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

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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 ($\Delta \Psi_m$). 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 ($\Delta \Psi_m$) (18). Moreover, it was shown that the mito-
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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 hepatoma 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 x 10⁻⁵ M). Recombinant human TNF-α with an activity of 5 x 10⁷ 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 10⁶ 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 followed 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 x 10⁵ cells/well) and permitted to grow at 37°C. Cells were then incubated 1 h with test substances and removed by scraping. They were
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>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>400</td>
<td>25</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α (10 pg/ml)</td>
<td>400</td>
<td>25</td>
<td>16</td>
<td>7 ± 0.4</td>
</tr>
<tr>
<td>TNF-α (1000 pg/ml)</td>
<td>360</td>
<td>24</td>
<td>15</td>
<td>35 ± 2.7</td>
</tr>
<tr>
<td>TNF-α (10000 pg/ml)</td>
<td>260</td>
<td>25</td>
<td>10.4</td>
<td>95 ± 9.0</td>
</tr>
<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>
</tr>
<tr>
<td>Homo (1 mM)</td>
<td>410</td>
<td>35</td>
<td>11.7</td>
<td>0</td>
</tr>
<tr>
<td>Homo + TNF-α (10 pg/ml)</td>
<td>407</td>
<td>34</td>
<td>11.9</td>
<td>6 ± 0.3</td>
</tr>
<tr>
<td>Ado + Homo (1 mM)</td>
<td>420</td>
<td>1200</td>
<td>0.35</td>
<td>0</td>
</tr>
<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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<tr>
<td>Ado + Homo (0.5 mM)</td>
<td>422</td>
<td>500</td>
<td>0.84</td>
<td>0</td>
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<tr>
<td>Ado + Homo (0.5 mM) + TNF-α (10 pg/ml)</td>
<td>420</td>
<td>490</td>
<td>0.86</td>
<td>68 ± 5.8</td>
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<td>Ado + Homo (0.1 mM)</td>
<td>410</td>
<td>250</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>Ado + Homo + TNF-α (10 pg/ml)</td>
<td>407</td>
<td>235</td>
<td>1.7</td>
<td>35 ± 2.9</td>
</tr>
</tbody>
</table>

To examine cytotoxicity L929 cells were seeded in 96-well plates at a density of 10⁴ 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 (2.5×10⁶ 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 determine the isoprenylcysteine carboxyl MTase activity in L929 cells, the Δψ was evaluated by flow cytometry using the fluorochrome 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (100 nM in PBS) (19). The percentage of cells with low Δψ was shown.

Experimental Animals
Female nude mice, 6–8 weeks old, were used (Swiss nu/nu; Iffa Credo, St Germain-sur-l’Arbresle, France).

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 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-

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 respectively.

Determination of the isoprenylcysteine carboxyl MTase activity
The test was based on the ability of C-terminal carboxyl MTase to use N-acetyl-S-farnesyl-l-cysteine (AFC) as substrate (28). Cells were incubated in Petri dishes (10⁶ cells/dish) and incubated for 24 h. Cells were then scraped off the plates, washed with PBS and lysed by sonication in buffer A (100 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.9). Cell extracts were incubated with AFC (100 µM) and [³H-methyl]AdoMet (1 µCi, sp. act. 60–80 Ci/mmol; DuPont NEN, Dreieich, Germany) in buffer A in a total volume of 100 µl for 25 min. The level of [³H-methyl]AFC methylester was determined by extraction with hexan (0.5 ml). Radioactivity in the hexan phase was then measured by scintillation counting.

Assessment of mitochondrial potential
To evaluate mitochondrial potential (Δψ), L929 (10⁶ cells/well) were incubated overnight in six-well plates. Cells were then washed with culture medium followed by addition of Ado and Homo (1 mM, each) and/or TNF-α at the indicated concentration. After the indicated periods of time the loss of the Δψ was evaluated by flow cytometry using the fluorochrome 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (100 nM in PBS) (19). The percentage of cells with low Δψ is shown.
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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 × 10⁶ cells/culture) was used to determine the content of AdoHcy and AdoMet employing HPLC. The second group (10⁶ 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 × 10⁴ c.p.m./10⁶ cells. Results are representative of three independent experiments. (B) Effect of AdoHcy on isoprenylcysteine protein carboxyl MTase activity. Cell extracts derived from 10⁶ 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/10⁶ cells AdoMet and ~26 pmol/10⁶ 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/10⁶ cells. In contrast, AdoHcy levels increased and after 1 h reached a maximum value of 990 pmol/10⁶ 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/10⁶ 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 (ΔΨ<sub>m</sub>) 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 DiOC<sub>6</sub>(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 ΔΨ<sub>m</sub>, 3 and 7 h after TNF-α addition. Similarly a modest disruption of ΔΨ<sub>m</sub> was noted when cells were treated with TNF-α (100 pg/ml) in the presence of actinomycin D (1 µg/ml). After 7 h almost 18% of the cells expressed low ΔΨ<sub>m</sub> (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 ΔΨ<sub>m</sub> in an efficient manner: At 3 h after addition of TNF-α (10 and 100 pg/ml) the percentages of cells with low ΔΨ<sub>m</sub> 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 ΔΨ<sub>m</sub>.

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

It was previously suggested that the collapse of the ΔΨ<sub>m</sub> 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.](http://intimm.oxfordjournals.org/)

Fig. 3. TNF-α-induced disruption of mitochondrial membrane potential in the presence and absence of Ado and Homo. L929 cells (10<sup>5</sup>/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 (ΔΨ<sub>m</sub>) was assessed by flow cytometry, using the fluorochrome (DiOC<sub>6</sub>(3)) (20). The results are representative of three experiments. Control mitochondria treated with the ΔΨ<sub>m</sub>-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
was very effective. Ado and Homo alone had no effect. From
with TNF-α
a representative experiment are shown in Fig. 6(A). Compared
and Homo (1 mg, each) were kept constant. The results of
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. 6(A). Compared
with TNF-α alone, the combination of TNF-α, Ado and Homo
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
prolonged (100 days) treatment with TNF-α. After 22 days all these mice were tumor-free. Again, after
prolonged (100 days) treatment 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 to 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 increased tumor size
more effectively than did TNF-α alone, indicating that these agents had no
inhibitory effect on tumor growth in vivo. The results of this
experiment are shown in Fig. 6(C).

In subsequent studies we examined whether the potentiating
effect of Ado and Homo on TNF-α-mediated cytotoxicity
occurs also in other tumor cells. Several murine and
human tumor cell lines were employed to study the effect of Ado and Homo on the cytotoxic activity of TNF-α. Three strains were
TNF-α sensitive (L929, WEHI and HeLa S) while five cells lines
(HeLa R3, mutant L929 M1, A549, PLC and SK-OV-3) were
TNF-α resistant. As shown in Fig. 5, combinations of Ado and
Homo enhanced TNF-α cytotoxicity in TNF-sensitive strains
and converted TNF-resistant into TNF-sensitive tumor cells.
In all cases, the effect of Ado and Homo was dose dependent.
At the concentration used (1 and 0.5 mM), Ado and Homo
did not affect cell viability in the absence of TNF-α.

Ado and Homo exert their enhancing potential on various
tumor cells

Subcutaneous injection of 10^6 TNF-α-sensitive L929 cells into
nude mice yielded fast-growing tumors. The tumors were
allowed to grow for 4 days. Mice received then Ado and
Homo, TNF-α, or TNF-α and 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. 6(A). 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
growth.

Fig. 4. Effect of BA on the TNF-α-induced cytotoxicity in the presence
and absence of Ado and Homo. L929 cells (10^4) were placed into
microtiter plates and incubated overnight. Cultures received then
graded amounts of TNF-α together with or without Ado and Homo
(1 mM, each) and BA (50 µM). Incubation was then continued and
after 18 h cytotoxicity was determined employing the MTT test. The results are representative for three experiments.

Fig. 5. Potentiation of TNF-α-mediated cytotoxicity in various tumor
cell lines by a combination of Ado and Homo. Cells were placed into
96-well microtiter plates at a cell density of 10^4 cells/well and allowed
to incubate for 18 h in culture medium. Incubation was then continued
in the presence of TNF-α (concentrations are indicated at the top of
each panel) in combination with various concentrations of Ado and Homo
(indicated at the bottom of each panel) and actinomycin D
(1 µg/ml). After a further 18 h cell viability was determined using the
MTT test. Ado and Homo alone at the concentrations used did
not affect cell viability. Results shown are representative of four
experiments.
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Fig. 6. Effect of TNF-α and/or Ado and Homo on the growth of s.c. tumors in nude mice. All mice (six per group) received $10^6$ tumor cells and tumors were allowed to grow. After day 4 following injection of tumor cells, mice received 0.1 ml injections of the solvent PBS, TNF-α (10 μg/mouse), Ado and Homo (1 mg/mouse each) or both TNF-α and Ado and Homo. The injections were conducted perilesionally for several periods on five consecutive days, interrupted by 2 days without treatment. Injections were placed near the tumor site but outside the nodule. Mean tumor size index (the product of the largest perpendicular diameters) is plotted against time for L929 (A), L929 M1 (B) and L929 M5 (D) tumors. (C) The viability of L929 M5 cells in the presence of TNF-α alone or together with Ado and Homo. Viability was determined employing the MTT test. All the animals were randomized and the reagent blinded. The figures show one representative out of three experiments.

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 ($\Delta \Psi_m$). Disruption of $\Delta \Psi_m$ 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 carboxyl 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 and 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 mediate 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

Regulation of TNF-α cytotoxicity by the cellular methylation state


