

TUMOR PRODUCTION OF ANGIOSTATIN IS ENHANCED AFTER EXPOSURE TO TNF- α

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Infection of tumors with an adenoviral vector expressing a chimeric gene composed of the CARG elements of the Egr-1 promoter and a cDNA encoding TNF- α (Ad.Egr-TNF) has previously been shown to result in the production of high intratumoral levels of TNF- α and thereby tumor regression. The antitumor effects of TNF- α were ascribed to vascular thrombosis. We and others, have reported that inhibition of tumor vessel thrombosis using anticoagulation therapy does not abrogate the antitumor effects after TNF- α treatment. To investigate the potential antiangiogenic effects of TNF- α , we studied the generation of angiostatin after intratumoral injection of Ad.Egr-TNF. We report an increase in plasma angiostatin levels both during and after treatment with Ad.Egr-TNF that parallel tumor regression. We also report that TNF- α enhances angiostatin production by inducing the activity of plasminogen activator and the release of MMP-9 by tumor cells. These studies support a model in which the antiangiogenic effects of TNF- α on the tumor microvasculature are mediated by generation of angiostatin.

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Tumor necrosis factor- α (TNF- α) is a multifunctional cytokine produced by a variety of immune cells, including activated macrophages and lymphocytes.¹ TNF- α exerts its antitumor effects through direct tumor cell cytotoxicity and damage to the tumor microvasculature.² Direct antitumor effects are mediated by binding of TNF- α to a 55 kD receptor, which trimerizes and associates with intracellular adaptor and effector molecules that signal apoptosis.³ Antiangiogenic effects of TNF- α specifically target proliferating tumor endothelial cells rather than quiescent vessels in normal tissues.^{4,5} In addition, TNF- α produces vascular damage by inducing endothelial cell apoptosis^{6,7} and intravascular thrombosis.^{8–11}

Despite potent antitumor effects in animal models, human trials employing systemic TNF- α have proven unsuccessful due to dose-limiting toxicities.¹² Although TNF- α administration significantly enhances the antitumor effects of radiotherapy in certain patients, toxicity has also restricted the overall utility of this combined approach.¹³ In an effort to increase the efficacy of radiotherapy by increasing intratumoral TNF- α levels, a gene therapy strategy was developed that employs the radiation-inducible promoter of the Egr-1 gene inserted upstream of the human TNF- α cDNA.¹⁴ The Egr-TNF construct was cloned into a replication deficient adenovirus to assess the regulation of TNF- α gene expression in irradiated tumors. When tumor-bearing mice were treated with Ad.Egr-TNF alone, or in combination with IR, a significant increase in tumor regression was observed when compared to IR alone.¹⁵ In addition, histological analysis of tumors from animals treated with Ad.Egr-TNF + IR revealed extensive necrosis and vascular thrombosis.¹⁶ Anticoagulation of tumor bearing animals with either coumadin or anecrod, however, did not block the antitumor effects of TNF- α (Weichselbaum *et al.*, unpublished data). These data are consistent with those reported by Watanabe *et al.*,² in which anticoagulation with heparin failed to influence TNF- α -mediated tumor growth inhibition and cure rate. We therefore investigated

additional potential mechanisms of the antitumor effects of TNF- α on the tumor vasculature.

Angiostatin is a potent endogenous inhibitor of angiogenesis. Angiostatin is generated from plasminogen by a variety of enzymatic mechanisms including, plasmin autoproteolysis in the setting of a free sulfhydryl donor,^{17–19} digestion of plasminogen by macrophage-derived metalloelastase (MMP-12),^{20,21} serine elastase,²² stromelysin-1 (MMP-3),²³ gelatinase A (MMP-2),²⁴ matrilysin (MMP-7) and gelatinase B (MMP-9).²⁵ The present experiments were performed to determine if treatment with Ad.Egr-TNF is associated with increased levels of circulating angiostatin. We report that treatment of SQ-20B tumors with Ad.Egr-TNF *in vivo* and exposure of SQ-20B tumor cells to TNF- α *in vitro* leads to increased generation of angiostatin. Exposure of SQ-20B cells to TNF- α results in increased activity of MMP-9 and PA in the tumor cell supernatant.^{17,25} These findings suggest that localized antitumor therapy may alter the production of antiangiogenic proteins that influence tumor progression.

MATERIAL AND METHODS

Tumor implantation

Female athymic nude mice (Frederick Cancer Research Institute, Frederick, MD) were injected subcutaneously (s.c.) in the right hind limb with human SQ-20B squamous carcinoma cells (5×10^6 cells in 100 μ l PBS). Tumors were permitted to grow and volume was determined by direct measurement with calipers as described.¹⁵ The initial tumor measurement represents the tumor volume at Day 0 and based on these measurements, mice were randomly assigned to treatment groups so that the mean tumor volume of each group was approximately equal. The care and treatment of experimental animals was in accordance with institutional guidelines.

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TNF- α injection

SQ-20B xenografts were injected with 2×10^8 PFU of the replication-deficient adenoviral vector Ad.Egr-TNF (GenVec, Inc., Gaithersburg, MD) twice weekly for 2 weeks as described previously.¹⁵

Irradiation

Xenografts were irradiated using a GE Maxitron 250 X-ray generator at a dose rate of 1.88 Gy/min. SQ-20B tumors were irradiated 4 times/week for 2.5 weeks, using 5 Gy fractions, to a total dose of 50 Gy.¹⁵ SQ-20B tumor cells receiving a single dose of 9 Gy were irradiated using a GE Maxitron X-ray generator operating as described above.²⁶

Plasma collection and storage

Mice were anesthetized using Metaflane inhalation. The abdomen was opened and blood was collected from the inferior vena cava using a 26-gauge intradermal bevel needle attached to a 1 ml latex free syringe containing 0.3 ml of 3.2% buffered citrate solution. Plasma was separated by centrifugation at 1,800 rpm for 20 min and stored at -80°C before analysis.

Western blot analysis of angiostatin levels

Protein levels were determined using the Bio Rad Micro Protein Assay (Bio Rad, Hercules, CA). Ten μg of plasma protein was loaded per well and electrophoresed in 12% polyacrylamide gels (Bio Rad, Hercules, CA) under reducing conditions. For serum free conditioned medium (CM) equal volumes were loaded and electrophoresed using the same protocol. Proteins were electrotransferred to a 0.45 μm polyvinylene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked overnight in blocking buffer (1% BSA in Tris-buffered saline and 0.1% Tween 20) and incubated for 1 hr with a 1:4,000 dilution of a monoclonal antibody to the kringle 1-3 (K1-3) fragment of human plasminogen (VAP 320L and 372PL, Enzyme Research Laboratories, Inc, South Bend, IN). After washing, membranes were incubated for 1 hr with peroxidase conjugated anti-mouse IgG secondary antibody (New England BioLabs, Beverly, MA). After washing, membranes were incubated with chemiluminescence developing substrate according to the manufacturer's protocol (Pierce, Rockford, IL) and exposed to Amersham ECLTM film. Developed films were scanned using a UMAX Astra 2400S scanner in transmission mode and images were saved in TIFF format. Films were quantified using NIH Image software. Significance between treatments was determined using 1-way analysis of variance (ANOVA).

Angiostatin generation by SQ-20B tumor cells

SQ-20B tumor cells were maintained in DMEM: F-12 (3:1) (Gibco, Grand Island, NY), 20% fetal bovine serum (Intergen, Purchase, NY), 0.4 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma, St. Louis, MO) and penicillin/streptomycin (Gibco, Grand Island, NY). Eighty percent confluent monolayers were washed twice with PBS and serum-free RPMI-1640 medium (Gibco) was added. Cultures were treated with 10 ng/ml of rhTNF- α 4 hr before IR exposure. Cultures were then incubated for 24 hr at 37°C and serum-free conditioned medium (SFCM) was collected. Two μg of human plasminogen (Enzyme Research Laboratories, Inc., South Bend, IN) was added to 100 μl aliquots of SFCM incubated for 48 hr and plasminogen conversion to angiostatin was assayed.

PC-3 prostate adenocarcinoma tumor cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium containing 10% fetal bovine serum and penicillin/streptomycin. Eighty percent confluent monolayers were washed twice with PBS and serum-free RPMI-1640 medium was added. Cultures were incubated for 24 hr at 37°C and SFCM was analyzed for angiostatin generation as described above.

Enzyme extraction from SQ-20B xenografts

Mice were injected with SQ-20B tumor cells as described above. Control tumors were injected with viral buffer (GenVec,

Gaithersburg, MD), IR tumors were treated with a total of 25 Gy of radiation (five 5 Gy fractions) and tumors receiving combined treatment were injected intratumorally twice with Ad.Egr-TNF.11D (5×10^{10} PU, GenVec, Gaithersburg, MD). On Day 8, after the initiation of treatment, tumors were excised and snap frozen in liquid nitrogen. For enzymatic analyses, tumors were homogenized in a buffer containing 50 mM Tris (pH 7.5), 0.15 M NaCl, 10 mM CaCl_2 , and 0.05% Brij 35.²⁷ Samples were centrifuged for 5 min at 12,000 rpm. Protein concentration in supernatants was determined using the Bio Rad Micro Protein Assay.

Gelatin zymography.

SQ-20B cells were grown to 50% confluence in 60 mm tissue culture dishes as described above. The cultures were washed with serum-free RPMI-1640 and 5 ml serum-free RPMI-1640 was added and incubated overnight. The following day, medium was removed and replaced with 1.5 ml serum-free RPMI-1640. Ten ng/ml of rhTNF- α was added. Cell free CM was collected at various time points and concentrated with Centricon 10 centrifugal filter devices (Millipore Corporation, MA). Protein concentration was determined as described above and equal amounts of protein from CM were analyzed. Zymography in 10% SDS-polyacrylamide gels containing 0.1% gelatin (Novex Corporation, San Diego, CA) was performed in the absence of chemical pre-activation of MMPs. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 hr and incubated overnight in a 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 10 mM CaCl_2 and 0.2% Brij 35 at 37°C . Gels were subsequently stained with 0.5% Coomassie brilliant blue and destained until gelatinolytic bands were clearly visible. Thereafter, the gels were dehydrated in 20% ethanol/10% glycerol and preserved in cellophane.

Plasminogen activator measurements

Plasminogen activation in CM was quantified spectrophotometrically using a coupled assay that monitors plasmin generation using the synthetic plasmin substrate VLK-pNA as previously described.²⁸ Briefly, plasminogen was incubated in the presence of serum-free CM. VLK-pNA was added and plasminogen activation was quantified based on the plasmin hydrolysis of VLK-pNA by monitoring the absorbance at 405 nm.

Inhibition studies

Plasminogen cleavage was determined in the presence of the following enzyme inhibitors: Pefabloc (4 mM) a serine protease inhibitor, (Sigma), TAPI-O (1 mM, $\text{IC}_{50} = 0.3\text{--}0.6 \mu\text{M}$) a hydroxamic acid-based inhibitor of zinc-dependent MMPs (Peptides International, Louisville, KY) and MMP inhibitor II (1 mM, $\text{IC}_{50} = 2.7 \text{ nM}$) a specific inhibitor of MMP-1, MMP-3, MMP-7 and MMP -9 (Calbiochem, San Diego, CA).

RESULTS

Plasma angiostatin levels are elevated after treatment with Ad.Egr-TNF

To determine if angiostatin generation is associated with the antitumor effects observed after treatment with TNF- α delivered using an adenoviral vector, we measured angiostatin levels in the plasma of mice bearing SQ-20B tumors after intratumoral injection of Ad.Egr-TNF with and without IR. Day 22 was chosen because this represents the time point when significant tumor growth inhibition is first observed.¹⁵ We hypothesized that if angiostatin is a component of the antitumor effects previously observed after treatment with Ad.Egr-TNF + IR, then differences in plasma angiostatin levels should be more apparent at this time point. We performed Western blot analysis on mouse plasma and angiostatin levels were estimated from optical density readings of developed films. We detected a 5-fold increase in angiostatin levels in the plasma of mice bearing SQ-20B tumors (4687 ± 721) compared to baseline levels in tumor-free animals (953 ± 167 ; $p = 0.002$). This finding is in agreement with published reports demonstrating the presence of circulating angiostatin in the serum

of mice with primary tumors.²² We measured plasma angiostatin levels after treatment with Ad.Egr-TNF alone or with Ad.Egr-TNF + IR combined treatment (mean initial tumor volume = 266.4 ± 66.4 mm³; $n = 16$). We detected a significant increase in angiostatin levels after treatment with Ad.Egr-TNF alone ($9,335 \pm 1,851$; 9.8-fold) and the combination of Ad.Egr-TNF + IR ($7,440 \pm 1,245$; 7.8-fold) compared to that in IR treated mice ($5,293 \pm 908$) and non-tumor bearing mice (953 ± 167 ; $p < 0.001$; Fig. 1). The observation that the angiostatin levels in the combined treatment group (Ad.Egr-TNF + IR) were lower than the angiostatin levels in the Ad.Egr-TNF group was anticipated. Because TNF- α enhances tumor cell killing by IR,¹⁵ tumors in the combined treatment had fewer viable tumor cells to participate in angiostatin generation.¹⁶ These data suggest that intratumoral treatment with TNF- α , delivered using the Ad.Egr-TNF vector, is associated with elevations in plasma angiostatin levels.

Increased plasma angiostatin levels correspond with tumor regression after treatment with Ad.Egr-TNF

We evaluated SQ-20B tumor growth at Day 22 and detected a significant decrease in mean tumor volume in animals treated with Ad.Egr-TNF and Ad.Egr-TNF + IR (Table I). Tumors in the untreated control (UTC) tripled in size reaching a mean volume of $1,009.8 \pm 236.6$ mm³ whereas those in the IR group were only slightly larger than Day 0 volumes (487.6 ± 114.7 mm³; 1.3% increase). By contrast, mean tumor volume was significantly reduced in animals treated with Ad.Egr-TNF alone (22.9% decrease compared to Day 0) and with Ad.Egr-TNF + IR (99.7% decrease compared to Day 0, $p = 0.001$, ANOVA). These data are consistent with our previous reports demonstrating significant tumor regression after combined treatment with Ad.Egr-TNF + IR.^{15,29} Increases in plasma angiostatin levels after treatment with Ad.Egr-TNF coincide with tumor regression and are consistent with continued intratumoral production of TNF- α up to Day 21 as reported previously.¹⁵ Elevations in plasma angiostatin levels after treatment with Ad.Egr-TNF in the SQ-20B xenograft model system were confirmed in separate experiments employing esophageal adenocarcinoma xenografts (data not shown).

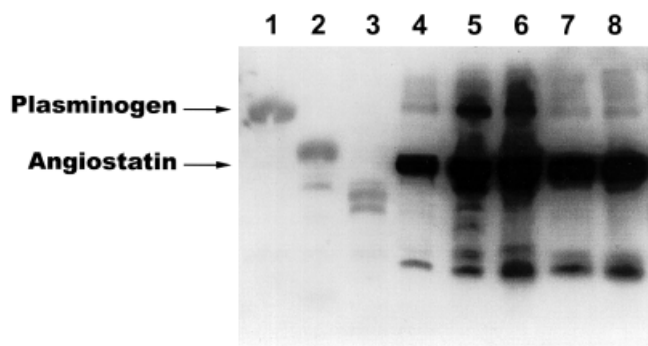


FIGURE 1 – Representative Western blot of Day 22 murine plasma angiostatin levels after treatment with Ad.Egr-TNF and IR. Gels were run under reducing conditions and analyzed as described in Material and Methods. Lane 1: Recombinant human plasminogen. Lane 2: Human angiostatin from plasma. Lane 3: Recombinant human angiostatin K1–3. Lane 4: Angiostatin in the plasma of untreated control mouse (UTC) bearing a SQ-20B xenograft in the right hind limb. Lane 5: Angiostatin in the plasma of an IR treated mouse (four 5 Gy fractions per week to a total dose of 50 Gy). Lane 6: Plasma angiostatin level after intratumoral injection of Ad.Egr-TNF (2×10^8 PFU, twice weekly for 2 weeks). Lanes 7, 8: Plasma angiostatin levels after intratumoral injections of Ad.Egr-TNF (2×10^8 PFU, twice weekly for 2 weeks) and exposure to IR (four 5 Gy fractions per week to a total dose of 50 Gy).

Plasma angiostatin levels peak during treatment with Ad.Egr-TNF

We next evaluated plasma angiostatin levels during treatment with Ad.Egr-TNF and compared them with that obtained after the termination of treatment. We assayed angiostatin in the plasma of animals bearing SQ-20B tumors treated with the Ad.Egr-TNF vector alone and the combination of Ad.Egr-TNF + IR at day 11 using Western blot analysis. Day 11 was selected for analysis because blood was collected from the animals 24 hr after the third intratumoral injection of the Ad.Egr-TNF vector. Plasma angiostatin levels were higher at Day 11 compared to Day 22. Angiostatin levels in the Ad.Egr-TNF alone group ranged from 7,185 to 21,714 at Day 11 (Fig. 2) compared to 7,489 to 11,180 at Day 22 (Fig. 1). In the Ad.Egr-TNF + IR combined treatment group, angiostatin levels ranged from 14,601 to 17,593 at Day 11 and from 4,218 to 9,155 at Day 22. The trend in elevated angiostatin levels at Day 11 may be due to enhanced *in vivo* angiostatin production in response to the TNF- α delivered using the Ad.Egr-TNF vector. Also because maximum regression does not occur until Day 22,¹⁵ more viable tumor cells are present at Day 11 compared to Day 22. We next investigated whether treatment with TNF- α *in vitro* would enhance the generation of angiostatin by SQ-20B tumor cells.

SQ-20B tumor cell conditioned medium converts plasminogen to angiostatin

It has been reported previously that tumor cells^{22,24,30} and inflammatory cells^{20,21,31} express angiostatin-generating activity. To determine if SQ-20B tumor cells exposed to rhTNF- α generate angiostatin from plasminogen,³⁰ we incubated human plasminogen with SQ-20B cell-derived conditioned medium (CM). CM collected from both control and IR treated cultures converted plasminogen to angiostatin confirming that SQ-20B cells possess angiostatin-generating activity. After exposure to TNF- α protein, conversion of plasminogen to angiostatin was enhanced by SQ-20B CM (Fig. 3). To verify our assay procedure, we also incubated PC-3 cell derived CM with human plasminogen and confirmed the conversion of plasminogen to angiostatin (data not shown) as previously described by Gately *et al.*³⁰ These findings demonstrate that SQ-20B tumor cells express enzymatic activity capable of converting plasminogen to angiostatin and that angiostatin generation is enhanced after TNF- α exposure.

Treatment with TNF- α increases MMP-9 release from SQ-20B tumor cells

It has been reported previously that TNF- α regulates the secretion of matrix metalloproteinases (MMPs).^{32,33} Both MMP-2 (gelatinase A/type IV collagenase) and MMP-9 (gelatinase B/type IV collagenase) have been reported to cleave plasminogen to angiostatin.^{24,25,34} We, therefore, asked if the levels of MMP-2 and MMP-9 are increased in the CM of SQ-20B tumor cells after exposure to TNF- α . Using gelatin zymography, we found that untreated control SQ-20B tumor cells constitutively release MMP-2, but not MMP-9. After exposure to TNF- α protein, we observed no detectable increase in MMP-2, however, we did detect an increase in MMP-9 levels in SQ-20B CM beginning at 24 hr. MMP-9 levels in TNF- α treated culture medium remained elevated at 48 and 72 hr (Fig. 4). These data demonstrate that treatment with TNF- α stimulates the release of MMP-9 by SQ-20B tumor cells.

Treatment with TNF- α increases PA activity in SQ-20B tumor cells

Previous studies have demonstrated that human prostate carcinoma cells (PC-3) release plasminogen activator (PA) activity and free sulfhydryl donors that are sufficient for angiostatin generation.¹⁷ We, therefore, tested the CM of SQ-20B tumor cells to determine if exposure to TNF- α protein affects PA activity. rhTNF- α was added to the culture medium at 0 hr and SQ-20B tumor cells were cultured in the presence of TNF- α for 72 hr.

TABLE 1 – SUMMARY OF PLASMA ANGIOSTATIN LEVEL AT DAY 22

Treatment	Day 0 Tumor vol. (mm ³) \pm SEM	Day 22 ² Tumor vol. (mm ³) \pm SEM	Plasma angiotatin level	Fold increase above baseline ³
UTC	349.0 \pm 162.7	1,009.8 \pm 236.6	4,687 \pm 721	4.9
50 Gy	363.8 \pm 152.8	487.6 \pm 114.7	5,293 \pm 908	5.6
Ad.Egr-TNF	115.0 \pm 28.2	88.7 \pm 58.9	9,335 \pm 1,851	9.8
Ad.Egr-TNF + 50 Gy	175.8 \pm 87.0	0.5 \pm 0.5	7,440 \pm 1,245	7.8

¹Mean tumor volume was significantly reduced in SQ-20B xenografts treated with Ad.Egr-TNF alone (22.9%) and Ad.Egr-TNF + IR (99.7%), $p = 0.001$. A significant increase in plasma angiotatin level above baseline was observed following treatment with Ad.Egr-TNF alone (9.8-fold) and Ad.Egr-TNF + IR (7.8-fold, $p < 0.001$). With injection of Ad.Egr-TNF alone plasma angiotatin level is increased 22.7-fold when compared with UTC. The greatest increase in angiotatin level is present in the plasma of mice receiving combined treatment with Ad.Egr-TNF + IR (3,207-fold increase when compared with UTC, and a 141.4-fold increase when compared with Ad.Egr-TNF alone).²Day 22 represents the first point on the growth curve which demonstrates significant regression ($p = 0.002$).³Baseline angiotatin levels (953 \pm 167) were determined in non-tumor bearing nude mice. Quantification was performed on developed films using NIH Image software.

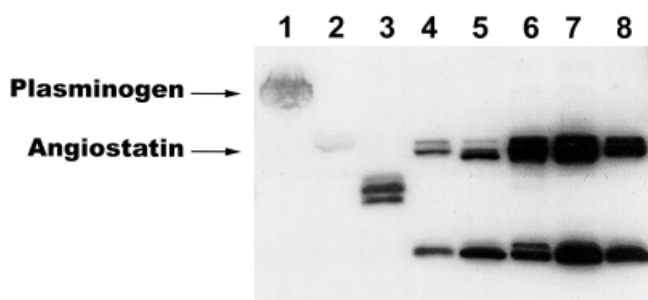


FIGURE 2 – Representative Western blot of Day 11 murine plasma angiotatin levels after treatment with Ad.Egr-TNF and IR. Gels were run under reducing conditions and analyzed as described in Materials and Methods. Lane 1: Recombinant human plasminogen. Lane 2: Human angiotatin from plasma. Lane 3: Recombinant human angiotatin K1-3. Lane 4: Angiotatin in the plasma of untreated control mouse (UTC) bearing a SQ-20B xenograft in the right hind limb. Lanes 5, 7: Plasma angiotatin level 24 hr after intratumoral injection of Ad.Egr-TNF. Lanes 6, 8: Plasma angiotatin level 24 hr after intratumoral injection of Ad.Egr-TNF and IR.

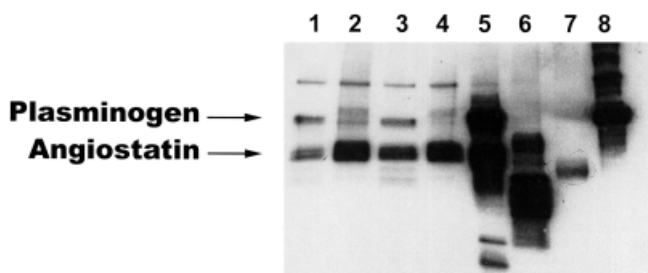


FIGURE 3 – Western blot analysis of serum free SQ-20B conditioned medium. SQ-20B cells were treated as described in Material and Methods. Angiotatin is generated from human plasminogen under the following conditions. Lane 1: Control. Lane 2: With rhTNF- α (10 ng/ml). Lane 3: With IR (900 cGy). Lane 4: With rhTNF- α (10 ng/ml) and IR (900 cGy). Lane 5: Human angiotatin from plasma. Lane 6: Recombinant human angiotatin K1-3. Lane 7: Recombinant murine angiotatin. Lane 8: Recombinant human plasminogen.

These experimental conditions *in vitro* were designed to mimic the tumor microenvironment in which SQ-20B tumor cells are continually exposed to TNF- α protein that is being expressed by tumor cells infected with Ad.Egr-TNF. We detected a significant increase in PA activity beginning at 24 hr in TNF- α treated cultures (34.05 \pm 0.19 mOD/min) when compared to untreated control cultures (0.93 \pm 0.32 mOD/min) and IR treated cultures (1.76 \pm 0.59 mOD/min; $p < 0.001$, ANOVA). PA activity in TNF- α treated cultures remained elevated at 48 and 72 hr. (Fig. 5).

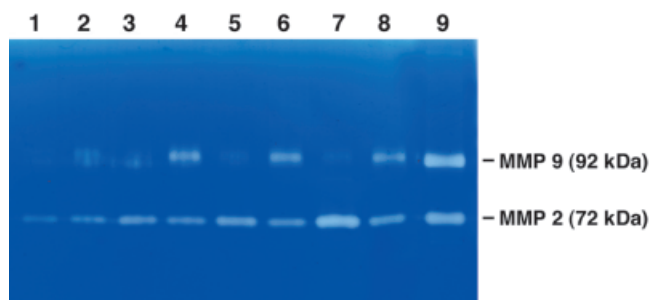


FIGURE 4 – Gelatin zymography of serum free conditioned medium from SQ-20B tumor cells. After treatment with rhTNF- α (10 ng/ml), media were collected and concentrated as described in Material and Methods. MMP-9 levels are increased over time after treatment with TNF- α . Lane 1: Control at 8 hr. Lane 2: With TNF- α at 8 hr. Lane 3: Control at 24 hr. Lane 4: With TNF- α at 24 hr. Lane 5: Control at 48 hr. Lane 6: With TNF- α at 48 hr. Lane 7: Control at 72 hr. Lane 8: With TNF- α at 72 hr. Lane 9: MMP marker.

These findings demonstrate that TNF- α enhances PA activity released by SQ-20B tumor cells.

Inhibitors of MMPs and serine proteinases block angiotatin generation by SQ-20B tumor cells

To elucidate the potential roles of MMP-9 and PA in TNF- α mediated conversion of plasminogen to angiotatin by SQ-20B tumor cells, we analyzed serum free CM from SQ-20B cells incubated with plasminogen and rhTNF- α in the presence of enzyme inhibitors. Conversion of plasminogen to angiotatin was partially inhibited by MMP inhibitor II, a specific inhibitor of MMP-1, MMP-3, MMP-7 and MMP-9, whereas no inhibition was observed in the presence of TAPI-0, a hydroxamic acid-based inhibitor of zinc-dependent matrix metalloproteinases.³⁵ We found that angiotatin production was completely inhibited in the presence of the serine protease inhibitor Pefablock (Fig. 6). These studies support roles for both MMP-9 and PA in the generation of angiotatin. The complete inhibition observed in the presence of Pefablock, however, suggests that PA may be the predominant enzyme involved in the conversion of plasminogen to angiotatin by SQ-20B tumor cells.

DISCUSSION

The present study demonstrates that combined treatment with the adenoviral vector Ad.Egr-TNF + IR is associated with an increase in plasma angiotatin levels. Angiotatin levels are elevated during and after cessation of treatment. The elevations in plasma angiotatin level coincide with significant tumor regression after combined treatment with Ad.Egr-TNF + IR compared to all other treatments. Angiotatin-generating enzymes produced by SQ-20B tumor cells are capable of cleaving plasminogen to an-

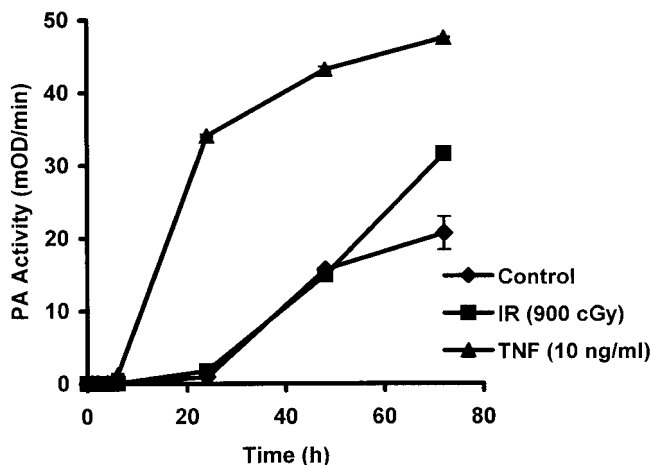


FIGURE 5 – Plasminogen activation by SQ-20B serum-free conditioned medium after treatment with rhTNF- α . Cell cultures were divided into three experimental groups as follows: control, 10 ng/ml rhTNF- α and 900 cGy. Medium was collected for each condition at 0, 1, 2, 4, 6, 24, 48 and 72 hr and stored at -80°C . PA activity (mOD/min) was significantly enhanced after TNF- α exposure when compared to control and IR treated cells beginning at 24 hr and continuing at 48 and 72 hr ($p < 0.001$, ANOVA).

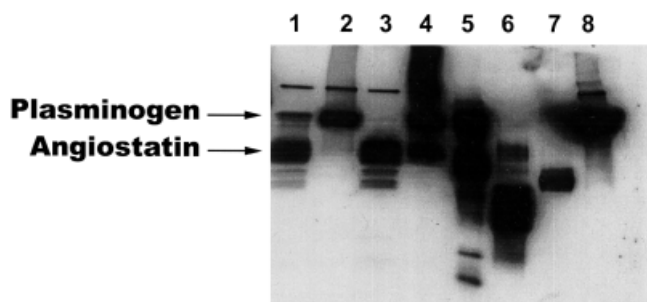


FIGURE 6 – Inhibition of plasminogen-angioctatin converting activity in serum-free conditioned media of SQ-20B tumor cells. Serum-free conditioned media treated with rhTNF- α (10 ng/ml) were exposed to various proteinase inhibitors 1 hr before the addition of recombinant human plasminogen. After 48 hr, samples were analyzed for the evidence of inhibition of angioctatin generation. Lane 1: Control. Lane 2: With Pefabloc, 4 mM. Lane 3: With TAPI-O, 1 mM. Lane 4: With MMP inhibitor II, 1 mM. Lane 5: Human angioctatin from plasma. Lane 6: Recombinant human angioctatin K1-3. Lane 7: Recombinant murine angioctatin. Lane 8: Recombinant human plasminogen.

giostatin. The elevation in plasma angioctatin is consistent with intratumoral TNF- α induction as described previously,^{15,26} Due to the inducibility of the Egr-1 promoter,³⁶ high intratumoral levels of TNF- α protein are maintained after the last IR fraction has been administered.¹⁵ These findings collectively support a model in which elevated intratumoral TNF- α levels are associated with elevated plasma angioctatin levels and that the elevated angioctatin may be a component of the antitumor effects of combined treatment with Ad.Egr-TNF and IR.

We examined the putative source for the enzymatic activity capable of converting plasminogen to angioctatin and found that

SQ-20B tumor cells generate angioctatin when exposed to TNF- α . Although these findings suggest that direct effects of TNF- α on tumor cells can contribute to the generation of angioctatin, other mechanisms may exist *in vivo*. For example, xenografts contain infiltrating lymphocytes,³⁷ and it has been demonstrated that macrophages express angioctatin generating enzymatic activity.²⁰ Thus, TNF- α may induce angioctatin generation through effects on tumor and other cell types. Also, in contrast to the *in vitro* setting and due to the constitutive and inducible nature of the Egr-1 promoter, SQ-20B and other cells in the xenograft may be exposed to transient and higher concentrations of TNF- α that further induce the activity of angioctatin generating enzymes.

Our results also demonstrate that SQ-20B tumor cells constitutively release MMP-2, but not MMP-9. Our findings are in agreement with those of other investigators who have reported constitutive MMP-2 release by tumor cell lines.^{24,38} Treatment with TNF- α did not alter MMP-2 expression by SQ-20B cells. Notably, when SQ-20B cells were treated with TNF- α , MMP-9 expression was enhanced beginning at 24 hr and remained elevated at 48 and 72 hr. These findings are in agreement with previous studies demonstrating that TNF- α stimulates MMP-9 gene expression in a dose- and time-dependent manner.³⁹ Our studies suggest a pathway by which treatment with TNF- α increases the generation of MMP-9 and thereby enhances angioctatin generation. We also examined the effects of TNF- α on PA activity and found that exposure to TNF- α is associated with a significant increase in PA activity in SQ-20B conditioned medium. The data from our enzyme inhibition studies verify that both MMP-9 and PA are involved in the conversion of plasminogen to angioctatin. Our data also suggest that PA activation may be the significant mechanism by which TNF- α treated SQ-20B tumor cells convert plasminogen to angioctatin. This observation may, however, not be universal, as the effects of TNF- α on angioctatin generating enzymes may be different *in vivo* than that observed *in vitro* and, additionally, may be tumor cell type specific. It was recently demonstrated by Lay et al.,⁴⁰ that when phosphoglycerate kinase, a glycolytic enzyme, was administered to tumor-bearing mice, an increase in plasma angioctatin levels was observed. These findings suggest additional enzyme systems may participate in angioctatin generation. Future experiments will be performed to determine if TNF- α treatment enhances phosphoglycerate kinase activity.

The data generated by the current studies provide an alternative hypothesis to vascular thrombosis as a mechanism for the antiangiogenic effects of TNF- α on the tumor microvasculature. The generation of angioctatin from plasminogen by tumor cells expressing MMP-9 and PA may provide the basis for the cytotoxic effects of TNF- α on proliferating endothelial cells. In addition, the present findings suggest that the efficacy of certain anticancer treatments may be due to the generation of antiangiogenic proteins that interact with conventional cytotoxins. The production of antiangiogenic agents by local radiotherapy also supports a potential strategy to suppress the formation of micrometastases without the use of additional adjuvant cytotoxic agents.

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