

Abstracts from AACR 2003 Meeting, Washington, D.C.

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In vitro and in vivo anti-angiogenic effects of VEGF121/rGel fusion toxin.

A unique fusion construct GrB/scFvMEL capable of targeted delivery of human pro-apoptotic granzyme B to human melanoma cells.

The anti-melanoma fusion toxin scFvMEL/TNF has a unique cytotoxic mechanism of action and delivers TNF to human tumor xenografts.

Targeting human pancreatic cancer cells with the anti-HER-2 fusion toxin scFv23/TNF.

In-vitro studies comparing the recombinant, single-chain immunotoxins scFv23/rGel and ML3.9/rGel which recognize the HER2/neu proto-oncogene.

Phase I clinical trial of the anti-CD-33 immunotoxin HuM195/rGel

VEGF₁₂₁ gelonin fusion protein inhibits breast cancer metastasis in nude mice.

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VEGF₁₂₁/rGel fusion protein is a vascular targeting agent composed of a non-heparin binding isoform of VEGF and the highly active plant toxin gelonin. Both receptors for VEGF-A (R1 and R2) are strongly up regulated on tumor neovasculature compared to normal endothelium. We have previously shown that VEGF₁₂₁/rGel was selectively cytotoxic to sub-confluent endothelial cells, displaying an IC₅₀ of 0.5 nM compared to 150 nM for free gelonin under the same conditions. Treatment of mice bearing various types of solid tumors with VEGF₁₂₁/rGel (17 mg/kg) inhibited the growth of primary tumors by 65-75% (Veenendaal et al., *PNAS* 99:7866-71, 2002). The goal of this study was to evaluate the effect of VEGF₁₂₁/rGel on metastatic growth of MDA-MB-231 tumor cells in nude mice. The MDA-MB-231 cells (0.5×10^6 per mouse) were injected i.v. and the treatment began 8 days after injection. Mice (6 per group) were treated 6 times either with VEGF₁₂₁/rGel (100 ug/dose) or with an equivalent amount of free gelonin. Three weeks after termination of the treatment, mice were sacrificed and their metastatic lungs as well as all other organs were harvested for examination. The number of surface lung foci in the VEGF₁₂₁/rGel – treated mice was reduced by 58 % as compared to gelonin control animals (mean numbers 22.4 and 53.3 for VEGF₁₂₁/rGel and control, respectively; $p < 0.05$). Immunohistochemical analysis revealed that the mean area of lung colonies from VEGF₁₂₁/rGel-treated mice was 50% smaller than that of the control mice ($210 \pm 37 \mu\text{m}$ versus $415 \pm 10 \mu\text{m}$ for VEGF₁₂₁/rGel and control, respectively; $p < 0.01$). In addition, the vascularity of metastatic foci from VEGF₁₂₁/rGel-treated mice was reduced compared to that of control colonies. The mean number of blood vessels per mm² in metastatic foci was 198 ± 37 versus 388 ± 21 for treated and control, respectively. Approximately 62 % of metastatic colonies from the VEGF₁₂₁/rGel-treated group had fewer than 10 vessels per colony as compared to 23 % in the control group. The VEGF receptor 2, a major receptor that mediates angiogenic effects of VEGF-A, was intensely detected on the metastatic vessels in the control but not on the vessels in the VEGF₁₂₁/rGel-treated group. The treatment was well tolerated. No significant morphological changes were visible in either VEGF₁₂₁/rGel-treated or gelonin control mice. These data strongly suggest that anti-tumor vascular effect of VEGF₁₂₁/rGel could be utilized not only for treating primary tumors but also for inhibiting metastatic spread. This research was funded in part by the Clayton Foundation for Research and DOD Breast Cancer Program.

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

In vitro and in vivo anti-angiogenic effects of VEGF₁₂₁/rGel fusion toxin.

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VEGF₁₂₁/rGel is a chimeric fusion toxin of VEGF₁₂₁ and the plant toxin gelonin (rGel). We have previously shown VEGF₁₂₁/rGel to target tumor neovasculature. Both receptors for VEGF₁₂₁, designated VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), are over-expressed on tumor vascular endothelium. VEGF₁₂₁/rGel was found to be cytotoxic to cells over-expressing KDR but not Flt-1. Cell ELISA using antibodies specific to either KDR or Flt-1 indicate equivalent binding of VEGF₁₂₁/rGel to the cell lines expressing each receptor. Studies with ¹²⁵I-VEGF₁₂₁/rGel demonstrated binding of the fusion toxin to both cell surfaces that was completed with cold VEGF₁₂₁/rGel but not cold gelonin. Cells transfected with each receptor (PAE/KDR and PAE/Flt-1) were incubated with VEGF₁₂₁/rGel for 1, 4, 16 and 24-hours, fixed and stained for intracellular rGel using an anti-rGel antibody. Internalization of VEGF₁₂₁/rGel into PAE/KDR cells was observed within one hour; however, no VEGF₁₂₁/rGel was detected in PAE/Flt-1 cells up to 24-hours after treatment. These studies demonstrate that while VEGF₁₂₁/rGel binds to both Flt-1 and KDR receptors, internalization of VEGF₁₂₁/rGel is mediated only by KDR and not Flt-1. In vivo effects of VEGF₁₂₁/rGel were evaluated using orthotopically implanted human 253J B-V bladder carcinoma cells. Tumor cells were allowed to establish and groups were treated (i.v. every other day) with a total of 20 mg/kg of VEGF₁₂₁/rGel, rGel or saline. The mice were necropsied 21 days after tumor implantation. No differences were observed in tumor weight from mice treated with saline or rGel. In contrast, tumors from mice treated with VEGF₁₂₁/rGel weighed 50% less than controls ($p < 0.05$) indicating significant suppression of bladder tumor growth. Immunohistochemical studies for the KDR receptor, blood vessel endothelium (CD-31) and rGel demonstrated co-localization of KDR with CD-31, indicating that the KDR receptor was localized only on endothelial cells and is not present on tumor cells. Immunofluorescence of tumor tissue sections with anti-CD31 and anti-rGel antibodies showed dramatic co-localization of VEGF₁₂₁/rGel on the tumor neovasculature but no co-localization in tumors treated with rGel. TUNEL staining of tissue sections show marked apoptosis of tumor cells treated with VEGF₁₂₁/rGel but not with rGel. These data suggest that VEGF₁₂₁/rGel fusion protein inhibited the growth of human bladder tumors by a cytotoxic effect directed against the tumor vascular supply and has a significant potential as a novel anti-angiogenic therapy for human bladder cancer. VEGF₁₂₁/rGel is also an important molecule useful to probe the biology of KDR and Flt-1 receptors. Research conducted, in part, by the Clayton Foundation for Research.

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

A unique fusion construct GrB/scFvMEL capable of targeted delivery of human pro-apoptotic granzyme B to human melanoma cells.

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Targeted therapeutic agents have a significantly greater biological impact when they can be engineered to deliver cytotoxic agents particularly those that act enzymatically. The serine protease granzyme B (GrB) is produced by cytotoxic T lymphocytes and induces apoptosis by directly activating caspases resulting in rapid DNA fragmentation, and/or direct damage non-nuclear structures through a caspase-independent mechanism. The purpose of this study was to determine whether a recombinant antibody could be utilized to deliver GrB to target cells in amounts sufficient to generate an apoptotic event. Human GrB gene was cloned from Hut-78 cells, then attached to the single-chain anti-melanoma antibody scFvMEL (anti-gp240) via a short, flexible tether (G_4S) by PCR. The fused gene was cloned into the pET32 vector. The GrB/scFvMEL fusion protein was expressed in E.coli and purified by Ni-NTA metal affinity chromatography. The His-tag was removed after digestion with recombinant enterokinase leaving the first residue Ile of mature GrB exposed. Western blotting using either anti-scFvMEL or anti-GrB antibody confirmed incorporation of both scFvMEL and GrB proteins into the construct. The purified fusion construct bound specifically to human A-375 melanoma cells by ELISA and was able to rapidly deliver GrB to the cytosol as assessed by immunofluorescence. The intact GrB enzymatic activity of the fusion construct ($SA = 2.6 \times 10^5$ units/ μ mole) was similar to that of native GrB ($SA = 4.8 \times 10^5$ units/ μ mole). Against log-phase melanoma cells, the construct demonstrated an $I.C._{50}$ of 20 nM but demonstrated minimal cytotoxicity to non-target cells at doses up to 1 μ M. The cytotoxic effects of the fusion construct were similar to that of the previous-described scFvMEL/rGel fusion toxin ($I.C._{50} \sim 20$ nM). The cytotoxicity of GrB/scFvMEL against melanoma cells was effectively competed by addition of parental murine ZME-018 antibody. TUNEL assay showed the construct developed impressive apoptotic effects by 16 h after treatment of target cells. Mediation of the apoptotic effects included release of cytochrome c from the mitochondria to the cytosol. It is likely both caspase dependent and caspase-independent mechanisms are all responsible for the observed cytotoxic/apoptotic events. These studies demonstrate that delivery of human pro-apoptotic GrB to tumor cells may have significant therapeutic potential for cancer treatment and represents a new class of targeted therapeutic agents with a defined mechanism of action. Research conducted, in part, by the Clayton Foundation for Research.

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

The anti-melanoma fusion toxin scFvMEL/TNF has a unique cytotoxic mechanism of action and delivers TNF to human tumor xenografts.

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ScFvMEL/TNF is composed of a single-chain Fv recognizing the gp240 antigen fused to the human cytokine TNF. Purified scFvMEL/TNF specifically bound to human melanoma cells and trimerizes in solution. Scatchard analysis revealed two binding sites with dissociation constants (Kd) of 1.9 nM (~ 4000 sites/cell) and the other with a Kd of 15.6 nM and $\sim 1.6 \times 10^5$ sites/cell. The fusion construct was selectively more cytotoxic to TNF-sensitive (A375-M) cells compared to TNF alone (I.C.₅₀ ~ 60 pM and 1000 pM respectively), and were also cytotoxic to TNF-resistant melanoma (AAB-527) cells (I.C.₅₀ 20 nM and > 1000 nM for scFvMEL/TNF and TNF respectively). The mechanism behind this effect is not completely understood. We employed cDNA microarray analysis to identify genes exhibiting differential expression between samples of untreated AAB-527 cells and those treated for 24 h with either TNF or scFvMEL/TNF. Array analysis of 2500 genes indicated that there were 67 genes down regulated and 63 genes up regulated by both scFvMEL/TNF and TNF treatment. In addition, 155 genes were down regulated and 132 genes up regulated uniquely by scFvMEL/TNF. Those genes most involved in cell surface receptor linked signaling, intracellular signaling cascade, stress response and intracellular protein traffic and transport. The scFvMEL/TNF fusion construct down regulated specific genes involved in cell cycle and cell proliferation as well as genes regulating nucleotide metabolism. These studies suggest that the construct delivers TNF into the cytosol and appears to activate different intracellular pathways compared to TNF alone. Pharmacokinetic studies of scFvMEL/TNF were performed using ¹²⁵I-labeled material administered to Balb/c mice (5 μ g total protein/mouse) and blood samples were obtained at various times after administration. The fusion construct was cleared triphasically with a $t_{1/2\alpha} = 0.38$ h, a $t_{1/2\beta} = 3.9$ h, and a $t_{1/2\gamma} = 17.6$ h. The iodinated construct was then administered to nude mice bearing well-developed A-375 xenografts and the animals were sacrificed at 24, 48 and 72 h after administration. Tumor: blood ratios increased over time up to ~ 7.0 at 72 h. The highest normal tissue content was found in kidney and spleen with intestine and hind quarters having the lowest content at all times studied. These studies suggest that the scFvMEL/TNF fusion construct has a mechanism of cytotoxic action which is unique compared to TNF and can be successfully targeted to tumors in vivo. Research conducted, in part, by the Clayton Foundation for Research

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

Targeting human pancreatic cancer cells with the anti-HER-2 fusion toxin scFv23/TNF.

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Pancreatic cancer is one of the most aggressive human malignancies with an extremely poor prognosis and HER-2 over-expression confers chemoresistance to pancreatic cancer cells. We have previously developed a scFv23/TNF fusion protein composed of a single-chain antibody recognizing the external domain of the HER-2 protooncogene linked by flexible tether to TNF. Using a panel of human pancreatic cancer cell lines (MIA PaCa-2, AsPc-1, Capan-1, Capan-2, and L3.6pl), we characterized the expression of HER-2, EGFR, TNFR-1, TNFR-2, and p53 by Western blot analysis. We then investigated the cytotoxic effect of scFv23/TNF and TNF itself against these cell lines and examined the effects of various chemotherapeutic agents alone and in combination with scFv23/TNF. All cell lines were highly resistant to the cytotoxic effects of TNF ($I.C_{.50} > 400$ nM) with the exception of MIA PaCa-2 which displayed an $I.C_{.50}$ of 8 nM. This cell line was also the most sensitive to the scFv23/TNF with an $I.C_{.50}$ of 60 nM. The other cell lines displayed $I.C_{.50}$ values between 150 and 700 nM. However 5-fluorouracil, cis-platinum, and VP-16 showed $I.C_{.50}$ values between 1.2 and 300 μ M whereas doxorubicin showed $I.C_{.50}$ values between 20 and 500 nM in five human pancreatic cancer cell lines. HER-2-over-expressing pancreatic cancer cell lines with EGFR over-expression were the most sensitive to the scFv23/TNF but not to TNF itself or chemotherapeutic agents. We found no correlation between expression of p53, TNFR-1, TNFR-2 and response of cells to scFv23/TNF, TNF or chemotherapeutic agents. Studies combining scFv23/TNF and various chemotherapeutic agents demonstrated a synergistic effect of scFv23/TNF and 5-fluorouracil in all cell lines tested. Some cell lines displayed an additive effect of scFv23/TNF with cis-platinum, VP-16 and doxorubicin while other cell lines showed a synergistic effect of these combinations. Therefore, our results suggest that targeting HER-2 expressing tumor cells using the scFv23/TNF fusion toxin may be an effective therapy for pancreatic cancer especially when utilized in combination with specific chemotherapeutic agents. Research conducted, in part, by the Clayton Foundation for Research. Research supported by DAMD17-02-1-0457-1

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

In-vitro studies comparing the recombinant, single-chain immunotoxins scFv23/rGel and ML3.9/rGel which recognize the HER2/*neu* proto-oncogene.

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The single-chain antibody scFv23, derived from a murine Mab, recognizes the HER2/*neu* cell surface domain, as does the human, phage-display-derived single-chain antibody ML3.9 (originally scFvC6.5, J. D. Marks). Genes for both were fused to the gene encoding the recombinant toxin rGelonin (rGel) using PCR. Each fusion construct was then ligated into a bacterial expression plasmid (pET32) for protein synthesis. Induction of bacterial expression resulted in the generation of new and soluble proteins at the expected molecular weights of 58 kDa (scFv23/rGel), and 56 kDa (scFvML3.9/rGel). As assessed by SDS-PAGE, both proteins were purified to homogeneity using IMAC and both were Western-positive for gelonin. A cell-free protein synthesis assay showed that both proteins have inhibitory activity similar to that of the native toxin (I.C.₅₀ of 53 pM, 92 pM and 60 pM for the scFv23/rGel, ML3.9/rGel and rGel respectively), suggesting that no loss of toxin activity occurred in the fusion molecules. ELISA assays showed that both fusion constructs specifically bound to HER2/*neu*-positive cells compared to recombinant gelonin. Cytotoxicity studies against antigen-positive SK-BR-3 cells in log phase culture demonstrate that both fusion constructs have I.C.₅₀ values of 21.6 nM and 15.6 nM, which were at least 100-fold lower than free rGel (I.C.₅₀ of 2213 nM), compared with approximately identical values using antigen-negative ME-180 cells (I.C.₅₀ of 240 nM, 1160 nM and 627 nM, respectively). Both constructs retain the specificity of the original antibody, as well as the biological activity of the original rGelonin toxin. Our data suggests that that immunotoxins derived from phage-generated antibodies appear to be equivalent to immunotoxins containing recombinant constructs derived from existing antibodies. Further in vitro and in vivo studies are planned for these constructs. Research conducted, in part, by the Clayton Foundation for Research

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.

Phase I clinical trial of the *anti*-CD-33 immunotoxin HuM195/rGel.

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The immunotoxin HuM195/rGel is composed of the humanized anti-CD-33 antibody HuM195 chemically conjugated to the recombinant plant toxin gelonin (rGel). Pre-clinical studies have demonstrated that this agent is specifically cytotoxic to CD-33 expressing human tumor cells growing both in vitro and as subcutaneous xenografts in nude mice. In addition, this agent was effective in vitro against primary tumor cells obtained from 9 AML patients. A dose-escalation Phase I study was initiated at MDACC employing a 1 hr i.v. infusion of HuM195/rGel. One course consisted of 4 infusions spaced 72 hrs apart followed by a 2-week observation period. Dose levels were 10,12,18,28 and 40 mg/m²/course. Twenty-one patients have been entered thus far completing the 28 mg/m² dose level. Two patients received 2 courses of the drug. Thus far, there were no major drug-related toxicities. Fever and chills were noted during the infusion. Six of 21 patients demonstrated a reduction in peripheral blood (PB) CD-33 count. One patient (12 mg/m²) had a complete clearance of PB CD-33 cells. Evidence for a substantial reduction in PB blasts were noted in 6/21 patients. 5/6 of these responses were noted at doses at 18 mg/m² and above. In bone marrow, there was a modest(10%) reduction in CD-33 cells noted in 3 pts at 10 mg/m² and in 2 at patients at 28 mg/m². In two patients at 28 mg/m², there was nearly complete clearance of CD-33 positive cells from bone marrow (94% to 1% and 85% to 15%). Pharmacokinetic analysis demonstrated that the highest achieved blood levels were 200-300 ng/ml which cleared with a half-life of ~20 hrs. Only 2/20 patients (10%) developed antibodies to the rGel portion of the drug. These studies suggest that the HuM195/rGel immunotoxin appears to be safe and well-tolerated with evidence of biological activity in some patients. Dose escalation studies are continuing. Research conducted, in part, by the Clayton Foundation for Research.

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Citation for abstracts scheduled for publication: Proceedings of the AACR, Volume 44, 2nd ed., July 2003.

Citation for abstracts not scheduled for publication: Proceedings of the AACR, Volume 44, 1st ed., March 2003.
