Enhancement of tumor thermal therapy using gold nanoparticle–assisted tumor necrosis factor-α delivery

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Abstract

Tumor necrosis factor-α (TNF-α) is a potent cytokine with anticancer efficacy that can significantly enhance hyperthermic injury. However, TNF-α is systemically toxic, thereby creating a need for its selective tumor delivery. We used a newly developed nanoparticle delivery system consisting of 33-nm polyethylene glycol–coated colloidal gold nanoparticles (PT-cAu-TNF-α) with incorporated TNF-α payload (several hundred TNF-α molecules per nanoparticle) to maximize tumor damage and minimize systemic exposure to TNF-α. SCG mammary carcinomas grown in A/J mice were treated with 125 or 250 μg/kg PT-cAu-TNF-α alone or followed by local heating at 42.5°C using a water bath for 60 minutes, 4 hours after nanoparticle injection. Increases in tumor growth delay were observed for both PT-cAu-TNF-α alone and heat alone, although the most dramatic effect was found in the combination treatment. Tumor blood flow was significantly suppressed 4 hours after an i.v. injection of free TNF-α or PT-cAu-TNF-α. Tumor perfusion, imaged by contrast enhanced ultrasonography, on days 1 and 5 after treatment revealed perfusion defects after the injection of PT-cAu-TNF-α alone and, in many regions, complete flow inhibition in tumors treated with combination treatment. The combination treatment of SCG tumors in vivo reduced the in vivo/in vitro tumor cell survival to 0.05% immediately following heating and to 0.005% at 18 hours after heating, suggesting vascular damage–mediated tumor cell killing. Thermally induced tumor growth delay was enhanced by pretreatment with TNF-α–coated gold nanoparticles when given i.v. at the proper dosage and timing. [Mol Cancer Ther 2006;5(4):1014–20]

Introduction

Thermal therapy has continued to gain the attention of clinicians and researchers for cancer treatment because of reduced morbidity and mortality, minimally or noninvasive therapy, and advances in devices and techniques (1–4). However, it has been difficult to achieve complete tumor destruction particularly near large blood vessels because of heat dissipation through blood perfusion, hindering the thermal dose delivered to tumor (5, 6). The possible solutions for augmenting thermal injury without increasing the energy deposition are to reduce tumor blood flow before heating and to increase the thermal sensitivity of the tumor and the tumor endothelium. There is a large body of literature showing the use of various antivascular agents [tumor necrosis factor-α (TNF-α), interleukin-α, arsenic trioxide, prostaglandin E1, etc.] as therapeutic adjuvants to low- and moderate-temperature hyperthermia (7–10). These adjuvants predominantly target the vasculature and accentuate the thermal injury by various mechanisms like altering the blood flow or inhibiting the heat shock protein response, to name a few (8, 11).

Of these therapeutics, TNF-α has been of particular interest for many years because it can affect both the cell and vasculature of the tumor with high potency. Stand alone treatment with TNF-α injected i.t. has shown significant reduction of tumor volume (12, 13). We have shown that in combination with hyperthermia or cryotherapy, TNF-α can induce significant tumor response (10, 14). Unfortunately, systemic administration of TNF-α has dose-limiting toxicity (15, 16). Therefore, there is a need for selective tumor delivery of TNF-α to minimize systemic toxicity. Delivery of TNF-α using a liposomal delivery system has shown some promise in animal cancer models but requires very high doses of the drug (17, 18). Gene therapy with TNF-α has also been explored, but the limitations are lack of selective transduction of tumor cells to minimize toxicity and efficient gene transfer to every cell to prevent proliferation of nontransduced cells (19–22). Recently, nanoparticle delivery systems (usually 20–100 nm) capable of escaping phagocytic clearance by the reticuloendothelial system have been investigated (23–25). By design, such delivery systems preferentially extravasate the tumor vasculature to accumulate TNF-α within the tumor interstitium while concomitantly reducing the accumulation of TNF-α in healthy organs that maintain greater control of vascular permeability than...
Materials and Methods

**In vitro Clonogenic Assay**

The SCK murine mammary carcinoma model originally obtained from a spontaneous tumor in an A/J mouse was used in this study. About 200 SCK cells were plated in 25-cm² tissue culture flasks and were incubated overnight. The cells were then incubated in a medium containing various concentrations of PT-cAu, free TNF-α, or PT-cAu-TNF-α for 4 hours at 37°C. The control and treated flasks were further incubated for 1 hour at 37°C, or those to be heated were immersed in a water bath preheated to 42.5°C for 1 hour. Immediately afterwards, the cells in all flasks were gently rinsed with 4 mL of drug-free medium and cultured in fresh medium in a 5%/95% air incubator at 37°C for 7 to 10 days to allow colony formation. The culture medium was drained, the flasks were rinsed quickly with saline, and the colonies were fixed with 10 mL of a 10:1 methanol/acetic acid solution for 10 to 15 minutes and stained with 1% crystal violet. Colonies containing >50 cells were counted, and survival curves were constructed.

**Tumor**

After resuspension in the appropriate volume of medium without serum, about 2 × 10⁵ SCK cells in 0.05 mL serum-free medium were injected s.c. into the shaved right hind limbs of 20 to 23 g male A/J mice (27). When tumors had grown to 7 to 8 mm in diameter, which took 7 to 9 days, animals were randomly assigned to each treatment group. All animal procedures and care were done using protocols approved by the University of Minnesota Institutional Animal Care and Use Committee in accordance with federally approved guidelines.

**Animals**

After randomization, nine groups were assigned: control, PT-cAu (a control gold particle with polyethylene glycol-thiol but no TNF-α; 125 μg/kg), PT-cAu-TNF-α (125 μg/kg), PT-cAu-TNF-α (250 μg/kg), free TNF-α (250 μg/kg), hyperthermia (42.5°C, 60 min), combination of PT-cAu (125 μg/kg) and hyperthermia (42.5°C, 60 minutes), and combination of PT-cAu-TNF-α and hyperthermia [(a) 125 μg/kg, 4 hours, 42.5°C, 60 minutes; (b) 250 μg/kg, 4 hours, 42.5°C, 60 minutes].

**Drug Treatment (Gold Nanoparticles/PT-cAu-TNF-α)**

The drug PT-cAu-TNF-α was kindly supplied by CytoImmune, Inc. (Rockville, MD). A fresh preparation of drug was made before each experiment by dissolving the necessary amount in PBS to get a concentration corresponding to 125 and 250 μg TNF-α/kg in 0.1 mL. Injections were made i.v. through the tail vein.

**Anesthesia**

The mice were anesthetized with an i.p. injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine during all hyperthermia treatments, ultrasound imaging, and isotope injections. When necessary, a second dose of 20 mg/kg ketamine and 2 mg/kg xylazine was given.

**Hyperthermia**

Each anesthetized mouse was placed on a specifically designed Plexiglas jig, and the tumor-bearing leg was vertically extended and loosely anchored to a support on the jig with masking tape. The jigs bearing the mice were then placed on a Plexiglas shelf positioned over a water bath, and the anchored legs were immersed into water preheated to 42.5°C by a precisely controlled heating unit with an accuracy of ±0.1°C. The temperature in the tumors during heating is typically 0.3°C to 0.5°C below the water temperature, depending on exact point in tumor (8). However, it is possible that in PT-cAu-TNF-α–treated tumors, the heating was slightly higher because of blood flow shutdown.

**In vivo/In vitro Cell Survival Assay**

After treatment and at the designated time points, the mice were sacrificed by cervical dislocation, the tumors were dissected and minced, and the fragments were stirred for 30 minutes in 30 mL of RPMI 1640 containing 0.25% trypsin and 10 μg/mL DNase I (Sigma, St. Louis, MO) for tumor cell dissociation. The dissociated cells were filtered, washed, centrifuged, suspended, and plated at known concentrations in 25-cm² plastic tissue culture flasks (28). The cells were cultured for 8 days in an incubator at 37°C to allow colony formation, and the colonies were then stained as described above in in vitro clonogenic assay section.

**Growth Delay**

The tumors were measured using metric-scale calipers (Scientecware, Pequannock, NJ), and the tumor volume was...
calculated with the formula \(a^2b/2\), where \(a\) and \(b\) are the shortest and the longest diameter of the tumor, respectively. After treatment, the tumor size was measured until the mean volume for each group reached at least 5-fold the mean volume on the day of treatment.

**Contrast Enhanced Ultrasonography**

The contrast enhanced ultrasonography was done on days 1 and 5 after treatment using a modified Technos MPX system from ESAOTE S.p.A. (Genoa, Italy). A linear array probe (LA522, one-cycle transmit pulse centered at 5.5 MHz) was used. When acquiring the *in vivo* power Doppler images, the transmit pulse was set to one cycle, and the transducer was operated at 8 MHz with electrical focus at 25 mm. Each data set was 1 second of frame data (nine frames per second). The contrast agent, BR14 (Bracco Research S.A., Geneva, Switzerland), which is a new experimental agent consisting of high molecular weight perfluorobutane gas bubbles (2 \(\mu\)m diameter) encapsulated by a flexible phospholipids shell and suspended in saline, was given via tail vein at a concentration of 0.0025 mL/kg of body weight.

**\(^{86}\text{Rb} Uptake Assay**

The blood perfusion in tumors was measured with the \(^{86}\text{Rb}\) uptake method. Tumor-bearing mice were anesthetized, and about 15 minutes later, 5 \(\mu\)Ci of \(^{86}\text{RbCl}\) in 0.1 mL PBS was injected through the lateral tail vein, and the mice were sacrificed 60 seconds later by cervical dislocation. The tumor from each mouse was removed and weighed, and the \(^{86}\text{Rb}\) activity in a reference aliquot of the injected dose and the tissues was counted in a well-type gamma counter (1282 Compugamma, Pharmacia LKB Wallac, Turku, Finland). By comparing the activity in the tumor to that in the total amount injected, the percent uptake per gram of tumor tissue was calculated. Animals in which the tail activity exceeded 5% of the total injected dose were excluded from the data analysis.

**Statistics**

Data sets were analyzed using a commercially available software package (InStat 2.03, GraphPad Software, San Diego, CA). A mean of measured values \(\pm SE\) was calculated for all groups. The Student’s *t* test was used to determine the validity of the differences between control and treatment data sets. *P* \(\leq 0.05\) was considered significant.

**Results**

**Effect of PT-cAu-TNF-\(\alpha\) on Thermosensitivity of SCK Cells *in vitro***

The effect of 10 to 1,000 ng/mL PT-cAu, free TNF-\(\alpha\), and PT-cAu-TNF-\(\alpha\) on the thermosensitivity of tumor cells in culture is shown in Fig. 1. The clonogenicity of cells treated with drug or heat alone or combined is normalized to the clonogenicity of nontreated control cells. An exposure to PT-cAu, free TNF-\(\alpha\), or PT-cAu-TNF-\(\alpha\) at a concentration of 10 to 1,000 ng/mL did not cause significant change in cell clonogenicity. Heating the cells to 42.5°C for 1 hour without TNF-\(\alpha\) decreased clonogenicity to 64% of control. Pretreatment with PT-cAu or free TNF-\(\alpha\) for 4 hours before heating did not significantly change clonogenicity of cells compared with the heating alone. However, pretreatment with TNF-\(\alpha\) delivered using gold nanoparticles (PT-cAu-TNF-\(\alpha\)) followed with heating reduced the cell survival to 21% to 26% of the control value. There was no significant dose response of TNF-\(\alpha\) or PT-cAu or PT-cAu-TNF-\(\alpha\) alone or in combination with heating apparent in our results.

**Effect of PT-cAu-TNF-\(\alpha\) and Hyperthermia on Tumor Cell Survival (*in vivo/in vitro* Cell Survival Assay)**

The survival of SCK cells assessed using *in vivo/in vitro* cell survival assay following treatment with PT-cAu-TNF-\(\alpha\) and hyperthermia alone or combined is shown in Fig. 2. In this study, the “0” hour time point represents the tumors dissected 5 hours after PT-cAu-TNF-\(\alpha\) treatment or immediately after heating, which was applied 4 hours after PT-cAu-TNF-\(\alpha\) injection. Similarly, the “18” hour time point represents the tumors dissected 23 hours after PT-cAu-TNF-\(\alpha\) treatment or 18 hours after heating, which was applied 4 hours after PT-cAu-TNF-\(\alpha\) injection. For control tumors, the plating efficiency of SCK cells was 22%. Five or 23 hours after an injection of PT-cAu-TNF-\(\alpha\), survival did not change significantly compared with control. The exposure of tumors to heating at 42.5°C for 60 minutes reduced the tumor cell survival to 0.2% to 0.4%, which was significantly less than that in control tumors or in tumors treated with PT-cAu-TNF-\(\alpha\) alone. The combined treatment with PT-cAu-TNF-\(\alpha\) and hyperthermia markedly reduced the cell survival (0.05%) at the 0 hour time point compared with hyperthermia alone (0.2–0.4%). After 18 hours of combined treatment, the cell survival was further reduced by an additional order of magnitude (0.005%).
Effect of PT-cAu-TNF-α on Heat-Induced Tumor Growth Delay

The SCK tumor growth delay caused by heating at 42.5 °C for 60 minutes alone or combined with 125 or 250 μg/kg PT-cAu-TNF-α treatment, 4 hours before heating is shown in Figs. 3 and 4. Control tumors grew about 3-fold the starting volume in 5 days. Tumors treated with heat alone grew to 3-fold of the starting volume by 7 days, a growth delay of 2 days. In animals treated with 125 μg/kg of PT-cAu-TNF-α + heat, tumor volume increased to 3-fold in 8 days, a growth delay of 3 days. The tumor growth was delayed 6 days in animals treated with 250 μg/kg of PT-cAu-TNF-α combined with heat.

In addition, to the heat alone and combination groups shown in Fig. 3, 125 μg/kg PT-cAu alone, 125 μg/kg PT-cAu-TNF-α alone, 250 μg/kg PT-cAu-TNF-α alone, or combined with heating were also studied. The delay in days for tumors to grow 3-fold their pretreatment volume for each treatment group from control tumor growth is shown in Fig. 4. The treatment with PT-cAu vector (gold particles without TNF-α) or PT-cAu-TNF-α alone did not delay the tumor growth significantly from control. The tumor growth was delayed by 2 to 3 days when the animals were treated with either hyperthermia or a combination of PT-cAu vector and hyperthermia or a combination of 125 μg/kg PT-cAu-TNF-α and hyperthermia. There was no statistically significant difference between these groups. The growth delay with the combined treatment of 250 μg/kg PT-cAu-TNF-α and heat was supra-additive.

Effect of PT-cAu-TNF-α on Tumor Perfusion Defects

Tumor perfusion defects visualized using contrast enhanced Doppler ultrasound, 1 and 5 days after treatment with PT-cAu-TNF-α alone or heat alone or their combination, are shown in Fig. 5. The vascularity, or blood flow index, of tumors 1 day after either treatment was reduced compared with control. Compared with tumors treated with heat alone, the perfusion was markedly defective in tumors treated with PT-cAu-TNF-α alone and with combination of PT-cAu-TNF-α and hyperthermia on day 1. The tumor perfusion recovered 5 days after treatment with PT-cAu-TNF-α alone or heat alone. However, the perfusion defect persisted in tumors treated with combination of PT-cAu-TNF-α and hyperthermia.

Effect of PT-cAu-TNF-α on Tumor Blood Perfusion

The changes in blood perfusion in SCK tumors at various time points following administration of free TNF-α, or 125 or 250 μg/kg PT-cAu-TNF-α measured with the 86Rb uptake method, is shown in Fig. 6. Four hours after an i.v. injection of the free or gold TNF-α, the 86Rb uptake decreased to 20% to 30%. The 86Rb uptake decreased to around 22%, 32%, and 22% of control level, respectively, at 2, 3, and 4 hours after an i.v. injection of 250 μg/kg PT-cAu-TNF-α, and there was partial recovery 4 to 16 hours after the PT-cAu-TNF-α injection. However, the tumor blood flow remained lower than untreated control tumors at 24 hours.

Discussion

The present study shows that pretreatment with TNF-α delivered using gold nanoparticles significantly affects tumor physiology and enhances hyperthermic injury. The tumor growth delay plots (Figs. 3 and 4) clearly indicate that the combined treatment of PT-cAu-TNF-α (250 μg/kg) and hyperthermia was more potent than PT-cAu-TNF-α or hyperthermia alone in suppressing tumor growth.

The accentuation of thermal injury with PT-cAu-TNF-α is also reflected in the in vivo/in vitro tumor cell survival assay (Fig. 2). In vivo/in vitro tumor cell survival after
combination treatment progressively decreased by two to three orders of magnitude compared with PT-cAu-TNF-α or hyperthermia alone over 18 hours after treatment. After 18 hours of treatment, the tumor cell survival in PT-cAu-TNF-α or hyperthermia alone group did not change significantly. The fact that the cell survival in tumors treated with the combination treatment progressively dropped suggested that the inflammatory action of PT-cAu-TNF-α in combination with heat-induced injury causes significant vascular damage, which deprives tumor cells from the circulation and causes increasing amounts of cell death after treatment.

Unresolved thermal injury was also observed a few days after treatment in contrast enhanced ultrasound images (Fig. 5). Tumor perfusion defects increased 5 days after combination treatment, whereas the blood flow reappeared in tumors treated with PT-cAu-TNF-α or hyperthermia alone. It should be pointed out that with the contrast enhanced ultrasonography technique, only vessels with diameter ≥200 μm are likely to be clearly visible in tumors.

This conclusion is based on our ability to measure 200-μm cellulose channels with a LA 522 high-frequency probe \textit{in vitro} (data not shown). The contrast-enhanced ultrasonography data was also dependent on the plane through which the tumor was bisected by the ultrasound wave. Thus, contrast enhanced ultrasonography was considered a qualitative measure of tumor vascular physiology, and for quantitative assessment of perfusion defects, we used the $^{86}$Rb uptake method.

The mechanism of vascular injury caused by TNF-α has been suggested by several possible scenarios. TNF-α has long been known to induce hemorrhagic necrosis (29, 30). The inflammatory pathways cause shutdown of tumor microvasculature and macrovasculature, ultimately leading to stasis (31, 32) and thus depriving the tumor microenvironment of nutrients and oxygen, eventually leading to cell death. With the reduction of blood perfusion, the cells are subjected to increased acidity as a result of higher anaerobic glycolysis and build-up of acidic byproducts (33). In the present study, drastic vascular shutdown was observed in solid tumors 4 hours after i.v. injection of free or gold-bound TNF-α (Fig. 6). Apparently, the microvascular shutdown and stasis caused by TNF-α made the tumor tissue more susceptible to thermal injury, and large numbers of tumor cells were cut off from the circulation causing massive cell death and tumor regression, thereby explaining the results of tumor growth delay and \textit{in vivo/in vitro} cell survival assay.

The enhancement of hyperthermic damage by PT-cAu-TNF-α was also observed \textit{in vitro} (Fig. 1). Tumor cell survival after hyperthermia alone (64% survival) and after combination treatment with PT-cAu-TNF-α (21–26% survival) was significantly different, suggesting that TNF-α delivered using gold nanoparticles enhances hyperthermic injury to SCK cells \textit{in vitro}. As previously reported (24), gold nanoparticles seem to increase the TNF-α loading in the tumor cells by 10-fold versus loading of the same TNF-α dose in native form. Interestingly, the treatment with TNF-α alone (free or bound) did not reduce tumor cell survival \textit{in vitro} indicating that TNF-α alone could not cause significant direct cell injury at the doses used. Furthermore, there was no dose response of
TNF-α (free or bound) in vitro, whereas in vivo, tumor response was dose dependent. Our data suggest that with higher PT-cAu-TNF-α dose, uptake of TNF-α by the tumor cells and vasculature in vivo increases, which likely increases the thermal injury. The in vivo dose response of TNF-α, which was not observed in vitro, is hypothesized to be attributed to (a) the inflammatory reaction caused by TNF-α in vivo (34), (b) cell-matrix interactions that are absent in vitro (35), and (c) increased TNF-α uptake due to hyperthermia-induced nanoparticle extravasation (36, 37). We are currently studying these variables in our models.

PT-cAu-TNF-α is a multivalent drug manufactured by covalently linking molecules of human recombinant TNF-α and thiol-derivatized polyethylene glycol onto the surface of the 33-nm colloidal gold particles (24). Each gold particle is loaded with several hundred TNF-α molecules. TNF-α being coated on the surface of the particle not only acts as a therapeutic but also as a ligand to facilitate receptor targeting. The polyethylene glycol moiety serves to hydrate and shield the particle from detection and clearance by the reticuloendothelial system, thus prolonging its circulation time in the body and facilitating the localization of TNF-α in murine tumors with no other toxicities observed (24). The gold particles are speculated to be taken up in tumor interstitium passively through leaky vasculature due to their overall size (20–100 nm) and actively by receptor-mediated endocytosis on tumor cells with TNF-α receptors (24). We believe that the gold nanoparticles increase the amount of TNF-α that reaches the cancer cells in the hind limb tumor model relative to other tissues. This is an important advantage because native TNF-α can cause systemic toxicity (34). We have conducted some experiments with free TNF-α (250 μg/kg dose) in combination with or without hyperthermia (42.5°C, 60 minutes) and have found that for the group treated with free TNF-α alone, the survival was only 60%, a sign of systemic toxicity of TNF-α. For the group treated with free TNF-α and hyperthermia, there was redness, inflammation, and massive swelling on the tumor-bearing leg. The swelling became worse over days causing the animal to limp. Due to these conditions, animals had to be sacrificed. In the present study, a 100% animal survival was observed following the i.v. injection of gold nanoparticles, suggesting that it may be safe to use the particles. However, complete toxicity studies need to be conducted to justify the safe use of the particles. The biodistribution and effect on tumor vascular physiology of TNF-α with and without the gold nanoparticles in vivo is currently being measured by our group because we can not rule out the possibility that TNF-α is released by the particles in vivo before reaching the tumor cells. In addition, studies to determine the lowest active dose of PT-cAu-TNF-α versus native TNF-α are warranted to further the true clinical potential of these particles.

**Conclusion**

PT-cAu-TNF-α in combination with hyperthermia significantly delayed tumor growth, reduced tumor cell survival, and reduced tumor blood perfusion at doses of TNF-α previously found to be toxic. Optimization of PT-cAu-TNF-α tumor delivery and use as part of multimodality therapy warrants continued study.

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**References**


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