Cancer Chemother Pharmacol (2003) 52: 391–398
DOI 10.1007/s00280-003-0665-1

ORIGINAL ARTICLE

**Masami Niiya · Kenji Niiya · Toru Kiguchi
Misako Shibakura · Noboru Asaumi · Katsuji Shinagawa
Fumihiko Ishimaru · Katsuyuki Kiura · Kazuma Ikeda
Hiroshi Ueoka · Mitsune Tanimoto**

Induction of TNF-α, uPA, IL-8 and MCP-1 by doxorubicin in human lung carcinoma cells

Received: 2 December 2002 / Accepted: 30 April 2003 / Published online: 8 August 2003

Abstract *Purpose*: We have previously demonstrated doxorubicin-induced urokinase (uPA) and interleukin-8 (IL-8) expression in human H69 small-cell lung carcinoma (SCLC) cells by a microarray technique using Human Cancer Chip version 2, in which 425 human "cancer-related" genes are spotted on the plates. The microarray analysis also revealed a significant induction of tumor necrosis factor-alpha (TNF-α), and doxorubicin-induced macrophage chemoattractant protein-1 (MCP-1) expression was demonstrated by an RNase protection assay. We extended the study by testing the effects of doxorubicin on the induction of TNF-α, uPA, IL-8 and MCP-1 in other types of lung carcinoma cells. *Methods*: We investigated the effects of doxorubicin on the expression of TNF-α, uPA, IL-8 and MCP-1 in 12 human lung carcinoma cell lines, including five SCLC, three adenocarcinoma and four squamous cell carcinoma cells. The surface expression of their receptors was also investigated. *Results*: TNF-α was significantly induced in three cell lines, H69, SBC-7 (SCLC) and PC-9 (adenocarcinoma), uPA in five cell lines, H69, SBC-7, EBC-1 (squamous cell), EBC-2 (squamous cell), and Sq-1 (squamous cell), IL-8 in three cell lines, H69, PC-9 and EBC-1, and MCP-1 in five cell lines, H69, SBC-3 (SCLC), SBC-7, PC-9 and Sq-1. In H69 cells, TNF-α antigen levels were increased approximately fivefold in the conditioned medium of doxorubicin-treated cells, in parallel with an increase in mRNA levels. As with uPA and IL-8, the maximum induction was observed at the "sublethal" concentrations of 2 and 4 μ*M* at which cell growth was slightly inhibited 24 h after treatment. Furthermore, the cells did not express receptors including types I and II TNF-α receptors, uPA receptor (uPAR), C-x-C-chemokine receptor-1 (CXCR-1), or C-C-chemokine receptor-2, corresponding to TNF-α, uPA, IL-8 and MCP-1, respectively, that were induced by doxorubicin in the cells, although SBC-7 cells expressed uPAR, and EBC-1 cells expressed CXCR-1. *Conclusions*: TNF-α, uPA, IL-8 and MCP-1 induced and secreted from tumor cells upon doxorubicin stimulation may activate surrounding cells expressing the receptors such as neutrophils and monocytes/macrophages in a paracrine fashion. TNF-α is a major proinflammatory cytokine, and IL-8 and MCP-1 are major chemoattractants for neutrophils and monocytes/macrophages, respectively. Furthermore, uPA activates matrix metalloproteinase 9 which can truncate and activate IL-8. Thus, the simultaneous induction of TNF-α, uPA, IL-8 and MCP-1 may enhance the interaction between tumor and inflammatory/immune cells, and augment cytotoxicity.

Keywords Tumor necrosis factor-α (TNF-α) · uPA · IL-8 · MCP-1 · Doxorubicin · Lung cancer · Gene expression

Abbreviations *BSA* Bovine serum albumin · *C-C* Cysteine-cysteine · *C-x-C* Cysteine-x-cysteine · *CCR-2* C-C chemokine receptor-2 · *CXCR-1* C-x-C chemokine receptor-1 · *ELISA* Enzyme-linked immunosorbent assay · *IL-1* Interleukin-1 · *IL-6* Interleukin-6 · *IL-8* Interleukin-8 · *MCP-1* Macrophage chemoattractant protein-1 · *MTT* (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide · *PBS* Phosphate-buffered saline · *ROS* Reactive oxygen species · *SCLC* Small-cell lung cancer · *TNF-α* Tumor necrosis factor-alpha · *TNFRI* Type I tumor necrosis factor-alpha receptor · *TNFRII* Type II tumor necrosis

M. Niiya · K. Niiya (✉) · T. Kiguchi · N. Asaumi · K. Shinagawa
F. Ishimaru · K. Kiura · K. Ikeda · H. Ueoka · M. Tanimoto
Department of Medicine II, Okayama University Medical School,
2-5-1 Shikatacho, 700-8558 Okayama, Japan
E-mail: kniiya@md.okayama-u.ac.jp
Tel.: +81-86-2357225
Fax: +81-86-2357225

M. Shibakura
Faculty of Health Sciences,
Okayama University Medical School, Okayama, Japan

factor-alpha receptor · *uPA* Urokinase-type plasminogen activator · *uPAR* Urokinase-type plasminogen activator receptor

Introduction

We have previously demonstrated the induction of uPA by IL-1 and lipopolysaccharide with the involvement of ROS [27]. We have also found that the ROS-generating anticancer agent, anthracycline, induces uPA and IL-8 in human H69 small-cell lung carcinoma (SCLC) cells by a microarray technique using Human Cancer Chip version 2 (Takara Shuzou, Kyoto, Japan) [14, 37]. The microarray analysis also revealed a significant induction of tumor necrosis factor-alpha (TNF-α), and doxorubicin-induced macrophage chemoattractant protein-1 (MCP-1) expression was detected by a ribonuclease protection assay [37].

uPA catalyzes the conversion of zymogen plasminogen to the broad-spectrum serine protease plasmin. In addition to its role in fibrinolysis, plasmin not only cleaves extracellular matrix substrates but also activates promatrix metalloproteinase zymogens [4], and therefore plays a central role in many aspects of cellular regulation such as tissue remodeling, inflammation, cell migration and tumor progression. IL-8 is a cysteine-x-cysteine (C-x-C) chemokine and acts as a chemoattractant and an activator of neutrophils [2, 44], whereas MCP-1 is a cysteine-cysteine (C-C) chemokine and functions mainly as a chemoattractant of monocytes/macrophages [11, 22]. IL-8 and MCP-1 released from tumors may reduce the rate of tumor growth by inducing the infiltration of neutrophils and monocytes/macrophages to the tumor site [20, 28], although their roles in tumor biology are controversial because both chemokines are important regulators in tumor progression and act by enhancing angiogenesis and metastasis [5, 15, 38].

TNF-α is a pleiotropic cytokine initially identified as a protein released by endotoxin-stimulated macrophages, although many other types of cells are also able to synthesize small amounts [24]. The term tumor necrosis factor originates from the observation that this protein plays a crucial role in the killing of tumor cells, and it can induce both necrotic and apoptotic (programmed) forms of cell death [19, 21]. TNF-α itself induces the synthesis of other cytokines and chemokines such as IL-1, IL-6, IL-8 and platelet-activating factor [3, 26, 29, 43]. Its biosynthesis is induced by many inflammatory stimuli such as endotoxins, exotoxins, enterotoxins, phorbol esters and ionizing radiation, and the regulation occurs at the transcriptional and post-transcriptional levels [1]. Current evidence suggests that these stimuli use ROS as signaling messengers to activate transcription factors, such as nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), and induce the expression of a number of genes [9, 32].

Anthracycline is one of the most widely used antitumor agents and is a key drug for treatment of malignant lymphoma and SCLC. It is readily activated to the semiquinone radical, and generates ROS [30]. Currently, increasing evidence suggests that at pharmacological concentrations, anthracyclines kill tumor cells through apoptosis by activating a metabolic pathway, but not through oxidative damage to DNA by crippling cellular metabolism [10]. Doxorubicin-induced apoptosis appears to be dependent on RNA synthesis and it is inhibited by antioxidants, suggesting ROS involvement in doxorubicin-induced apoptosis [25].

We extended the study by testing the effects of doxorubicin on the induction of TNF-α, uPA, IL-8 and MCP-1, in other types of lung carcinoma cells. We screened a total of 12 human lung carcinoma cell lines, including five SCLC, three adenocarcinoma and four squamous cell carcinoma cell lines, and found that upon doxorubicin stimulation TNF-α, uPA, IL-8 and MCP-1 were induced in three, five, three, and five cell lines, respectively, but their corresponding receptors were expressed only at low levels in these cell lines, suggesting that these factors may influence neutrophils and monocytes/macrophages infiltrating to tumor sites in a paracrine fashion.

Material and methods

Cell lines

The human lung cancer cell lines used in these experiments were as follows: five SCLC lines, NCI-H69 (ATCC HTB-119), LK79, LC-65A, SBC-3 (JCRB0818) and SBC-7 (not yet registered), three adenocarcinoma cell lines, PC-9, ABC-5 (not yet registered) and 11-18, and four squamous cell lines, EBC-1 (not yet registered), EBC-2 (not yet registered), Sq-1 and LC-1Sq. LK79, LC-65A, PC-9, 11-18, Sq-1 and LC-1Sq were kindly provided by Dr. E. Nakayama (Department of Immunology, Okayama University Medical School, Okayama Japan) [39], and SBC-3, SBC-7, ABC-5, EBC-1 and EBC-2 were established in our laboratory [12].

Cell culture

Cells were grown in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated BSA (Whittaker Bioproducts, Walkersville, Md.), and 100 U/ml penicillin. Before the experiment, cells were washed once with PBS, resuspended in serum-free RPMI-1640 (at approximately $1–2\times10^6$/ml) and cultured in a 24-well culture plate (1 ml/well) with or without doxorubicin. After 24–48 h, cells were mixed well by pipetting and 20 µl of the mixture from each well was transferred to a 96-well plate containing 80 µl RPMI-1640, and the cell density was determined by the colorimetric MTT assay as described by Mosmann [23].

Microarray analysis

To identify the doxorubicin-induced cancer-related genes, we performed a microarray analysis using Human Cancer CHIP, version 2 (Takara Shuzou, Kyoto, Japan) in which 425 human "cancer-related" genes and 11 control housekeeping genes, listed on the

home page of Takara Shuzo (http//bio.takara.co.jp/catalog/DNAchip_Download.htm), were spotted on three separate glass plates. H69 cells were cultured in fetal calf serum-free RPMI-1640 culture medium in the absence or presence of 2 μ*M* doxorubicin for 9 h. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method, and then further purified to mRNA by passing through an oligo-dT column (Oligotex-dT30, Takara Shuzo). A fluorescent probe was synthesized by incorporating Cy3- or Cy5-dUTP (Amersham Life Science, Arlington Heights, Ill.) with the use of 1 μg of the above mRNA as a template and 50 U AMV reverse transcriptase (Takara Shuzou) as described previously [14].

uPA activity

uPA activities in the conditioned medium were measured using a synthetic uPA substrate S-2444 and a plasminogen-containing fibrin plate (fibrin zymography) as previously described [14].

TNF-α, IL-8 and MCP-1 antigen levels

TNF-α, IL-8 and MCP-1 antigen levels in the conditioned medium were measured using ELISA kits (Biosource International, Burlingame, Calif.). Briefly, cells were washed once with PBS and resuspended in serum-free RPMI-1640 at approximately $1–2\times10^6$/ml. The cells were cultured in a 24-well culture plate (1 ml/well) in the presence of various concentrations of doxorubicin for 24–48 h.

Northern blot analysis

Total RNA was isolated from cells by the acid guanidinium thiocyanate-phenol-chloroform method and further purified to mRNA using an oligo-dT column as described above. The mRNA (2 μg) was subjected to Northern blot analysis as described by Sambrook et al. [34]. A cDNA probe for TNF-α was labeled with ^{32}P-dCTP by a random-primed DNA labeling technique. Levels of mRNA were quantified by counting radioactivity using a BAS 2000 image analyzer (Fuji-film Corporation, Tokyo). As a control for differences in RNA sample loading, filters were rehybridized with a radiolabeled β-actin cDNA probe.

Flow cytometric analysis for detecting the surface expression of TNFRI and TNFRI, uPAR, CXCR-1 and CCR-2

Approximately 2×10^6 cells/ml were washed twice with PBS and suspended in PBS containing 2% BSA. The cells were incubated with monoclonal mouse antibodies (IgG) against human TNFRI or TNFRII (Sigma, St. Louis, Mo.), uPAR (American Diagnostic, Hauppauge, N.Y.) and CXCR-1 (Genzyme-Techne Corporation, Minneapolis, Minn.) for 30 min at 4°C, and then washed twice with PBS containing 2% BSA. The cells were further treated with an FITC-conjugated anti-mouse IgG goat antibody (Organon Teknika Corporation, West Chester, Pa.) for 30 min at 4°C, and after washing twice with PBS containing 2% BSA, the cells were analyzed on a FACScan (Becton Dickinson, Mountain View, Calif.). For detecting CCR-2, a phycoerythrin-conjugated mouse monoclonal anti-human CCR-2 antibody (Genzyme-Techne) was used.

Statistical analysis

Statistical analysis was done on a DOS/V computer (Sharp Computer, Tokyo, Japan). The results are expressed as means ± SE. Statistical significance was determined using Student's *t*-test to compare unpaired data.

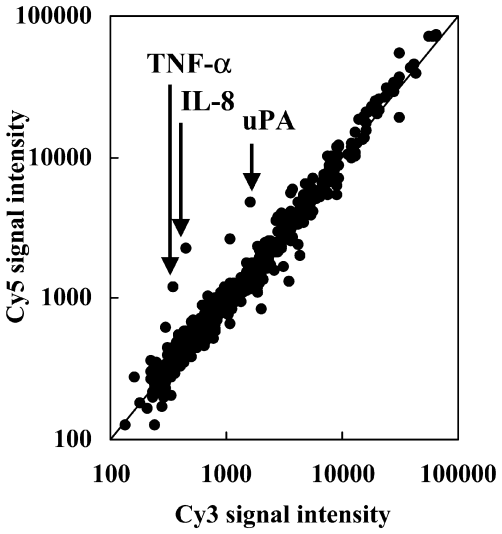


Fig. 1 Microarray analysis of changes in gene expression following doxorubicin treatment. Messenger RNA isolated from H69 cells cultured in the presence and absence of 2 μ*M* doxorubicin for 9 h was subjected to microarray analysis using Human Cancer CHIP, as described in Materials and methods. The TIFF files created by the Affimetrix 418 Array Scanner were colored using Adobe Photoshop software (Mountain View, Calif.) and this scatter graph was drawn using Excel software. Horizontal and vertical axes indicate the color intensity of Cy3 and Cy5 on a log scale, respectively. Cy3 and Cy5 indicate mRNA levels in the doxorubicin-untreated and treated cells, respectively

Results

Microarray analysis

We have previously reported doxorubicin-induced uPA and IL-8 expression in H69 SCLC cells as determined by microarray analysis [14, 37]. Microarray analysis using Takara's human Cancer CHIP, version 2, showed that, not only uPA and IL-8, but also TNF-α genes were markedly increased (more than fourfold) after stimulation with 2 μ*M* doxorubicin for 9 h (Fig. 1).

Doxorubicin-induced TNF-α expression in lung carcinoma cells

Next, to confirm the TNF-α induction, we measured TNF-α antigen levels in the conditioned medium of doxorubicin-stimulated H69 cells by ELISA as described in Materials and methods. As shown in Fig. 2A and B, doxorubicin increased TNF-α antigen levels in the H69 conditioned medium in a time- and dose-dependent manner. The peak induction was observed at a sublethal concentration (2 μ*M*) at which the cell density was slightly decreased 24 h after treatment. Northern blotting using 2 μg mRNA purified through an oligo-dT column revealed that TNF-α mRNA levels were increased 2 and 9 h after stimulation with doxorubicin (Fig. 2C). We then treated 11 other lung cancer cell

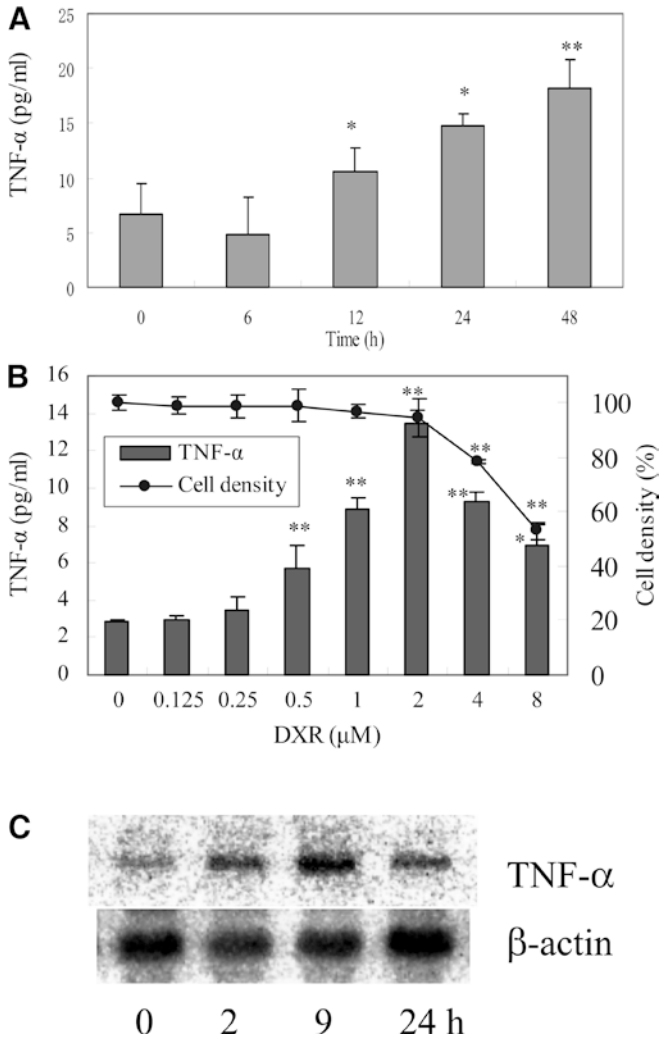


Fig. 2A–C Doxorubicin-induced TNF-α in H69 cells. **A** Time-dependent TNF-α induction by doxorubicin stimulation. Approximately 1×10^6 cells/ml per well of H69 SCLC cells were cultured in the presence of 2 μ*M* doxorubicin for up to 48 h, and TNF-α antigen levels were measured using an ELISA kit as described in Materials and methods. Each experiment was conducted at least twice in triplicate, and the results were reproducible. **B** Dose-dependent doxorubicin-induced TNF-α expression. Approximately 1×10^6 cells/ml/well of H69 cells were cultured in the presence of various concentrations of doxorubicin for 24 h, and TNF-α antigen levels in the conditioned medium and cell density were measured using an ELISA kit and the MTT assay, respectively, as described in Materials and methods. Means ± SE of triplicate values are shown. $*P<0.05$, $**P<0.01$ vs control values. **C** TNF-α mRNA levels in H69 cells after stimulation with 2 μ*M* doxorubicin. TNF-α mRNA levels were measured by Northern blotting as described in Materials and methods. The results of one representative of three independent experiments are shown. A *β*-actin cDNA probe was used as an internal control. mRNA (20 μg each) was extracted from H69 cells collected at the times shown, and subjected to Northern blotting

lines, including four SCLC (LK79, LC-65A, SBC-3 and SBC-7), three adenocarcinoma (PC-9, ABC-5 and 11-18), and four squamous (EBC-1, EBC-2, Sq-1 and LC-1Sq) cell lines, with 2 and 4 μ*M* doxorubicin for 24 h and measured TNF-α antigen levels in the conditioned medium. The growth of these cells was slightly inhibited 24 h after treatment with 2 or 4 μ*M* doxorubicin, and longer treatment had killed most cells by 3 days (data not shown). TNF-α antigen levels were increased in the conditioned medium of H69, SBC-7 and PC-9 cells , and the maximum increases were approximately 5-, 2.7- and 2.5-fold, respectively (Fig. 3).

uPA accumulation in doxorubicin-treated lung cancer cells

As shown in Fig. 4, uPA activities were significantly increased after doxorubicin stimulation, and the maximum increases in H69, SBC-7, EBC-1 EBC-2 and Sq-1 cells were approximately 3.6-, 2.4-, 16-, 3.3-, and 11.5-fold, respectively. This was also confirmed by fibrin zymography (data not shown).

IL-8 and MCP-1 antigen levels in doxorubicin-treated cells

Basal IL-8 antigen levels varied between cell types (Fig. 5A), and upon doxorubicin treatment were markedly increased in H69 cells and significantly increased in PC-9 and EBC-1 cells (Fig. 5B). As shown in Fig. 6, basal MCP-1 antigen levels also varied between cell types, and upon doxorubicin stimulation were markedly increased in H69 cells and significantly increased in SBC-3, SBC-7, PC-9 and Sq-1 cells.

Cell surface expression of TNFR I, TNFR II, uPAR, CXCR-1 and CCR-2

The cell-surface expression of TNFR I, TNFR II, uPAR, CXCR-1 and CCR-2 was determined using monoclonal antibodies against TNFR I, TNFR II, uPAR, CXCR-1 and CCR-2 by flow cytometry as described in Materials and methods. As summarized in the Table 1, uPAR was expressed only on SBC-7 cells, and CXCR-1 was expressed on EBC-1, but TNFRI, TNFRII and CCR-2 were not expressed on the surface of any of the cell types.

Discussion

Studies on the mechanism of doxorubicin-induced cytotoxicity have demonstrated that doxorubicin can induce either apoptosis or necrosis through alteration of mitochondrial structure and activation of the downstream apoptotic enzyme, caspase-3 [31], or by directly intercalating into DNA and destroying its structure, although the former mechanism appears to be dominant at pharmacological concentrations [25]. As demonstrated in this study, doxorubicin induced TNF-α, uPA, IL-8 and MCP-1 in a number of lung cancer cells

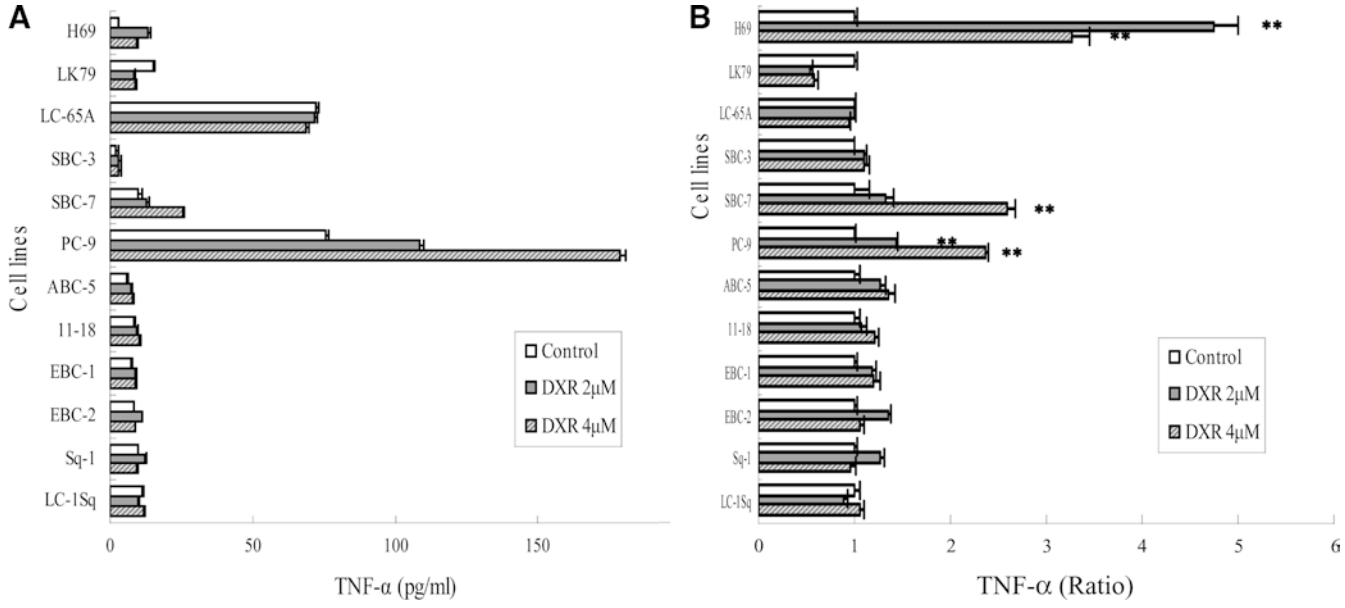
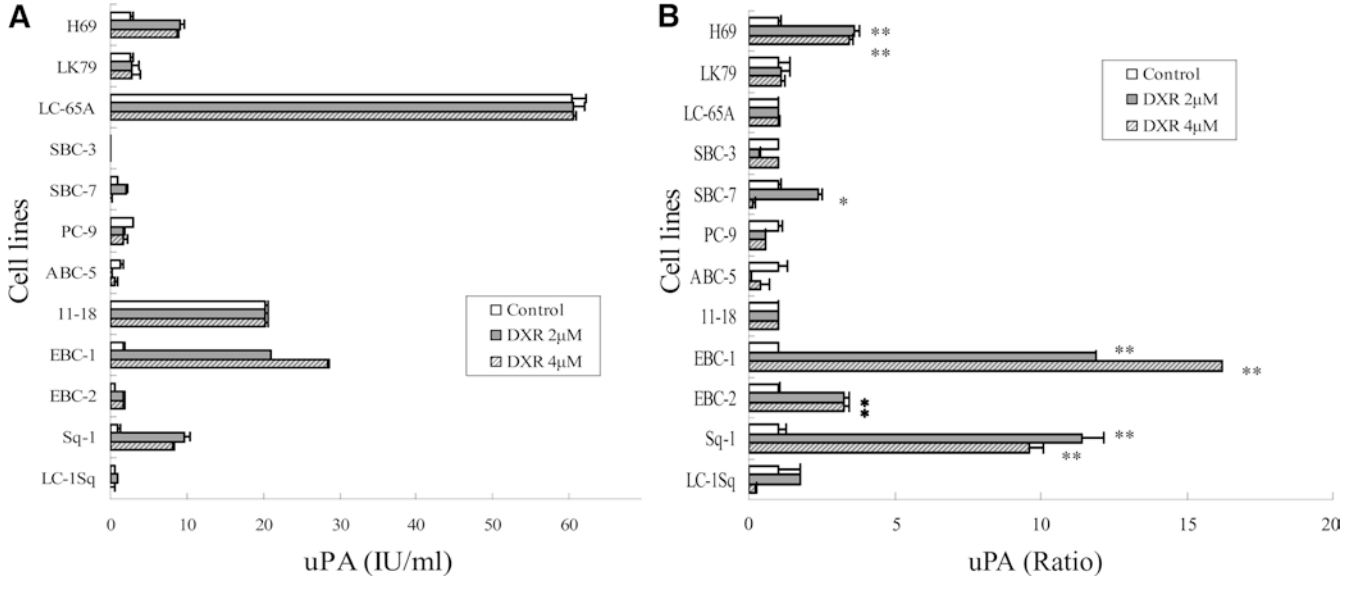


Fig. 3A,B TNF-α antigen levels in doxorubicin-treated lung cancer cells. Approximately 1–2×10^6 cells/ml per well of lung cancer cells were cultured in a 24-well plate for 24 h in the presence or absence of 2 or 4 μ*M* doxorubicin. **A** TNF-α antigen levels in the conditioned medium were measured by ELISA method as described in Materials and methods. **B** The relative ratio of antigen levels in doxorubicin-treated cells was calculated by dividing by the corresponding value obtained from control unstimulated cells and the results expressed with the control values arbitrarily set at 1.00. $^{**}P < 0.01$ vs control values

Fig. 4A, B uPA accumulation in doxorubicin-treated lung cancer cells. Approximately 1–2×10^6 cells/ml per well of lung cancer cells were cultured in a 24-well plate for 24 h in the presence or absence of 2 or 4 μ*M* doxorubicin. **A** uPA activities in the conditioned medium were measured using a synthetic uPA substrate S-2444 as described in Materials and methods. **B** The relative ratio of uPA levels in doxorubicin-treated cells was calculated by dividing by the corresponding value obtained from control unstimulated cells and the results expressed with the control values arbitrarily set at 1.00. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control values

maximally at ''sublethal'' concentrations. Changes in gene expression before and after doxorubicin stimulation have been previously examined in human malignant cells using the microarray technique, and a number of genes have been shown to be upregulated by either transient or persistent stimulation [17, 41]. Most of them overlap, and are directly related to fundamental cell functions such as cell cycling, signal transduction, transcription and/or metabolism, and their induction appears to reduce the apoptotic response to doxorubicin stimulation. Thus, it may contribute to drug resistance, although neither report refers to TNF-α, uPA, IL-8 or MCP-1.

However, these four genes among many ''cancer-related'' genes were induced by doxorubicin stimulation as shown in Fig. 1. Thus, these genes must have a similar gene expression mechanism. We have previously suggested the involvement of PEA3 transcription factor in



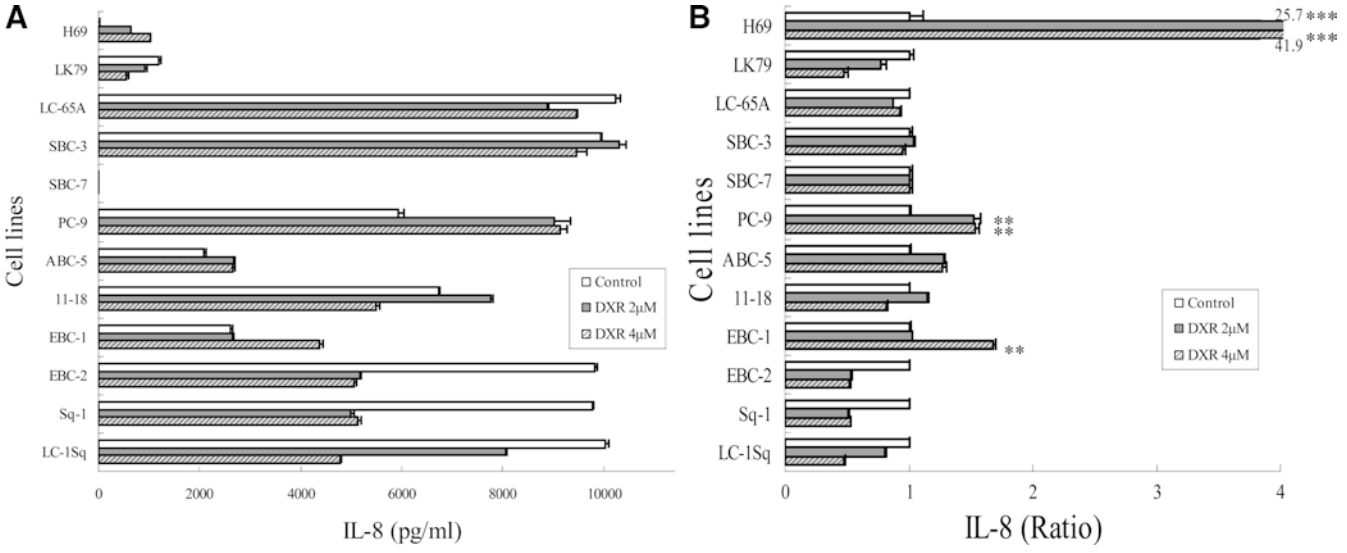


Fig. 5A, B IL-8 antigen levels in doxorubicin-treated lung cancer cells. Approximately 1–2×10^6 cells/ml per well of lung cancer cells were cultured in a 24-well plate for 24 h in the presence or absence of 2 or 4 μ*M* doxorubicin. **A** IL-8 antigen levels in the conditioned medium were measured by an ELISA method as described in Materials and methods. **B** The relative ratio of antigen levels in doxorubicin-treated cells was calculated by dividing by the corresponding value obtained from control unstimulated cells and the results expressed with the control values arbitrarily set at 1.00. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control values

doxorubicin-induced IL-8 expression in H69 cells [37], and in fact the consensus motif of PEA3 binding is found in the promoter region of all TNF-α, uPA, IL-8 and MCP-1 genes [8, 16, 33, 40]. However, these four proteins were not uniformly induced in the doxorubicin-treated cells as shown in Table 1, and it is not currently known why the cells express different levels of these factors upon doxorubicin stimulation, although epigenetic alteration such as methylation in the promoter regions of uPA has been reported to be responsible for changes in gene expression levels [42].

Over-expression of uPA, as well as IL-8 and MCP-1, has been shown in chemotherapy-resistant cancer cells [5, 6, 35], and a close association between high levels of these factors in tumor tissue and the malignant phenotype of the tumor has been reported [13, 15, 38]. Neovascularization in tumor tissue is essential in invasion and progression of the tumor, and it is regulated by various angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor and epidermal growth factor, as well as IL-8 and MCP-1 [43].

However, the effects of uPA, IL-8 and MCP-1 on tumor progression are unknown. uPA activates matrix

Fig. 6A,B MCP-1 antigen levels in doxorubicin-treated lung cancer cells. Approximately 1–2×10^6 cells/ml per well of lung cancer cells were cultured in a 24-well plate for 24 h in the presence or absence of 2 and 4 μ*M* doxorubicin. **A** MCP-1 antigen levels in the conditioned medium were measured by an ELISA as described in Materials and methods. **B** The relative ratio of antigen levels in doxorubicin-treated cells was calculated by dividing by the corresponding value obtained from control unstimulated cells and the results expressed with the control values arbitrarily set at 1.00. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control values

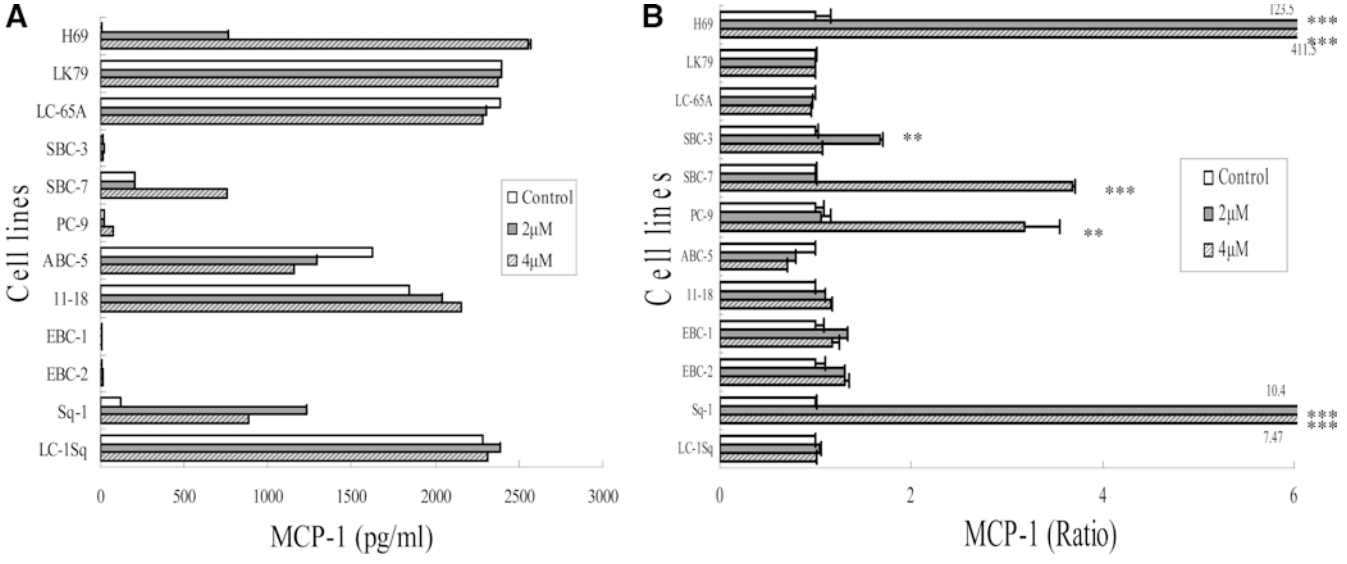


Table 1 TNF-α, uPA, IL-8 and MCP-1 expression following doxorubicin stimulation and the expression of the corresponding receptors such as TNFR I, TNFR II, uPAR, CXCR-1 and CCR-2 in 12 human lung carcinoma cell lines. The relative ratio of maximum expression levels with doxorubicin stimulation compared to that from unstimulated control cells is shown in this table. ($p < 0.05$, $p < 0.01$, $p < 0.001$ compared to control values.) The maximum expression levels with doxorubicin stimulation were compared with the levels in unstimulated control cells and the results expressed as *P* values

	TNFα	sTNFR I	TNFRII	uPA	uPAR	IL-8	CXCR-1	MCP-1	CCR-2
H69	$P < 0.01$	–	–	$P < 0.01$	–	$P < 0.001$	–	$P < 0.01$	–
LK79	–			–		–		–	
LC-65A	–			–		–		–	
SBC-3	–			–		–		$P < 0.01$	–
SBC-7	$P < 0.01$	–	–	$P < 0.05$	+	–		$P < 0.001$	–
PC-9	$P < 0.01$	–	–	–		$P < 0.01$	–	$P < 0.01$	–
ABC-5	–			–		–		–	
11-18	–			–		–		–	
EBC-1	–			$P < 0.01$	–	$P < 0.01$	+	–	
EBC-2	–			$P < 0.01$	–	–		–	
Sq-1	–			$P < 0.01$	–	–		$P < 0.001$	–
LC-1Sq	–			–		–		–	

metalloproteinase 9 and both can cleave the extracellular matrix and induce cell detachment. Detached tumor cells become more sensitive to cytotoxic drugs [36]. IL-8 and MCP-1 released from tumors may reduce the rate of tumor growth by inducing the infiltration of neutrophils and monocytes/macrophages to the tumor sites [15, 20]. As summarized in Table 1, uPAR was expressed only on SBC-7 cells, and CXCR-1 was expressed on EBC-1, but TNFR I, TNFR II, and CCR-2 were not expressed on the surface of any of the cell types, suggesting that TNF-α, uPA, IL-8 and MCP-1 secreted from tumor cells upon doxorubicin stimulation essentially act on the inflammatory/immune cells expressing the receptors in a paracrine fashion. Thus, the significance of the doxorubicin-induced factors appears to be much more prominent in the in vivo setting than in simple cell experiments such as those performed here, because these factors directly and indirectly interact with each other and may provoke interaction between tumor and inflammatory/immune cells.

TNF-α is a major proinflammatory cytokine and can kill tumor cells, and IL-8 is a major chemokine for neutrophils and is also chemotactic for T lymphocytes [18]. uPA activates matrix metalloproteinase 9 which can truncate and activate IL-8. MCP-1 is a major chemoattractant for monocytes/macrophages and tumor cell contact itself activates macrophages, and tumor-associated macrophages can secrete TNF-α, IL-1, IL-8, proteases and ROS to augment cytotoxicity. Furthermore, increased responsiveness of specific cytotoxic T cells against implanted lymphoma cells has been shown in animals treated with combinations of doxorubicin and TNF-α, and this was correlated with survival [7]. Therefore, simultaneous expression of these factors may enhance the antitumor effects of doxorubicin when used clinically, even if these factors were maximally expressed at sublethal concentrations of doxorubicin in a simple cell experiment, although it should be clarified whether the efficacy of doxorubicin depends on the inducibility of these factors in tumor cells.

Acknowledgements This work was supported in part by a Grant-in-Aid (no. 12771476) for Encouragement of Young Scientists (to M.S.) and a Grant-in-Aid (no. 10670937) for Scientific Research (C) (to K.N.) from the Ministry of Education, Sciences, Sports and Culture, Japan. Northern blotting was carried out in the Radioisotope Center of Okayama University.

References

1. Aggarwal BB, Natarajan K (1996) Tumor necrosis factors: developments during the last decade. Eur Cytokine Netw 7:93
2. Baggiolini M, Dewald B, Moser B (1994) Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. Adv Immunol 55:97
3. Bussolino F, Camussi G, Baglioni C (1988) Synthesis and release of platelet-activating factor by human vascular endothelial cells treated with tumor necrosis factor or interleukin 1 alpha. J Biol Chem 263:11856
4. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D (1997) Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet 17:439
5. De Larco JE, Wuertz BR, Manivel JC, Furcht LT (2001) Progression and enhancement of metastatic potential after exposure of tumor cells to chemotherapeutic agents. Cancer Res 61:2857
6. Duan Z, Feller AJ, Penson RT, Chabner BA, Seiden MV (1999) Discovery of differentially expressed genes associated with paclitaxel resistance using cDNA array technology: analysis of interleukin (IL) 6, IL-8, and monocyte chemotactic protein 1 in the paclitaxel-resistant phenotype. Clin Cancer Res 5:3445
7. Ehrke MJ, Verstovsek S, Maccubbin DL, Ujhazy P, Zaleskis G, Berleth E, Mihich E (2000) Protective specific immunity induced by doxorubicin plus TNF-alpha combination treatment of EL4 lymphoma-bearing C57BL/6 mice. Int J Cancer 87:101
8. Evans CP, Stapp EC, Dall'Era MA, Juarez J, Yang JC (2001) Regulation of u-PA gene expression in human prostate cancer. Int J Cancer 94:390
9. Feng L, Xia Y, Garcia GE, Hwang D, Wilson CB (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. J Clin Invest 95:1669
10. Fisher DE (1994) Apoptosis in cancer therapy: crossing the threshold. Cell 78:539
11. Furutani Y, Nomura H, Notake M, Oyamada Y, Fukui T, Yamada M, Larsen CG, Oppenheim JJ, Matsushima K (1989)

Cloning and sequencing of the cDNA for human monocyte chemotactic and activating factor (MCAF). Biochem Biophys Res Commun 159:249
12. Hiraki A, Kaneshige T, Kiura K, Ueoka H, Yamane H, Tanaka M, Harada M (1999) Loss of HLA haplotype in lung cancer cell lines: implications for immunosurveillance of altered HLA class I/II phenotypes in lung cancer. Clin Cancer Res 5:933
13. Inoue K, Slaton JW, Kim SJ, Perrotte P, Eve BY, Bar-Eli M, Radinsky R, Dinney CP (2000) Interleukin 8 expression regulates tumorigenicity and metastasis in human bladder cancer. Cancer Res 60:2290
14. Kiguchi T, Niiya K, Shibakura M, Miyazono T, Shinagawa K, Ishimaru F, Kiura K, Ikeda K, Nakata Y, Harada M (2001) Induction of urokinase-type plasminogen activator by the anthracycline antibiotic in human RC-K8 lymphoma and H69 lung-carcinoma cells. Int J Cancer 93:792
15. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258:1798
16. Kramer B, Wiegmann K, Kronke M (1995) Regulation of the human TNF promoter by the transcription factor Ets. J Biol Chem 270:6577
17. Kudoh K, Ramanna M, Ravatn R, Elkahloun AG, Bittner ML, Meltzer PS, Trent JM, Dalton WS, Chin KV (2000) Monitoring the expression profiles of doxorubicin-induced and doxorubicin-resistant cancer cells by cDNA microarray. Cancer Res 60:4161
18. Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K (1989) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science 243:1464
19. Laster SM, Wood JG, Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J Immunol 141:2629
20. Lee LF, Hellendall RP, Wang Y, Haskill JS, Mukaida N, Matsushima K, Ting JP (2000) IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. J Immunol 164:2769
21. Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104:487
22. Montovani A, Sozzani S, Proost P, van Damme J (1996) The monocyte chemoattractant protein family. In: Horuk R (ed) Chemoattractant ligands and their receptors: pharmacology and toxicology: basic and clinical aspects, CRC Press, Boca Raton, FL, p 169
23. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55
24. Mueller H (1998) Tumor necrosis factor as an antineoplastic agent: pitfalls and promises. Cell Mol Life Sci 54:1291
25. Muller I, Jenner A, Bruchelt G, Niethammer D, Halliwell B (1997) Effect of concentration on the cytotoxic mechanism of doxorubicin—apoptosis and oxidative DNA damage. Biochem Biophys Res Commun 230:254
26. Nawroth PP, Bank I, Handley D, Cassimeris J, Chess L, Stern D (1986) Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363
27. Niiya K, Shinbo M, Ozawa T, Hayakawa Y, Sakuragawa N (1995) Modulation of urokinase-type plasminogen activator gene expression by inflammatory cytokines in human pre-B lymphoma cell line RC-K8. Thromb Haemost 74:1511
28. Nokihara H, Nishioka Y, Yano S, Mukaida N, Matsushima K, Tsuruo T, Sone S (1999) Monocyte chemoattractant protein-1 gene modification of multidrug-resistant human lung cancer enhances antimetastatic effect of therapy with anti-P-glycoprotein antibody in SCID mice. Int J Cancer 80:773
29. Pober JS, Gimbrone MA Jr, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA (1986) Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol 137:1893
30. Powis G (1989) Free radical formation by antitumor quinones. Free Radic Biol Med 6:63
31. Rebbaa A, Chou PM, Emran M, Mirkin BL (2001) Doxorubicin-induced apoptosis in caspase-8-deficient neuroblastoma cells is mediated through direct action on mitochondria. Cancer Chemother Pharmacol 48:423
32. Rizzardini M, Carelli M, Cabello Porras MR, Cantoni L (1994) Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by N-acetylcysteine. Biochem J 304 (Pt 2):477
33. Roebuck KA (1999) Regulation of interleukin-8 gene expression. J Interferon Cytokine Res 19:429
34. Sambrook J, Fritsh EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory
35. Schmitt M, Harbeck N, Thomssen C, Wilhelm O, Magdolen V, Reuning U, Ulm K, Hofler H, Janicke F, Graeff H (1997) Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb Haemost 78:285
36. Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, Chilvers ER, Dransfield I, Donnelly SC, Strieter R, Haslett C (1999) Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med 5:662
37. Shibakura M, Niiya K, Kiguchi T, Kitajima I, Niiya M, Asaumi N, Huh NH, Nakata Y, Harada M, Tanimoto M (2003) Induction of IL-8 and monocyte chemoattractant protein-1 by doxorubicin in human small cell lung carcinoma cells. Int J Cancer 103:380
38. Strieter RM, Polverini PJ, Arenberg DA, Walz A, Opdenakker G, Van Damme J, Kunkel SL (1995) Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. J Leukoc Biol 57:752
39. Takaki T, Hiraki A, Uenaka A, Gomi S, Itoh K, Udono H, Shibuya A, Tsuji T, Sekiguchi S, Nakayama E (1998) Variable expression on lung cancer cell lines of HLA-A2-binding MAGE-3 peptide recognized by cytotoxic T lymphocytes. Int J Oncol 12:1103
40. Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T (1997) Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. J Biol Chem 272:31092
41. Watts GS, Futscher BW, Isett R, Gleason-Guzman M, Kunkel MW, Salmon SE (2001) cDNA microarray analysis of multidrug resistance: doxorubicin selection produces multiple defects in apoptosis signaling pathways. J Pharmacol Exp Ther 299:434
42. Xing RH, Rabbani SA (1999) Transcriptional regulation of urokinase (uPA) gene expression in breast cancer cells: role of DNA methylation. Int J Cancer 81:443
43. Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M (1997) Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol 17:4015
44. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ (1987) Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci U S A 84:9233