Cancer Chemother Pharmacol (2002) 50: 213–222
DOI 10.1007/s00280-002-0496-5

ORIGINAL ARTICLE

Witold Lasek · Tomasz Świtaj · Jacek Sieńko
Monika Kasprzycka · Grzegorz Basak
Piotr Mikłaszewicz · Małgorzata Maj · Dominika Nowis
Tomasz Grzela · Jakub Gołąb · Izabela Młynarczuk
Ahmad Jalili · Bożena Kamińska
Magdalena Dziembowska · Krzysztof Czajkowski
Maria Nowaczyk · Agata Górska
Zygmunt Kazimierczuk

Stimulation of TNF-α production by 2-(1-adamantylamino)-6-methylpyridine (AdAMP) – a novel immunomodulator with potential application in tumour immunotherapy

Received: 2 January 2002 / Accepted: 24 June 2002 / Published online: 31 July 2002

Abstract The immunomodulatory effects of a recently synthesized adamantane derivative of aminopyridine – 2-(1-adamantylamino)-6-methylpyridine (AdAMP) – were tested on normal and neoplastic cells in vitro. When incubated with TNF-α gene-transduced mouse melanoma cells (B78/TNF), AdAMP significantly enhanced basal production of TNF-α by these cells, both by "high" and "moderate" TNF-α-producer cells. A similar TNF-α production-enhancing effect was observed in cultures of human ovarian carcinoma cells (CAOV1) which spontaneously produce TNF-α but not in cultures of tumour cells incapable of TNF-α secretion. RT-PCR analysis showed that the enhancement of TNF-α production by AdAMP was associated with an increase in TNF-α mRNA expression in the treated cells. The results of an electrophoretic mobility shift assay (EMSA) showed that AdAMP significantly activated nuclear factor κB (NF-κB) in both CAOV1 and B78/TNF cells. The role of NF-κB in enhancement of TNF-α production was confirmed in experiments in which MG132, an inhibitor of NF-κB activation, reversed the effect of AdAMP. Unexpectedly, dexamethasone, a potent antiinflammatory agent and a strong inhibitor of TNF-α production in vivo, increased both spontaneous and AdAMP-augmented production of TNF-α in in vitro cultures of ovarian carcinoma cells and B78/TNF cells. AdAMP also enhanced TNF-α secretion by LPS-induced monocytes. AdAMP-induced augmentation of TNF-α production by B78/TNF cells was accompanied by morphological changes in the treated cells and a decrease in their adherence to fibrinogen and collagen IV. In view of these properties, AdAMP seems to be a therapeutically promising compound with potential application as an adjuvant augmenting the efficacy of cancer vaccine-based therapies or in the local treatment of certain tumours.

Keywords TNF-α · Adamantane · Tumour immunotherapy

This work was supported by the Foundation for Development of Diagnostics and Therapy, the Foundation for Polish Science, and Grant 1 M19/W2 from the Medical University, Warsaw, Poland.

W. Lasek (✉) · T. Świtaj · G. Basak · P. Mikłaszewicz
M. Maj · D. Nowis · J. Gołąb · I. Młynarczuk · A. Jalili
Department of Immunology, Centre of Biostructure,
Medical University, Chalubinskiego 5, 02-004 Warsaw, Poland
E-mail: wlasek@ib.amwaw.edu.pl
Tel.: +48-22-6226306
Fax: +48-22-6226306

J. Sieńko · K. Czajkowski
II Department and Clinic of Obstetrics and Gynecology,
Medical University, Warsaw, Poland

M. Kasprzycka · M. Nowaczyk
Department of Clinical Immunology,
Transplantation Institute, Medical University, Warsaw, Poland

T. Grzela
Department of Histology and Embryology,
Centre of Biostructure, Medical University, Warsaw, Poland

B. Kamińska · M. Dziembowska
Department of Cellular Biochemistry,
Nencki Institute of Experimental Biology, Warsaw, Poland

A. Górska · Z. Kazimierczuk
Institute of Chemistry, Agricultural University, Warsaw, Poland

Introduction

Tumour necrosis factor α (TNF-α) is a cytokine primarily produced by cells of the mononuclear system, and is recognized as one of the key mediators of immune and inflammatory responses (for review see reference 1).

Among many biological effects of TNF-α some are interesting in the context of tumour therapy. These include: (a) destruction of tumour vasculature and induction of haemorrhagic necrosis in vivo [10], (b) direct cytotoxic effect on some tumour cells in vitro [26, 40], and (c) activation of cells of the immune system to exert antitumour effects [42]. However, despite its spectacular activity against murine tumours, TNF-α has been found to be toxic when administered systemically in cancer patients with disseminated disease [17]. For this reason, the clinical use of TNF-α as an antitumour agent is at present limited to regional administration, e.g. in isolated limb perfusion regimens. In this approach TNF-α is successfully used in combination with chemotherapy and IFN-γ, under hyperthermic conditions, to treat patients with in transit melanomas or inoperable sarcomas confined to the extremities [28].

One of the strategies for improving the therapeutic index of TNF-α is to stimulate its endogenous production. A number of natural agents (for example bacterial wall components such as lipopolysaccharides, LPS [5]) and synthetic low molecular weight chemical compounds exhibit TNF-α-inducing activity. The latter group includes alkylphthalimides [39], Taxol [7, 8], and derivatives of xanthenone of which 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is currently in clinical trials [18].

Recently, we have synthesized several adamantylamino-pyridine and -pyrimidine derivatives with TNF-α production-enhancing properties [22, 31]. In the present study we examined the effects of AdAMP (Fig. 1), the most potent of these adamantane derivatives, on some biological functions and TNF-α production by normal and neoplastic cells.

Materials and methods

Tumour cells

The B78H1 melanoma cell line transduced with the gene for human TNF-α (herein named B78/TNF) has been described previously [27]. A DCCMV-TNF-α vector was generated by blunt end ligation of human TNF-α, excised as EcoRI fragment from the MSCV-TNF retroviral vector, into a filled SalI site of DCCMV between the hCMV-IE promoter and the EMCV IRES-Neo cassette [27]. Two TNF-α-producing clones (B78/TNF/3, a "high producer", and B78/TNF/9, a "moderate producer") were used in this study. Other established cell lines included SK-OV-3 (ATCC no. HTB-77), MDAH 2774 (ATCC no. CRL-10303), and CAOV1 human ovarian carcinoma cells. We also used the human ovarian cell lines OVA-2, OVA-4, OVA-11 that were established in our laboratory.

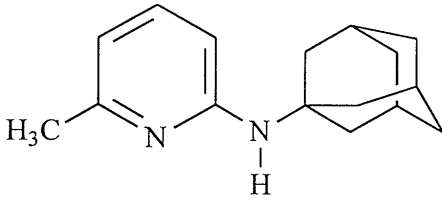

Fig. 1. Chemical structure of AdAMP

All of the cell lines were cultured in Dulbecco's MEM with 4.5 g/l glucose, sodium pyruvate and Glutamax-1 (high glucose DMEM) supplemented with antibiotic-antimycotic, 50 μ*M* 2-mercaptoethanol and 10% fetal calf serum (FCS) (all from Gibco BRL, Life Technologies, Paisley, UK). Cells were maintained in 25-cm^2 tissue flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere containing 5% CO_2 and were passaged two or three times weekly.

Reagents

AdAMP was synthesized at the Institute of Chemistry, Agriculture University of Warsaw, as described previously [22]. The crystallographic structure and other details of AdAMP are reported elsewhere [31]. A stock solution of AdAMP (100 m*M*) was prepared in DMSO and was diluted to the required concentrations in culture medium. Water-soluble actinomycin D (actinomycin D-mannitol), N-CBZ-Leu-Leu-Leu-al (MG132), pyrrolidine dithiocarbamate, rolipram, fibronectin, collagen IV and LPS from *E. coli* 055:B5 (prepared by trichloroacetic acid precipitation) were purchased from Sigma (St. Louis, Mo.). Forskolin was purchased from Calbiochem (La Jolla, Calif.), and dexamethasone (Dexaven) was from Jelfa (Jelenia Gora, Poland).

Determination of TNF-α in culture supernatants

Tumour cells were cultured in 24-well plates in the presence of different concentrations of AdAMP for 24 h (1 or 2×10^5 cells/ml). The concentration of human TNF-α in culture supernatants was measured using a DuoSet ELISA development system (R&D Systems, Minneapolis, Minn.) according to the protocol provided by the manufacturer. The capture antibody was mouse anti-human TNF-α, and the detection antibody was biotinylated goat anti-human TNF-α. Occasionally, tumour cells were incubated with DMSO at concentrations comparable with those in AdAMP-treated cultures (1:1000 and lower). No enhancing effects of DMSO on TNF-α production were found. In some experiments, the activity of TNF-α in culture supernatants was measured in parallel using the L929 bioassay in which L929 cells were incubated with actinomycin D (1 μg/ml) and serial dilutions of supernatants. The amount of TNF-α measured by ELISA correlated with its activity in the bioassay.

Preparation of nuclear protein extracts and electrophoretic mobility shift assay (EMSA)

Cells were untreated or treated with 10 μM AdAMP for 4, 8 or 24 h. At specific time points cells were washed with cold phosphate-buffered saline (PBS), and nuclear extracts were isolated as previously described [32]. Double-stranded oligonucleotides containing consensus recognition sites for NF-κB transcription factors were labelled with γ-[^{32}P]dATP using polynucleotide kinase (Promega, Madison, Wis.) and purified on spun columns. EMSA was performed by incubation of protein extracts (10 μg) in EMSA buffer (25 m*M* Hepes, pH 7.9, 40 m*M* KCl, 0.1 m*M* EDTA, 3 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 20 μg/ml poly(dI-dC), 10% glycerol) with 0.2 ng labelled oligonucleotide for 20 min at room temperature. Samples were resolved on 6% native polyacrylamide gels, and visualized by autoradiography. The oligonucleotide probe was NF-κB-5′-AGTTGAGGGGACTTTCCCAGGC-3′.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

The expression of specific mRNA for TNF-α in the B78/TNF/3 clone and CAOV1 cell line after AdAMP treatment (100, 10, 1 or 0.1 μ*M*) was determined using a semiquantitative RT-PCR method with β-actin as internal standard. Following a 24-h incubation

(2×10^5 cells/ml), cells were washed twice in ice-cold PBS and total RNA was isolated using TRIzol reagent (Gibco, San Diego, Calif.), according to the protocol provided by manufacturer. The amount and quality of the RNA preparations were estimated by spectrophotometric measurement at the UV wavelengths of 260 nm and 280 nm. Then 2 μg of total RNA was subjected to reverse transcription using oligo-deoxythymidine primers (dT_{15}) and AMV reverse transcriptase (both from Promega, Madison, Wis.). PCR was performed using complementary DNA templates, 0.2 m*M* dNTPs, specific forward and backward primers (see below) for TNF-α and β-actin (0.4 μ*M* of each) and Taq polymerase (Promega) [35, 38]. The forward and backward primers, respectively, for β-actin were 5′-TAC ATG GCT GGG GTG TTG AA and 3′-AAG AGA GGC ATC CTC ACC CT, and for TNF-α were 5′-CTT CTG CCT GCT GCA CTT TGG A and 3′-TCC CAA AGT AGA CCT GCC CAG A.

The PCR conditions for TNF-α were 94°C for 20 s, 55°C for 20 s and 72°C for 20 s, and for β-actin were 94°C for 20 s, 58°C for 20 s and 72°C for 20 s. Each run was followed by 5 min of final elongation at 72°C. The numbers of amplification cycles for TNF-α and β-actin were 35 and 30, respectively. These numbers of cycles were established as being always in the linear part of the reaction product amount curve. The PCR amplification products were separated in 1.0% agarose gels with 0.2 μg/ml of ethidium bromide and pictures were taken using a digital image capture system (GDS9000; Ultra-Violet Products, Cambridge, UK). The relative expression of cytokine-specific mRNA is presented in relation to the respective amount of β-actin transcript.

Monocyte cultures

Heparinized blood samples diluted 1:1 (v/v) with PBS were centrifuged with Gradisol (Polfa, Kutno, Poland) to separate mononuclear cells from granulocytes and erythrocytes. After aspiration of the mononuclear cell fraction, the cells were washed three times in PBS, resuspended in RPMI-1640 medium containing 10% FCS and incubated overnight to allow separation of mononuclear cells from thrombocytes. The supernatant was then discarded gently, the cell pellet was resuspended in 10 ml RPMI-1640 medium containing 2% FCS and left in Petri dishes for 30 min at 37°C in an atmosphere containing 5% CO_2 to allow adherence of monocytes. Nonadherent cells were removed by washing the dishes with warm (37°C) culture medium. Finally, the Petri dishes were washed extensively with cold (4°C) RPMI-1640 and recultured in 24-well plates (2.5×10^5 cells/ml) with or without 1 μg/ml of LPS and/or appropriate concentrations of AdAMP. After 24 h, the culture supernatants were collected and frozen. The amounts of TNF-α in the supernatants were determined within 3 months.

[^{3}H]Thymidine incorporation assay

B78/TNF/3 melanoma cells were cultured for 20 h at a concentration of 1×10^4 cells/200 μl culture medium per well in a 96-well flat-bottomed microtitre plate (Nunc) in the presence of serially diluted AdAMP (in triplicate). The cells were then pulsed with [^{3}H]thymidine (Polatom, Otwock-Swierk, Poland) (1 μCi per well) for 5 h, trypsinized and automatically harvested onto glass-fibre strips using a Skatron harvester. [^{3}H]Thymidine incorporation was determined using a Wallac liquid scintillation counter (1450 Microbeta Plus; Wallac, Gaithersburg, Md.).

Matrix adhesion assay

Adhesion of B78/TNF/9 cells to matrix proteins was tested in a matrix adhesion assay [14]. Briefly, aliquots of fibronectin or collagen IV dissolved in PBS (10 μg/ml) were added to wells of a 96-well flat-bottomed plastic plate (Nunc) and incubated for 18 h at 4°C (in quadruplicate). After three washes, the plates were blocked with 1% bovine serum albumin for 2 h at room temperature. Next, B78/TNF/9 cells were added to each well (4×10^4 cells/well) and incubated with different concentrations of AdAMP for 20 h at 37°C. Nonadherent cells were washed off and the remaining cells were stained with 0.1% crystal violet in 20% methanol for 10 min at room temperature. The unbound dye was washed off and the plates were air-dried. The bound dye was solubilized in methanol and the absorbance was measured at 600 nm in an ELISA reader (Spectra).

Statistical analysis

Data are expressed as means ± SD. Differences between samples were analysed for significance using Student's *t*-test (two-tailed).

Results

Effect of AdAMP on the production of TNF-α by B78/TNF cells

Preliminary studies, using TNF-α gene-modified tumour cells (B78/TNF), had shown that AdAMP is the most potent stimulator of TNF-α production among the adamantylamino-pyridines and -pyrimidines synthesized in the laboratories of the Institute of Chemistry, Agriculture University of Warsaw [22]. As shown in Fig. 2, AdAMP strongly enhanced TNF-α production by TNF-α gene-transduced B78H1 cells. In the presence of 10 μ*M* AdAMP, an increase in TNF-α production was observed both in cultures of clone 3 of this cell line (B78/TNF/3, producing large amounts of TNF-α) (Fig. 2A) and in cultures of clone B78/TNF/9 (''moderate producer clone'') (Fig. 2B): 249% and 355% in relation to the amount produced in control unstimulated cultures of B78/TNF, respectively. The induction of TNF-α production by AdAMP was dose-dependent, and at concentrations as low as 100 n*M* production of TNF-α by B78/TNF/9 melanoma cells was significantly augmented (5.2 vs 3.3 ng/ml, $P < 0.01$).

Effect of AdAMP on the viability of B78/TNF/3 cells

The amount of TNF-α produced by genetically modified tumour cells incubated with 100 μ*M* AdAMP was significantly lower than that produced in cultures with 10 μ*M*. To determine whether the decrease in TNF-α production could have resulted from a toxic effect of AdAMP, the viability of B78/TNF/3 cells was tested in a standard 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay. Results of experiments in which B78/TNF/3 cells were incubated with different concentrations of AdAMP confirmed this supposition. Incubation of tumour cells at concentrations of 100 μ*M* and higher induced a significant reduction in tumour cell viability (data not presented). The results of the [^{3}H]thymidine incorporation assay (Fig. 3) revealed that, independently of TNF-α production-enhancing activity, AdAMP had moderate cytostatic properties.

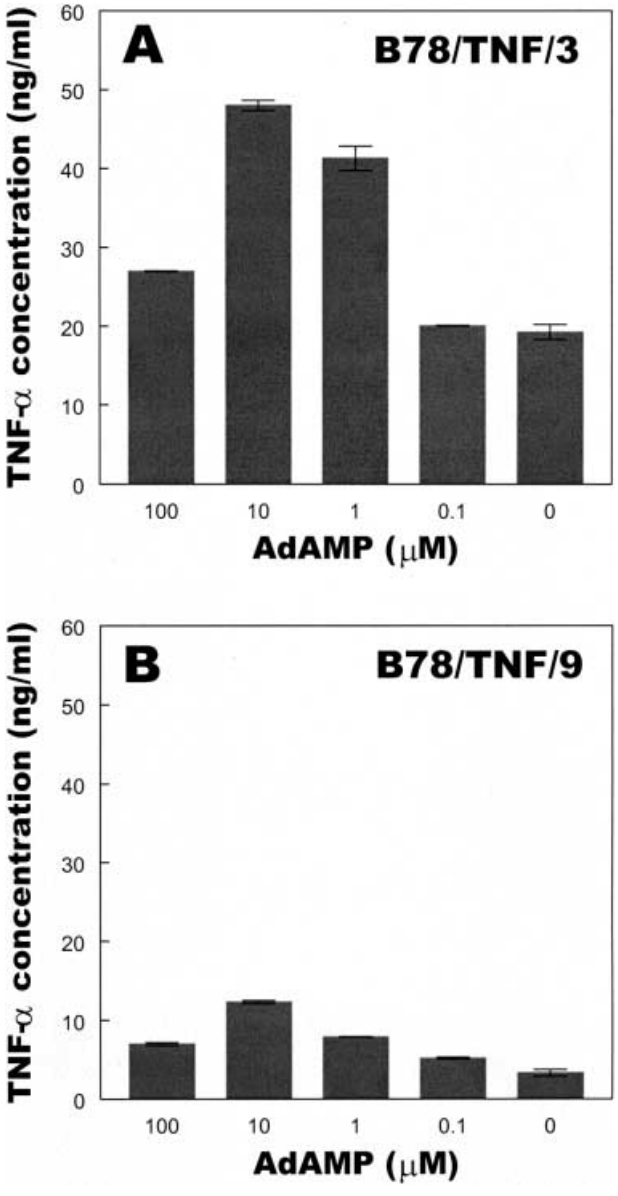

Fig. 2A, B. Effect of AdAMP on TNF-α production by TNF-α gene-transduced B78 melanoma cells (B78/TNF). Cells of the clone B78/TNF/3 (high producer) (**A**) and B78/TNF/9 (moderate producer) (**B**) were incubated (2×10^5 cells/ml) for 24 h in the presence of different concentrations of AdAMP. The amounts of TNF-α released into the culture medium were measured by ELISA. Data are means ± SD. The results are representative of four individual experiments

Effect of AdAMP on TNF-α production by human ovarian cancer cells

Some tumour cells express or spontaneously release cytokines, including TNF-α. For example, expression of TNF-α by ovarian cancers and their ability to secrete TNF-α is well documented [34, 41]. To determine whether AdAMP could stimulate TNF-α production by ovarian cancer cells, several human ovarian cancer cell lines were tested in preliminary experiments. We found that AdAMP did not stimulate TNF-α secretion by

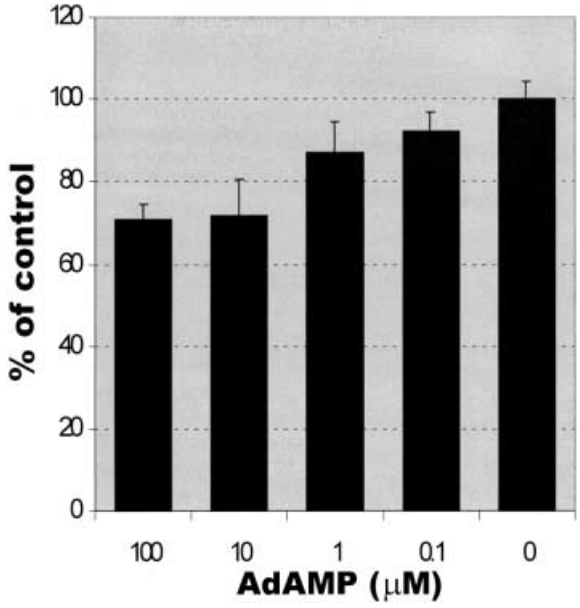

Fig. 3. Antiproliferative effect of AdAMP on B78/TNF/3 melanoma cells. Tumour cells were incubated with different concentrations of AdAMP for 24 h. The rate of proliferation was assayed in a standard [^{3}H]thymidine incorporation assay, as described in Materials and methods. Data are means ± SD

primarily non-producing tumour cells (e.g. MDAH 2774). However, both low- (SK-OV-3, OVA-4), moderate- (OVA-2), and high-producing (CAOV1, OVA-11) cell lines were found to be susceptible to the TNF-α-stimulating action of AdAMP. A representative, dose-dependent pattern of TNF-α secretion by CAOV1 ovarian cancer cells incubated with AdAMP is presented in Fig. 4. As shown in Fig. 4, AdAMP significantly stimulated production of TNF-α by CAOV1 cells at concentrations as low as 100 n*M* (14.3 vs 11.1 ng/ml in control cultures, $P < 0.01$). Interestingly, CAOV1 ovarian carcinoma cells were found to be much more susceptible to the cytostatic/cytotoxic effects of AdAMP than B78/TNF cells (Figs. 2 and 4).

Effect of AdAMP on TNF-α production by LPS-stimulated human monocytes

Since TNF-α is mainly produced by cells of the monocyte/macrophage lineage, we tested the ability of AdAMP to stimulate TNF-α production by LPS-activated human monocytes (Table 1). The basal production of TNF-α by monocytes (33 pg/ml) was augmented in cultures containing 1 μg/ml LPS (to 59 pg/ml TNF-α) and was additionally enhanced in the presence of 1 or 10 μ*M* of AdAMP to 95 pg/ml ($P < 0.01$) and 90 pg/ml ($P < 0.05$) TNF-α, respectively. These relatively low concentrations of TNF-α (in comparison with levels of TNF-α in cultures of CAOV1 and B78/TNF cells) could be explained by the prolonged procedure of monocyte preparation and/or incomplete maturation of these cells in in vitro cultures. Higher concentrations of AdAMP (100 μ*M*) were found to be toxic to monocytes (Table 1).

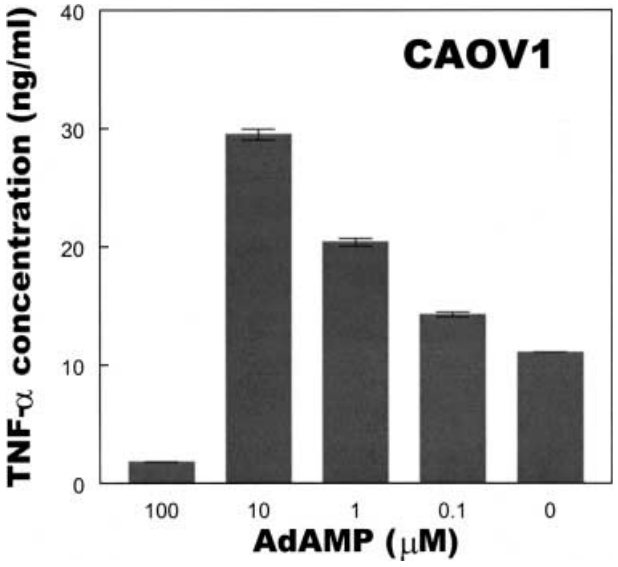

Fig. 4. Effect of AdAMP on TNF-α production by CAOV1 ovarian carcinoma cells. Tumour cells (2×10^5 cells/ml) were incubated for 24 h in the presence of different concentrations of AdAMP. The amounts of TNF-α released into the culture medium were measured by ELISA

Table 1. Production of TNF-α by human monocytes incubated with AdAMP and/or LPS. Monocytes (2.5×10^5/ml) were incubated in the presence of different concentrations of AdAMP for 18 h, and the amounts of TNF-α in the supernatants were measured by ELISA. Values are means ± SD

AdAMP (μ*M*)	TNF-α production in monocyte culture (pg/ml)	
	Without LPS	With LPS (1 μg/ml)
100	0[a]	10 ± 1
10	36 ± 4	90 ± 14
1	35 ± 4	95 ± 4
0	33 ± 3	59 ± 8

[a]Below sensitivity of the assay

AdAMP increases mRNA expression in B78/TNF/3 and CAOV1 cells

To get some information on the biochemical events in cells treated with AdAMP, B78/TNF/3 and CAOV1 cells were incubated with different concentrations of this compound and were subjected to RT-PCR analysis for TNF-α mRNA. As shown in Fig. 5, incubation with AdAMP markedly increased expression of mRNA for TNF-α both in B78/TNF/3 (Fig. 5A) and CAOV1 (Fig. 5B) cells.

MG132 inhibits production of TNF-α by CAOV1 ovarian carcinoma cells

To investigate the potential role of NF-κB in AdAMP-induced stimulation of TNF-α production, CAOV1 cells, either untreated or treated with 10 μ*M* AdAMP, were incubated with MG132, an inhibitor of NF-κB

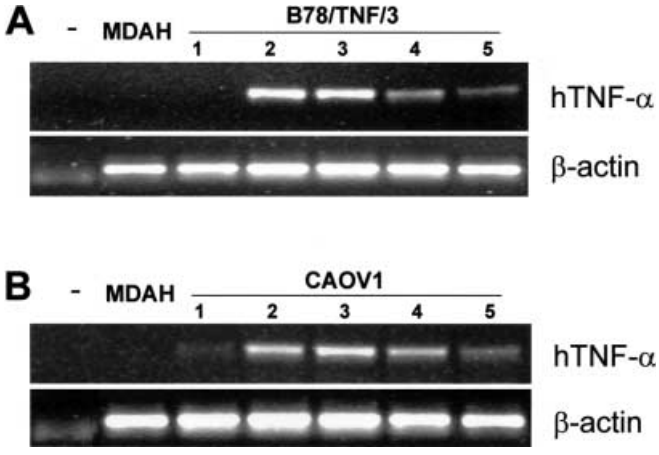

Fig. 5A, B. RT-PCR analysis of TNF-α and *β*-actin mRNA expression in B78/TNF/3 and CAOV1 cells treated with AdAMP. *Lane* – negative control containing all the components of the PCR except the template DNA; *lanes 1, 2, 3, 4, 5* cells treated, respectively, with 100, 10, 1 and 0.1 μ*M* AdAMP, and untreated; *lane MDAH* MDAH 2774 cells, non-secreting TNF-α as assayed by ELISA

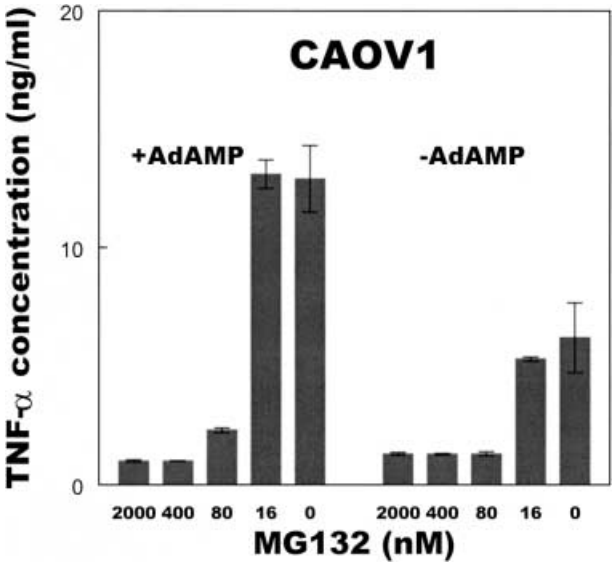

Fig. 6. Effect of MG132 on basal and AdAMP-stimulated TNF-α production by CAOV1 ovarian carcinoma cells. The cell culture conditions and determination of the TNF-α levels were as described for Fig. 2

activation. As shown in Fig. 6, MG132 (at concentrations of 80 n*M* and higher) potently inhibited spontaneous production of TNF-α and reversed the effect of AdAMP. This inhibition of TNF-α production was not due to a toxic effect of MG132 since at the concentrations tested, MG132 induced only a marginal loss of CAOV1 cell viability (as tested by the MTT assay; not presented). Similar inhibitory effects on TNF-α production by CAOV1 cells were observed when another NF-κB inhibitor – pyrrolidine dithiocarbamate – was used instead of MG132 (not shown).

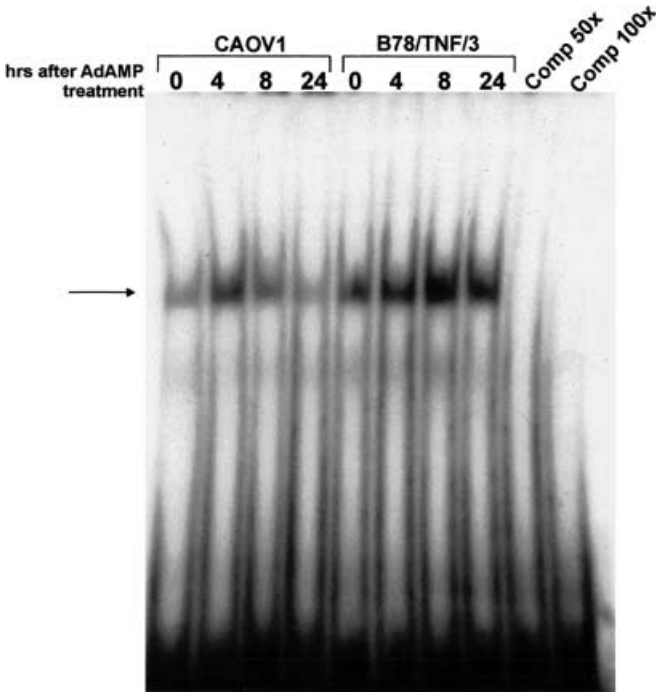

Fig. 7. AdAMP induces DNA NF-κB binding activity. CAOV1 and B78/TNF/3 cells were treated with AdAMP (10 μM) and nuclear extracts were isolated at various times after treatment. An EMSA assay was performed by incubation of nuclear extracts with the specific ^{32}P-labelled oligonucleotides carrying NF-κB binding sites. The reaction products were resolved by electrophoresis and the gels were dried and exposed to X-ray films. The *arrow* indicates the band corresponding to specific DNA/protein complex. An unlabelled competitor (*Comp*) was used in 50-fold and 100-fold excess

AdAMP activates NF-κB transcription factor in CAOV1 and B78/TNF/3 cells

To shed more light on the role of NF-κB in AdAMP-induced TNF-α production, EMSA was employed to determine DNA-binding activity of NF-κB transcription factor after AdAMP stimulation. The increase in NF-κB activity occurred 4 h after treatment in CAOV1 cells and had decreased by 8 h (Fig. 7). In B78/TNF/3 cells, NF-κB binding activity remained elevated after 24 h. The specificity of binding to the respective DNA binding site was confirmed by preincubation of extracts with 50-fold or 100-fold excesses of unlabelled probes (Fig. 7, lanes labelled Comp).

Effects of rolipram and dexamethasone on production of TNF-α

Both rolipram and dexamethasone exert inhibitory effects on the production of proinflammatory cytokines, including TNF-α, in vivo [6, 11, 15, 16, 19]. Rolipram, a type IV phosphodiesterase inhibitor, augments the levels of cAMP [24] and inhibits transcription of certain genes, including those coding for TNF-α [25], while the effects

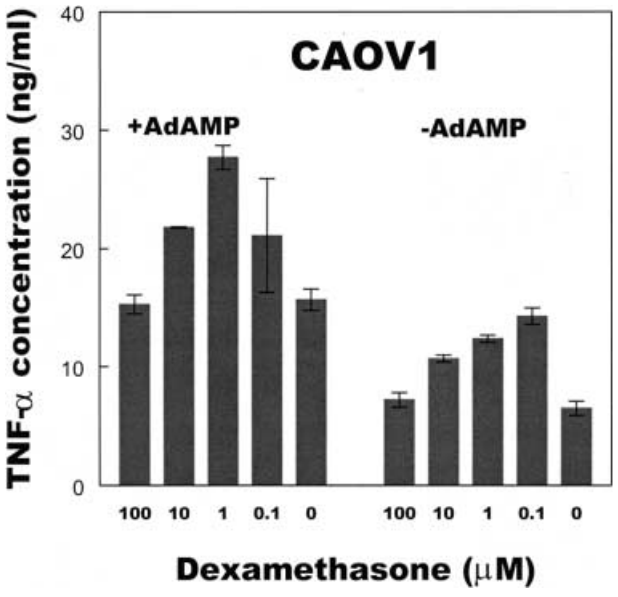

Fig. 8. Effect of dexamethasone on basal and AdAMP-stimulated TNF-α production by CAOV1 ovarian carcinoma cells. The cell culture conditions and determination of the TNF-α levels were as described for Fig. 2

of dexamethasone on TNF-α production are more complex and include suppression of TNF-α gene expression and inhibition of mRNA translation [6, 11]. In our studies, rolipram significantly suppressed TNF-α production by both untreated and AdAMP-treated B78/TNF/9 cells but was ineffective in cultures of the human ovarian carcinoma line CAOV1 (data not shown). Similarly, forskolin (a specific activator of protein kinase A) did not alter TNF-α production in cultures of CAOV1 cells incubated either alone or in the presence of AdAMP (not shown). Unexpectedly, dexamethasone significantly stimulated production of TNF-α by CAOV1 cells both in untreated cultures and in cultures incubated with AdAMP. It is interesting to note that the dose-response curve had a bell-shaped character (Fig. 8). This stimulatory effect and the type of response was confirmed in cultures of OVA-2 ovarian carcinoma cells and B78/TNF/9 cells (data not shown).

AdAMP modifies adhesive properties of tumour cells to extracellular matrix proteins

When incubated with AdAMP (especially at concentrations of 10 μ*M* and higher), B78/TNF/9 tumour cells no longer grew in monolayers (Fig. 6A) but tended to aggregate in large clusters (Fig. 6B, C). A similar phenomenon was observed in AdAMP-treated cultures of CAOV1 and B78H1 cells (not shown). No effect on cell aggregation was observed in untreated B78H1 cultures supplemented with TNF-α at concentrations as high as 1 μg/ml, indicating that the cell-grouping phenomenon was not a consequence of TNF-α production. As

revealed in the MTT assay, cluster formation was not accompanied by loss of viability (data not presented). To study this phenomenon more deeply, B78/TNF/9 cells were cultured in plastic plates covered with different stromal proteins (fibronectin and collagen IV) in the presence of different concentrations of AdAMP. As shown in Fig. 7 (confirmed by direct microscopic inspection), AdAMP at a concentration of 10 μM slightly, but statistically significantly, inhibited the adhesive properties of B78/TNF/9 cells both to fibronectin and collagen IV (91% and 86% of control adhesion for fibronectin and collagen IV, $P < 0.05$ and $P = 0.01$, respectively). No inhibitory effect of AdAMP on cell adhesion to matrix proteins was observed in cultures of unmodified (wild-type) B78H1 melanoma cells (data not presented).

Discussion

In the present study we demonstrated a strong TNF-α production-enhancing activity of AdAMP – the most potent immunomodulator among a series of adamantylamino-pyrimidine and -pyridine derivatives synthesized in our laboratory [22]. Adamantane derivatives have diverse biological activities. Amantadine (1-aminoadamantane), the best known adamantane derivative, is used for prophylaxis and treatment of type A influenza [23]. Amantadine is also used in the treatment of Parkinson's disease [13]. Some adamantane moiety-containing compounds have been reported to exert direct antibacterial and antitumour activity [36, 44]. Another 1-aminoadamantane-derived compound, *N*-adamantylphthalimide, has recently been described as a costimulator of TNF-α production by PMA-treated human leukaemia HL-60 cells [39].

The exact cellular target of AdAMP has not been established, but results of EMSA (Fig. 7) strongly suggest that AdAMP-induced augmentation of TNF-α production could be mediated by NF-κB. This was confirmed in experiments in which MG132 (a proteasome inhibitor blocking IκB degradation) significantly reduced both spontaneous and AdAMP-induced TNF-α production by CAOV1 cells (Fig. 6). Unexpectedly, dexamethasone, a synthetic glucocorticoid, used with the aim of reducing TNF-α production, produced the opposite effect: it significantly augmented both spontaneous and AdAMP-induced secretion of TNF-α (Fig. 8). At the molecular level, glucocorticoids act by binding to cytoplasmic receptors which are then transported into the nucleus to modulate (either positively or negatively) the expression of specific genes [4].

There are some data showing that glucocorticoids at physiological concentrations can increase production of some proinflammatory cytokines in vivo [45], which may partly explain our observations of stimulatory effects of low doses of dexamethasone on TNF-α production in our in vitro system (Fig. 8). We are not able to offer a convincing hypothesis explaining this phenomenon. It has been suggested that glucocorticoids can increase the expression of TNF-α receptors [4, 19] and it may be, bearing in mind that TNF-α can activate NF-κB [21], that this cytokine enhances its own production in dexamethasone-treated cells in an autocrine/paracrine manner. In experiments in which AdAMP-stimulated B78/TNF/9 melanoma cells were cultured with rolipram (a specific inhibitor of type IV phosphodiesterase that elevates cAMP [21]), a significant decrease in TNF-α secretion was observed, but this effect was not reproducible in cultures of CAOV1 cells (not presented). Similarly, another cAMP-augmenting agent, forskolin (an activator of protein kinase A [24]), did not influence the levels of TNF-α in cultures of CAOV1 cells stimulated with AdAMP (data not shown). These results show that the cAMP-mediated pathway is not critical for the effect of AdAMP.

The possibility that AdAMP operates at more than one level of regulation of TNF-α production, together leading to the accumulation of mRNA for TNF-α (Fig. 5) and enhanced secretion of this cytokine, cannot be excluded. Certainly, stimulation of TNF-α production is not the only property of AdAMP and, as shown in the studies on the effects of AdAMP on the adhesive properties of tumour cells (Figs. 9 and 10), this interesting agent probably regulates expression of a wide spectrum of genes.

TNF-α is a potent proinflammatory cytokine that, when produced in increased amounts in different disease states, can severely disturb the function of tissues and organs. Recently, TNF-α-targeted proteins (infliximab, anti-TNF-α monoclonal antibodies and the sTNF-R-Fc fusion protein etanercept) have been approved for the treatment of patients with Crohn's disease and rheumatoid arthritis [11, 12, 43]. Agents with proven anti-TNF-α effects also include synthetic chemical compounds such as pentoxifylline and thalidomide and its analogues [11, 15, 30]. On the other hand, TNF-α is still attracting attention as a potential antitumour agent because it exhibits immunomodulatory effects [42] and striking selective cytotoxicity against various tumour cell lines [26, 40]. Unacceptably severe side effects induced by TNF-α have made its use in the systemic treatment of cancer impossible [17]. However, using locoregional strategies for TNF-α administration, for example the procedure of isolated limb perfusion in which TNF-α is used in combination with melphalan and/or IFN-γ and hyperthermia, very encouraging results have been obtained in patients with in transit melanomas and soft tissue sarcomas [28]. Another approach aimed at improving the therapeutic index of TNF-α is the generation of so-called muteins (mutants) of the TNF-α molecule. The results of animal studies have shown that such modified forms of TNF-α are characterized by reduced untoward effects but still retain the therapeutic antitumour potential of native TNF-α [29]. The therapeutic potential of TNF-α can be also improved by PEGylation [20].

One of the most encouraging strategies for exploiting the antitumour potential of TNF-α is using low molec-

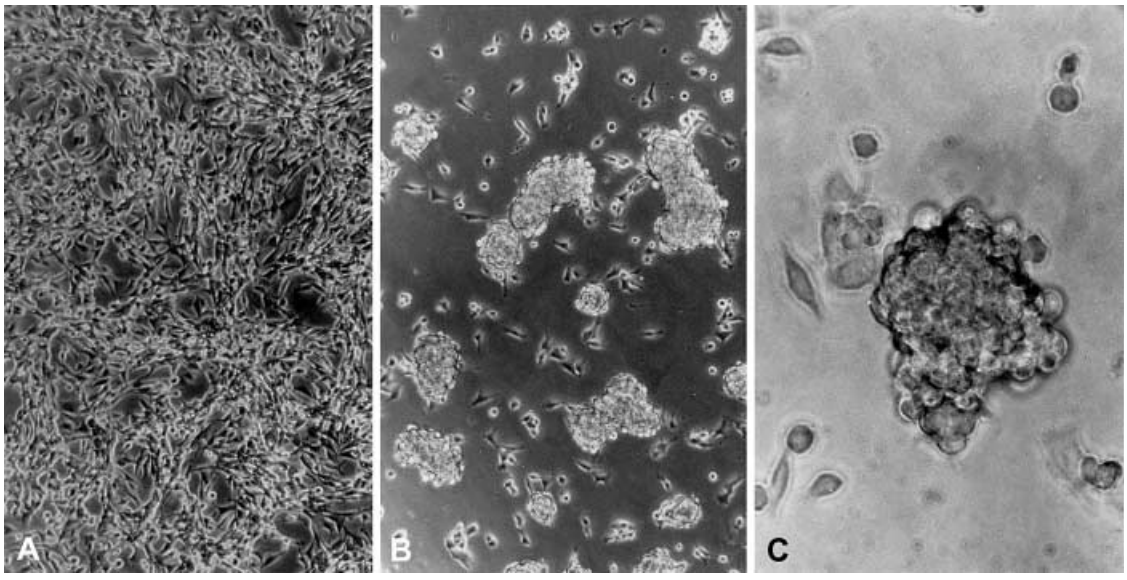

Fig. 9A–C. Morphological changes in B78/TNF/9 melanoma cells treated with AdAMP. Phase contrast images of B78/TNF/9 cultures incubated in the absence (**A**, ×100) or presence of 10 μ*M* AdAMP (**B**, ×100 and **C**, ×400). Note the strong tendency of the tumour cells to form clusters in cultures incubated with AdAMP. A similar phenomenon was observed in B78/TNF/3 melanoma cell cultures

ular weight chemical compounds to enhance the local production of TNF-α. The representative agent of this group of chemicals is DMXAA. This interesting well-characterized compound has been shown to stimulate production of TNF-α in leucocytes and tumour cells and to induce haemorrhagic necrosis of tumours in mice [18]. Recent data suggest that DMXAA, in addition to its necrosis-inducing properties, may also exert antiangiogenic effects [9]. DMXAA is currently being tested in a phase I clinical trial. We have no definite answer as to whether AdAMP could have similar antitumour activity but there are suggestions that this compound could show activity in vivo as potent as that of DMXAA. On a molar basis, in comparison with DMXAA, AdAMP has been found to be a more potent stimulator of TNF-α production in our in vitro model of melanoma cells transduced with the gene for TNF-α (unpublished data). Further, in our preliminary experiments AdAMP enhanced production of IL-12 by IL-12 gene-transduced melanoma cells. Moreover, in pilot in vivo studies in mice, AdAMP has been found to be relatively nontoxic (no lethality was observed after i.p. administration of a dose of 100 mg/kg). However, unlike DMXAA, AdAMP has very low water solubility. Recently, we complexed AdAMP with β-cyclodextrins to increase its bioavailability after administration in vivo [31].

The efficacy of cancer therapies based on cytokine gene-transduced tumour cell vaccines is strictly dependent on the amount of cytokine produced by the tumour cells [33, 37]. In this context, the property of AdAMP to enhance TNF-α production (and also IL-12 secretion by IL-12 gene-transduced B78H1 melanoma

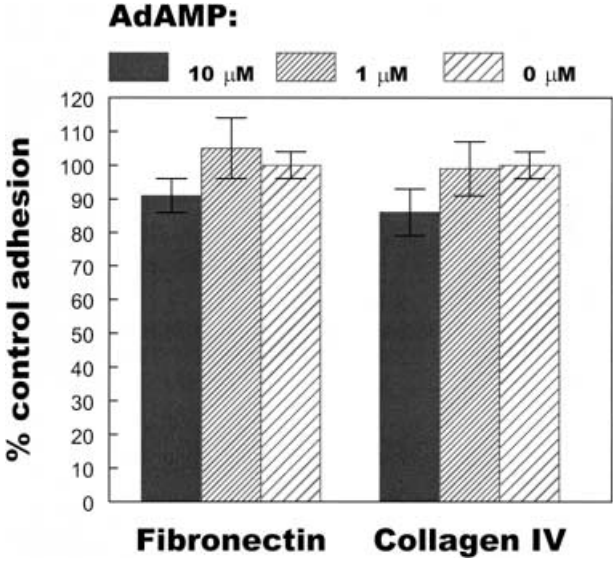

Fig. 10. Effect of AdAMP (1 or 10 μM) on the adhesion of B78/TNF/9 melanoma cells to extracellular matrix proteins fibronectin and collagen IV. For details see Materials and methods

cells; unpublished data) by genetically modified tumour cells could be the basis for its application in cancer gene therapy. As shown in Fig. 4, AdAMP also enhanced TNF-α production by ovarian carcinoma cells. Our previous studies [26] and many others have demonstrated that some chemotherapeutic agents, especially targeted at DNA topoisomerase II, can show strong synergistic antitumour effects (including in human ovarian carcinoma models) when combined with TNF-α [2, 3]. It would be interesting to determine whether AdAMP, by augmenting the levels of TNF-α at the tumour site, could potentiate the effect of chemotherapy as far as cytokine-secreting tumours are concerned.

In summary, we demonstrated interesting immunomodulatory properties of AdAMP, as a representative

1-amino-adamantane-derived compound. Because of the strong stimulatory effect of AdAMP on cytokine production by gene-transduced and unmodified tumour cells as well as cells of the mononuclear phagocyte system this compound shows potential for use as an adjuvant in cancer gene therapy and in the treatment of some types of tumours.

Acknowledgements We thank Ms. A. Czerepinska and Mrs. E. Gutowska for their excellent technical assistance and Dr. M. Siedlar for providing the CAOV1 ovarian carcinoma cell line. We thank also Dr. M. Jakóbisiak for constructive review of the manuscript and helpful suggestions.

References

1. Aggarwal BB, Natarajan K (1996) Tumour necrosis factors: Developments during the last decade. Eur Cytokine Netw 7:93
2. Alexander RB, Nelson WG, Coffey DS (1987) Synergistic enhancement by tumor necrosis factor of in vitro cytotoxicity from chemotherapeutic drugs targeted at DNA topoisomerase II. Cancer Res 47:2403
3. Aluigi MG, Debernardis D, Cimoli G, Ottoboni C, Parodi S, Russo P (1995) Tumor necrosis factor and DNA topoisomerase II inhibitors in human ovarian cancer: potential role in chemotherapy. Int J Oncol 7:461
4. Asadullah K, Schacke H, Cato ACB (2002) Dichotomy of glucocorticoid action in the immune system. Trends Immunol 23:120
5. Barsig J, Kusters S, Vogt K, Volk H-D, Tiegs G, Wendel A (1995) Lipopolysaccharide-induced interleukin-10 in mice: role of endogenous tumor necrosis factor-α. Eur J Immunol 25:2888
6. Bloom JW (1999) New insights into the molecular basis of glucocorticoid action. Immunol Allergy Clin North Am 19:653
7. Bogdan C, Ding A (1992) Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor alpha and interleukin-1 in macrophages. J Leukoc Biol 52:119
8. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A (2001) The role of MyD88 and TLR in the LPS-mimetic activity of taxol. Eur J Immunol 31:2448
9. Cao Z, Baguley BC, Ching L-M (2001) Interferon-inducible protein 10 induction and inhibition of angiogenesis in vivo by the antitumor agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA). Cancer Res 61:1517
10. Carswell WA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A 72:3660
11. Eigler A, Sinha B, Hartmann G, Endres S (1997) Taming TNF: strategies to restrain this proinflammatory cytokine. Immunol Today 18:487
12. Feldmann M, Maini RN (2001) Anti-TNFα therapy of rheumatoid arthritis: what have we learned? Annu Rev Immunol 19:163
13. Ferreira JJ, Rascol O (2000) Prevention and therapeutic strategies for levodopa-induced dyskinesias in Parkinson's disease. Curr Opin Neurol 13:431
14. Gorski A, Castronovo V, Stepien-Sopniewska B, Grieb P, Ryba P, Mrowiec T, Korczak-Kowalska G, Wierzbicki P, Matysiak W, Dybowska B, Piekarczyk J, Gawalkiewicz J, Kryst P, Borkowski A, Tolloczko T (1994) Depressed immune surveillance against cancer: role of deficient T cell: extracellular matrix interactions. Cell Adhes Commun 2:225
15. Gutschow M, Hecker T, Thiele A, Hauschild S, Eger K (2001) Aza analogues of thalidomide: synthesis and evaluation as inhibitors of tumor necrosis factor-α production in vitro. Bioorg Med Chem 9:1059
16. Hartmann G, Bidlingmaier C, Siegmund B, Albrich S, Schulze J, Tschoep K, Eigler A, Lehr HA, Endres S (2000) Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. J Pharmacol Exp Ther 292:22
17. Jones AL, Selby P (1989) Tumor necrosis factor: clinical relevance. Cancer Surv 8:817
18. Joseph WR, Cao Z, Mountjoy KG, Marshall ES, Baguley BC, Ching L-M (1999) Stimulation of tumors to synthesize tumor necrosis factor-α in situ using 5,6-dimethylxanthenone-4-acetic acid: a novel approach to cancer therapy. Cancer Res 59:633
19. Joyce DA, Kloda A, Steer JH (1997) Dexamethasone suppresses release of soluble TNF receptors by human monocytes concurrently with TNF-α suppression. Immunol Cell Biol 75:345
20. Kamada H, Tsutsumi Y, Yamamoto Y, Kihira T, Kaneda Y, Mu Y, Kodaira H, Tsunoda S, Nakagawa S, Mayumi T (2000) Antitumor activity of tumor necrosis factor-α conjugated with polyvinylpyrrolidine on solid tumors in mice. Cancer Res 60:6416
21. Karin M, Lin A (2002) NF-κB at the crossroads of life and death. Nat Immunol 3:221
22. Kazimierczuk Z, Gorska A, Switaj T, Lasek W (2001) Adamantylamino-pyrimidines and -pyridines are potent inducers of tumor necrosis factor-α. Bioorg Med Chem Lett 11:1197
23. Khare MD, Sharland M (2000) Influenza. Expert Opin Pharmacother 1:367
24. Kim DH, Lerner A (1998) Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood 92:2484
25. Lalli E, Sassone-Corsi P (1994) Signal transduction and gene regulation: the nuclear response to cAMP. J Biol Chem 269:17359
26. Lasek W, Giermasz A, Kuc K, Wankowicz A, Feleszko W, Golab J, Zagozdzon R, Stoklosa T, Jakobisiak M (1996) Potentiation of the anti-tumor effect of actinomycin D by tumor necrosis factor α in mice: correlation between in vitro and in vivo results. Int J Cancer 66:374
27. Lasek W, Mackiewicz A, Czajka A, Switaj T, Golab J, Wiznerowicz M, Korczak-Kowalska G, Balkowiec-Iskra EZ, Gryska K, Izycki D, Jakobisiak M (2000) Antitumor effects of the combination therapy with TNF-α gene-modified tumor cells and interleukin 12 in a melanoma model in mice. Cancer Gene Ther 7:1581
28. Lejeune FJ, Ruegg C, Lienard D (1998) Clinical applications of TNF-α in cancer. Curr Opin Immunol 10:573
29. Lucas R, Montesano R, Pepper MS, Hafner M, Sablon E, Dunant Y, Grau GE, De Baetselier P, Mannel D, Fransen L (2001) Lectin-deficient TNF mutants display comparable antitumor but reduced pro-metastatic potential as compared to the wild-type molecule. Int J Cancer 91:543
30. Mariott JB, Muller G, Dalgleish AG (1999) Thalidomide as an emerging immunotherapeutic agent. Immunol Today 20:538
31. Maurin JK, Lasek W, Gorska A, Switaj T, Wamil M, Mlynarczuk I, Kazimierczuk Z (2001) Synthesis, structure and tumor necrosis factor-α production enhancing properties of novel adamantylamino heterocyclic derivatives. Anticancer Drug Des 16:73
32. Mosieniak G, Pyrzynska B, Kaminska B (1998) Nuclear factor of activated T cells (NFAT) as a new component of the signal transduction pathway in glioma cells. J Neurochem 71:134
33. Nanni P, Rossi I, de Giovani C, Landuzzi L, Nicoletti G, Stoppacciaro A, Parenza M, Colombo MP, Lollini PL (1998) Interleukin 12 gene therapy of MHC-negative murine melanoma metastases. Cancer Res 58:1225
34. Naylor MS, Stamp GWH, Foulkes WD, Eccles D, Balkwill FR (1993) Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. J Clin Invest 91:2194
35. Oka M, Hirose K, Iizuka N, Aoyagi K, Yamamoto K, Abe T, Hazama S, Suzuki T (1995) Cytokine mRNA expression pattern in human esophageal cancer cell lines. J Interferon Cytokine Res 15:1005

36. Orzeszko A, Gralewska R, Starosciak BJ, Kazimierczuk Z (2000) Synthesis and antimicrobial activity of new adamantane derivatives I. Acta Biochim Pol 47:87
37. Parmiani G, Rodolfo M, Melani C (2000) Immunological gene therapy with ex vivo gene-modified tumor cells; a critique and a reappraisal. Hum Gene Ther 11:1269
38. Pisa EK, Pisa P, Hansson M, Wigzell H (1992) OKT3-induced cytokine mRNA expression in human peripheral blood mononuclear cells measured by polymerase chain reaction. Scand J Immunol 36:745
39. Shibata Y, Shichita M, Sasaki K, Nishimura K, Hashimoto Y, Iwasaki S (1995) N-alkylphthalimides: structural requirement of thalidomidal action on 12-O-tetradecanoylphorbol-13-acetate-induced tumor necrosis factor alpha production by human leukemia HL-60 cells. Chem Pharm Bull 43:177
40. Sugarman BJ, Aggarval BB, Hass PE, Figari IS, Palladino MA Jr, Shepard HM (1985) Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. Science 230:943
41. Takeyama H, Nobutaka W, O'Hara C, Arthur K, Niloff J, Kufe D (1991) Tumor necrosis factor expression by human ovarian carcinoma in vivo. Cancer Res 51:4476
42. Talmadge JE, Phillips H, Schneider M, Rowe T, Pennington R, Bowersox O, Lenz B (1988) Immunomodulatory properties of recombinant murine and human tumor necrosis factor. Cancer Res 48:544
43. Van Assche G, Rutgeerts P (2000) Anti-TNF agents in Crohn's disease. Expert Opin Investig Drugs 9:103
44. Wang JJ, Wang SS, Lee CF, Chung MA, Chern YT (1997) In vitro antitumor and antimicrobial activities of N-substituents of maleimide by adamantane and diamantane. Chemotherapy 43:182
45. Wilckens T, De Rijk R (1997) Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today 18:418