The regulation of human adrenomedullin (AM) and tumor necrosis factor α (TNF-α) receptors on human epithelial carcinoma (HeLa) cells. The role of AM secretion in tumor cell sensitivity*

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The cytostatic cytokine tumor necrosis factor-α (TNF-α) and proliferative hormone adrenomedullin (AM) are abundantly expressed in human tumors. However, little is known about mechanism(s) through which TNF-α and AM exert their regulatory effects, especially in the regulation of proliferative activity in malignant cells. Also the role played by TNF-α in pathogenesis and treatment of cancer (targeted cancer therapy) remains less understood. The purpose of this study was therefore to characterize the significance of TNF-α induced apoptosis with down-regulation of plasma-membrane TNF-α receptors and up-regulation of AM receptors with increased production of human AM mRNA, i.e. mechanisms that subsequently control aberrant cellular proliferation in malignant cells. Cytotoxicity, and the whole cell ligand binding assays for TNF-α and AM receptors, and RIA-assays of AM production were accomplished in control experiments using pharmacologically pretreated HeLa cells. AM increased proliferation of HeLa cells and AM antagonist $(Ala^{6,21})AM_{(22-52)}$ significantly antagonized this increase. TNF-α inhibitor of cell growth actinomycin-D significantly increased cytotoxicity of TNF-α in HeLa cells. Hypoxia increased TNF-α production and increased surface-membrane [^{125}I]AM binding. Tumor promotor PMA and histamine down-regulated specific binding of [^{125}I]TNF-α on HeLa cells. Mitogenic peptide endothelin-1 increased and specific ET-1 antagonist BQ123 and significantly reduced AM binding. Production of AM in HeLa cells markedly increased after exposure to hypoxia >ET-1 >PMA. BAY11-7082 at concentrations that inhibited I_kB phosphorylation and thus nuclear translocation and surface membrane TNF-α expression increased AM specific binding. Pretreatment of cells inhibitor of HMGCoA reductase inhibitor VULM1457 significantly increased the total number of specific [^{125}I]AM binding sites on HeLa cells. These results suggest relative and contradictory TNF-α and AM surface-membrane receptor signaling in HeLa cells and findings reveal a novel proliferative mechanisms that control AM production and thus oncogenic signaling in cells. This implies that several putative inhibitors of TNF-α and AM signaling may be considered in oncology for treatment of tumors otherwise nonresponding to cytostatic therapy.

Key words: TNF-α, adrenomedullin, receptors, AM production, proliferation, HeLa, epithelial carcinoma cells, cells in culture

How tumor cells utilize their membrane receptor machinery to drive neoplastic growth is still not clearly understood. Autocrine/paracrine signals cells captured by membrane receptors are transduced inward to control cell proliferation and gene expression. Tumor necrosis factor-α (TNF-α) was originally identified as an agent inducing necrosis in tumor tissue. TNF-α is acting in a paracrine or autocrine fashion on target cells via at least two specific surface-membrane receptors [1]. TNFR1 is a member of death receptor group, it induces endogenous TNF-α and apoptosis. Dissociation of TNF-α from TNFR-1 was found to be quite slow [2]. It has been indicated that TNFR-2 is a receptor without death domain, with the rapid association-dissociation kinetics probably does not generate true signal in cells, but instead "it passes the TNF-α to TNFR1 receptor" [3, 4]. Several reports have indicated that tumor cells express also hormone

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adrenomedullin (AM), and AM receptors [5]. It was suggested that the executive apparatus of AM induction could be the cause of protection of tumor cells from TNF- mediated apoptosis and reason for tumor survival, but this has not been directly shown. Human cervical carcinoma (HeLa) cells have been shown to express exclusively TNFR-1 receptors [6]. In addition there is a support for "TNF- shedding" i.e. for control of TNF- function in cells by histamine-induced release of a soluble form of TNFR-1 [7].The TNF- release, AM induction and behavior of tumorigenic cells suggest that not all proliferative properties could be explained by deregulated over-activation of oncogenes, but inactivation of tumor supressor genes, or a recently described category of "caretaker genes", seems to be also crucial. In an attempt to further study the distribution of TNF- and AM receptors on tumor cells and to show their role played in cellular function, we used cytotoxic and ligand binding activity of tumorigenic cell line to analyze signaling machinery from cell surface-receptor proteins. Here we describe the detection of specific high-affinity binding sites (TNF- and AM receptors) on the HeLa cells. The quantitative expression of TNF- has been compared with the sensitivity of Hela cells and AM production and expression of AM receptors on HeLa cells, which may contribute towards the apoptosis and necrotic cell death or to proliferation and survival of tumorigenic cells.

Material and methods

All experimental procedures with human cell cultures were performed in accordance with the Helsinki Declaration of 1975 and studies were performed in compliance with the Principles, formulated by the National Society for Medical Research. The experiments were realized in the Laboratory of Cardiovascular Pharmacology, Institute of Experimental Pharmacology, Slovak Academy of Sciences and they were approved by the Ethic Committee No.11/04.

Chemicals. Human (h)adrenomedullin$_{(1-52)}$, (Calbiochem), adrenomedullin$_{(22-52)}$ (Bache), (h)angiotensin-II (Calbiochem), BAY11-7082 (Calbiochem), BQ123 (Calbiochem), (h)endothelin-1, (Sigma) Sep-Pack C_{18} (Amprep), (h)tumor necrosis factor alpha (Bachem), Amprep Minicolumns (Amersham Pharmacia Biotech), 13-O-tetrahydro-decanoyl-phorbol-13-acetate (Serva), VUM1457 (1-(2,6-diisopropylphenyl)-3-[4-4'-nitrophenylthio) phenyl] urea, (Inst for Drug Res Modra, SK).

Radiochemicals. ([^{125}I]-Iodotyrosyl)-Adrenomedullin and [^{125}I]-Tumor Necrosis Factor Alpha (both Amersham Biosciences, Buckinghamshire, UK), ([^{125}I]-Iodotyrosyl)- Adrenomedullin (h)-RIA Kit (Peninsula Lab. Inc., Ca., USA)

Cells. HeLa cell line was obtained as a gift from the Institute of Virology, Slovak Academy of Sciences, Slovak Republic. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) in a humidified 95% air, 5% CO_2 incubator. The cells were grown as monolayer cultures and maintained in Costar flasks containing 10 ml of a medium supplemented with FCS, non-essential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin under standard cell culture incubation conditions (room air, 5% CO_2, 37 °C) or under hypoxic (24 h, 1% O_2 balanced with 95% N_2, 5% CO_2) conditions. The cells were transferred to low serum media (1% FCS) 2 h before the treatment. The cell binding assays were performed in triplicate with cells ($2.5x10^5$ cells/well) under polarized (whole cell binding) conditions (Buffer I, in mmol/l): NaCl 135, $MgCl_2$ 1.0, KH_2PO_4 0.44, NaH_2PO_4 0.34, $NaHCO_3$ 2.6, HEPES 20.0, glucose 5.6, pH=7.4. In equilibrium binding assays aliquots of HeLa cells (0.8–1.1 mg of protein) were incubated with 10 increasing concentrations (from 0.5 to 3.0 nmol/l) of human recombinant [^{125}I]TNF-α specific activity 400 Ci/mmol, or (h)([^{125}I]-Iodotyrosyl) Adrenomedullin$_{(1-52)}$, specific activity 2000 Ci/mmol (both Amersham Biosciences, UK). Nonspecific binding was routinely determined in the presence of 0.1 µmol/l of unlabeled AM. After incubation (60 min) the mixtures were filtered over 25 mm Whatman GF/C glass-fiber filters and the bound radioligand was separated.

Radioimmunoassays of human Adrenomedullin. AM present in the culture media was extracted by using Sep-Pak C_{18} cartridges (Waters, Millford, MA, USA), eluted with Buffer II (60% acetonitrile, HPLC grade, in 1% TFA) and the extract was reconstituted with RIA Buffer (Buffer III).The concentrations of AM in the medium were measured by commercial Competitive Adrenomedullin Radioimmunoassay Kit (Peninsula Lab. Inc. Belmont, CA.) with two overnight incubations.

ELISA-TNF-α detection. For ELISA of endogenously produced TNF-α, cells were treated as indicated, harvested, and lysed in 300 µl of 20 mM Tris, pH=7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100 complemented with a protease inhibitor mixture for 20 min on ice. Calibrated lysates, corresponding cell culture supernatants were analyzed with TNF-α specific ELISA according to the manufacturer's recomendations (Endogen, Boston, MA).

Cell viability and proliferation assays. Cells were plated on 96-well plates (Corning, NY, USA) at a density 10^5 cells/well, in 100 µl of FCS/DMEM without phenol red and incubated for 24 h. Shortly before cell stimulation, cell media were replaced with 90 µl of 1% FCS/DMEM. Pharmacologically active compounds were pretreated for 24 h. Cell viability was assessed by Trypan Blue Dye exclusion test. To produce actively growing cells sub-confluent populations of HeLa cells were then stimulated to proliferate by feeding with 2 ml/Petri Dish of a medium containing 10% FBS and 0.1 µCi/ml [^{3}H]thymidine (21.0 Ci/mmol, Amersham). Apoptosis was induced by treatment of cells with varying concentrations of TNF-α in the presence of 10 $\mu g.ml^{-1}$ cycloheximide for 4 h. Cell viability was assessed by trypan blue dye exclusion test. To produce quiescent cells, subconfluent populations were changed to serum free medium with 0.1% FBS. Cell proliferation was assessed by measure-

ment of [^{3}H]thymidine uptake. Protein concentration was determined according to BRADFORD [8] with use of bovine serum as standard.

Cytotoxicity measurements. Cytotoxic cell death measurements were performed according to the method desribed by MACEWAN [9]. The cells were aliquoted into 96-well culture plates at the density of 10^5 cells/ml, cultured for 24 h then treated with the required combination of agents and actinomycin-D (100 ng/ml) for another 24 h. Colorimetric determination of the attached cell number was performed by microplate reader.

Ligand binding studies. Association and dissociation kinetics of [^{125}I]TNF-α: For kinetic analysis whole-cell bindig assays were performed in triplicate with HeLa cells ($2.5x10^5$ cells/well) in PBS solution with 0.1% BSA. Cells were incubated with 100 pmol/l of TNF-α (in a final volume of 250 µl at 25±0.1 °C, up to 4 h) and at 10 min intervals aliquots were removed, washed and counted for radioactivity. In association studies, incubated samples were washed at predetermined times (two times with 1.5 ml of cold PBS), filtered through Whatmann GFC Glass Fiber Filters and radioactivity retained on filters was measured by using Packard (Tri-Carb-300CD, IL, USA) scintillation counter. Nonspecific binding was determined in the presence of 1 µmol/l of nonlabeled TNF-α. The off rates of [^{125}I]TNF-α from TNF-receptor were performed after incubation (120, 180 and 360 min) by addition of 0.1 µmol/l of unlabeled TNF-α.

Saturation experiments with [^{125}I](h)AM and (h)[^{125}I]TNF-α. In equilibrium binding assays aliquots of HeLa cells ($2x10^5$ cells) were incubated with 10 increasing concentrations of (h) peptides (for details see [10] §4), [^{125}I]AM (from 0.5 to 2.4 nmol/l) or [^{125}I]TNF-α (from 0.7 to 4.0 nmol/l) in other experiments. Nonspecific binding (not shown) was routinely determined in the presence of 0.1 µmol/l of nonlabeled AM or TNF-α. After incubation (60 min.) the mixtures were filtered over 25 mm Whatman GF/C Glass Fiber Filters with 3x1.5 ml of ice-cold Buffer I and the bound radioligand was separated. Statistical analysis was performed with Student's t-test elaborating experimental data by means of Inplot, and Origin Programs.

Results

To examine the effects of TNF-α on cell death in HeLa cell line we studied its various concentrations (from 5.0 to 100 ng/ml) alone and with preincubation of 100 ng/ml actinomycin-D. The EC_{50} concentration of TNF-α alone was 64.7±14 ng/ml. The combined cell treatment with TNF-α and actinomycin-D showed a 11.4 fold increase in cytotoxicity (Fig. 1). With the TNF-α concentration of 5.0 ng/ml or higher, death was more rapid and nearly all cells were nonviable 15 h after addition of these substances to the mixture. Synchronized HeLa cells (experiments employing serum-deprivation as a means of cell cycle synchronization, for details see methods and [10]) were assessed for [^{3}H]thymidine incorporation induced by AM in a wide range of concentrations (from 0.1 pmol/l to 10 nmol/l) both in the absence and presence of the AM receptor antagonist (^{6,21}Ala)-adrenomedullin$_{(22-52)}$).

As seen in Figure 2, the putative adrenomedullin receptor antagonist (Ala-derivative of AM$_{(22-52)}$ significantly ($p<0.05$) reduced the increase in [^{3}H]thymidine incorporation produced by (h)adrenomedullin. Specific binding of [^{125}I]TNF-α was measured as a function of ligand concentration on polarized (in whole cell binding assays) HeLa cells. To determine the specificity of the binding aliquots of HeLa cells, they were

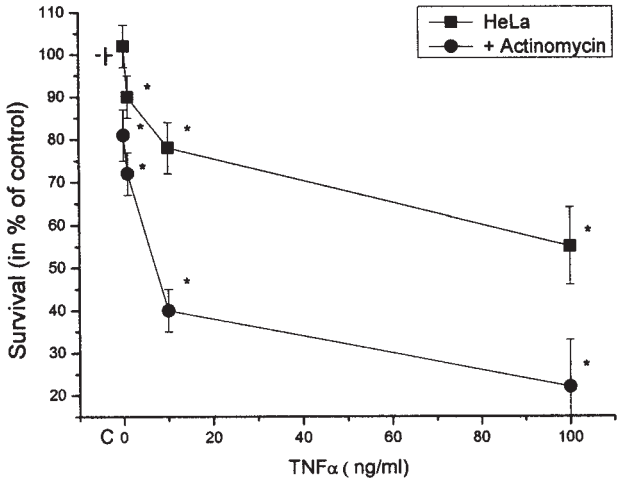

Figure 1. Concentration-response of TNF-α-induced cell death in HeLa cells in the absence and in the presence of actinomycin D (100 ng/ml). Values are mean ±SEM of 5 experiments.
***Statistical significance ($p<0.05$). C = control.**

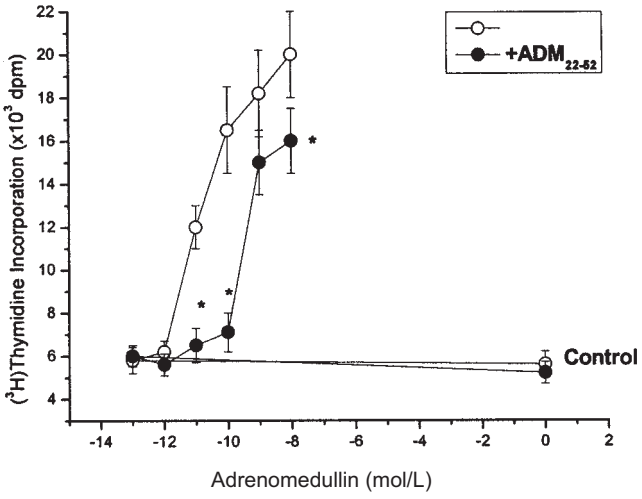

Figure 2. Growth effects of human (h)AM in HeLa cells and growth-inhibitory effects of preincubation of HeLa cells with 1.0 µmol/l (h)AM(22-52), a putative AM receptor antagonist (n=8). Explanation and symbols as in preceding figure.

incubated with 10 increasing concentrations of radiolabeled TNF-α in the presence and absence of an excess unlabeled TNF-α. The binding curves are shown in Figure 3.

Kinetics of TNF-α association and dissociation on HeLa cells. The specific association of TNF-α with receptors on HeLa cells was found to be rapid ($t_{1/2}$ = 11 min., not shown). In contrast, dissociation of TNF-α from HeLa cells was found to be slow, with a half life >3.5 h (not shown). The course and characteristics of saturation curves in our control experiments confirmed the presence of a single class of high affinity [^{125}I]TNF-α binding sites with the (K_d = 0.53±0.10 nmol/l and density (B_{max} = 77±3.5 fmol/mg of protein (n=6). Values are given throughout the paper as mean ± standard deviation). The experiments with HeLa cells preincubated with an activator proteinkinase C and strong inducer of $NF_κB$, phorbol-myristate-acetate (for details see methods), showed significant down-regulation of [^{125}I]TNF-α specific binding (with the density of [^{125}I]TNF-α binding $B_{max\ PMA}$ = 35.4±2.5 fmol/mg of protein, p<0.05) at unchanged affinity (K_d = 0.54±0.20). For comparison, the preincubation of HeLa cells with the BAY11-7082 selective and irreversible inhibitor of TNF-α inducible phosphorylation of I_KB-, (resulting in a decreased expression of nuclear factor NF_KB), significantly reduced [^{125}I]TNF-α binding density (to 62±8% of its control value, p<0.05) at markedly reduced affinity (nominal values of K_D in the range of + 258±86%). Treatment of confluent HeLa cells with 10.0 and 100.0 μmol/l of histamine that caused increased shedding of TNF-α significantly reduced the [^{125}I]TNF-α specific binding to 72±6 and 54±5%, (p<0.05), (not shown). Similarly, the long-term preincubation of HeLa cells with the endothelin$_{A/B}$ antagonist BQ123 significantly reduced [^{125}I]TNF-α binding density. Production of TNF-α in HeLa cells was markedly induced by hypoxia and lipopolysaccharide (Tab. 1). When HeLa cells were stimulated with LPS the production of TNF-α significantly increased. Hypoxia induced HeLa cells to secrete significantly more of TNF-α and stimulation of HeLa cells with LPS under hypoxic conditions produced further increase in the level of TNF-α in cell supernatant.

Binding of [^{125}I]AM to quiescent HeLa cells was found to be specific and to have a saturable high affinity component with K_d = 0.63±0.11 and B_{max} = 139±4 fmol/mg of protein (Fig. 3). The specific binding of [^{125}I]AM significantly increased after long-term preincubation of HeLa cells with selective and irreversible inhibitor of NF_KB, BAY 11-7082, (B_{maxBAY} = 251±19%, p<0.05) and surprisingly also after preincubation of HeLa cells with the hydroxy-methyl-glutaryl-acyl-CoA reductase (HMGCoA) inhibitor VULM1457 (0.1 and 1.0 μmol/l, $B_{maxVULM}$ 128±4 and 145±10%, respectively, p<0.05). Specific binding of [^{125}I]AM on HeLa cells was completely inhibited by unlabeled (h)AM and also by 1.0 μmol/l of AM antagonist $^{Ala}AM_{22-52}$ (not shown). The total number of specific [^{125}I]AM_{1-52} binding sites (B_{max}) increased markedly after short-term exposure of HeLa cells to hypoxia (4) h ($B_{max\ Hypox}$ = +262±12%, p<0.05) and also after

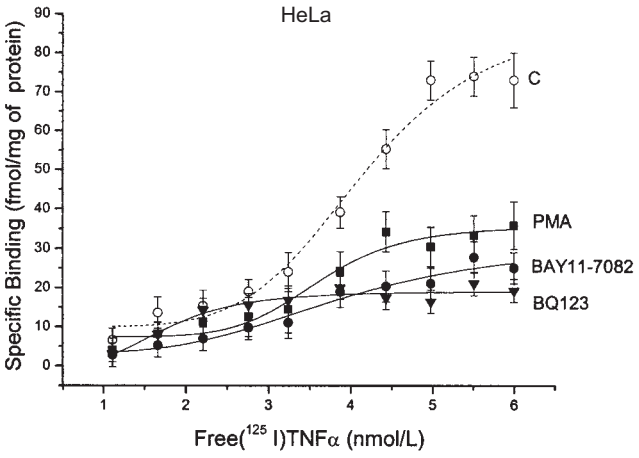

Figure 3. Whole cell saturation binding isotherms of human [^{125}I] TNF-α in control HeLa cells and cells preincubated with activator protein kinase C and tumor promotor phorbol-12-myristate-13-acetate (PMA, 1.0 μmol/l), selective and irreversible inhibitor of TNF-α-inducible phosphorylation of $I_κB$ phosphorylation (BAY11-7082, 10 μmol/l), and endothelin ET_A/ET_B antagonist (BQ123, 1.0 μmol/l).

Table 1. Production of TNF-α in HeLa cells

TNF-α production in cell supernatant (pg/ml) (n = 24)			
21 ± 5	48 ± 6*	29 ± 8	66 ± 12*
Control Normoxic	Stimulated Normoxic	Unstimulated Hypoxic	Stimulated Hypoxic

preincubation of 100 pmol/l of endothelin-1 ($B_{max\ ET-1}$ = +182±9%, p<0.05). The preincubation of HeLa cells with the activator of cytosolic Ca^{2+} dependent tyrosine-kinase angiotensin-II (50 μg/l), was without any effect on the total number of sites. As shown in Figure 4, in control HeLa cells incubated 24 h under normoxic conditions the production of AM was low. However when cells were made hypoxic by incubation 24 h in DMEM with 1% of oxygen, respective AM levels in the medium of HeLa cells increased significantly. In a separate experiment, when cells were incubated 24 h under "normoxic" conditions, and were exposed to endothelin-1, or PMA, in other experiments, the production of AM in the media increased significantly. (Fig. 5).

The experiments with acid wash (AW) to remove the surface-membrane bound TNF-α receptors showed that after 120 min of incubation an approximately 17±5% of activity was retained in HeLa cells (Fig. 6) representing down-regulated (internalized) TNF-α receptors.

Discussion

To examine the relation of TNF-α cytotoxicity, adrenomedullin(AM)-induced cell proliferation and malignant cell

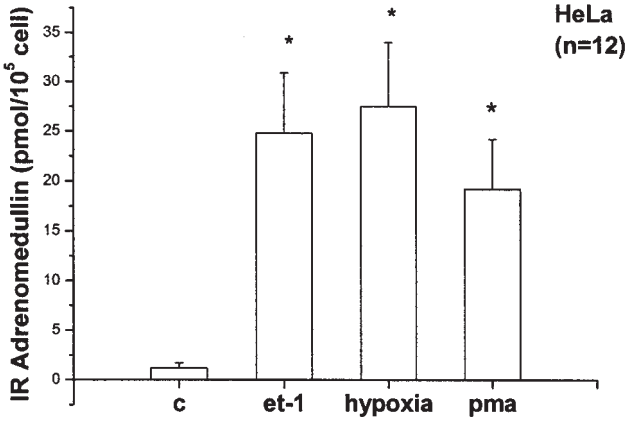

Figure 4. Competitive radioimmunoassays of IR[^{125}I]-AM in the conditioned media of HeLa cells cultured under normoxia and hypoxia (1% O_2, 5% CO_2, and 94% N_2), incubated 24 h in DMEM. *Significant increase ($p<0.01$) in accumulated IR-AM in cultured media of HeLa cells preincubated with mitogenic peptide endothelin-1 (et-1), hypoxia and phorbol-myristate-acetate (pma). For details see Methods. Values are mean and corresponding S.E.M.

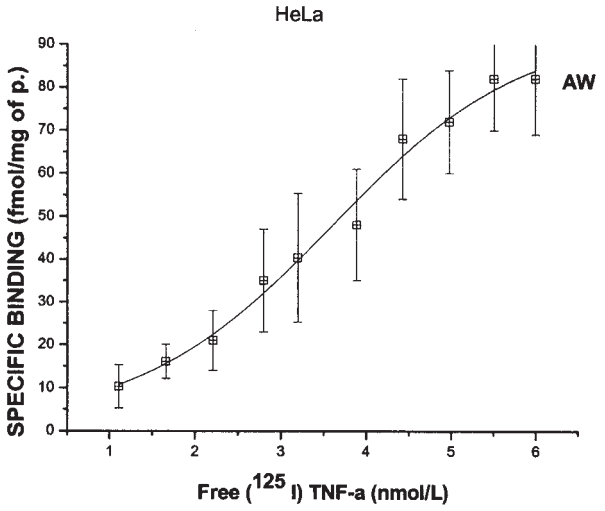

Figure 6. Removal of specific [^{125}I]TNF-α binding activity from the cell surface of HeLa cells with mild acid wash (AW). Procedure is frequently used to wash out membrane-bound receptor fraction, i.e. that fraction of receptors that was not subjected to internalization. An approximately 20% of specific activity binding in the present study represent remnant, i.e. internalized TNF-α receptors (n=6).

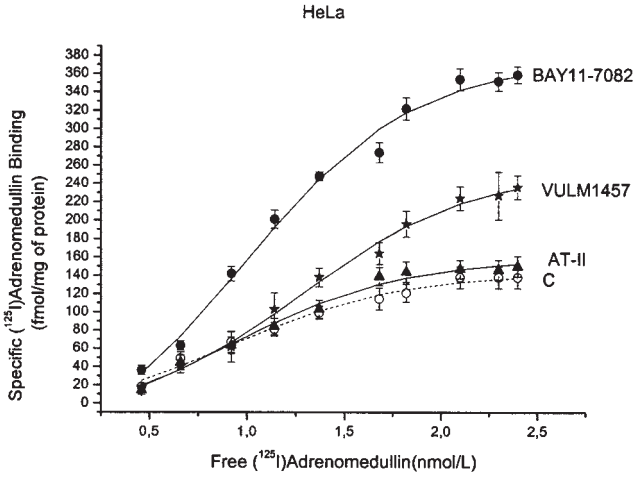

Figure 5. Specific whole cell saturation isotherms of (h)[^{125} I]AM(1-52) binding on HeLa cells exposed (24 h) to selective and irreversible inhibitor of TNF-α-induced phosphorylation of $I_κB$, BAY11-7082 (10 μmol/l), resulting in reduced expression of $NF_κB$, to acylCoA: cholesterol-acyl-transferase and AM-inhibitor VULM1457 (1.0 μmol/l), and to stimulative peptide angiotensin (A 50 ng/ml).

survival, we investigated the characteristics of membrane-bound TNF-α and AM receptors, TNF-α and AM production in epithelial carcinoma (HeLa) cells. Here we report the contribution of nuclear factor-kappaB ($NF_κB$) activation to this process. Our data clearly showed a direct correlation of the excessive TNF-α signaling with down-regulation of membrane-bound TNF-α receptors, reduced cellular sensitivity against TNF-α with increased adrenomedullin production and with resultant increased proliferation in HeLa cells. A major finding of this investigation was that BAY11-7082, the selective and irreversible blocker of TNF-α inducible phosphorylation ($I_κB$ is an inhibitory factor of $NF_κB$ and nuclear translocation in cells), significantly down-regulated TNF-α receptors. In an alternative model with experiments on endothelial cells [11], it was suggested that TNF-α induced degradation of $I_κB$ through TNFR-1. In the present study, we therefore used an inhibitor of phosphorylation $I_κB$ protein BAY11-7082, and TNF-R1 exclusive binding of [^{125}I]TNF-α in HeLa cells. Acyl-CoA: Cholesterol-Acyl-Transferase (ACAT) is an important cellular protein in the pathways of cholesterol (CH) esterification and membrane CH insertion. Pharmacological evidence showed that ACAT inhibitor F-1394 reduced atherosclerosis in apolipoprotein-E deficient mice [12]. A new specific ACAT inhibitor VULM1457 significantly reduced atherogenic activity in animal experimental model of atherosclerosis [13, 14]. The second major finding of this investigation was that the HMGCoA reductase inhibitor VULM1457 and also the selective and irreversible inhibitor of the TNF-α induced phosphorylation of $I_κB$ (BAY11-7082), (resulting in decreased expression of nuclear factor $NF_κB$ [15] when preincubated, solely and also in combination, significantly up-regulated AM receptors on HeLa cells. One possible explanation of these results is that AM is a component of a so far not described selective mechanism that protects human HeLa cells against TNF-α induced cytotoxicity. A second possibility is that resultant AM release and reduced sensitivity of HeLa cells to TNF-α are specific phenomena of trans-

formed tumorigenic cells. A third possibility would be that posttranslationally modified signal proteins (TNF-α and AM) may vary from the original ones expressed on control HeLa cells. In the present study in synchronized HeLa cells an inhibitor of cell growth actinomycin-D produced apoptosis and increased cytotoxicity of TNF-α. Whether the same concentration of inhibitor is capable of inducion AM production remains open question. It is also important to note that when cells in the present study were preincubated with PMA and in other experiments with BAY11-7082, the reductions in B_{max} for TNF-α always correlated well with decreased affinity of TNF-α for the membrane receptors. KALTSCHMIDT [16] reported that PMA is a strong inducer of $NF_{\kappa}B$ activity and since BAY11-7082 and also HMGCoA inhibitor VULM1457 in the present study induced AM production in cells and significantly increased AM binding capacity, the blockade of phosphorylation of "inhibitory factor of nuclear translocation (IkB)" may well (via $NF_{\kappa}B$ proteins) increase nuclear transduction, and AM secretion and in turn the level of oncogenic signaling in tumorigenic cells. The majority of TNF-α receptor molecules are probably localized on the cell surface and to the Golgi apparatus. Soluble forms of TNF-α receptors can be shed from the cell surface and inhibit TNF-α actions [7]. However, the relationships among cell surface, Golgi-associated and shed forms of TNF-α receptors are unclear at present. Thus cytotoxic activity of TNF-α and proliferative effects of AM, two diverse processes may well be under the same general controlling mechanisms in tumorigenic cells.

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