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Molecular Mechanism of a Synergistic Interaction between scFv23/TNF and 5-Fluorouracil in L3.6pl.

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Immunocytokines are novel class of recombinant agents composed of normal cytokines fused to antibodies capable of re-directing their biological effects to target specific cells and to prevent non-target toxicity. 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 the cytokine TNF and the scFV23/TNF has demonstrated remarkable anti-tumor activity against HER-2/neu positive tumor cells which are resistant to TNF itself. We investigated the cytotoxic effects of scFv23/TNF alone and in combination with various chemotherapeutic agents against a variety of human pancreatic cancer cell lines which expresses HER-2. We found that L3.6pl cells were the most sensitive to the scFv23/TNF but were relatively insensitive to conventional chemotherapeutic agents. However, in combination of scFv23/TNF, we found a synergistic effect between the fusion construct and 5-fluorouracil (5-FU) whereas cisplatin, VP-16, doxorubicine, and gemcitabine showed additive effects against L3.6pl cells. Capan-1 and Capan-2 cells displayed an additive effect of scFv23/TNF with 5-FU, cisplatin, VP-16, and gemcitabine. Interestingly, AsPc-1 cell line showed no additional effects of scFv23/TNF with doxorubicine and gemcitabine. We next examined the molecular mechanisms which might be responsible for this synergistic interaction in L3.6pl. Exposure of the cells to the scFv23/TNF plus 5-FU combination resulted in increased apoptosis as demonstrated by an increase in PARP cleavage, caspase-8 and caspase-3 activity. We additionally found a significant decrease in Akt phosphorylation at 48 hr. Additional mechanisms relating to apoptosis and the biochemical effects of 5-FU and TNF are currently being explored to determine whether other pathways may also be responsible for these observations. Theses 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.

Category and Subclass: ET7-03 Apoptosis: therapeutic manipulation

Keyword: Immunocytokine; HER-2; Synergism; Pancreatic cancer

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Vascular Targeting withVEGF$_{121}$/rGel Inhibits Angiogenesis: Specific Effects Assessed Using Micro-Array Analysis

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$VEGF_{121}$/rGel, a fusion protein of $VEGF_{121}$ and the plant toxin gelonin (rGel) targets the tumor neovasculature and exerts impressive cytotoxic effects by inhibiting cellular protein synthesis. We have previously shown that in vivo administration of this molecule inhibits tumor growth in melanoma, bladder, breast and prostate models. Further studies characterizing this molecule demonstrated that $VEGF_{121}$/rGel inhibited tube formation of endothelial cells over-expressing VEGFR-2 (PAE/KDR) on matrigel-coated plates. A concentration of 1 nM reduced by over 50% the number of tubes formed. In contrast, 100 nM of unconjugated gelonin resulted in the same degree of reduction of tube formation. Endothelial cells expressing VEGFR-1 (PAE/FLT-1) were not as sensitive to $VEGF_{121}$/rGel as PAE/KDR cells, requiring 100 nM $VEGF_{121}$/rGel to inhibit tube formation by 50%. PAE/KDR cells pre-treated with 1 nM $VEGFR_{121}$/rGel prior to plating on matrigel showed significant reduction in tube formation that was dependent upon the length of pre-treatment. We investigated the effects of $VEGFR_{121}$/rGel on angiogenesis in the chicken chorio-allantoic membrane (CAM) assay. CAMs of 9-day chicken embryos were stimulated using bFGF, and simultaneously treated with $VEGFR_{121}$/rGel at a dose of 1 or10 nM. Three days later vascular density was analyzed. Treatment with $VEGFR_{121}$/rGel significantly inhibited the bFGF-mediated angiogenesis by 30% ($P < 0.001$, t-test, double sided). $VEGFR_{121}$/rGel treatment decreased newly-sprouting vessels. As expected, the control protein gelonin, at equivalent concentrations, had no effect. We examined the mechanism of $VEGF_{121}$/rGel-induced cytotoxicity against cells in culture. Treated cells were TUNEL-negative and we found no evidence of PARP or caspase-3 cleavage and we concluded that the effects of this fusion construct were necrotic rather than apoptotic. To further delineate the activity of this construct, HUVECs treated with an IC_{50} dose of $VEGF_{121}$/rGel for 24 hours were harvested and the effect of $VEGF_{121}$/rGel on intracellular events was examined by extraction of mRNA and microarray analysis of genes involved in signal transduction, stress response, cell cycle control, hypoxia and metastasis. The results were validated by RT-PCR. Our data suggests that $VEGF_{121}$/rGel induces expression of genes known to be induced by VEGF alone, in addition to genes involved in inflammation, chemotaxis and transcription regulation. Research conducted, in part, by the Clayton Foundation for Research and supported by DAMD 17-02-1-0457.

Category: CB14-01 Angiogenesis inhibitors

Key words: VEGF, gelonin, angiogenesis, microarray

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The vascular targeting agent, $VEGF_{121}$/rGel, inhibits the growth of human MDA-MB-231 breast tumors in the lungs of SCID mice

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Tumor neovascularization plays a key role in tumor development and metastatic spread. The cytokine vascular endothelial growth factor (VEGF) and its associated receptor fetal liver kinase-1 (Flk-1/KDR/VEGFR2), appear to play a central role in tumor neovascularization. In solid tumor biopsy specimens, Flk-1 is found frequently to be expressed in tumor vasculature at levels significantly higher than those found in the vasculature of adjacent normal tissue. Previous studies have defined a novel fusion construct of $VEGF_{121}$ and the highly cytotoxic recombinant plant toxin gelonin (rGel). The $VEGF_{121}$/rGel fusion toxin was highly toxic to endothelial cells overexpressing Flk-1/KDR, but not toxic to cells expressing the related Flt-1/FLT-1 receptor. Furthermore, $VEGF_{121}$/rGel was able to inhibit the growth of human melanoma and prostate tumor xenografts in mice. In this study, we demonstrated that $VEGF_{121}$/rGel, injected i.v. into SCID mice bearing orthotopic human MDA-MB-231 breast tumors, localizes specifically to the tumor vasculature. In addition, we evaluated the effect of $VEGF_{121}$/rGel on the growth of human breast tumor cells in the lungs of SCID mice. Mice were injected i.v. with human MDA-MB-231 breast tumor cells and, following an eight day establishment period, treated six times with $VEGF_{121}$/rGel (100 µg/dose) or free gelonin. Three weeks after completion of treatment, mice were sacrificed and lungs were harvested for examination. $VEGF_{121}$/rGel treatment reduced surface lung foci by 58% compared to gelonin controls (means were 22.4 and 53.3, respectively; $p<0.05$). The mean area of lung colonies from $VEGF_{121}$/rGel-treated mice was 50% less than in control mice (210 ± 37 μm^2 versus 415 ± 10 μm^2 for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, pulmonary tumor foci vascularity in $VEGF_{121}$/rGel-treated mice was reduced by 50% relative to control mice (198 ± 37 versus 388 ± 21 vessels per mm^2 for VEGF/rGel-treated and control, respectively; $p<0.02$). Lung tumor foci also had a 3-fold lower proliferation (Ki-67 labeling) index than did control tumors. These data suggest strongly that the vascular targeting action of $VEGF_{121}$/rGel might be utilized, not only for treating primary tumors, but also for inhibiting the development and vascularization of metastases. This research was conducted, in part, by the Clayton Foundation for Research, the Longenbaugh Foundation, and supported by DAMD 17-02-1-0457.

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Development of "Designer Toxins" with Reduced Antigenicity and Size

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Ribosome inhibitory proteins(RIPs) derived from plant or bacterial sources have tremendous potential as payloads for antibodies or other cell-targeting carriers but they potentially have immunogenicity drawbacks which could complicate long-term or repeated therapy. Recombinant gelonin(rGel) is a RIP which retains its biological activity as a fusion construct and does not require release from its cell-targeting carrier in contradistinction to other toxins such as RTA and PE. We initially mapped the antigenic domains of rGel by first generating 10 linear peptides spanning the amino acid sequence of rGel. We detected and isolated human polyclonal anti-rGel antibodies from 2 patients who had developed antibodies to rGel after systemic administration of

an rGel-based immunotoxin(HuM195/rGel). Using an ELISA format, we utilized this antisera to identify 5 antigenic regions on the molecule. We then generated and purified six N-terminal deletion mutants and 2 C-terminal deletion mutants of rGel and examined the cell-free protein synthesis inhibitory activity(RRLA) of these molecules. Individual deletions significantly reduced the RRLA compared to native rGel. However, the molecule with deletion of AA's #43-71 showed the greatest activity. Molecules containing combined N- and C-terminal deletions were also active. The deletion mutant with the greatest RRL activity(designated N-68/C-201) is almost 50% smaller than native rGel (14,410 vs 28,490 daltons respectively). To test for cytotoxicity against mammalian cells, we fused these mutants to the $VEGF_{121}$ cytokine and tested the fusion constructs against PAE cells over-expressing FLT-1 and FLK-1 receptors. All of these agents demonstrated IC_{50} values in the low nM range and were similar in activity to that of the $VEGF_{121}$/rGel native molecule. Western analysis probing with both rabbit and human polyclonal anti-rGel antisera demonstrated that several these highly modified toxins showed no reactivity and that the antigenic domains of this toxin can be engineered out while maintaining biological and enzymatic activity.

Category and Subclass: CL6-01 Antibodies/immunoconjugates

Keyword: Gelonin; Fusion Proteins; Targeted Therapy; Vascular Targeting

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Targeted Delivery Granzyme B to Melanoma Cells: Directed Apoptotic Effects, Anti-tumor Activity and Synergy with Chemotherapeutic Agents

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The novel fusion construct GrB/scFvMEL is composed of the single chain antibody scFvMEL recognizing the gp240 antigen and the human serine protease Granzyme B (GrB). GrB initiaties apoptosis by multiple mechanisms which include directly activating caspases, inducing DNA fragmentation, activating the mitochondrial death pathway, and directly cleaving the nuclear matrix. The GrB moiety of the fusion construct was shown to be efficiently delivered into the cytosol of gp240 antigen-positive A375-M melanoma cells after treatment with GrB/scFvMEL as assessed by confocal microscopy. Treatment with the original anti-gp240 antibody ZME-018 completely suppressed internalization of this agent demonstrating that antibody binding of the construct to gp240 on the cell surface is responsible for internalization. GrB/scFvMEL demonstrated an $I.C._{50}$ of 20 nM against log-phase A375-M cells and minimal cytotoxicity to non-target SKBR3 cells at doses of up to 1000 nM. Co-administration of exogenous perforin to cells resulted in a light increase in the cytotoxic effects of the fusion construct on A375-M cells and a significant increase in cytotoxicity to SKBR3 cells. The construct produced impressive apoptotic effects by 8 h after treatment of target cells. The construct was shown to mediate capspase 3 cleavage and release of cytochrome c into the cytosol compartment from the mitochondrial compartment. Co-administration GrB/scFvMEL and chemotherapeutic agents (adriamycin, vincristine sulfate, etoposide, cisplatin or cytorabine) to A375-M cells for 72 hours,

demonstrated synergistic antitumor activity with adriamycin, vincristine or cisplatin and additive effects in combination with etoposide or cytorabine. Against highly metastatic A375-SM cells, we found synergistic effects of the fusion construct in combination with all chemotherapeutic agents except cisplatin which showed additive activity. Pre-treatment with GrB/scFvMEL for 6 h followed by exposure to these chemotherapeutic agents for 72 hours showed significantly inhibited growth as compared to pre-treatment with drugs followed by fusion construct treatment ($p< 0.01$). Human tumor xenograft studies are progressing and will be reported. These studies demonstrate that the GrB/scFvMEL fusion construct demonstrates impressive antitumor activity and enhances the sensitivity of human melanoma cells to chemotherapy. Targeted delivery of human pro-apoptotic GrB to tumor cells may therefore have a significant potential for cancer treatment. Research conducted, in part, by the Clayton Foundation for Research.

Category and Subclass: ET7-03 Apoptosis: therapeutic manipulation

Key words: Fusion Proteins, Granzyme B, Melanoma, Immunotoxin

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