Synergistic Antitumor Activity of Cisplatin, Paclitaxel, and Gemcitabine with Tumor Vasculature-Targeted Tumor Necrosis Factor-α

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Abstract

Purpose: Subnanogram doses of NGR-tumor necrosis factor (TNF), a TNF-α derivative able to target tumor neovessels, can enhance the antitumor activity of doxorubicin and melphalan in murine models. We have examined the antitumor activity of NGR-TNF in combination with various chemotherapeutic drugs acting via different mechanisms, including, besides doxorubicin and melphalan, cisplatin, paclitaxel, and gemcitabine.

Experimental Design: Chemotherapeutic drugs were tested alone and in combination with NGR-TNF (0.1 ng) in murine lymphoma, fibrosarcoma, and mammary adenocarcinoma models. Different administration schedules have been tested and the effects on tumor growth, animal weight, tumor perfusion, and cell cytotoxicity have been investigated.

Results: Pretreatment with NGR-TNF enhanced the response to all these drugs although to a different extent. The increased efficacy was not accompanied by increased toxicity at least as judged from the loss of animal weight. The synergistic effect was transient, maximal synergism being observed with a 2-hour delay between NGR-TNF and drug administrations in all models and with all drugs tested. NGR-TNF did not increase the *in vitro* cytotoxicity of chemotherapeutic drugs against tumor cells, suggesting that the *in vivo* synergism depends on NGR-TNF effects on host cells rather than on tumor cells.

Conclusions: Targeted delivery of low doses of NGR-TNF to the tumor vasculature can increase the efficacy of various drugs acting via different mechanisms. Optimal administration schedule requires 2 hours of pretreatment with NGR-TNF independently from the mechanism of drug cytotoxicity. This work could provide important information for designing clinical studies with NGR-TNF in combination with chemotherapeutic drugs.

Tumor necrosis factor-α (TNF) is currently used in combination with melphalan or, less frequently, with doxorubicin in isolated limb or hepatic perfusion of patients with tumors confined to the extremities or to the liver (1–5). Unfortunately, the clinical use of TNF as an anticancer drug is limited to locoregional treatments because of systemic toxicity (6, 7). We have shown previously that systemic administration of picogram doses of NGR-TNF, a TNF derivative able to target the tumor vasculature (8), can enhance the antitumor activity of melphalan and doxorubicin in mouse models, with no evidence of increased toxicity (9). NGR-TNF was prepared by coupling TNF with the tumor-homing peptide Cys-Asn-Gly-Arg-Cys (CNGRC), a ligand of a CD13 (aminopeptidase N) isoform expressed by endothelial cells in tumor vessels (8, 10, 11). Studies on the mechanism of action showed that targeted delivery of very low doses of NGR-TNF to the tumor vasculature is a valuable strategy for overcoming major TNF counterregulatory mechanisms and for increasing the penetration of doxorubicin in murine B16 melanomas and RMA lymphomas (9).

In view of the potential clinical application of NGR-TNF in combination with different chemotherapeutic drugs, this work was undertaken to examine the antitumor activity of NGR-TNF in combination with various chemotherapeutic drugs acting via different mechanisms, including, besides doxorubicin and melphalan, cisplatin, paclitaxel, and gemcitabine. Different administration schedules have been tested in RMA lymphoma-bearing mice, the effect of NGR-TNF on tumor vessel and microenvironment being characterized previously in this model (8, 9, 12) as well as in other murine fibrosarcoma and mammary adenocarcinoma models.

We show that NGR-TNF can exert synergistic effects not only with melphalan and doxorubicin but also with cisplatin, gemcitabine, and paclitaxel, provided that the timing of administration is carefully scheduled. Moreover, we provide data to suggest that the response strongly depends on the *in vivo*

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tumor sensitivity to the chemotherapeutic drug and much less to the *in vitro* sensitivity, pointing to a role of tumor microenvironment elements and tumor-host interactions in the overall response.

Materials and Methods

Tumor cell lines, drugs, and reagents. Mouse RMA and RMA-T lymphoma, TS/A mammary adenocarcinoma, and WEHI-164 fibrosarcoma cells were cultured as described previously (8, 13, 14). Melphalan (Alkeran) was obtained from Glaxo-Wellcome (London, United Kingdom); doxorubicin (Adriblastina) was purchased from Pharmacia-Upjohn (Milan, Italy); cisplatin (Cisplatino Teva) was from Teva Pharma Italia S.r.l. (Milan, Italy); paclitaxel (Taxol) was from Bristol-Myers Squibb S.r.l. (Sermoneta, Italy); and gemcitabine (Gemzar) was from Ely Lilly Italia S.p.A. (Sesto Fiorentino, Italy). Human and murine TNF and NGR-TNF (consisting of TNF fused with the COOH terminus of CNGRCG) were prepared by recombinant DNA technology and purified from *Escherichia coli* cell extracts as described (8). Murine NGR-TNF was used in *in vivo* and *in vitro* studies involving murine models. Human NGR-TNF was used in *in vitro* cytotoxicity studies with human umbilical vein endothelial cells.

In vitro *cytotoxicity assays.* RMA cells were plated in RPMI 1640 containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (6×10^4, 100 μL) and incubated overnight at 37°C, 5% CO_2. NGR-TNF (1 ng/mL in complete RPMI 1640) was added to each well (50 μL) followed 2 hours later by chemotherapeutic drugs solutions at various concentrations (50 μL) and incubated for 48 hours at 37°C, 5% CO_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (10 μL, 5 mg/mL in PBS) was added to each well and left to incubate for 3 hours. Then, the cells were treated with 10% (w/v) SDS, 50% (v/v) *N*,*N*-dimethylformamide, 0.025 mol/L HCl, and 0.35 mol/L acetic acid solution (100 μL/well, 24 hours at 37°C). The absorbance of each well at 570 and 650 nm (reference) was then measured using a microplate reader.

The same procedure was applied for cytotoxicity assays involving TS/A, WEHI-164, and human umbilical vein endothelial cells.

In vivo *studies.* Studies on animal models were approved by the Ethical Committee of the San Raffaele H. Scientific Institute and done according to the prescribed guidelines. C57BL/6/N female, 8 weeks old, were from Charles River Laboratories Italia S.p.A. (Calco, Italy). BALB/c female, 8 weeks old, were from Harlan Italy (San Pietro al Natisone, Italy).

Mice were challenged with s.c. injection in the left flank of 7×10^4 RMA cells (C57BL/6), 10^5 TS/A cells (BALB/c), or 10^6 WEHI-164 cells (BALB/c); 9 to 10 days later, mice were treated with NGR-TNF solutions (100 μL) in 0.9% NaCl containing 100 μg/mL endotoxin-free human serum albumin (Farma-Biagini, Lucca, Italy) followed by administration of chemotherapeutic drug solution (100 μL) diluted with 0.9% NaCl. All drugs were given i.p. Tumor growth was monitored daily by measuring tumor volumes with calipers as described previously (15). Animals were sacrificed before the tumors reached 1.0 to 1.5 cm in diameter. Tumor sizes are shown as mean ± SE (five animals per group). Statistical analysis was done by two-tailed *t* test. Differences between groups were considered significant when $P < 0.05$.

The lack of palpable tumors after therapy was considered complete tumor rejection.

Tumor perfusion with patent blue. C57BL/6 and BALB/c mice bearing RMA or WEHI-164 tumors (diameter, 1.0-1.4 cm) were treated with or without murine NGR-TNF (0.1 ng i.p.) followed 2 or 5 hours later by patent blue VF (Sigma, St. Louis, MO; 12.5 mg/mL, 0.1 mL i.v.). After 5 minutes, the animals were sacrificed and the tumors were excised. Each tumor was weighed, homogenized, resuspended in cold PBS containing 1% Triton X-100 (1 mL/g tumor), and incubated for 1 hour on ice. The suspension was then centrifuged (14,000 × *g*, 4°C, 15 minutes) and the supernatant was mixed with trichloroacetic acid [10% (v/v), final concentration]. The product was centrifuged again (14,000 × *g*, 4°C, 15 minutes) and the absorbance at 405 nm of the supernatant was measured using a spectrophotometer.

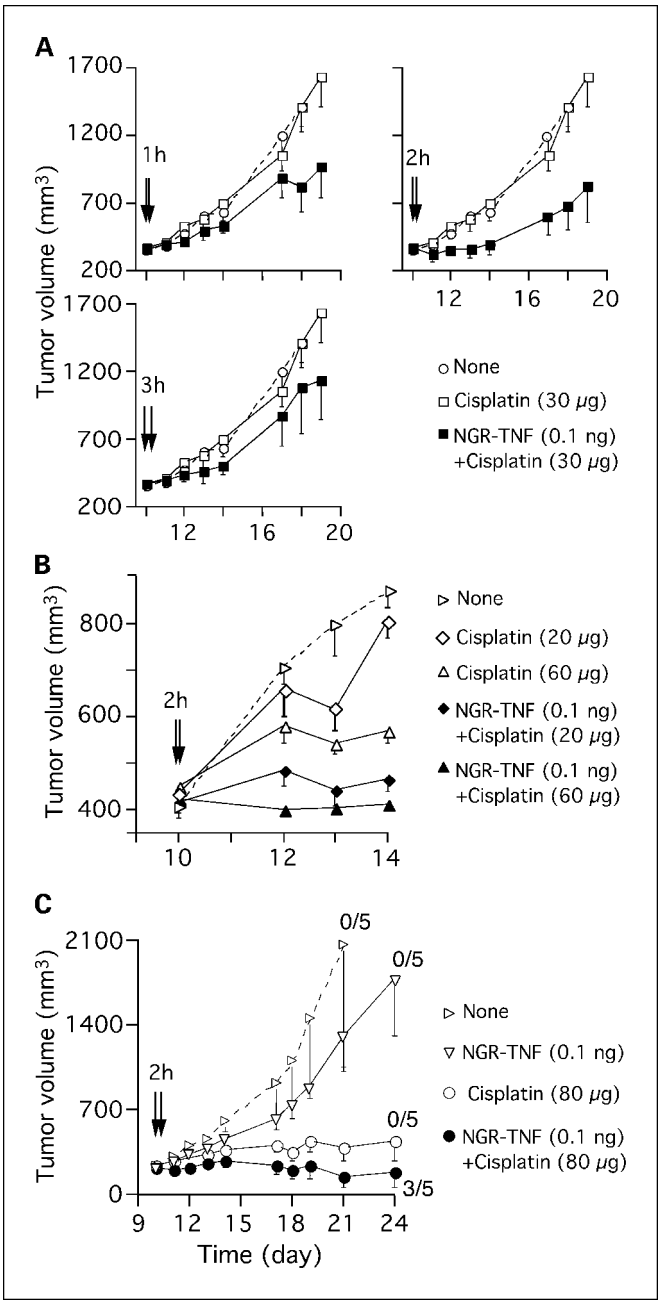

Fig. 1. Effect of NGR-TNF in combination with cisplatin on RMA lymphoma growth *in vivo*. Animals bearing RMA tumors (five mice per group) were treated i.p. with NGR-TNF in combination with cisplatin at day 10 after tumor implantation. Tumor volumes after treatment are shown for three separate experiments (*A-C*). The schedule of treatments (timing and doses) is indicated. NGR-TNF was given 1, 2, or 3 hours before cisplatin (*arrows*). *A,* right, ■ versus □ at day 18 ($P < 0.01$). *B,* ♦ versus◇ at day 14 ($P < 0.01$); ▲ versus △ at day 14 ($P < 0.0001$). *C,* numbers, tumor-free mice at day 30.

Results

Antitumor activity of NGR-TNF in combination with cisplatin in the RMA and RMA-T lymphoma models. In previous work,

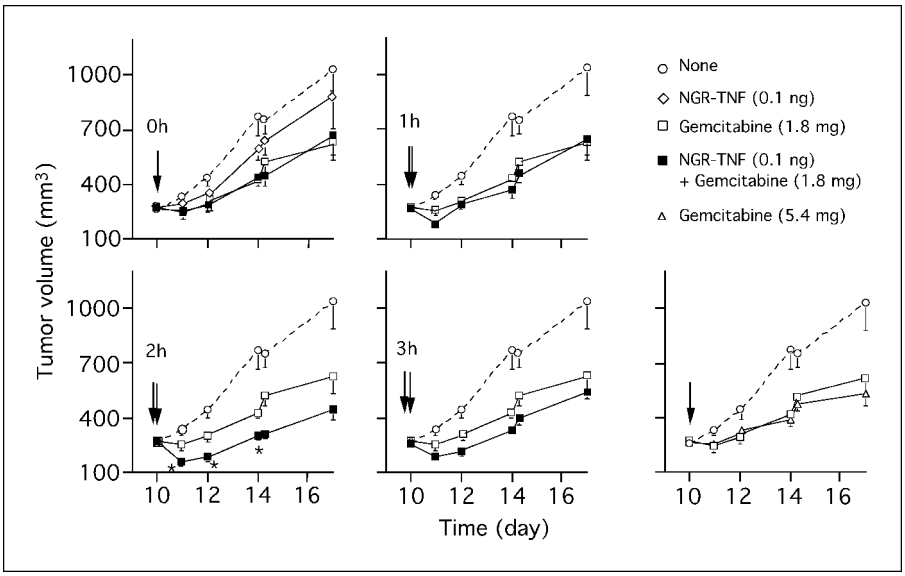

Fig. 2. Effect of different administration schedules of NGR-TNF in combination with gemcitabine on RMA tumors. Tumor-bearing mice (five mice per group) were treated with NGR-TNF in combination with gemcitabine at day 10 after tumor implantation. NGR-TNF and gemcitabine were given with 0, 1, 2, or 3 hours intervals at the indicated doses. ■ versus □. *, $P < 0.05$.

we showed that 0.1 ng NGR-TNF given to RMA tumor-bearing mice, although poorly active alone, is sufficient to increase the antitumor activity of melphalan and doxorubicin, whereas 10^4 to 10^5 greater doses of TNF are necessary to achieve comparable results (9). The same dose of NGR-TNF (0.1 ng) was therefore used to investigate the effect of this cytokine in combination with cisplatin on RMA tumors. In this model, 30 μg cisplatin induced little or no effects when used alone. However, a significant antitumor response was observed when NGR-TNF was given 2 hours before cisplatin, whereas the response was lower at 1- or 3-hour intervals (Fig. 1*A*).

In another experiment, we compared the effect of 20 and 60 μg cisplatin in animals bearing RMA tumors pretreated, 2 hours before, with or without NGR-TNF. Again, we observed stronger responses in animals pretreated with NGR-TNF (Fig. 1*B*). Noteworthy, the effect of NGR-TNF plus 20 μg cisplatin was stronger than that of 60 μg cisplatin alone. Remarkably, the increased antitumor effect observed with each drug combination was not associated with a greater body weight loss compared with cisplatin alone (data not shown).

No complete tumor rejection was observed with cisplatin alone, even when a higher dose (80 μg) was used in the RMA-T model, a more immunogenic tumor, whereas three of five mice showed complete tumor rejection in this model by the combination treatment (Fig. 1*C*).

These results suggest that NGR-TNF can exert synergistic effects with cisplatin, provided that this chemotherapeutic drug is given 2 hours after the cytokine.

Antitumor activity of NGR-TNF in combination with paclitaxel or gemcitabine in the RMA lymphoma model. To assess whether the same phenomenon could occur also with other chemotherapeutic drugs, we tested the effect of NGR-TNF in combination with paclitaxel or gemcitabine, with various administration schedules. Also in this case, we observed significantly higher antitumor effects, compared with each drug alone, when NGR-TNF was given 2 hours before gemcitabine (1.8 mg) and less, or not at all, with 0-, 1-, or 3-hour intervals (Fig. 2).

Similar results were obtained also with paclitaxel (60 and 80 μg; Fig. 3*A* and *B*). These results suggest that the timing of administration is very critical for the antitumor activity of all combination tested. The increase of the antitumor activity of gemcitabine and paclitaxel by NGR-TNF was apparently modest in this model compared with cisplatin. However, when the dose of gemcitabine was increased to 5.4 mg alone, we obtained only a modest increase of activity (Fig. 2, *right*). Moreover, the increase of the dose of paclitaxel from 60 to 180 μg was associated with higher loss of body weight (data not shown) and a lower response (Fig. 2*A*). These results suggest that RMA tumors do not respond to gemcitabine or paclitaxel in a linear manner. Nevertheless, despite the lower response rates with these drugs compared with cisplatin, it is remarkable that NGR-TNF combined with 1.8 mg gemcitabine produced stronger effects than 5.4 mg gemcitabine alone and that NGR-TNF combined with 60 μg paclitaxel produced stronger effects than 180 μg paclitaxel alone without causing loss of body weight.

Antitumor activity of NGR-TNF in combination with melphalan in the RMA lymphoma model. The NGR-TNF (0.1 ng)/melphalan (90 μg) combination has been tested previously in the B16 and RMA-T models, showing good synergism (9). To provide comparable data with cisplatin, paclitaxel, and gemcitabine, we have tested this combination in RMA tumor-bearing mice. When mice were treated with 50 or 150 μg melphalan alone, we observed a dose-dependent response (Fig. 4). Preadministration (2 hours) of NGR-TNF markedly increased the effect of 50 μg melphalan, apparently much more than observed with gemcitabine, paclitaxel, or cisplatin. However, it is noteworthy that the effect of NGR-TNF/melphalan (50 μg) was similar to that of 150 μg melphalan alone in terms of both tumor mass reduction and survival increase. We estimate, therefore, that also in this case a >3-fold potentiation activity occurred as observed with cisplatin and the other drugs. These results suggest that the apparent potentiation of drug efficacy by NGR-TNF is strongly dependent on the *in vivo* assay sensitivity, which is likely drug and model dependent.

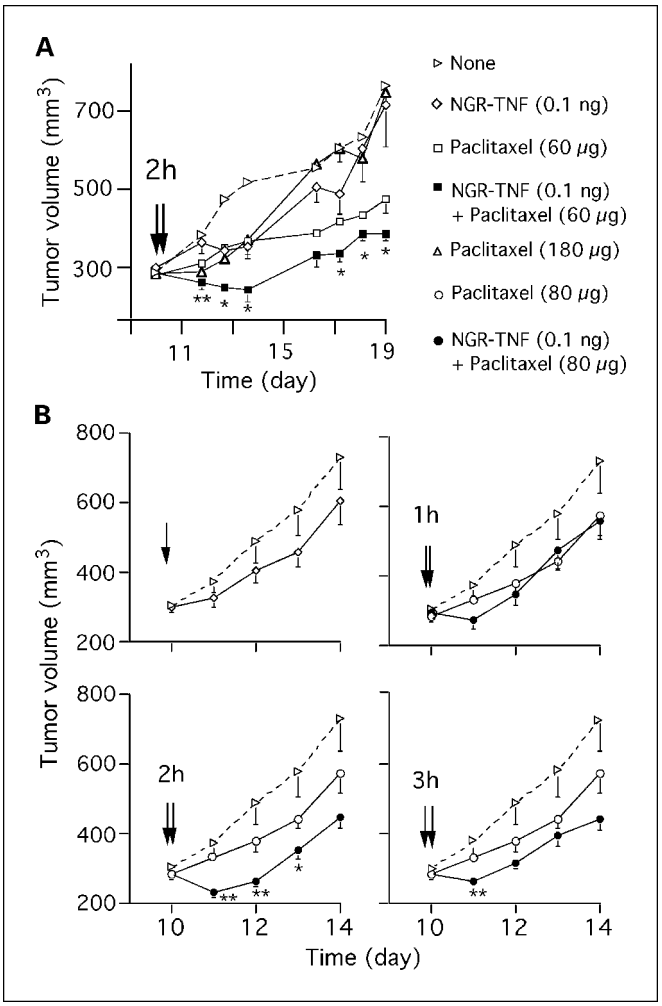

Fig. 3. Effect of different administration schedules of NGR-TNF in combination with paclitaxel on RMA tumor growth. Tumor-bearing mice (five mice per group) were treated with NGR-TNF in combination with paclitaxel at day 10 after tumor implantation. The schedules of treatment (timing and doses) are indicated in each panel for two independent experiments (*A* and *B*). ■ versus □. *, $P < 0.05$; **, $P < 0.01$.

Antitumor activity of NGR-TNF in combination with chemotherapeutic drugs in WEHI-164 fibrosarcoma and TS/A adenocarcinoma models. We then addressed whether the optimal administration schedule was drug and model dependent. To this aim, we tested the effect of NGR-TNF/doxorubicin in the TS/A adenocarcinoma and NGR-TNF/melphalan in the WEHI-164 model with 1-, 2-, and 3-hour delay between drug administrations. Maximal effect was observed when doxorubicin was given 2 hours after NGR-TNF in the TS/A model as observed with cisplatin, gemcitabine, and paclitaxel in the RMA model (data not shown). Similar results were obtained also with melphalan in the WEHI-164 model. In this case, NGR-TNF (0.1 ng) alone could induce a 3- to 4-day delay in tumor growth. Synergistic effects were observed only when melphalan was given 2 hours after NGR-TNF. Apparently, the optimal administration schedule is independent from drug and model, suggesting a common mechanism of action in all models.

NGR-TNF does not increase the toxicity of chemotherapeutic drugs as judged from the loss of animal weight. To provide information on toxicity caused by the combined treatments, we measured the animal weight, before and after treatment with the various drugs, for few days. No evidence of increased toxicity due to pretreatment with NGR-TNF was observed with all drug combinations in all models tested at least as judged from animal weight loss (data not shown). For instance, in the experiment reported in Fig. 2, the weight of mice before treatment and 1 day after treatment with gemcitabine (1.8 mg) were 21.5 ± 0.3 and 20.6 ± 0.2 g, respectively, whereas the weight of mice treated with NGR-TNF in combination with gemcitabine at 2 hours were 21.8 ± 0.3 and 21.1 ± 0.4 g, respectively. NGR-TNF alone caused no loss of weight.

Effect of NGR-TNF on the* in vitro *cytotoxic activity of chemotherapeutic drugs. To assess whether the synergistic effect between the various chemotherapeutic drugs and NGR-TNF in the RMA model was related to a direct mechanism of tumor cell killing or to an indirect mechanism, we then tested the effect of each drug alone or in combination with NGR-TNF in *in vitro* cytotoxicity assays. RMA cells were pretreated with NGR-TNF (1 ng/mL) for 2 hours and then with various doses of chemotherapeutic drugs. Cell viability was measured 48 hours later by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All drugs, including cisplatin, paclitaxel, gemcitabine, doxorubicin, and melphalan, were able to kill RMA cells although with different potency (Fig. 5*A*). Pretreatment of cells with NGR-TNF did not increase the cytotoxic effect of these drugs (Fig. 5*B*).

Similar experiments were carried out to assess whether the synergism with doxorubicin and melphalan in the TS/A and WEHI-164 models, respectively, was related to indirect mechanisms. Also in this case, no significant changes were induced by NGR-TNF in the cytotoxic effects of doxorubicin and melphalan on TS/A cells WEHI-164, respectively (data not shown).

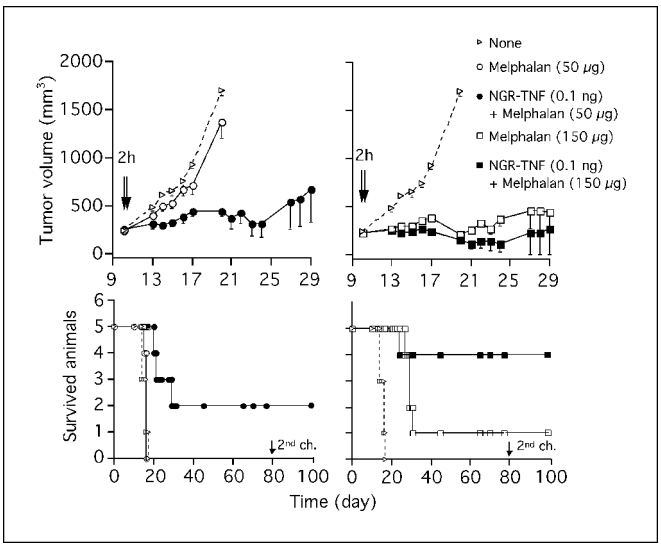

Fig. 4. Effect of NGR-TNF in combination with melphalan on RMA tumors. Mice bearing RMA tumors (five mice per group) were treated i.p. with NGR-TNF in combination with melphalan at day 10 after tumor implantation. NGR-TNF was given 2 hours before melphalan at the indicated doses. Top, tumor volume; bottom, animal survival. Mice that rejected the tumor rejected also a second challenge at day 80 (*2nd ch.*) with fresh tumor cells. Top, ● versus ○ at day 20 ($P < 0.001$); ■ versus □ at day 20 ($P < 0.05$).

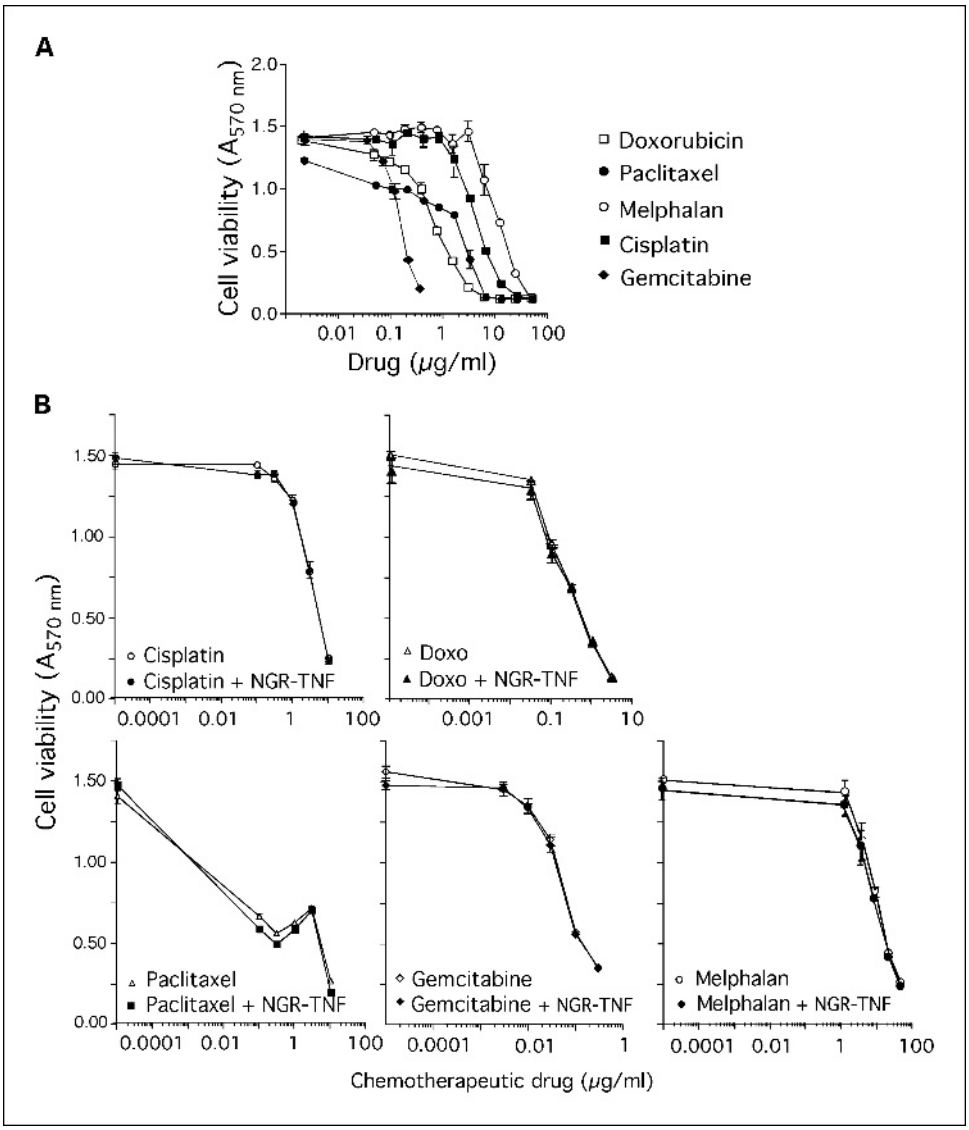

Fig. 5. *In vitro* cytotoxic activity of different chemotherapeutic drugs alone and in combination with NGR-TNF. RMA cells were treated for 48 hours with various doses of doxorubicin (*Doxo*), paclitaxel, melphalan, cisplatin, and gemcitabine (*A*). In another experiment, cells were pretreated for 2 hours with NGR-TNF (1 ng/mL) and then with various doses of chemotherapeutic drugs for 48 hours (*B*). Points, mean of four wells; bars, SD.

Because RMA, TS/A, and WEHI-164 tumor cells are likely exposed to a lower dose of NGR-TNF *in vivo* (0.1 ng/mouse) compared with *in vitro* (1 ng/mL), these results suggest that the main mechanism of action of the *in vivo* synergism was related to an indirect effect presumably against components of the tumor vasculature.

To assess whether NGR-TNF could affect the cytotoxic activity of drugs against endothelial cells, we then analyzed the cytotoxicity of doxorubicin and melphalan against human umbilical vein endothelial cells. The cytotoxic activity of various doses of doxorubicin and melphalan was not changed by human NGR-TNF even when we used this cytokine at concentrations as high as 10 ng/mL in cell culture (data not shown).

Thus, increased tumor or endothelial cell cytotoxicity of chemotherapeutic drugs does not seem to be a major mechanism for the *in vivo* synergism with NGR-TNF.

Effect of NGR-TNF on RMA and WEHI-164 tumor perfusion. TNF is known to induce intravascular coagulation and vascular damage, leading to vessel occlusion. To investigate whether the decrease in the synergism with chemotherapeutic drugs given with >3-hour delay was related to vascular occlusion and, consequently, to decreased tumor perfusion, we have given 0.1 ng NGR-TNF to RMA and WEHI-164 tumor-bearing mice and, 2 or 5 hours later, patent blue, a nontoxic dye. As positive controls, some mice were treated with 10,000 ng NGR-TNF, a dose that can cause marked vascular damage and occlusion. The animals were sacrificed 5 minutes later and the amount of dye in the tumor was analyzed by spectrophotometric techniques. Whereas the high dose of NGR-TNF almost completely inhibited tumor perfusion, as expected, the low dose of NGR-TNF could not significantly inhibit tumor perfusion in both models (Fig. 6). In another experiment carried out in the RMA model, we tested the effect of heparin, an anticoagulant, on the combination with NGR-TNF/doxorubicin, the latter given with a 4-hour delay. Heparin (30 IU) was given 2 hours before doxorubicin. No synergism between NGR-TNF and doxorubicin was observed even when heparin was included in the treatment (data not shown). These results suggest that intravascular coagulation is not a major

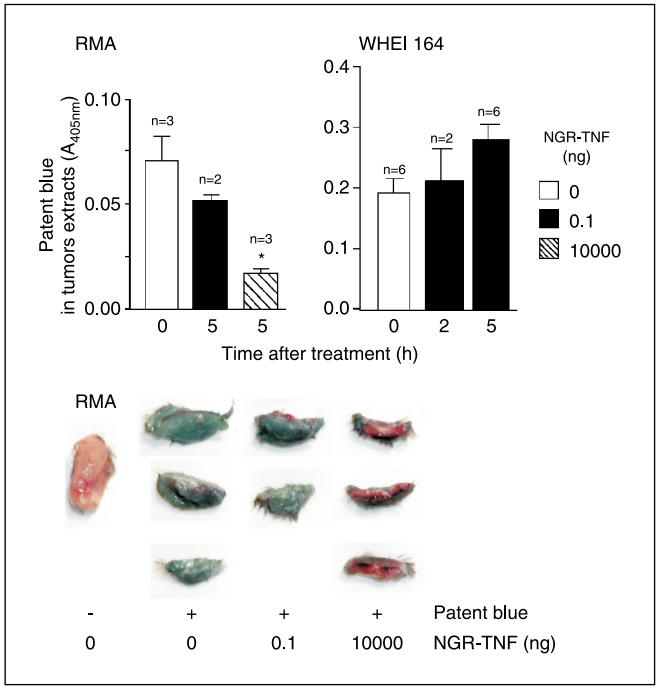

Fig. 6. Effect of NGR-TNF on tumor perfusion with patent blue. C57BL/6 and BALB/c mice bearing RMA or WEHI-164 tumors (diameter, 1.0-1.4 cm), respectively, were treated with murine NGR-TNF (0.1 or 10,000 ng) followed 2 or 5 hours later by patent blue. After 5 minutes, the animals were sacrificed and the tumors were excised, weighed, disaggregated, resuspended in PBS, and centrifuged. The amount of patent blue in tissue extract was then quantified by spectrophotometric measurements. The number of animals treated is indicated. Columns, mean; bars, SE. *, $P < 0.05$. Bottom, visual inspection of tumor sections 5 hours after treatment.

mechanism for the decreased synergism between NGR-TNF and chemotherapy observed at administration intervals greater than 2 hours.

Effect of repeated treatment with NGR-TNF on the synergism with drugs. To assess whether the lack of synergism at >3-hour time points was related to tumor unresponsiveness to drugs or to NGR-TNF clearance from circulation, we have treated one group of tumor-bearing mice with NGR-TNF at time 0 hour followed by cisplatin at 2 and 4 hours later. In parallel, another group of mice received NGR-TNF at 0 and 2 hours and cisplatin at 2- and 4-hour time points. The response was greater in animals that received two treatments of NGR-TNF (Fig. 7), supporting the hypothesis that lack of synergism between NGR-TNF and cisplatin with 4-hour delay was due to NGR-TNF clearance.

However, when a similar experiment was carried out with doxorubicin, the responses to one or two treatments with NGR-TNF were similar (Fig. 7), pointing to a different behavior of these drugs in repeated treatments.

Discussion

In previous work, we have shown that a low dose (0.1 ng) of NGR-TNF is sufficient to increase the efficacy of melphalan and doxorubicin in animal models (9). The main finding of this work is that targeted delivery of a low amount of NGR-TNF to tumor vasculature can enhance the response of tumors to other drugs, including cisplatin, gemcitabine, and paclitaxel. These drugs act via different mechanisms: for instance, cisplatin is a DNA-damaging agent, gemcitabine is a pyrimidine analogue with antimetabolic activity, and paclitaxel is an antimicrotubule agent; moreover, melphalan is an antitumor alkylating agent and doxorubicin is a DNA intercalator and a topoisomerase interactive compound (16). Remarkably, the optimal administration schedule for maximal synergism required a 2-hour delay between NGR-TNF and chemotherapeutic drug administration irrespective of drug and tumor model used. Thus, the synergy, despite being strongly dependent on administration schedule, is apparently independent from the mechanism of cytotoxic activity and pharmacokinetics, the 2-hour delay being required for all drugs. We have also found that synergism occurs *in vivo* but not in *in vitro* cytotoxicity assays with cultured tumor cells. This suggest that the *in vivo* synergism is not related to an effect of NGR-TNF on tumor cells. More likely, NGR-TNF affects host components that are critical for the activity of all drugs tested.

Previous studies showed that TNF can rapidly increase endothelial permeability (17, 18) and can decrease interstitial fluid pressure in tumors (19), both believed to be important barriers for drug penetration in tumors (20). Tumor vessel

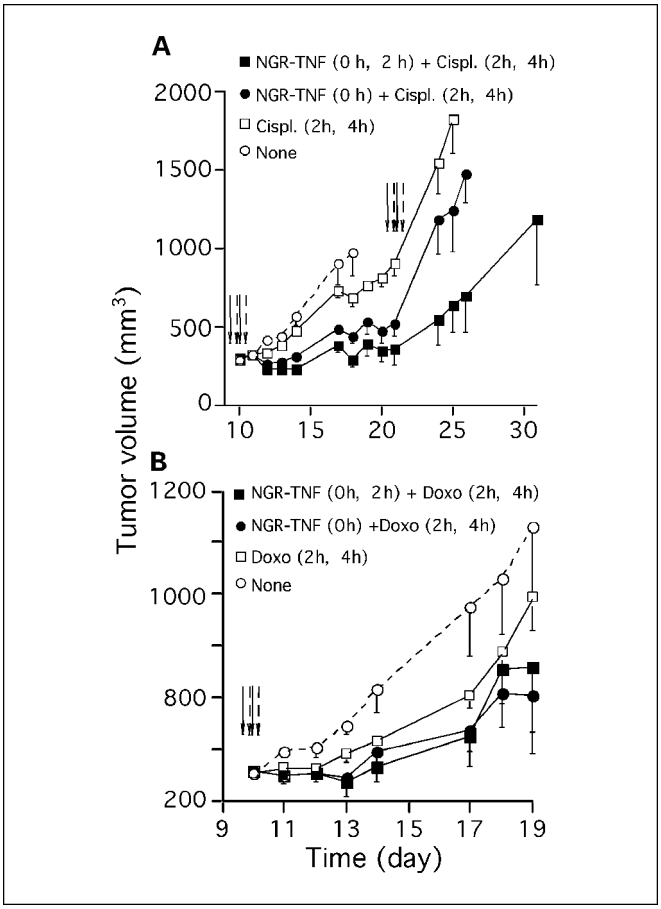

Fig. 7. Effect of repeated treatments with NGR-TNF and chemotherapeutic drugs on RMA tumors. Tumor-bearing mice were treated at day 10 twice with 0.1 ng NGR-TNF alone or in combination with 40 μg cisplatin at the indicated times (*A*). A similar experiment was done in combination with 40 μg doxorubicin (*B*). Mice treated with NGR-TNF/cisplatin received a second treatment at day 20.

damage and increased drug penetration are thought to be important mechanisms of the synergy between high doses of TNF with melphalan and doxorubicin in animal models and in patients (2, 3, 19, 21–23). This mechanism is likely to apply also for tumor vasculature-targeted TNF. According to this view, we have shown previously that preadministration of low doses of NGR-TNF (0.1 ng) increases the penetration of doxorubicin in murine B16 melanomas and RMA-T lymphomas (9). Moreover, magnetic resonance imaging of RMA tumors showed a larger leakage of ultrasmall iron oxide particles, a contrast agent, from the vasculature to the interstitium in NGR-TNF (0.1 ng)–treated tumors compared with controls (12). It is therefore possible that this mechanism occurs also with other chemotherapeutic drugs, such as cisplatin, gemcitabine, and paclitaxel. Likely, the optimal 2-hour delay is the time necessary for NGR-TNF to affect the tumor vessels and therefore is independent from the chemotherapeutic drug. However, other mechanisms could contribute to the synergism with chemotherapeutic drugs at 2-hour time points. For instance, it has been shown previously that administration of NGR-TNF (0.1 ng) to RMA tumor-bearing mice increases cell proliferation in tumor hypoxic areas possibly due to transient normalization of the vascular function in certain tumor areas (12). This effect was transient, lasting probably few hours, as we typically observed a reduction in tumor growth 1 day after treatment. Considering the importance of cell proliferation on chemotherapeutic drug activity, this mechanism could contribute, along with an increased vascular permeability and drug penetration, to the overall activity of drug combinations.

Why the effect of TNF is so short and synergism decreases at >3- to 4-hour time points? This phenomenon could have several explanations. For instance, it is well known that gap formation and endothelial barrier alteration can be followed by rapid recovery of barrier function through active processes (24). Second, NGR-TNF-induced vascular leakage, documented by magnetic resonance imaging measurements 2 hours after treatment (12), could lead, at later time points, to increased protein extravasation and increased interstitial pressure, which could reduce the transvascular transport of drugs in the tumor interstitium. Furthermore, given that TNF can induce intravascular coagulation (25), it is also possible that vessel occlusion and reduction of tumor perfusion reduce drug delivery. However, this mechanism is likely to play a minor role, as we observed significant vessel occlusion and inhibition of perfusion with patent blue only when mice were treated with a high dose of NGR-TNF (10 μg) and not with a low dose (0.1 ng). Furthermore, the synergism with doxorubicin was transient even when animals were treated with heparin.

The loss of synergism at times >3 to 4 hours could also be related to rapid clearance of NGR-TNF from circulation (8) and consequently to a decrease of its vascular effects. This hypothesis is supported by the observation that a second administration of NGR-TNF, 2 hours after the first, was synergistic with cisplatin given at 4-hour time point. This also implies that tumors do not became resistant to cisplatin 4 hours after NGR-TNF administration. In contrast, no synergism was observed with doxorubicin in a similar experiment, pointing to the importance of drug pharmacokinetics and pharmacodynamics for the synergism at 4 hours. To explain this behavior, we should keep in mind that different biological effects are likely induced by NGR-TNF at different time points, which may or may not affect chemotherapy depending on drug properties. For instance, increased protein extravasation and interstitial pressure could reduce the transvascular transport of drugs in the tumor interstitium depending on drug pharmacokinetics, pharmacodynamics, and plasma protein binding as well as tumor blood flow and oxygenation (26–30). Considering the different effects that hypoxia may induce on chemotherapeutic drug activity (31), this factor could also contribute to the differential behavior of these drugs at time points >3 to 4 hours.

In conclusion, we have shown that targeted delivery of low doses of NGR-TNF to the tumor vasculature can increase the efficacy of various chemotherapeutic drugs, in a transient manner, in different tumor models. Evidence was obtained to suggest that 2 hours is the time necessary for NGR-TNF to induce optimal biological effects independently from chemotherapeutic drug pharmacokinetics and pharmacodynamics. Rapid NGR-TNF clearance and changes in the tumor responsiveness could account for the lower synergism at later times in a manner depending on drug properties. The increase of efficacy was not accompanied by an increased toxicity at least as judged from the loss of animal weight. Considering that NGR-TNF is currently tested in clinical trials, this work could provide important information for designing clinical studies with NGR-TNF in combination with chemotherapeutic drugs.

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