2×10^7 /well), with 8 wells per group, and cultured for 3 days in the presence of doxorubicin (0.125 μ g/ml) or cisplatin (0.3 μ g/ml). Supernatants were collected and cells were counted. The concentrations of TPFs in the supernatants were analyzed using a multiplex immunobeads kit and data were calculated as pg/ 1×10^6 cells.

Experiment A2: Cells collected from the control and drug-treated groups (experiment A1) were seeded into a 96-well plate (1×10^4 cells/well) in fresh media without any drug. After 3 days, supernatants were collected and the numbers of cells were estimated using the One Solution Cell Proliferation Kit (Promega).

* Differences between control and experimental groups were significant ($p < 0.05$).

Table 3

Concentration of TPFs in medium conditioned by tumor cells (TCM), by doxorubicin-treated tumor cells (DTCM) and in fresh medium (FM) supplemented with tumor cell lysate

Media	Cytokines (pg/1×10 ⁶ cells)					
	IL-6	CXCL8	CCL2	CXCL1	VEGF	
TCM	59,044	13,934	178	182	2098	
DTCM	108,263*	198,000*	271*	447*	2968*	
FM + tumor cells lysates	122	68	12	78	4	
FM + drug-treated cell lysates	150	67	10	60	4	

H460 tumor cells were cultured in a 6-well plate (2×10⁶/well/2 ml) in the presence or absence of doxorubicin (0.125 µg/ml) for 24h. Supernatants were collected, remaining cells were harvested and sonicated, and tumor lysates were centrifuged to remove cell debris. Tumor cell lysates (equivalent to 1×10⁶ tumor cells) were added to 1 ml of fresh culture medium. The concentrations of cytokines in media conditioned by untreated tumor cells (TCM), doxorubicin-treated tumor cells (DTCM) or fresh media (FM) conditioned by tumor lysates were determined using a Luminex multiplex assay.

* Significantly (p<0.05) differ from other groups.