Modulation of tumor necrosis factor-α-mediated cytotoxicity by changes of the cellular methylation state: mechanism and in vivo relevance

Frank Ratter, Christoph Gaßner, Vladimir Shatrov and Volker Lehmann

German Cancer Research Center, Division of Immunochemistry, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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Abstract

A combination of adenosine (Ado) and homocysteine (Homo) enhances tumor necrosis factor (TNF)-α cytotoxicity in vitro and in vivo in several tumor cells. Ado and Homo at concentrations that enhanced TNF-α-mediated cytotoxicity accumulated S-adenosylhomocysteine (AdoHcy) and as consequence decreased the cellular methylation state, i.e. the ratio of S-adenosylmethionine to AdoHcy. This decrease led to inhibition of the isoprenylcysteine carboxyl methyltransferase (MTase), an enzyme that catalyzes carboxyl methylation of C-terminal cysteine residues on isoprenylated proteins. The effect of Ado and Homo on TNF-α cytotoxicity was at least partly mimicked by S-farnesylthioacetic acid, a selective inhibitor of the isoprenylcysteine carboxyl MTase, suggesting involvement of methylations of prenylated proteins in TNF-α-mediated cytotoxicity. Blockage of methylation reactions was associated with an enhancement of the TNF-α-induced disruption of the mitochondrial membrane potential (ΔΨm). In nude mice, a combination of Ado, Homo and TNF-α led to TNF-α-induced hemorrhagic necrosis and growth inhibition of TNF-sensitive L929 tumors, whereas little effect was observed with TNF-α alone. Even more important, the TNF-resistant L929 M1 tumors were rendered TNF-sensitive by the combined action of Ado and Homo. We conclude that Ado and Homo together enhance the effectiveness of TNF-α in vitro and in vivo, results that may have therapeutic implications.

Introduction

Tumor necrosis factor (TNF)-α is a cytokine that is capable of inducing hemorrhagic necrosis in vivo (1). In vitro, TNF-α exerts cytostatic and cytotoxic activity against a number of human and murine tumor cells, but spares most non-transformed cell lines (2). TNF-α-induced cytotoxicity emanates from TNF-α receptor 1 (55–60 kDa) (3–5). Upon oligomerization of TNF-α receptor 1 by trimeric TNF-α, TRADD is recruited to the receptor complex. TRADD then binds FADD/MORT1 (6), which in turn recruits caspase-8 (FLICE, MACH, Mch5) (7) to the receptor complex. Caspase-8 is considered to play a role in the activation of other proteases that are responsible for the execution of cell death. Recently, it has been proposed that TNF-α-mediated cell death in L929 cells proceeds in a caspase-independent manner (8). TNF-α-induced cytotoxicity includes also the G-protein coupled activation of phospholipase A2 (9), the generation of reactive oxygen intermediates (10,11) and DNA damage (12). Many tumor cells resist the cytotoxic action of TNF-α. However, TNF-α sensitivity can be induced in most of these cells by chemically blocking de novo protein synthesis (13). This effect is due to inhibition of the synthesis of proteins that protect cells against the cytotoxic action of TNF-α. These proteins include the TNF-α-inducible manganese superoxide dismutase (MnSOD) that destroys superoxide radicals (14,15) or the inhibitor of apoptosis proteins c-IAP1 and c-IAP2 that inhibit specific caspases (16,17).

It has also been demonstrated that TNF-α induces the opening of mitochondrial permeability transition (PT) pores, resulting in a reduction of the mitochondrial transmembrane potential (ΔΨm) (18). Moreover, it was shown that the mito-
chondrial PT constitutes a critical early event of the death process (19,20). PT pores function as sensors for multiple physiological effectors including ATP, ADP, NAD, pH, thiols and peptides, and are thereby capable of integrating information on the metabolic and redox state of the cell. The exact composition of the PT pore is not known. However, it appears that at least one inner mitochondrial transmembrane protein, the adenine nucleotide translocator (ANT), is involved in PT formation (21). ANT associates with several molecules of the outer mitochondrial membrane, including the voltage-dependent anion channel. ANT ligands such as bongkrekic acid (BA) reduce the probability of PT (22).

It was previously suggested that TNF-α-induced cytotoxicity is influenced by S-adenosylmethionine (AdoMet)-dependent methylation reactions (23). Such processes may include methylation of proteins with isoprenylated cysteine residues at their C-terminus (24). Many of these proteins function in signal transduction cascades across the plasma membrane. There are two types of isoprenyl groups, C15 farnesyl and the more abundant C20 geranylgeranyl groups. Proteins in the 21 and 28 kDa class, which include members of the Ras superfamily of small guanine nucleotide-binding proteins (G-proteins), are mainly geranylgeranylated, while p21ras itself, the γ-subunit of the heterotrimeric large G-proteins, the cGMP phosphodiesterase and the nuclear lamins are farnesylated. Isoprenylated proteins initially contain a C-terminal sequence, CAAX, where A is an alphaic amino acid and X any amino acid. A C15 farnesyl or a C20 geranylgeranyl group is then added to the cysteine residue to form a thioether. The terminal AAX is next removed to produce a C-terminal isoprenylated cysteine. Finally, the modified C-terminal cysteine is then carboxyl methylated in a AdoMet-dependent reaction. The methyl esterification is catalyzed by the isoprenylcysteine protein carboxyl methyltransferase (MTase), an enzyme that also methylates isoprenylated analogs of cysteine (24). Unlike other classes of carboxyl MTase that are cytosolic, the isoprenylated protein MTase is membrane associated. The carboxyl methylated cysteine is reversible and may, therefore, play a regulatory role in signal transduction.

Transmethylation reactions utilize AdoMet as a methyl donor, converting it to S-adenosylhomocysteine (AdoHcy), which in turn is a potent inhibitor of AdoMet-dependent transmethylation reactions (25). The immediate metabolic fate of AdoHcy involves its hydrolysis to adenosine (Ado) and L-homocysteine (Homo) by the enzyme AdoHcy-hydrolase. An important property of this hydrolysis reaction is that it is readily reversible. Thus, for AdoHcy hydrolase to remove potentially inhibitory AdoHcy, it is necessary that either Ado or Homo be further metabolized in an efficient manner. In mammalian cells, the primary pathways for Homo metabolism are trans-sulfuration to cysteine or methionine. Ado is converted to either Ado phosphate by the Ado kinase or to inosine by Ado deaminase. Moreover, under conditions of Ado and Homo excess, reversibility of the hydrolytic reaction causes accumulation of AdoHcy and as consequence inhibition of methylation reactions.

We have used exogenous Ado and Homo as a tool to study the role of the cellular methylation state in TNF-α-mediated cytotoxicity. The present paper shows that a decrease of this state enhances TNF-α-mediated cytotoxicity in vitro. It provides evidence that the modulating effect is caused by a series of events that include a blockade of carboxyl methylations of prenylated proteins and an enhancement of the TNF-α-induced disruption of the mitochondrial membrane potential. Moreover, we show that the Ado and Homo-specific TNF-α potentiation can be extended to the in vivo antitumor action of TNF-α.

Methods

Cell lines

The TNF-resistant cell lines L929 M1, L929 M5 and HeLa R3 (3) were isolated in our laboratory. Both L929 cell lines are derivatives of the TNF-α-sensitive L929 cell line (unpublished work). L929 M1 cells express increased amounts of MnSOD. L929 M5 cells are resistant to TNF-α and to the combined action of Ado, Homo and TNF-α. The HeLa R3 cell line is a derivative of the TNF-α-sensitive HeLa S cell line (3). All these strains were obtained by culturing highly TNF-α-sensitive parent strains in the presence of TNF-α (L929 M1 and HeLa R3) or in the presence of Ado, Homo and TNF-α (L929 M5). TNF-α-resistant or Ado/Homo and TNF-α-resistant clones were then isolated by limiting dilution. The properties of these clones will be described elsewhere. The TNF-sensitive cell lines, including murine L929 and WEHI fibrosarcoma cells, HeLa S cervix carcinoma cells as well as the TNF-resistant cell lines, including human PLC hepatitis cells, human A549 carcinoma cells and human ovarian adenocarcinoma SK-OV-3 cells, were obtained from the DFKZ collection.

All cell lines were incubated in culture medium: RPMI 1640 supplemented with 10% heat-inactivated (30 min at 56°C) newborn calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (5 mM) and mercaptoethanol (3×10⁻⁵ M). Recombinant human TNF-α with an activity of 5×10⁴ U/mg was obtained by courtesy of Bender (Vienna, Austria).

BA was kindly provided by Dr. Duine (Delft University of Technology, Delft, the Netherlands).

Cytotoxicity assay

The cytotoxic activity of TNF-α was determined by a colorimetric assay (MTT test) (26). L929 cells were seeded at a density of 10⁴ cells/well in 96-well microtiter plates (flat bottomed) and incubated for 18–24 h in 0.2 ml culture medium. The supernatant was then removed and replaced by fresh medium containing serial dilutions of TNF-α and actinomycin D (1 µg/ml). Incubation was continued for 18 h followed by addition of 10 µl of a MTT solution (5 mg/ml PBS). After another 4 h incubation, supernatants were removed by addition of 100 µl of a isopropanol:HCl solution (isopropanol and 1 N HCl, 24:1, v:v). The absorbance of each well was determined with an automated plate reader (SLT Easy Reader EAR 400 AT) at 550 nm. Survival was calculated as the percentage of the staining value of untreated cultures. Percent cytotoxicity is the difference between control (100%) and percent survival.

Determination of cellular AdoMet and AdoHcy

L929 cells were seeded in six-well plates (2.5×10⁶ cells/well) and permitted to grow at 37°C. Cells were then incubated 1 h with test substances and removed by scraping. They were
resuspended in 2 ml PBS and cell suspensions centrifuged at 7000 g for 20 s in an Eppendorf microfuge. Cell pellets were extracted with 200 µl ice-cold 2.5% sulfosalicylic acid and cell extracts centrifuged for 5 min at 10,000 g. The supernatants were stored at −70°C and measured (20 µl) within 48 h. Analysis was performed by HPLC (27), utilizing a DuPont liquid chromatography Model 850, a Partisil 10 SCX column as well as a DuPont UV spectrophotometer with a wavelength detector to measure absorbancy at 234 nm. AdoHcy was eluted isocratically at ambient temperature at a flow rate of 0.5 ml/min. The mobile phase was 100 mM and 500 ammonium phosphate, pH 2.6, each, for determination of AdoHcy and AdoMet, L929 cells were seeded in six-well microtiter plates (2.5×10³ cells/well) and permitted to grow at 37°C. Cells were then incubated for 2 h with test substances and then removed by scraping. They were suspended in PBS followed by centrifugation. Cell pellets were extracted with cold 2.5% sulfosalicylic acid. Analysis was performed by HPLC. The values were obtained from one representative out of three experiments.

To examine cytotoxicity L929 cells were seeded in 96-well plates at a density of 10⁵ cells/well. After 24 h test substances (1 mM each) were added followed by TNF-α and actinomycin D (1 µg/ml) and TNF-α. Incubation was continued for additional 18 h and cytotoxicity was determined by the MTT test. The results are the mean ± SEM of four determinations.

To measure AdoHcy and AdoMet, L929 cells were seeded in six-well microtiter plates (2.5×10³ cells/well) and permitted to grow at 37°C. Cells were then incubated for 2 h with test substances and then removed by scraping. They were suspended in PBS followed by centrifugation. Cell pellets were extracted with cold 2.5% sulfosalicylic acid. Analysis was performed by HPLC. The values were obtained from one representative out of three experiments.

### Table 1. Effect of cellular methylation state on TNF-α-mediated cytotoxicity in L929 cells

<table>
<thead>
<tr>
<th>Agents</th>
<th>AdoMet (pmol/10⁶ cells)</th>
<th>AdoHcy (pmol/10⁶ cells)</th>
<th>AdoMet:AdoHcy ratio</th>
<th>Cytotoxicity (%)</th>
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<tr>
<td>None</td>
<td>400</td>
<td>25</td>
<td>16</td>
<td>0</td>
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<tr>
<td>TNF-α (10 pg/ml)</td>
<td>400</td>
<td>25</td>
<td>16</td>
<td>7 ± 0.4</td>
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<td>TNF-α (1000 pg/ml)</td>
<td>360</td>
<td>24</td>
<td>15</td>
<td>35 ± 2.7</td>
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<tr>
<td>TNF-α (10000 pg/ml)</td>
<td>260</td>
<td>25</td>
<td>10.4</td>
<td>95 ± 9.0</td>
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<tr>
<td>Ado (1 mM)</td>
<td>400</td>
<td>100</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Ado + TNF-α (10 pg/ml)</td>
<td>400</td>
<td>100</td>
<td>4</td>
<td>12 ± 1.8</td>
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<tr>
<td>Homo (1 mM)</td>
<td>410</td>
<td>35</td>
<td>11.7</td>
<td>0</td>
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<tr>
<td>Homo + TNF-α (10 pg/ml)</td>
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<td>34</td>
<td>11.9</td>
<td>6 ± 0.3</td>
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<tr>
<td>Ado + Homo (1 mM)</td>
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<td>1200</td>
<td>0.35</td>
<td>0</td>
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<tr>
<td>Ado + Homo (1 mM) + TNF-α (10 pg/ml)</td>
<td>419</td>
<td>1100</td>
<td>0.38</td>
<td>99 ± 9.2</td>
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<td>490</td>
<td>0.86</td>
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<td>250</td>
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<td>0</td>
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<tr>
<td>Ado + Homo + TNF-α (10 pg/ml)</td>
<td>425</td>
<td>235</td>
<td>1.7</td>
<td>35 ± 2.9</td>
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</tbody>
</table>

To examine cytotoxicity L929 cells were seeded in 96-well plates at a density of 10⁵ cells/well. After 24 h test substances (1 mM each) were added followed by TNF-α and actinomycin D (1 µg/ml) and TNF-α. Incubation was continued for additional 18 h and cytotoxicity was determined by the MTT test. The results are the mean ± SEM of four determinations.

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### Results

#### Influence of the cellular methylation state on TNF-α-mediated cytotoxicity

The cellular methylation state is defined by the ratio of AdoMet to AdoHcy and determines the ability of cells to methylate target structures (25). This ratio is 16 in L929 cells growing in culture medium (see Table 1). When TNF-α was added to L929 cells, the ratio of AdoMet to AdoHcy declined within 2 h in a dose-dependent manner. Decrease was detectable at a concentration of 1000 pg/ml and maximum decrease (from 16 to 10.4) was observed at 10,000 pg/ml TNF-α. During this time period the internal pool of AdoHcy did not change but the amount of AdoMet decreased. This suggested that TNF-α decreased the AdoMet:AdoHcy ratio by inhibiting AdoMet synthesis or by stimulating the consumption of AdoMet.

We next modulated the TNF-α-induced decrease of AdoMet:AdoHcy and determined the effect of this modulation on TNF-α-mediated cytotoxicity. In order to achieve modulation, we used a combination of Ado and Homo that causes the reversible enzyme AdoHcy hydrolase to synthesize AdoHcy (23,28) and as a consequence to decrease the AdoMet:AdoHcy ratio. As shown in Table 1, combinations of Ado and Homo decreased the AdoMet:AdoHcy ratio in a dose-
Regulation of TNF-α cytotoxicity by the cellular methylation state

Fig. 1. (A) Changes in AdoHcy/AdoMet contents and isoprenylcysteine protein carboxyl MTase activity in response to Ado and Homo. Two groups of L929 cultures were set up. One group (2.5 x 10^6 cells/culture) was used to determine the content of AdoHcy and AdoMet employing HPLC. The second group (10^6 cells/culture) was used to measure isoprenylcysteine carboxyl MTase activity as follows. Cell extracts prepared by sonication were incubated with AFC (100 µM) and [3H-methyl]AdoMet (1 µCi) in buffer A at 37°C. The level of [3H-methyl]AFC methyl ester was determined by heptan extraction and scintillation counting. The 100% value represents 3.2 x 10^4 c.p.m./10^6 cells. Results are representative of three independent experiments. (B) Effect of AdoHcy on isoprenylcysteine protein carboxyl MTase activity. Cell extracts derived from 10^6 L929 cells were prepared by sonication and then exposed to various concentrations of AdoHcy. The carboxyl MTase was assayed as described in legend to (A). Results are representative of three independent experiments.

Changes in intracellular AdoHcy/AdoMet content and isoprenylcysteine carboxyl MTase activity in response to Ado and Homo

Decrease of the AdoMet:AdoHcy ratio leads to inhibition of AdoMet-dependent methylation reactions of various cellular target structures including proteins. Therefore, we asked what critical methylation reactions are involved in the potentiation of TNF-α-mediated cytotoxicity? To answer this question, we focused on methylation of prenylated proteins, which are thought to participate in signal transduction processes. Enzymes that mediate methylations of such proteins include the isoprenylcysteine carboxyl MTase. To examine the relationship between AdoMet:AdoHcy ratio and the activity of the isoprenylcysteine carboxyl MTase, two parallel groups of L929 cultures were set up and exposed to Ado and Homo (1 mM each). After various times the amounts of AdoMet and AdoHcy were determined in one group and carboxyl MTase activity in the other group. As expected, untreated L929 cells contained 400 pmol/10^6 cells AdoMet and ~26 pmol/10^6 cells AdoHcy (see Fig. 1A) resulting in a AdoMet:AdoHcy ratio of 15.3. When Ado and Homo (1 mM each) were added, the amount of AdoMet remained rather constant between 400 and 420 pmol/10^6 cells. In contrast, AdoHcy levels increased and after 1 h reached a maximum value of 990 pmol/10^6 cells, resulting in a cellular methylation ratio of 0.40. Moreover, accumulation of AdoHcy, induced by the combination of Ado and Homo, was closely associated with a decrease of the C-terminal isoprenylcysteine protein carboxyl MTase activity. Loss of enzyme activity was complete when AdoHcy rose to maximum levels. In contrast, Ado or Homo alone generated ~200 pmol/10^6 cells within 1 h and were unable to decrease this enzyme activity (not shown). Similarly, TNF-α (10 pg/ml) did not decrease enzyme activity or modulate Ado and Homo-induced changes of enzyme activity (data not shown).

Loss of carboxyl MTase activity was also achieved when cell extracts were directly exposed to AdoHcy. Decrease of enzyme activity was observed with 0.01 mM AdoHcy while complete loss of activity was observed with 1 mM (see Fig. 1B).
Farnesylcysteine analogs enhance TNF-α-mediated cytotoxicity

To further investigate the role of carboxyl methylation of isoprenylated proteins, we studied the effect of two farnesyl-cysteine analogs that were previously shown to enter cells and to inhibit carboxyl methylation of Ras and Ras-related proteins (24,28). These analogs included AFC, a substrate and competitive inhibitor of the C-terminal isoprenylcysteine carboxyl MTase as well as S-farnesylthioacetic acid (FTA), a poor substrate but good inhibitor of this enzyme (24). As shown in Fig. 2, both compounds modulated TNF-α-induced cytotoxicity. AFC at 20 µM, a concentration that equals the Michaelis constant of AFC for carboxyl MTase (28), enhanced TNF-α-mediated cytotoxicity. Doses of AFC up to 100 µM caused no cell death, but were less efficient than 20 µM AFC (data not shown). Similarly, FTA at a concentration of 10 µM enhanced TNF-α-mediated cytotoxicity.

Ado and Homo modulate the ability of TNF-α to destroy the mitochondrial membrane potential

Disruption of the mitochondrial membrane potential (ΔΨm) is thought to be a crucial event in the cellular commitment to the cell death program, including TNF-α-induced cytotoxicity (19,20). Therefore, we investigated the depolarization of the mitochondrial membrane in response to TNF-α in the presence or absence of Ado and Homo and actinomycin D. Accordingly, L929 cell cultures received test substances, and after 3 and 7 h of incubation changes of the mitochondrial membrane potential were evaluated by using the fluorochrome DiOC6(3) (19). As shown in Fig. 3(A–C), TNF-α alone at 10 and 100 pg/ml did not alter the percentage of cells with low ΔΨm. After 7 h, almost 18% of the cells expressed low ΔΨm (Fig. 3C). No change was observed with 10 pg/ml TNF-α and actinomycin D at the same time point. However, addition of TNF-α in combination with Ado and Homo destroyed ΔΨm in an efficient manner: At 3 h after addition of TNF-α (10 and 100 pg/ml) the percentages of cells with low ΔΨm were 29% (Fig. 3A and B) and 50% (Fig. 3A) respectively, and increased to 70 and 82% after 7 h (Fig. 3C). In contrast, the combination of Ado and Homo (1 mM, each), or actinomycin D without TNF-α did not decrease ΔΨm.

BA inhibits TNF-α-mediated cytotoxicity in the presence and absence of Ado and Homo

It was previously suggested that the collapse of the ΔΨm may be due to the opening of mitochondrial PT pores (19). In accord with this hypothesis it was observed that a specific ligand of the mitochondrial adenine nucleotide translocator

Fig. 3. TNF-α-induced disruption of mitochondrial membrane potential in the presence and absence of Ado and Homo. L929 cells (10^3/well) were placed in six-well plates and incubated overnight. Cells were then washed with culture medium, and then received Ado, Homo (1 mM, each) and/or TNF-α (10 or 100 pg/ml) and actinomycin D (1 µg/ml). Incubation was then continued. After 3 (A and B) and 7 (C) h mitochondrial potential of mitochondria (ΔΨm) was assessed by flow cytometry, using the fluorochrome (DiOC6(3)) (20). The results are representative of three experiments. Control mitochondria treated with the ΔΨm-disrupting protonophore m-chlorophenylhydrazine (CCCP) (100 µM) served as negative control (unfilled curve in control).
the second week on, most of the tumor exhibited signs of necrosis following treatment with either TNF-α or TNF-α and Ado and Homo. However, while growth inhibition induced by TNF-α was generally modest, no tumor growth was observed with TNF-α in the presence of Ado and Homo. In some cases tumor sizes increase and after the second week on decrease (Fig. 6A). After prolonged treatment (100 days) with TNF-α, Ado and Homo, 80% of these mice were completely free of tumors.

We next injected TNF-resistant L929 M1 cells s.c. into nude mice (10^6 cells/mouse) and treated mice as described above. The result of a representative experiment is shown in Fig. 6(B). In this experiment, TNF-α or Ado and Homo had no detectable effect on tumor growth. In contrast, a combined treatment with TNF-α, Ado and Homo led to growth inhibition. After 22 days all these mice were tumor-free. Again, after prolonged (100 days) treatment with TNF-α, Ado and Homo, 60% of the mice carried no tumor.

To examine whether the in vivo effect of Ado and Homo was direct or indirect, L929 M5 cells (10^6 per mouse) were employed that resist the combined action of Ado, Homo and TNF-α (Fig. 6C). The tumors were treated according to the protocol mentioned above. After 3 weeks neither treatment had a detectable effect on tumor growth. In contrast, a combined treatment with TNF-α, Ado and Homo perilesionally for several periods of five consecutive days, interrupted by 2 days without treatment. The doses of TNF-α (10 µg) and Ado and Homo (1 mg, each) were kept constant. The results of a representative experiment are shown in Fig. 6A. Compared with TNF-α alone, the combination of TNF-α, Ado and Homo was very effective. Ado and Homo alone had no effect. From the second week on, most of the tumor exhibited signs of necrosis following treatment with either TNF-α or TNF-α and Ado and Homo. However, while growth inhibition induced by TNF-α was generally modest, no tumor growth was observed with TNF-α in the presence of Ado and Homo. In some cases tumor sizes increase and after the second week on decrease (Fig. 6A). After prolonged treatment (100 days) with TNF-α, Ado and Homo, 80% of these mice were completely free of tumors.

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Discussion

This report shows that a combination of Ado and Homo modulates in vitro and in vivo the sensitivity of several tumor cells towards the cytotoxic action of TNF: it potentiates TNF-α-mediated cytotoxicity in TNF-sensitive tumor cells and converts TNF-resistant tumor cells into TNF-sensitive cells. Moreover, the paper provides information about the mechanism underlying the potentiating effect of Ado and Homo.

Ado and Homo were used as a tool to alter the methylation state. When these compounds are added they enter cells and force the reversible enzyme AdoHcy hydrolase to synthesize AdoHcy and to decrease the AdoMet:AdoHcy ratio (25).

Decrease of the AdoMet:AdoHcy ratio was associated with the inhibition of the isoprenylcysteine carboxyl MTase, an enzyme that catalyzes methylation of carboxyl groups of the C-terminal residues on isoprenylated proteins. To further investigate the role of carboxyl methylations in the modulation of TNF-α-mediated cytotoxicity, we studied the effect of farnesylcysteine analogs such as AFC and FTA. Both compounds were shown previously to enter cells and to inhibit carboxyl methylations of isoprenylated proteins (24). We observed that these compounds are able to mimic, at least partially, the potentiating effect of Ado and Homo, suggesting that carboxyl methylations are indeed involved in the modulation of the TNF-α-induced cytotoxicity.

Next we investigated the ability of Ado and Homo to influence the TNF-α-induced collapse of the mitochondrial membrane potential (ΔΨm). Disruption of ΔΨm is thought to be a central executioner for cell death and marks a point of no return for the induction of cell death (19,20). Here we confirm an earlier observation that TNF-α is capable of reducing the mitochondrial membrane potential (18), but more important we show that this process is modulated by the methylation status. We observed that the combination of Ado and Homo potentiated the TNF-α-induced disruption of the mitochondrial potential in L929 cells.

Both TNF-α and Ado/Homo/TNF-α-induced cytotoxicity are, at least partially, inhibited by BA, a specific ligand of the mitochondrial ANT. ANT is considered as a component of the permeability transition pore and cooperates with other proteins including the voltage-dependent channel proteins (22). Therefore, our findings suggest that the TNF-α- and the Ado/Homo/
TNF-α-induced disruption of the mitochondrial potential are partially due to the opening of mitochondrial permeability pores.

We have also addressed the question whether the cytotoxic effect in vitro and the antitumor effect in vivo are the result of the same bioactivity of TNF-α. We have shown that tumor cells, i.e. L929 M5 cells that are resistant to the combined action of Ado, Homo and TNF-α, fail to respond in vivo. This suggests that the in vivo action of TNF-α, Ado and Homo is mainly direct.

At the moment it is unclear how Ado and Homo-induced alterations of the cellular methylation status influence the TNF-α-mediated cytotoxicity. However, we propose the following working hypothesis. In the absence of Ado and Homo, the methylation status permits AdoMet-dependent methylation reactions, including carboxyl methylation of prenylated proteins that suppress TNF-α-inducible cytotoxic pathways. In the presence of Ado and Homo the cellular methylation state (ratio of AdoMet:AdoHcy) is decreased and as a consequence carboxylic methylations are blocked. This opens a TNF-α-inducible cytotoxic pathway that leads to the collapse of the mitochondrial membrane potential. Currently there is no evidence that Ado, Homo or AdoHcy act at the cell surface to induce some kind of signal that influences the methylation state. For instance agents that prevent binding of Ado to the Ado receptors do not block potentiation of TNF-α cytotoxicity. Moreover, it is known that AdoHcy is unable to enter the cell (24). When this compound was added to L929 cells no enhancement of TNF-α cytotoxicity was observed (unpublished results).

Proteins that have such C-terminal modifications include members of the Ras superfamily such as the γ subunit of the heterotrimeric G-binding proteins. Many of these proteins are involved in signal transduction processes. For instance, certain heteromeric G-binding proteins are quite effective in their methylated form in activating enzymes such as phosphoinositol-3-kinase and phospholipase C-β, whereas their unmethylated counterparts are virtually inert (29,30). Moreover, phosphoinositol-3-kinase and phospholipase C-β are implicated in a pathway that conveys survival signals from various cell surface receptors to mitochondria (31,32). Our current effort focuses on the identification of the prenylated protein that mediates the Ado and Homo-induced effect on TNF-α cytotoxicity.

In conclusion, our results provide evidence that a combination of Ado and Homo potentiates the TNF-α cytotoxicity by a mechanism which involves a decrease of the cellular methylation state, the down-regulation of the isoprenylcysteine protein MTase activity and, finally, a decrease of the threshold for the TNF-α-induced opening of mitochondrial PT pores. Moreover, the considerable potentiation of TNF-α cytotoxicity in vitro and in vivo by Ado and Homo might be helpful to develop new strategies to human cancer therapy.

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References


Abbreviations

Ado adenosine
AdoHcy S-adenosylhomocysteine
AdoMet S-adenosylmethionine
AFC N-acetyl-S-farnesyl-cysteine
ANT mitochondrial adenine nucleotide translocator
BA bongkrekic acid
mCCCP carbonyl cyanide m-chlorophenylhydrazone
carboxyl MTase carboxyl methyltransferase
DIOC6(3) 3,3′-dihexyloxacarbocyanine iodide
FTA S-farnesylthioacetic acid
Homo D,L-homocysteine
MnSOD manganese superoxide dismutase
ΔΨm mitochondrial transmembrane potential
PT permeability transition
TNF tumor necrosis factor
Regulation of TNF-α cytotoxicity by the cellular methylation state