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13. ABSTRACT (Maximum 200 Words)

The unique fusion toxin $VEGF_{121}$/rGel can specifically kill both log-phase and confluent vascular endothelial cells expressing the KDR receptor for VEGF(PNAS 99:7866,2002). We have discovered 19 unique genes upregulated(>5 fold) in endothelial cells treated with $VEGF_{121}$/rGel(confirmed by Western and RT-PCR).$VEGF_{121}$/rGel(i.v.) treatment had a dramatic cytotoxic effect in both orthotopic and metastatic human breast tumor models. Against the orthotopic model, tumor growth was significantly delayed by~50%. In addition, tumors completely regressed in 3/6 (50%) of treated mice. In the metastatic breast model, treatment with $VEGF_{121}$/rGel reduced both the number and area of lung foci by 58% and 50% respectively and we demonstrated $VEGF_{121}$/rGel(by IHC) on lung tumor vasculature but not normal vasculature. In addition, the number of blood vessels per mm^2 in metastatic foci was 198$\pm$ 37 versus 388$\pm$ 21 for treated and control respectively. Approximately 62% of metastatic colonies from the VEGF/rGel treated group had <10 vessels/colony compared to 23% in the control group. The flk-1 receptor on blood vessel endothelium was intensely expressed on control tumors, but not expressed on treated tumors. Metastatic foci had a 3 fold lower Ki-67 labeling index compared to control tumors. This suggests that $VEGF_{121}$/rGel has impressive antitumor activity in breast cancer.

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Evaluation of Novel Agents Which Target Neovasculature of Breast Tumors

Introduction

Biological studies examining the development of the vascular tree in normal development and in disease states have identified numerous cytokines and their receptors responsible for triggering and maintaining this process *(1-7)*. Tumor neovascularization is central not only to the growth and development of the primary lesion but appears to be a critical factor in the development and maintenance of metastases *(8-12)*. Clinical studies in bladder cancer *(9)* have demonstrated a correlation between micro-vessel density and metastases. In addition, studies of breast cancer metastases by Fox et al. and Aranda et al. *(11-12)* have demonstrated that microvessel count in primary tumors appears to be related to the presence of metastases in lymph nodes and micrometastases in bone marrow.

The cytokine vascular endothelial growth factor-A (VEGF-A) and its receptors Flt-1(Receptor 1, R1) and KDR(Receptor 2, R2) have been implicated as one of the central mediators of normal angiogenesis and tumor neovascularization *(13-20)*. Up-regulation or over-expression of the KDR receptors or the VEGF-A ligand itself have been implicated as poor prognostic markers in various clinical studies of colon, breast and pituitary cancers *(21-23)*. Recently, Padro et al. *(24)* have suggested that both VEGF-A and KDR may play a role in the neovascularization observed in bone marrow during AML tumor progression and may provide evidence that the VEGF/KDR pathway is important in leukemic growth particularly in the bone marrow.

For these reasons, there have been several groups interested in developing therapeutic agents and approaches targeting the VEGF-A pathway. Agents which prevent VEGF-A binding to its receptor, antibodies which directly block the KDR receptor itself and small molecules which block the kinase activity of the KDR and thereby block growth factor signaling are all under development *(25-37)*. Recently, our laboratory reported the development of a growth factor fusion construct of $VEGF_{121}$ and the recombinant toxin gelonin *(38)*. Our studies demonstrated that this agent was specifically cytotoxic only to cells expressing the KDR receptor and was not cytotoxic to cells over-expressing the Flt-1 receptor. In addition, this agent was shown to localize within tumor vasculature and caused a significant damage to vascular endothelium in both PC-3 prostate and MDA-MB-231 orthotopic xenograft tumor models.

The current study seeks to extend our original observations describing the in vitro biological effects of this novel fusion construct and we examined the effects of this agent in both orthotopic and metastatic tumor models.

Body

Original SOW:

1. **Establish In Vivo Activity of the $VEGF_{121}$/rGel Fusion Toxin in the MDA-MB231 Tumor Models**

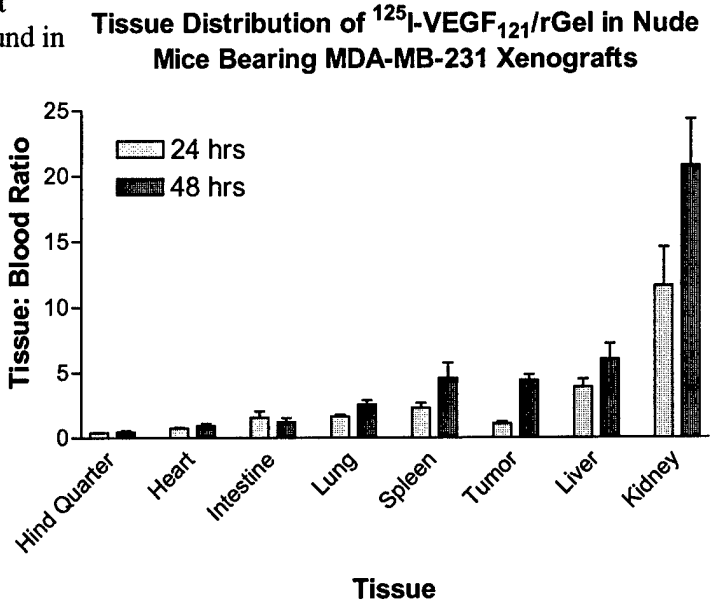
Task 1: Radiolabeling

Numerous methods were employed initially to label the target protein. We settled on using Bolton-Hunter reagent which generated the highest yield of material capable of specific binding to purified, immobilized KDR receptor. Highly purified $VEGF_{121}$/rGel was radiolabeled using ^{125}I with this reagent and the material was adjusted to a specific activity of 7.17 mg/mCi.

Task 2: Tissue Distribution

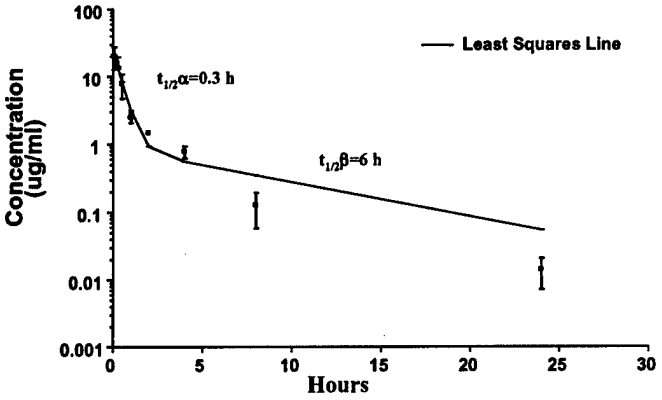
Mice bearing orthotopically-placed MDA-MB231 tumors were administered XXXmCi of $VEGF_{121}$/rGel(via iv tail vein). At 24 and 48 hrs after administration, groups of 6 mice were sacrificed and various organs were excised, weighed and counted to determine ^{125}I activity.

As shown in this figure, the highest concentration of radiolabel was found in kidney> liver>tumor> spleen. At 48 hrs, the tissue:blood ratio in these organs increased particularly for kidney and tumor. The high levels found in kidney may be related to high levels of the flt-1 receptor found in this organ.



The pharmacokinetics of $VEGF_{121}$/rGel were additionally described using this radiolabeled material. Balb/c mice were injected with 1 uCi of labeled material and at various times after administration, groups of 3 mice were sacrificed and blood samples were removed and centrifuged. Aliquots of plasma were counted to determine radioactivity and the results were analyzed for conventional pharmacokinetic analyses using conventional mathematical modeling(pK Analyst from MicroMath, Inc). As shown in the figure below, the $VEGF_{121}$/rGel cleared from plasma with initial and terminal half-lives of 0.3 and 6h respectively. Therefore this agent has a relatively long half-life despite the significant uptake in kidney.

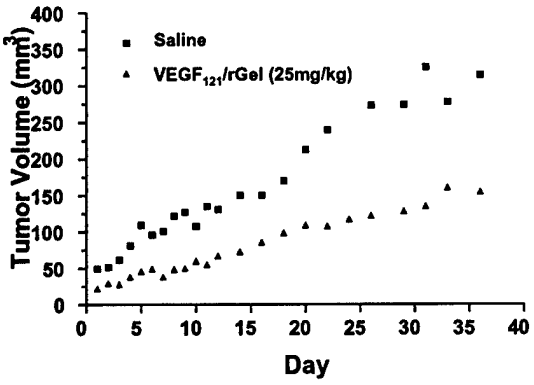
Clearance of $VEGF_{121}$/rGel From Plasma



Task 3: Antitumor Effects-Orthotopic Model

Given the pharmacokinetic and tissue distribution data described above, we designed a treatement schedule comprised of 5 injections spaced 48 hrs apart(10 day course). Using this schedule, we delineated the Maximum Tolerated Dose as this schedule to be ~25 mg/kg. The effect of $VEGF_{121}$/rGel administration on orthotopically-placed MDA MB231 tumor bearing mice(6 per group) is shown in the Figure below. As shown, treatment significantly retarded tumor growth. In addition, 3/6 mice in the treated group demonstrated complete disappearance of the tumor compared to 0/6 in the saline-treated group.

Effect of $VEGF_{121}$/rGel on Orthotopically Placed MDA-MB-231 Tumor Cells in Nude Mice

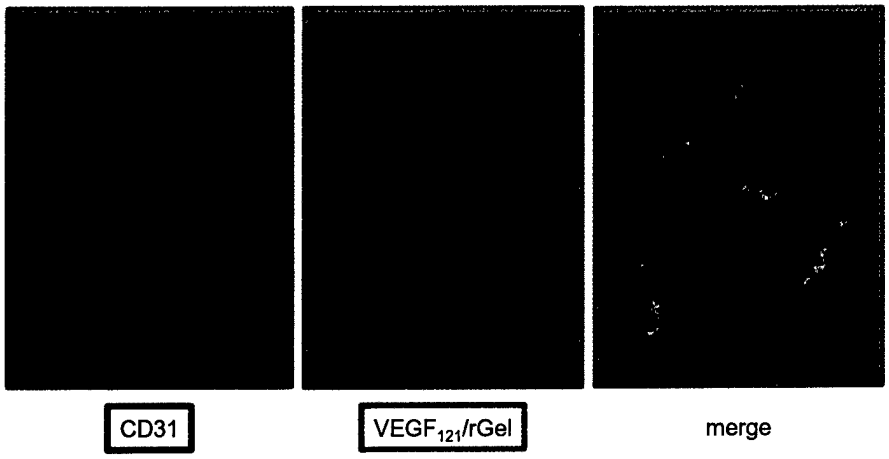


Task 4: Antitumor Effects-Metastatic Model

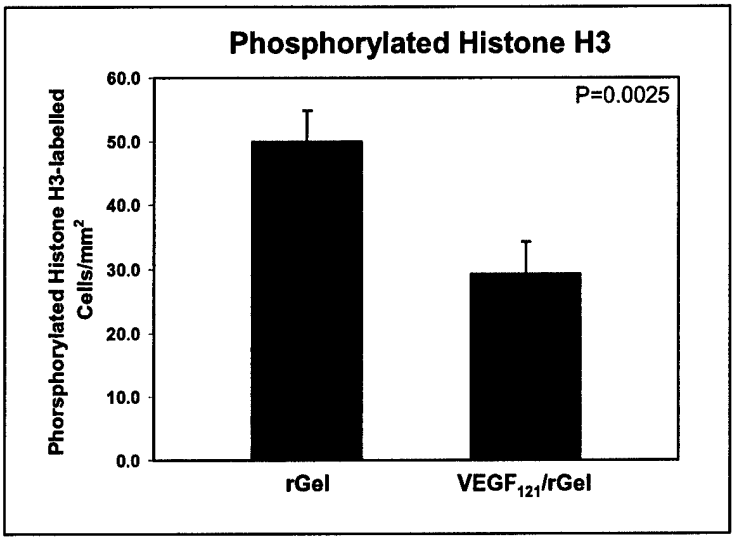
We evaluated the effect of $VEGF_{121}$/rGel fusion toxin treatment on the growth of metastatic MDA-MB-231 tumor cells in nude mice. Tumor cells ($0.5x10^6$ per mouse) were injected i.v. and 8 days after innoculation, mice (6 per group) were treated 6 times either with

$VEGF_{121}$/rGel (100 ug/dose) or free gelonin. Three weeks after treatment, mice were sacrificed and the lungs were harvested and examined. The surface lung foci in the $VEGF_{121}$/rGel – treated mice were reduced by 58 % as compared to gelonin control animals (means were 22.4 and 53.3 for $VEGF_{121}$/rGel and control, respectively; $p<0.05$). The mean area of lung colonies from $VEGF_{121}$/rGel-treated mice was also 50% smaller than control mice (210 ± 37 μm versus 415 ± 10 μm for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, the vascularity of metastatic foci as assessed by the mean number of blood vessels per mm^2 in metastatic foci was significantly reduced (198 ± 37 versus 388 ± 21 for treated and control, respectively). Approximately 62% of metastatic colonies from the $VEGF_{121}$/rGel-treated group had fewer than 10 vessels per colony as compared to 23% in the control group. The VEGF receptor(flk-1) was intensely detected on the metastatic vessels in the control but not on the vessels in the $VEGF_{121}$/rGel-treated group.

$VEGF_{121}$/rGel Localizes to Vasculature of Breast Tumor Foci in the Lungs of Mice



Mice bearing MDA-MB-435s lung tumor foci were injected i.v. with 50μg of $VEGF_{121}$/rGel or 20μg of free rGel (only tissues from $VEGF_{121}$/rGel injected mice are shown). One hour later, mice were sacrificed and tissues excised. Lung sections were double-stained using an anti-CD31 antibody and an anti-gelonin antibody to detect blood vessels (red) and localized $VEGF_{121}$/rGel (green), respectively. Co-localization of the stains is indicated by a yellow color. Free rGel did not localize to the vasculature of lung tumor foci (not shown). No $VEGF_{121}$/rGel staining was detected in any of the normal tissues examined (lung, liver, kidney, heart, spleen, pancreas, brain).



Frozen sections of lungs derived from $VEGF_{121}$/rGel- and rGel-treated mice bearing MDA-MB-231 lung tumor foci were stained with a phosphorylated histone H3 antibody. The number of tumor cells with phosphorylated histone H3 positive nuclei (mitotic cells) was counted in five high power fields of tumor sections from five mice per treatment group. The mean number per group ± SEM is presented and statistical significance was determined using the student's *t*-test.

Metastatic foci present in lung had a 3-fold lower Ki-67 labeling number compared to control tumors. These data strongly suggest that the anti-tumor vascular-ablative effect of $VEGF_{121}$/rGel could be utilized not only for treating primary tumors but also for inhibiting metastatic spread.

Task 5: Effects on Tissue Necrosis and Thrombosis

Please note that a complete manuscript detailing the impressive anti-metastatic effects of $VEGF_{121}$/rGel in this breast tumor model is attached in the Appendix #4 (The Vascular-Ablative Agent, $VEGF_{121}$/rGel, Inhibits Pulmonary Metastases of MDA-MB-231 Breast Tumors, by Ran et.al)

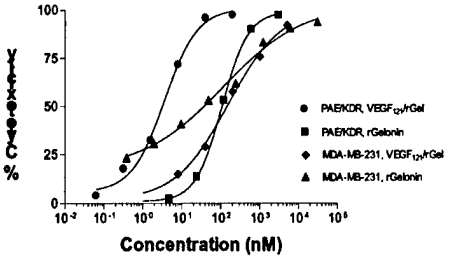
Task 6: Effects on Vascular Permeability and Task 6:Effects on Tumor Hypoxia

These studies are presently ongoing at MDACC and at UT Southwestern and the results will be reported in the next annual report.

Task 7: Co-Culture of Endothelial Cells and MDA-MB231 Tumor Cells

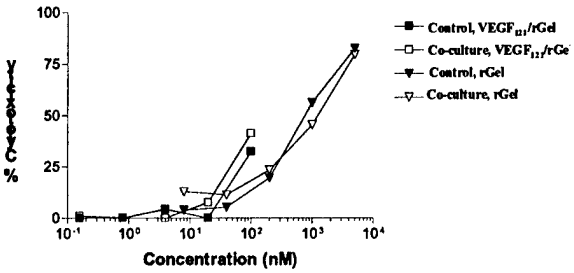
We utilized the Falcon multi-well culture plates to culture the MDA-MB231 tumor cells. Suspended above these cells, we placed an insert containing log-phase PAE/flk-1 cells. The 2 cell lines are cultured in the same DMEM/F-12 growth media, so no adaptation was required.

$VEGF_{121}$/rGel is not cytotoxic to MDA-MB-231 cells



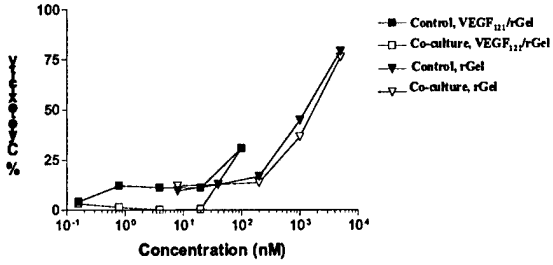
PAE/KDR and MDA-MB-231 cells were plated in 96-well plates and cultured over-night. Cells were then treated with $VEGF_{121}$/rGel or rGel for 72 hours, followed by determination of cytotoxicity by crystal violet staining. While $VEGF_{121}$/rGel is selectively cytotoxic to PAE/KDR cells compared to free gelonin, it does not show any targeted cytotoxicity towards MDA-MB-231 cells.

Effect of $VEGF_{121}$/rGel on MDA-MB-231 cells co-cultured with PAE/KDR cells (normoxia)



Forty thousand MDA-MB-231 cells were plated in 6-well plates and grown overnight. Cells were then co-cultured with twenty thousand PAE/KDR cells. Control cells were not co-cultured. Cells were treated with $VEGF_{121}$/rGel and rGel and incubated for a further 72 hours followed by determination of cytotoxicity by crystal violet staining. Co-culture of MDA-MB-231 cells with PAE/KDR cells does not effect the cytotoxicity of $VEGF_{121}$/rGel towards the breast tumor cells, even under $VEGF_{121}$/rGel concentrations cytotoxic towards PAE/KDR cells.

Effect of $VEGF_{121}$/rGel on MDA-MB-231 cells co-cultured with PAE/KDR cells (hypoxia)

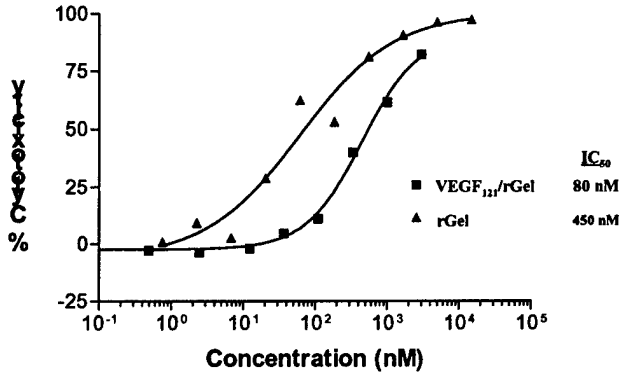


To determine if the cytotoxicity of $VEGF_{121}$/rGel towards MDA-MB-231 cells would change under hypoxic conditions in the presence and absence of PAE/KDR cells, we performed the co-culture experiment under hypoxic conditions. No specific effect of $VEGF_{121}$/rGel was observed as a result of hypoxia, either in the presence or absence of PAE/KDR cells, even under $VEGF_{121}$/rGel concentrations that were toxic to PAE/KDR cells.

Task 8: Studies of $VEGF_{121}$/rGel Effects on Endothelial and Breast Tumor Cells

In the co-culture chambers, the 2 cell lines were treated with various doses of the $VEGF_{121}$/rGel fusion construct targeting the flk-1 receptor on endothelial cells. The MDA-MB231 cells lack this receptor and were not affected by the doses utilized. At various times after drug administration, both cell lines were harvested, the RNA extracted and analyzed using the Gene Chip as described to assess the impact of treatment on over 7,000 genes including proteins involved in signal transduction, stress response, cell cycle control and metastasis. As shown below, we demonstrate the cytotoxic effects of the fusion construct on endothelial cells. Initial studies demonstrated that the initially-proposed PAE/flk-1 endothelial cells would be utilized, however, Gene Chip analysis showed no hybridization to isolated RNA samples. Troubleshooting demonstrated that there is insufficient homology of the porcine cell RNA to that of the human probes on the Gene Chip, therefore, HUVEC cells were substitued for this phase of study. As shown below, HUVEC cells showed specific cytotoxicity of the fusion construct compared to rGel itself although the magnitude of the differential is lower than that of the PAE/flk-1 cells.

Cytotoxicity of $VEGF_{121}$/rGel on HUVECs



As shown below, extensive micro-array analyses were performed on breast tumor cells as well as on endothelial cells as described in the original SOW. We identified a total of 22 unique genes upregulated by treatment with $VEGF_{121}$/rGel. These include genes involved in the control of cell adhesion, apoptosis, transcription regulation, chemotaxis and inflammatory response. These micro-array data were confirmed using Western analysis and RT-PCR and are further detailed in the attached manuscript (Appendix #5) submitted to the Journal of Biological Chemistry

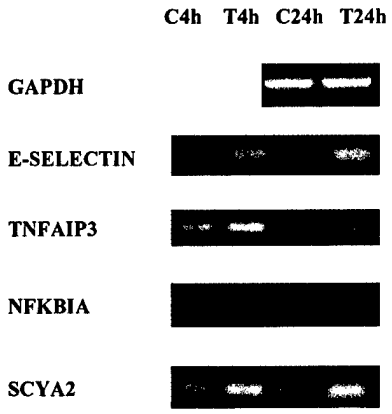
Genes overexpressed(>5X Control) in HUVECs by 24 hr treatment with VEGF121/rGel:

E Selectin (endothelial adhesion molecule 1)
Small inducible cytokine A2 (monocyte chemotactic protein 1)
Tumor necrosis factor, alpha-induced protein 3
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

Kinesin-like 5 (mitotic kinesin-like protein 1)
Small inducible cytokine A4
Jun B proto-oncogene
Nidogen 2
Prostaglandin-endoperoxide synthase 2
Dual specificity phosphatase 5
Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)
Plasminogen activator, urokinase
Human proteinase activated receptor-2 mRNA, 3'UTR
Ephrin-B2
Small inducible cytokine A7 (monocyte chemotactic protein 3)
Spermidine/spermine N1-acetyltransferase
Syndecan 4 (amphiglycan, ryudocan)
Chemokine (C-X-C motif), receptor 4 (fusin)
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)

All of the genes described above are known in the literature, however their association with cytotoxic events related to toxins such as rGel and activity on endothelial cells was previously unsuspected. To confirm these observations, these studies were repeated and RT-PCR was also employed to more closely assess the timelines for gene modulation. As shown below, our RT-PCR essentially confirmed the observations of the first 5 genes.

Up-regulation of mRNA in HUVECs treated with $VEGF_{121}$/rGel



C: No Treatment Controls

Tasks Still To Be Completed in the Next Year

Task 9: Effects of $VEGF_{121}$/rGel Exposure on Endothelial Cells Followed by Hypoxia

Task 10: Confirmation of In Vitro Gene Chip Findings With PCR Analysis of Tumor Samples

Task 11: Use of $VEGF_{121}$/rGel in combination with chemotherapeutic agents against MDA-MB231

Unanticipated, Novel Findings of related to $VEGF_{121}$/rGel and This Proposal

Breast cancer metastases to bone are associated with significant morbidity and mortality. Patients with advanced breast cancer experience frequent bone metastasis. However, the pathophysiological processes leading to the development of breast skeletal metastases remains poorly understood. Since breast skeletal metastases are essentially untreatable, improvements in our understanding of the biological mechanisms behind breast cancer metastases to bone could enhance the development of regimens to treat this disease. Therefore, we have initiated studies to improve our understanding of the development of breast skeletal metastases by examining how breast tumors remodel bone, which result in both osteoblastic and osteolytic lesions.

Osteoclastogenesis plays a central role in the development and maintenance of normal bone tissue, which requires osteoblastic matrix deposition and osteoclastic resorption to be closely coordinated. Interference with the process of osteoclastogenesis alters the kinetics of bone remodeling resulting in abnormal bone development. There is general consensus that the hematopoietically derived osteoclast is the pivotal cell in the degradation of the bone matrix. Osteoclast pre-cursor cells have been shown to be recruited to the future site of resorption by VEGF-A and RANKL, two cytokines that are expressed in the immediate vicinity of the bone surface. In addition, both of the major receptors of VEGF-A have been observed in osteoclasts, although some reports cite only the presence of Flt-1. The VEGF-Flt-1 interaction has been implicated in the recruitment process of osteoclast pre-cursor cells from hematopoietic tissue to the site of bone resorption. However, the role of each receptor, and its regulation, has yet to be established.

It has been hypothesized that osteoclasts play a critical role in the establishment of osteoblastic bone metastases by inducing bone resorption, which allows breast tumor cells to invade the bone and therefore promote tumor growth. VEGF plays an important role in the vascularization of bone tissues, as a mitogen for endothelial cells and as a chemo-attractant for both osteoblasts and osteoclasts. Therefore, establishing the precise role that each VEGF receptor plays in the maturation of osteoclast pre-cursor cells to osteoclasts is a critical step towards understanding the interaction that occurs between breast tumor cells and the bone microenvironment.

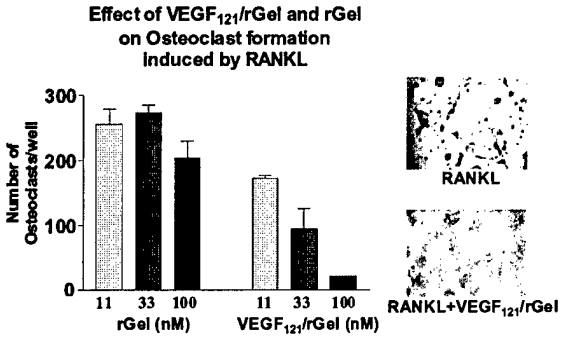


Figure 1. Effect of $VEGF_{121}$/rGel and rGel on RANKL-mediated osteoclast formation. Raw 264.7 cells were cultured overnight in 24-well plates. Osteoclast formation was induced by addition of 100 ng/ml RANKL with increasing concentrations of $VEGF_{121}$/rGel or rGel. Cells were allowed to differentiate for 96 hours followed by determination of the number of osteoclasts per well. Each experiment was performed in triplicate. The data shown is representative of three separate experiments. RANKL or RANKL + rGel-treated Raw 264.7 cells differentiate into large multi-nucleated TRAP-positive osteoclasts. In contrast, RANKL + $VEGF_{121}$/rGel-treated cells do not differentiate and do not stain for TRAP.

We have begun preliminary experiments examining the effect of $VEGF_{121}$/rGel on osteoclast formation in two model systems: (1) RAW 264.7 cells, cultured mouse osteoclast precursor cells that differentiate into mature osteoclasts upon stimulation with RANKL and (2) Bone marrow-derived macrophages (BMM), mouse primary cells that require stimulation with macrophage colony stimulating factor (MCSF) followed by RANKL for differentiation into osteoclasts. $VEGF_{121}$/rGel dramatically reduces osteoclastogenesis of both RAW 264.7 (Figure 1) and bone marrow-derived macrophages (Figure 2). Interestingly, a significantly lower concentration of $VEGF_{121}$/rGel is required

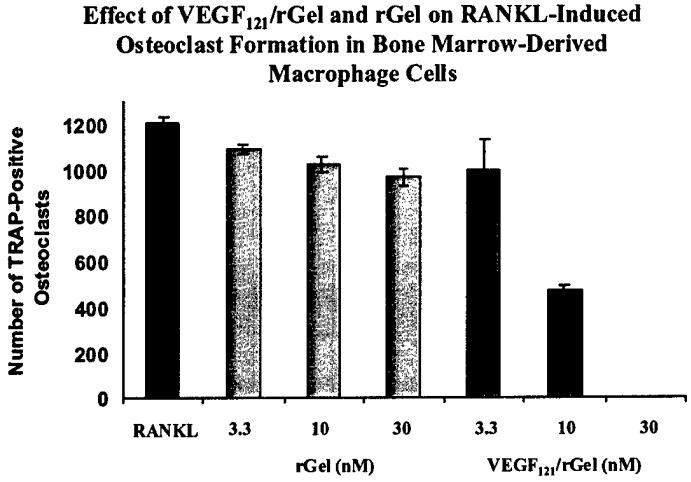
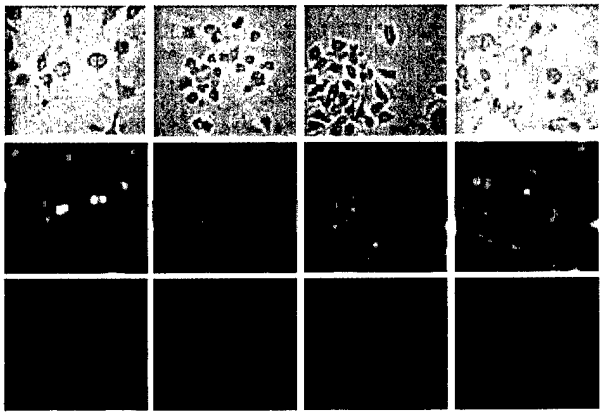


Figure 2. Effect of $VEGF_{121}$/rGel and rGel on RANKL-mediated osteoclast formation in bone marrow-derived macrophages (BMM). BMM cells were cultured overnight in 24-well plates with MCSF. Osteoclast formation was induced by addition of 100 ng/ml RANKL with increasing concentrations of $VEGF_{121}$/rGel or rGel. Cells were allowed to differentiate for 96 hours followed by determination of the number of osteoclasts per well. Each experiment was performed in duplicate. RANKL or RANKL + rGel-treated BMM cells differentiate into large multi-nucleated TRAP-positive osteoclasts. In contrast, RANKL + $VEGF_{121}$/rGel-treated cells do not differentiate and do not stain for TRAP to inhibit osteoclastogenesis in the primary bone marrow-derived cells than in RAW264.7 cells.

Treatment of Raw 264.7 cells with $VEGF_{121}$/rGel and rGel for 24 hours



[$VEGF_{121}$/rGel] (nM)	**10**	**-**	**-**	**100**
[rGel] (nM)	**-**	**1000**	**-**	**-**
Primary Ab	**Yes**	**Yes**	**Yes**	**No**
Secondary Ab	**Yes**	**Yes**	**Yes**	**Yes**

Figure 3. $VEGF_{121}$/rGel is internalized into RAW 264.7 cells. One thousand RAW 264.7 cells were plated into chamber slides and cultured overnight. Cells were then treated with $VEGF_{121}$/rGel and rGel for 24 hours. After the cell surface was stripped of membrane-bound $VEGF_{121}$/rGel or rGel, non-specific binding sites were blocked with 5% BSA and cells were permeabilized. Cells were treated with rabbit anti-gelonin (1:100) overnight followed by FITC-conjugated secondary antibody (1:80). Nuclei were stained with Propidium Iodide (middle row). $VEGF_{121}$/rGel is specifically internalized into RAW 264.7 cells (Column 1) as a 100-fold increase in rGel results in significantly lower internalizion. Columns 3 and 4 serve as negative controls without antigen or primary antibody respectively.

In attempt to understand if the inhibition of osteoclastogenesis by $VEGF_{121}$/rGel is mediated by entry of the molecule into the cell or solely by disruption via cell signaling, we performed immunohistochemistry experiments on RAW 264.7 cells. Our results, shown in Figure 3, indicate that $VEGF_{121}$/rGel is internalized into the osteoclast pre-cursor cells within 24 hours, and that the internalization is due to the presence of $VEGF_{121}$, not gelonin. Thus, it is

likely that inhibition of osteoclastogenesis is mediated by one of the receptors for $VEGF_{121}$, Flt-1 (VEGFR-1) or Flk-1/KDR (VEGFR-2). In support of our hypothesis that inhibition of osteoclastogenesis is not solely due to disruption of signaling at the cell surface, we have observed that activation of ERK1/2 occurs in cells treated with RANKL with or without $VEGF_{121}$/rGel or rGel.

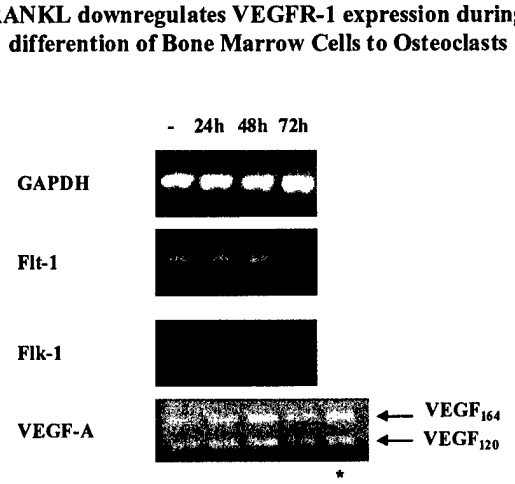


Figure 4. Flt-1 levels are down-regulated during osteoclastogenesis. Bone marrow-derived macrophage (BMM) cells were isolated and cultured for 72 hours with MCSF with or without stimulation with RANKL for 24, 48 and 72 hours. Total RNA was harvested and subjected to PCR analysis using primers specific for Flk-1, Flt-1 and four isoforms of VEGF-A. BMM cells do not express Flk-1. In contrast, PCR analysis suggests that Flt-1 is expressed by BMM cells, and that these levels decline during osteoclastogenesis. BMM cells also express $VEGF_{120}$ and $VEGF_{164}$ isoforms, but not the $VEGF_{189}$ and $VEGF_{144}$ isoforms, similar to MDA-MB-231 cells (indicated by *).

In order to understand whether the effect of $VEGF_{121}$/rGel is receptor-mediated, we have begun experiments to identify the presence of $VEGF_{121}$ receptors, and their role during osteoclastogenesis. This information is critical because the receptor target through which $VEGF_{121}$/rGel effects are mediated on osteoclasts is not known. This is significant because $VEGF_{121}$/rGel may inhibit breast cancer osteoblastic and osteolytic lesions in bone as a result of osteoclastogenesis inhibition. We have identified the presence of Flt-1, but not Flk-1, in BMM cells by PCR analysis (Figure 4). Interestingly, the levels of Flt-1 appear to decrease during osteoclast formation. We will examine the role of Flt-1 in this process by testing if $VEGF_{121}$/rGel effects the level of Flt-1 during osteoclastogenesis, both by PCR and by Western blot. We also plan on performing co-culture experiments with breast tumor cells.

Key Research Accomplishments

- **Described the Pharmacokinetics and Tissue Distribution of $VEGF_{121}$/rGel to allow rational development of an optimal therapeutic schedule on which to base both murine and eventual clinical studies.**

- **Described significant in vivo antitumor effects of $VEGF_{121}$/rGel against orthotopicially-placed breast tumor xenografts. Demonstrated complete regression of primary orthotopic breast tumors in 50% of treated mice(3/6).**
- **Identified significant vascular-ablative effects of $VEGF_{121}$/rGel on breast metastatic foci present in lung. Identified the impact this agent appears to have in suppressing the development of tumor metastases by destruction of tumor vascular endotheluim and drug-induced downregulation of flk-1 receptors in tumor endothelium.**
- **Identified a unique aspect of therapy using $VEGF_{121}$/rGel in that lung metastases of treated mice have virtually no vasculature and appear to grow to the approximate limit of oxygen diffusion for avascular tissues.**
- **Identified 20 unique genes associated with the development cytotoxic effects of the rGel toxin component on vascular endothelial cells.**
- **Confirmed the Gene Chip results via RT-PCR and Western analysis. Provided a unique gene "fingerprint" for intoxication of cells by $VEGF_{121}$/rGel which provides information as to the exact molecular mechanism of action of this agent and may provide a molecular rationale for combinations with other therapeutic agents.**
- **Provided significant rationale for continued pre-clinical development of $VEGF_{121}$/rGel as a vascular-ablative agent in breast cancer.**
- **Described the unique ability of this fusion construct to inhibit skeletal tumor metastases in vivo. The mechanism of action appears to be through direct inhibition of normal osteoclast function which appears to be essential for bone remodeling in the development of skeletal metastases.**
- **Identified an unanticipated mechanism of action of this fusion construct which indicates a role for VEGF in development of skeletal metastases.**

Reportable Outcomes

Abstracts:

1. Luster, T. A., Ran, S., Mohamedali, K. A., Rosenblum, M. G., and Thorpe, P. The vascular targeting agent, $VEGF_{121}$/rGel, inhibits the growth of human MDA-MB-231 breast tumors in the lungs of SCID mice. AB3001, AACR, 2004 (See Appendices #1).

2. Mohamedali, K. A., Gomez-Manzano, C., Ramdas, L., Xu, J., Cheung, L., Luster, T., Thorpe, P., and Rosenblum, M. G. Vascular targeting with $VEGF_{121}$/rGel inhibits angiogenesis: specific effects assessed using micro-array analysis. AB2572, AACR, 2004 (See Appendices #2).

Complete Manuscripts:

1. Mohamedali, K. A., Ran, S., Cheung, L., Marks, J., Hittelman, W., Waltenberger, J., Thorpe, P., and Rosenblum, M. G. Mechanistic and internalization studies of $VEGF_{121}$/rGel: cytotoxicity on endothelial cells mediated by VEGFR2 but not by VEGFR1. International Journal of Cancer, accepted (See appendices #3).

2. Ran, S., Mohamedali, K., Thorpe, P., Rosenblum, M G. The vascular-ablative agent $VEGF_{121}$/rGel inhibits pulmonary metastases of MDA-MB-231 breast tumors. Cancer Research, accepted (See appendices #4).

3. Mohamedali, K. A., Gomez-Manzano, C., Ramdas, L., Xu, J., Cheung, L., Zhang, W., Thorpe, P., and Rosenblum, M. G. Targeting the KDR receptor with $VEGF_{121}$/rGel fusion toxin inhibits vascular endothelial growth: specific effects assessed using microarray analysis. The Journal of Biological Chemistry, submitted (See Appendices #5).

Conclusions:

Vascular targeting as an approach to tumor therapy holds significant promise for the treatment of solid tumors. However, many current approaches attempting to inhibit the neovascularization process through small molecule inibitors of VEGFR signaling, antibodies to VEGF itself or to the VEGFR2 have not met with success. This is due in part to the multiply-redundant and robust process which tumor vascularization represents. On the other hand, lethal damage to tumor endothelium using the $VEGF_{121}$/rGel fusion toxin is a comparatively unique approach. This construct has remarkable and long-term antitumor effects in xenograft models as opposed to other agents which have limited activity in their own right. Dr. Louise Gorchow , Head of CTEP has indicated in a public presentation that the $VEGF_{121}$/rGel fusion toxin is one of a very few agents with these properties. The data presented above and in the attached Appendix demonstrates that this agent can reduce the growth of both orthotopic breast tumors and can significantly limit the metastatic spread of a breast metastatic model. In addition, the lung metastases which do survive appear to have a much lower vascular supply and a limited tumor cell turnover rate suggesting a reduced growth and metastatic potential. No other vascular targeting agents have thus far demonstrated such unique effects in an in vivo model. Of interest will be to examine the impact this fusion toxin will have on survival in this metastatic model. In addition, our findings examining the mechanism of direct action of the fusion construct on endothelial cells has significance in more exactly understanding how toxins work at the molecular level and may be an important first step in understanding how to more effectively employ these agents for therapeutic advantage. Furthermore, the importance of understanding how vascular targeting agents affect tumor cells indirectly may also have therapeutic significance in understanding the rationale for combinations of these vascular targeting agents with conventional chemotherapeutic agents, or with radiotherapeutic or biological agents. Finally, we have identified that development of breast tumor skeletal metastases apparently require VEGF and the VEGF-receptor pathway. Our studies have demonstrated that the $VEGF_{121}$/rGel fusion construct can significantly inhibit skeletal metastases through a unique inhibition of osteoclast maturation and function in vitro and probably in vivo. This suggests that the VEGF/rGel fusion construct may therefore be useful for the prevention and treatment of skeletal metastases in breast cancer and perhaps for skeletal metastases in other types of cancers as well.

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Appendices

1. Luster, T. A., Ran, S., Mohamedali, K. A., Rosenblum, M. G., and Thorpe, P. The vascular targeting agent, $VEGF_{121}$/rGel, inhibits the growth of human MDA-MB-231 breast tumors in the lungs of SCID mice. AB3001, AACR, 2004.

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Abstract Number: 3001

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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Abstract Number: 2572

Vascular targeting with $VEGF_{121}$/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.

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MECHANISTIC AND INTERNALIZATION STUDIES OF $VEGF_{121}$/rGEL: CYTOTOXICITY ON ENDOTHELIAL CELLS MEDIATED BY VEGFR2 BUT NOT BY VEGFR1

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Running Title: $VEGF_{121}$/rGel cytotoxicity mediated by VEGFR2

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Keywords: fusion toxin, vascular targeting, gelonin, VEGF, KDR

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Abstract

We have previously reported the targeting of the neovasculature of solid tumors with $VEGF_{121}$/rGel, a chimeric fusion toxin of $VEGF_{121}$ and recombinant gelonin, a low molecular weight single chain toxin with a mechanism of action similar to that of ricin A-chain. While several studies have shown both receptors of $VEGF_{121}$, namely VEGFR1(FLT-1) and VEGFR2 (KDR/Flk-1), to be over-expressed on the endothelium of tumor vasculature, our *in vitro* studies have shown $VEGF_{121}$/rGel to be cytotoxic to cells over-expressing KDR (IC_{50} = 0.5 nM) but not Flt-1 (IC_{50} = 300 nM), compared to gelonin alone (IC_{50} = 300 nM). Cell ELISA using antibodies specific to either KDR or FLT-1 indicate binding of $VEGF_{121}$/rGel to the both cell lines. Incubation of PAE/KDR and PAE/FLT-1 with ^{125}I-$VEGF_{121}$/rGel demonstrated binding of the fusion toxin to both cell surfaces that was competed with cold $VEGF_{121}$/rGel but not cold gelonin. Both PAE/KDR and PAE/FLT-1 cells were incubated with $VEGF_{121}$/rGel for 1 hr, 4hr, 16 hr and 24 hrs and subjected to immunofluorescence using an anti-rGel antibody. Internalization of $VEGF_{121}$/rGel into PAE/KDR cells within one hour of treatment was observed. However, no $VEGF_{121}$/rGel was detected in PAE/FLT-1 cells up to 24 hours after treatment. Cells treated with rGel only did not immunofluoresce. The cytotoxic mechanism activated as a result of $VEGF_{121}$/rGel internalization is not clear; TUNEL assay over 72 hours did not result in cell death nor was PARP cleavage activated over 48 hours. We conclude that while $VEGF_{121}$/rGel binds to both FLT-1 and KDR, internalization of $VEGF_{121}$/rGel is mediated only by KDR and not FLT-1. $VEGF_{121}$/rGel is also an important molecule useful to probe the biology of KDR and FLT-1 receptors.

Introduction

Angiogenesis is a hallmark of cancer. Vascular endothelial growth factor-A (VEGF-A) plays a key role as the primary stimulant of vascularization in solid tumors[1-3]. VEGF-A enhances endothelial cell proliferation, migration, and survival[3-6] and is essential for blood vessel formation[7]. Other roles of VEGF include wound healing[8], vascular permeability and the regulation of blood flow[9-11]. The VEGF-A family of isomers range from 121 to 206 amino acids in length with varying receptor and heparin binding affinities[25] as a result of alternate splicing[12,13].

VEGF is released by a variety of tumor cells and studies have demonstrated that the VEGF receptor KDR/Flk-1, over-expressed on the endothelium of tumor vasculature, is primarily responsible for mediating the tumor neovascularization properties of VEGF. KDR/Flk-1 is almost undetectable in the vascular endothelium of adjacent normal tissues. $VEGF_{121}$ exists in solution as a disulfide linked homodimer and binds to KDR and FLT-1 in a heparin-independent manner. It does not bind neuropilin-1 or neuropilin-2. $VEGF_{121}$ has been shown to contain the full biological activity of the larger variants.

We have previously defined a novel fusion construct of $VEGF_{121}$ and the highly cytotoxic plant toxin Gelonin (rGel). Gelonin is a 28.5 kDa single-chain toxin with a mechanism of action similar to that of ricin A-chain. The $VEGF_{121}$/rGel fusion toxin was shown to be highly cytotoxic to endothelial cells that over-express the KDR/Flk-1 receptor, but not to cells that over-express the FLT-1 receptor even though $VEGF_{121}$ binds to FLT-1 with greater affinity than to KDR[25]. In this report, we extend our initial findings and further characterize the biological effects of this fusion toxin on endothelial cells. We determined the binding profile of $VEGF_{121}$/rGel to receptors expressed on the cell surface

as well as investigated the internalization of $VEGF_{121}$/rGel.

Materials and Methods

Materials: Bacterial strains, pET bacterial expression plasmids and recombinant enterokinase were obtained from Novagen (Madison, WI). All other chemicals were from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). TALON metal affinity resin was obtained from Clontech laboratories (Palo Alto, CA). Other chromatography resin and materials were from Pharmacia Biotech (Piscataway, NJ). Endothelial cell growth supplement (ECGS) from bovine neural tissue was obtained from Sigma Chemical Company. Murine brain endothelioma (bEnd.3) cells were provided by Professor Werner Risau (Max Plank Institute, Munich, Germany). Tissue culture reagents were from Gibco BRL (Gaithersburg, MD) or Mediatech Cellgro (Herndon, VA). Rabbit anti-gelonin antisera was obtained from the Veterinary Medicine Core Facility at MDACC. Anti-flt-1 (sc-316), anti-flk-1 (sc-504), and anti-PARP (sc-8007) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture:. Porcine aortic endothelial cells transfected with the KDR receptor (PAE/KDR) or the FLT-1 receptor (PAE/FLT-1) were a generous gift from Dr. J. Waltenberger. Cells were maintained as a monolayer in F12 Nutrient Media (HAM) suplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum and passaged twice weekly. Cells were harvested by treatment with Versene (0.02% EDTA).

Purification of $VEGF_{121}$/rGel: Construction and purification of $VEGF_{121}$/rGel was essentially as described, with minor modifications[26]. E. coli cells were lysed with 100 ml 0.1mm glass beads (BioSpec Products, Inc) in a Bead Beater (BioSped Products, Inc) for

eight cycles of 3 minutes each. The lysate was ultracentrifuged at 40,000 rpm for 90 minutes at 4°C. The supernatant was carefully collected and adjusted to 40 mM Tris-HCl (pH 8.0), 300 mM NaCl, and incubated at 4°C with metal affinity resin. The resin was washed with 40 mM Tris-HCl (pH8.0), 0.5 M NaCl buffer containing 5 mM Imidazole and eluted with buffer containing 100 mM Imidazole. After pooling fractions containing $VEGF_{121}$/rGel, the sample was dialyzed against 20 mM Tris (pH 8.0), 200 mM NaCl and digested with recombinant Enterokinase at room temperature. Enterokinase was removed by agarose-linked soybean trypsin inhibitor. Other proteins of non-interest were removed by Q Sepharose Fast Flow resin and metal affinity resin as described previously[26]. $VEGF_{121}$/rGel was concentrated and stored in sterile PBS at –20°C.

Rabbit Reticulocyte Lysate Assay: The functional activity of rGel and $VEGF_{121}$/rGel were assayed using a cell-free protein translation inhibition assay kit from Amersham Biotech (Arlington Heights, IL) as described by the manufacturer.

ELISA Analysis: The ability of the chimeric fusion protein to bind to Flk-1 was tested on microtiter plates coated with soluble mouse Flk-1. All steps of the ELISA procedure were performed at room temperature unless stated otherwise. Plates were treated with 2 μg/ml of NeutrAvidin (Pierce, Rockford, IL) for 6 h. The extracellular domain of Flk-1 was expressed in Sf9 cells, purified to homogeneity[28], and biotinylated. Biotinylated receptor (1 μg/ml) was incubated with NeutrAvidin-coated wells for 2 h. $VEGF_{121}$ or $VEGF_{121}$/rGel was added to the wells at concentrations ranging from 0.002 to 2 nM in the presence of 2% BSA diluted in PBS or normal mouse serum. After 2 h of incubation, plates were washed

and incubated with non-blocking mouse monoclonal anti-VEGF antibody, 2C3[29] or rabbit polyclonal anti-gelonin IgG. For competition studies of $VEGF_{121}$/rGel and $VEGF_{121}$, binding of the $VEGF_{121}$/rGel fusion protein was detected using a rabbit anti-gelonin antibody. In competition experiments, a 10-fold molar excess of $VEGF_{121}$ was premixed with $VEGF_{121}$/rGel before it was added to the plate. Mouse and rabbit IgG were detected by HRP-labeled goat anti-mouse and anti-rabbit antibodies, respectively (Daco, Carpinteria, CA). Peroxidase activity was measured by adding O-phenylenediamine (0.5 mg/ml) and hydrogen peroxide (0.03% v/v) in citrate-phosphate buffer (pH 5.5). The reaction was stopped by addition of 100 µl of 0.18 M of H_2SO_4. The absorbance was read at 490 nM. Presence of KDR and FLT-1 on cells was tested as follows: 50,000 cells (PAE/KDR or PAE/FLT-1) were aliquoted per well and dried overnight at 37°C. Non-specific binding sites were blocked for 1 hour with 5% BSA. Wells were treated with anti-KDR or anti-FLT-1 antibodies, followed by incubation with HRP-conjugated secondary antibody (1:5000) for 1 hour.

Western Blot Analysis: Whole cell lysates of PAE/KDR and PAE/FLT-1 cells were obtained by lysing cells in Cell Lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol) supplemented with protease inhibitors (leupeptin (0.5%), aprotinin (0.5%) and PMSF (0.1%)). Protein samples were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF memberane overnight at 4°C in transfer buffer (25 mM Tris-HCl, pH 7.6, 190 mM glycine, 20% HPLC-grade methanol). The samples were analyzed for KDR with rabbit anti-flk-1 polyclonal antibody and FLT-1 using an anti-flt-1 polyclonal antibody. The membranes

were then incubated with goat-anti-rabbit IgG horseradish peroxidase (HRP), developed using the Amersham ECL detection system and exposed to X-ray film.

Immunoprecipitation: Cells were lysed as described (see western protocol). 500 μg MDA-MB-231 and L3.6pl cell lysates were mixed with 2 μg anti-flk-1 antibody in a final volume of 250 μl and incubated for two hours at 4°C. 100 μg PAE/KDR and PAE/FLT-1 cell lysates were immunoprecipitated as controls. The mixtures were then incubated overnight with 20 μl Protein A beads that had been blocked with 5% BSA. The beads were washed 4 times in lysis buffer and the samples, along with 30 μg PAE/KDR cell lysate, were run on a gel, transferred overnight onto a PVDF membrane and probed using an anti-flk-1 polyclonal antibody.

Binding of Radiolabeled $VEGF_{121}$/rGel to PAE/KDR and PAE/FLT-1 cells: 100 μg of $VEGF_{121}$/rGel was radiolabeled with 1mCi of NaI^{125} using Chloramine T[27] for a specific activity of 602 Ci/mMol. Cells were grown overnight in 24-well plates. Non-specific binding sites were blocked for 30 minutes with PBS/0.2% gelatin followed by incubation for 4 hours with ^{125}I-$VEGF_{121}$/rGel in PBS/0.2% gelatin solution. For competition experiments, cold $VEGF_{121}$/rGel or gelonin were pre-mixed with ^{125}I-$VEGF_{121}$/rGel. Cells were washed four times with PBS/0.2% gelatin solution, detached and bound cpm was measured.

Cytoxicity of $VEGF_{121}$/rGel and rGel: Cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase PAE/KDR cells was performed as described[26]. Cells were grown in 96 well flat-

bottom tissue culture plates. Purified $VEGF_{121}$/rGel and rGel were diluted in culture media and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 M sodium citrate, pH 4.2 in 50% ethanol). Absorbance was measured at 630 nm. To assess if the activity of $VEGF_{121}$/rGel was affected by the exposure time to endothelial cells, log-phase PAE/KDR cells were grown and treated with $VEGF_{121}$/rGel as above. Media containing the cytotoxic agent was removed at varying time-points and the cells were washed once with 200 µl culture media. Fresh culture media was added to the wells and the cells were then returned to the incubator. Seventy-two hours after the start of the experiment, the number of remaining adherent cells was assessed using crystal violet and Sorensen's buffer as described above.

Internalization of $VEGF_{121}$/rGel into PAE/KDR cells: PAE/KDR and PAE/FLT-1 cells were incubated with 4 µg/ml $VEGF_{121}$/rGel at the timepoints indicated. After stripping the cell surface, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Non-specific binding sites were blocked with 5% BSA in PBS. Cells were then incubated with a rabbit anti-gelonin polyclonal antibody (1:200) followed by a TRITC-conjugated anti-rabbit secondary antibody (1:80). Nuclei were stained with propidium iodide (1µg/ml) in PBS. The slides were fixed with DABCO media, mounted and visualized under fluorescence (Nikon Eclipse TS1000) and confocal (Zeiss LSM 510) microscopes.

TUNEL assay: Log phase PAE/KDR and PAE/FLT-1 cells were diluted to 2000 cells/100 μl. Aliquots (100 μl) were added in 16-well chamber slides (Nalge Nunc International) and incubated overnight at 37°C with 5% CO_2. Purified $VEGF_{121}$/rGel was diluted in culture media and added at 72, 48 and 24 hour time points at a final concentration of 1 nM (twice the IC_{50}). The cells were then processed and analyzed for TUNEL as described by the manufacturer of the reagent. Positive control cells were incubated with 1mg/ml DNAse for 10 minutes at 37°C.

PARP Cleavage: Effects of $VEGF_{121}$/rGel on PARP-mediated apoptosis were investigated by pre-incubating PAE/KDR cells with 100 mM Na_2VO_4 for 5 minutes at 37°C followed by stimulation with $VEGF_{121}$/rGel or $VEGF_{121}$ for 5 minutes, 30 minutes, 4 h, 24 h and 48 h. Cells were washed and lysed and the cell lysate was analyzed by Western using an anti-PARP antibody.

Results

We have previously demonstrated the successful use of $VEGF_{121}$/rGel fusion construct for the targeted destruction of tumor vasculature in vivo. The combination of $VEGF_{121}$ and recombinant gelonin was originally prepared in two different orientations (Figure 1) with both orientations displaying similar cytotoxicity profiles. However, the orientation with $VEGF_{121}$ at the N-terminus results in a higher yield following purification from bacteria (data not shown), and is used in subsequent experiments.

Biological Activity of the rGel Component: The ability of $VEGF_{121}$/rGel and rGel to inhibit translation in a cell-free system was determined by using a rabbit reticulocyte translation assay (Figure 2). The purified $VEGF_{121}$/rGel and rGel had IC_{50} values of 47 and 234 pM, respectively, showing that fusion of rGel and $VEGF_{121}$ did not reduce the activity of the toxin component.

Binding of $VEGF_{121}$/rGel to Soluble Flk-1 Receptor: The fusion protein was tested for its ability to bind to the Flk-1 receptor by ELISA. The extra-cellular domain of recombinant Flk-1 was purified, biotinylated and incubated with wells coated with NeutrAvidin. The receptor was treated with $VEGF_{121}$ or $VEGF_{121}$/rGel, and binding of the ligand to the receptor was assessed by anti-VEGF and anti-gelonin antibodies. Figure 3 shows that $VEGF_{121}$/rGel and native human $VEGF_{121}$ bind equally well to Flk-1 at all concentrations, indicating that the VEGF component of the fusion protein is fully capable of binding to Flk-1. To confirm that the binding of $VEGF_{121}$/rGel to Flk-1 was specific,

free $VEGF_{121}$ (10-fold molar excess) was used to compete with $VEGF_{121}$/rGel for binding to the receptor, followed by detection of $VEGF_{121}$/rGel binding by an anti-gelonin antibody. Binding of $VEGF_{121}$/rGel to the Flk-1 extra-cellular domain was dramatically reduced in the presence of $VEGF_{121}$ (Figure 4) indicating that the $VEGF_{121}$ domain of the fusion toxin retained activity and that binding to the receptor was specific.

$VEGF_{121}$/rGel binds to both KDR and FLT-1: $VEGF_{121}$ has been shown to bind to the FLT-1 receptor with greater affinity than to KDR[25]. Because cytotoxicity of $VEGF_{121}$/rGel to KDR-expressing cells was found to be nearly 600-fold greating than for FLT-1 expressing cells, we investigated the relative binding of $VEGF_{121}$/rGel to PAE cells expressing each of the receptors. ELISA analysis was performed to confirm the expression of both receptors on the cell surface using receptor-specific antibodies (data not shown). Expression of VEGFR-1 (FLT-1) and VEGFR-2 (KDR) was confirmed by western blot (Figure 5a). In order to confirm that $VEGF_{121}$/rGel bound to human VEGFR-1 and VEGFR-2 and that the presence of recombinant gelonin did not interfere with the binding properties of $VEGF_{121}$, we investigated the binding of radiolabeled $VEGF_{121}$/rGel to both KDR and FLT-1 receptors expressed on the surface of PAE cells. Our results (Figure 5b) show that the binding of ^{125}I-$VEGF_{121}$/rGel to both cells was nearly identical. In order to confirm that the binding on the cell surface was receptor-specific, competition studies of ^{125}I-$VEGF_{121}$/rGel with unlabeled $VEGF_{121}$/rGel and rGel were performed. Binding of $VEGF_{121}$/rGel to both PAE/KDR and PAE/FLT-1 cells was competed by unlabeled $VEGF_{121}$/rGel but not by unlabeled gelonin indicating that binding of $VEGF_{121}$/rGel was mediated by $VEGF_{121}$ and, therefore, receptor-specific.

Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells. We investigated the internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells using immunofluorescence staining. Cells were attached, treated with $VEGF_{121}$/rGel or rGelonin at various time points, and then treated with polyclonal rabbit anti-gelonin primary antibody and FITC-conjugated secondary antibody. $VEGF_{121}$/rGel was detected in PAE/KDR cells within 1 hour of treatment with the immunofluoresence signal progressively increasing to 24 hours (Figure 6). As expected, cell density also decreased over the 24 hour time period. No $VEGF_{121}$/rGel was detected in PAE/FLT-1 cells up to 24 hours after treatment with the fusion toxin. Treatment of cells with the same concentration of rGelonin showed no internalization, confirming that entry of $VEGF_{121}$/rGel into PAE cells was specifically via the KDR receptor.

Exposure duration of $VEGF_{121}$/rGel on endothelial cells: The IC_{50} of $VEGF_{121}$/rGel incubated for 72 hours on log-phase PAE/KDR cells has been shown to be about 1 nM[26]. However, $VEGF_{121}$/rGel internalizes into these cells within one hour of incubation. To study the cytototoxic effect of $VEGF_{121}$/rGel as a function of exposure time of this agent on endothelial cells, we incubated cells with $VEGF_{121}$/rGel from 1-72 hours and assessed its cytotoxicity on PAE/KDR cells at the end of the 72-hour period. While $VEGF_{121}$/rGel retained cytotoxicity even after a one hour incubation, appreciable cytotoxicity was observed after 24 hours and maximal cytotoxic effect of $VEGF_{121}$/rGel on PAE/KDR cells

was observed after 48 hours (Figure 7). The cytotoxic effect of $VEGF_{121}$/rGel on PAE/FLT-1 cells was also affected as a function of exposure duration (data not shown).

TUNEL assay and PARP Cleavage. In order to investigate the mechanism of cytotoxicity of $VEGF_{121}$/rGel to PAE/KDR cells, we performed a TUNEL assay for 24, 48 and 72 hours. No TUNEL staining was observed with PAE/KDR cells exposed to $VEGF_{121}$/rGel up to 72 hours (Figure 8). In contrast nuclei of positive control cells showed intense staining, indicating that the mechanism of cytotoxicity of $VEGF_{121}$/rGel is not apoptotic. PARP cleavage was tested on PAE/KDR cells by treating cells with $VEGF_{121}$/rGel or $VEGF_{121}$ for periods ranging from 5 minutes to 48 hours. Western blot analysis of these cells by an anti-PARP antibody shows that $VEGF_{121}$/rGel did not activate PARP-mediated apoptosis (Figure 9).

Discussion

Agents targeting the neovascularization process in tumors have significant potential for therapeutic impact. Molecules which interfere with the growth and development of vascular endothelial cells by targeting the VEGF/receptor complex have an additional advantage since these agents do not have to penetrate into the tumor parenchyma and the receptor targets are expressed on the luminal surface of tumor vascular endothelium.

Although this study demonstrates that the VEGF/rGel fusion can bind to both the KDR and FLT-1 receptors, we found that only cells which express KDR were able to internalize the construct thereby delivering the toxin component to the cytoplasmic compartment. Zheng et al [3] suggest that it is the interaction of VEGF with the KDR receptor but not the FLT-1 receptor which is responsible for the growth proliferative signal on endothelial cells and other studies suggest that the KDR receptor is primarily responsible for mediating the vascular permeability effects of VEGF-A [14]. Studies suggest that the FLT-1 receptor can modulate signaling of the KDR receptor[3] and may impact monocyte response to VEGF[15], but its role in neovascularization has not been well-defined.

The possible binding of VEGF-containing constructs to the neuropilin receptor could be a source of unwanted toxicity and mis-targeting of the complex, however, studies by Gluzman et al.[16] have demonstrated that the $VEGF_{121}$ fragment as opposed to other forms of VEGF-A does not appear to bind to this receptor.

Another important aspect of this study was the observation that the cytotoxic effects of the construct on vascular endothelial cells did not involve an apoptotic mechanism. This is in sharp contrast to studies of other toxins such as ricin A chain (RTA)

and pseudomonas exotoxin (PE) which demonstrate generation of apoptotic effects which appear to be mediated, at least in part, by caspase activation[17-19]. Recently, Keppler-Hafkemeyer, et al[20] have suggested that PE toxins may generate cytotoxic effects through both caspase-dependant and protein synthesis inhibitory mechanisms. Despite the sequence homology of RTA and rGel[21] and the known similarities in their mechanism of action[22,23], it appears that these two toxins differ in their pro-apoptotic effects. One possible explanation for the observed differences in apoptotic effects between RTA and rGel toxin could be in the cell types examined. The cells targeted in the current study of rGel are non-transformed endothelial cells while those in the RTA study were tumor cells.

The exposure duration studies for the $VEGF_{121}$/rGel fusion toxin demonstrate that as little as 1 hr exposure to target cells is required to develop a cytotoxic effect 72 hrs later. However, continual exposure for up to 48 hrs was shown to improve the cytotoxic effect by almost 10 fold. Should pharmacokinetic studies demonstrate a relatively short plasma half-life for this agent, this may suggest that optimal therapeutic effect could be achieved by maintaining blood concentrations of drug at therapeutic concentrations for at least 48 hrs. This could be achieved by frequent interval dosing or continuous infusion but may be important in the development of pre-clinical and clinical dosing strategies.

Figure Legends

Figure 1: Design and construction of $VEGF_{121}$/rGel. Constructs of the targeting molecule ($VEGF_{121}$) to the cytotoxic agent (gelonin) were expressed in two orientations, with either $VEGF_{121}$ or gelonin at the N-terminus. A G4S tether was used to fuse $VEGF_{121}$ and gelonin and reduce steric hindrance.

Figure 2: Rabbit reticulocyte assay to determine the ability of $VEGF_{121}$/rGel and rGel to inhibit translation in a cell-free system. The fusion of $VEGF_{121}$ and recombinant gelonin does not reduce the activity of the toxin component.

Figure 3: ELISA demonstrating that $VEGF_{121}$/rGel binds to the receptor. $VEGF_{121}$/rGel, $VEGF_{121}$ and rGel were incubated with biotinylated mouse flk-1 receptor attached to NeutrAvidin-coated plates. Binding was assessed using anti-gelonin and anti-VEGF antibodies.

Figure 4: Binding to the anchored flk-1 receptor is specific for $VEGF_{121}$/rGel. $VEGF_{121}$/rGel or $VEGF_{121}$ was incubated with flk-1 receptor as described in Materials and Methods. Binding of $VEGF_{121}$/rGel was competed with $VEGF_{121}$ and a rabbit anti-gelonin antibody was used for detection. $VEGF_{121}$ specifically reduced binding of $VEGF_{121}$/rGel to flk-1. $VEGF_{121}$ was not detected by the anti-gelonin antibody (data not shown).

Figure 5: Expression of KDR and FLT-1. (A) Whole cell lysate (30 μg) of PAE/KDR and PAE/FLT-1 was run on an SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted using the appropriate antibody. Expression of both receptors on their respective cell-lines was confirmed. (B) Receptor-specific binding of radio-labeled $VEGF_{121}$/rGel is demonstrated on cells expressing these receptors. Binding was reduced with unlabeled $VEGF_{121}$/rGel but not by unlabeled gelonin.

Figure 6: Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells. PAE/KDR cells were incubated with 4 μg/ml $VEGF_{121}$/rGel at the timepoints indicated. Cells were then incubated with an anti-gelonin polyclonal antibody (1:200) followed by a FITC-conjugated secondary antibody (1:80). Nuclei were stained with propidium iodide. $VEGF_{121}$/rGel enters PAE/KDR cells within one hour of treatment. However, PAE/FLT-1 cells did not internalize $VEGF_{121}$/rGel even after 24 hours of incubation with $VEGF_{121}$/rGel.

Figure 7: Effect of exposure time of $VEGF_{121}$/rGel on PAE/KDR cells on cytotoxicity. $VEGF_{121}$/rGel was incubated with PAE/KDR cells for varying lengths of time, as described in Materials and Methods. While $VEGF_{121}$/rGel retained cytotoxicity towards PAE/KDR cells even with a 1 h exposure time, cytotoxicity of this fusion toxin was markedly enhanced by an exposure time of 48 hours.

Figure 8: Cytotoxicity of $VEGF_{121}$/rGel to PAE/KDR cells does not result in apoptosis. PAE/KDR cells were grown overnight. 1 nM $VEGF_{121}$/rGel (twice the IC_{50})

was added and incubated for 24, 48 and 72 hours. The cells were analyzed for TUNEL. Positive control cells were incubated with 1 mg/ml DNAse for 10 minutes at 37°C.

Figure 9: Treatment of PAE/KDR cells with $VEGF_{121}$/rGel does not result in PARP cleavage. PAE/KDR cells were stimulated with $VEGF_{121}$/rGel or $VEGF_{121}$ for the times indicated. Cells were washed and lysed and the cell lysate was analyzed by Western using an anti-PARP antibody. No PARP cleavage was observed.

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Orientation "A"

Orientation"B"

Figure 1

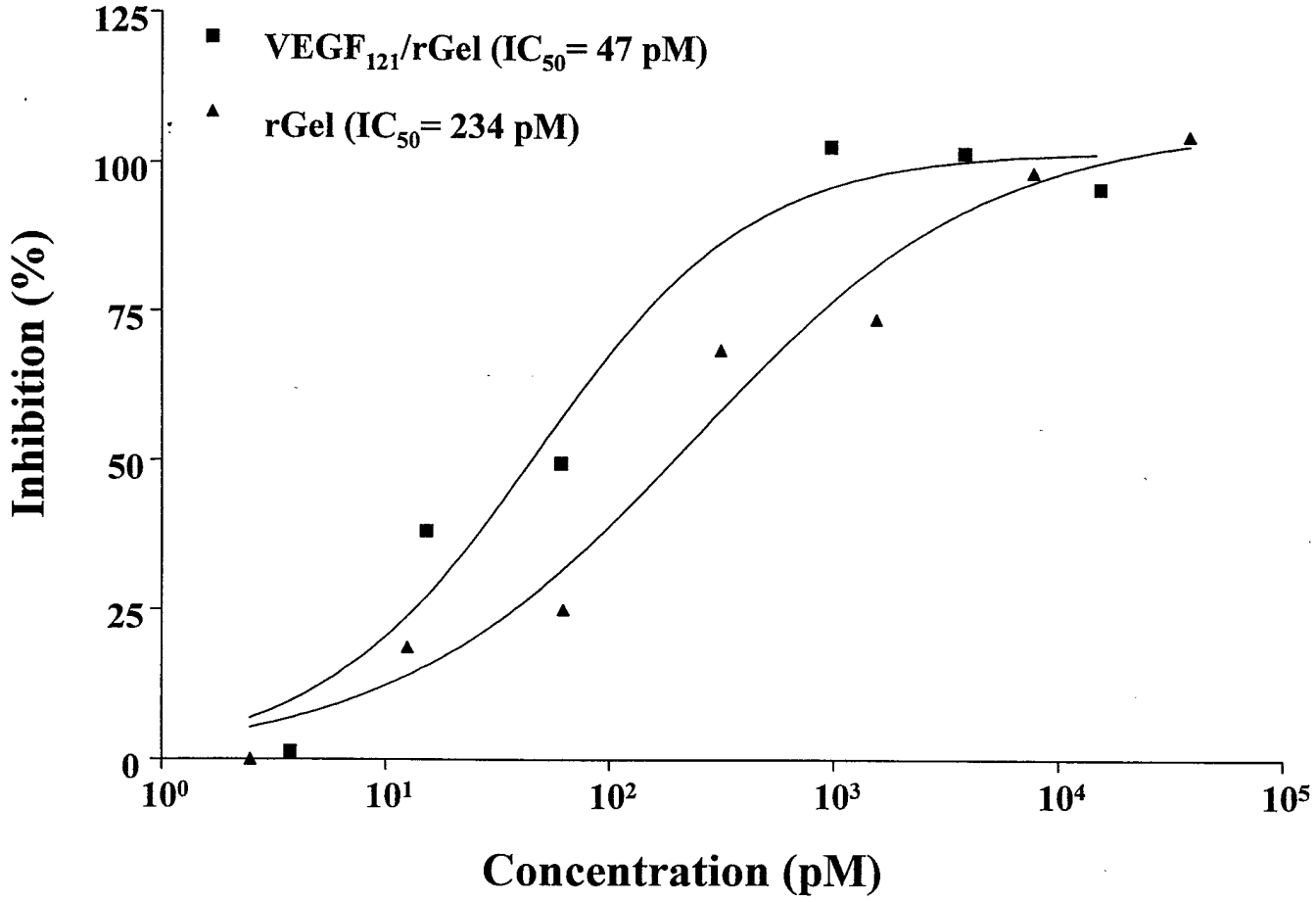

125
100
75
50
25
0
Inhibition (%)
VEGF121/rGel (IC50= 47 pM)
rGel (IC50= 234 pM)
10^0
10^1
10^2
10^3
10^4
10^5
Concentration (pM)

Figure 2

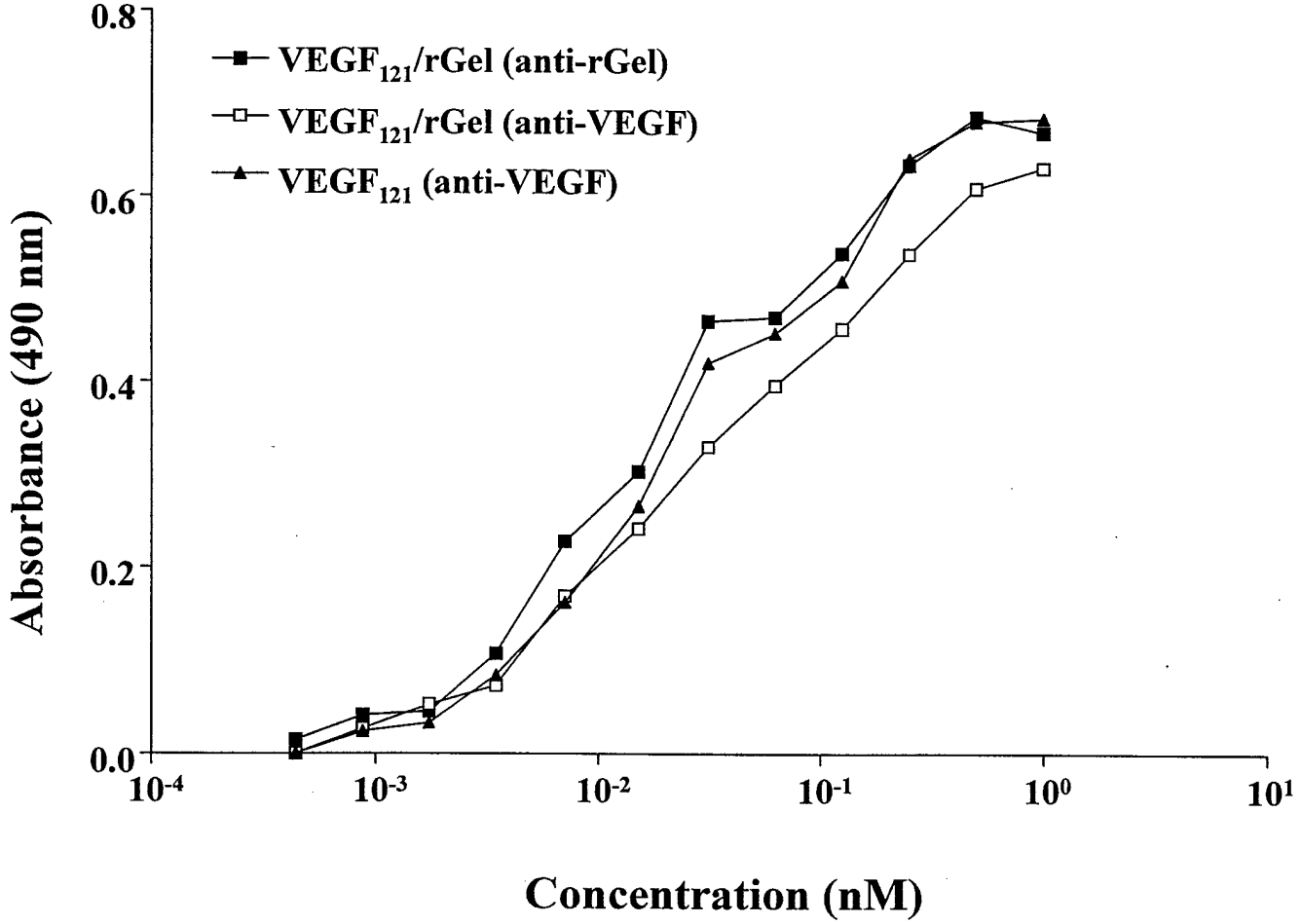

0.8
0.6
0.4
0.2
0.0
Absorbance (490 nm)
VEGF121/rGel (anti-rGel)
VEGF121/rGel (anti-VEGF)
VEGF121 (anti-VEGF)
10-4
10-3
10-2
10-1
100
101
Concentration (nM)

Figure 3

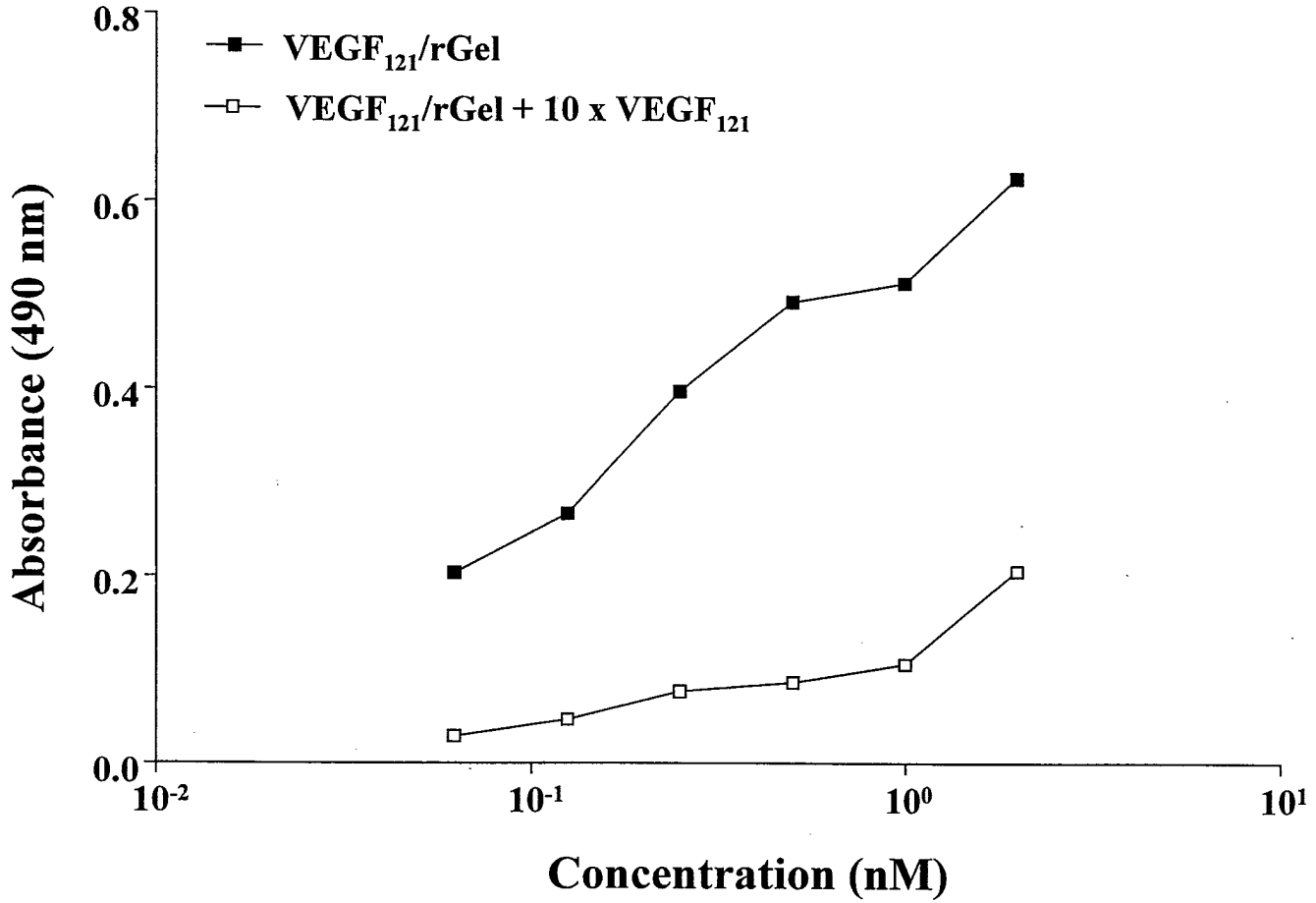
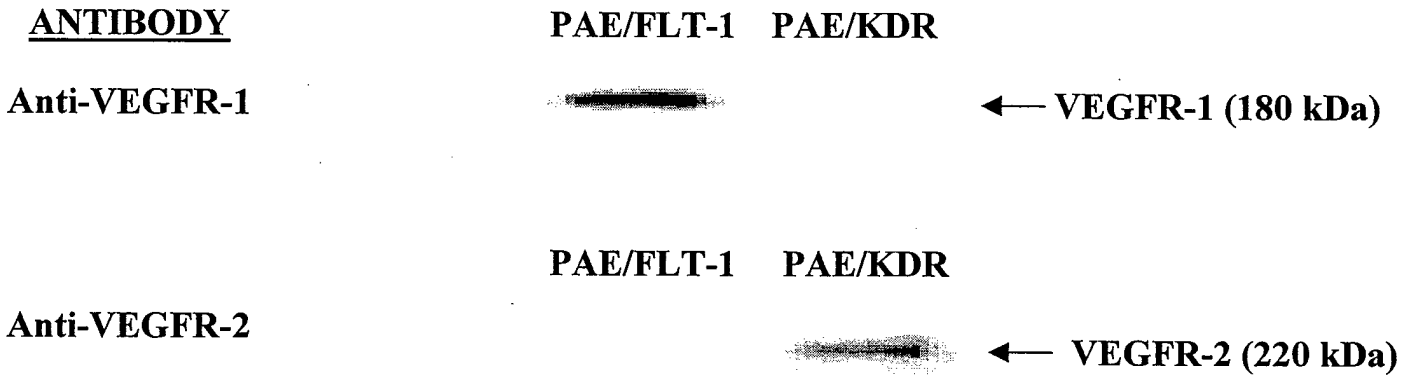

0.8
0.6
0.4
0.2
0.0
Absorbance (490 nm)
VEGF121/rGel
VEGF121/rGel + 10 x VEGF121
10-2
10-1
100
101
Concentration (nM)

Figure 4

Western Analysis of VEGF Receptors



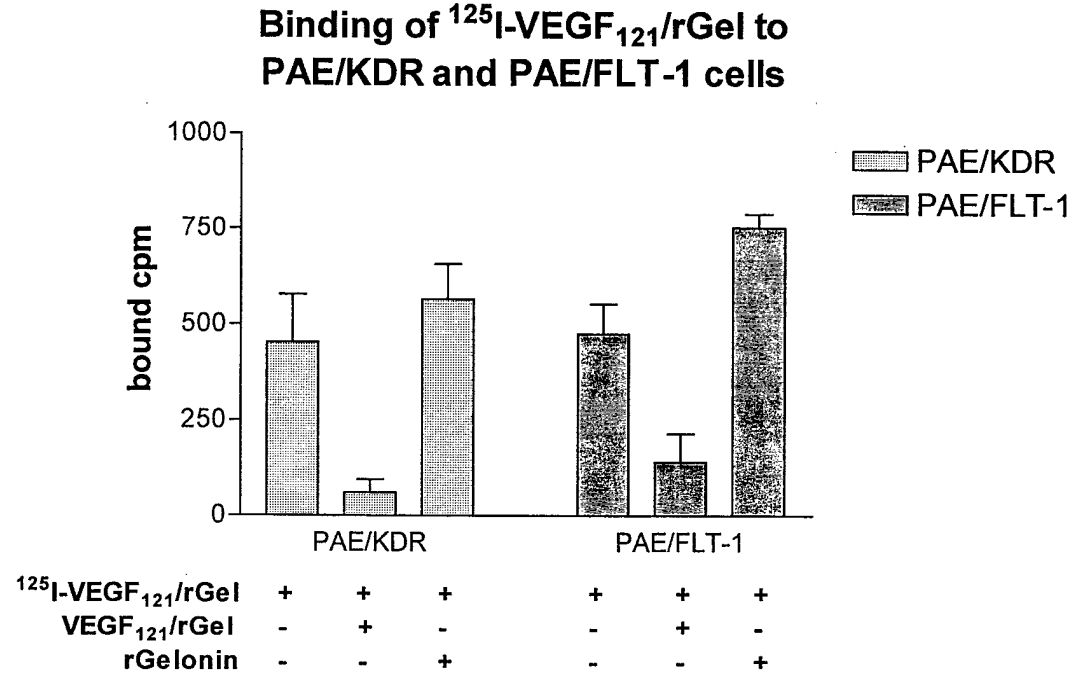


Figure 5

Internalization of $VEGF_{121}$/rGel into PAE/KDR and PAE/FLT-1 cells

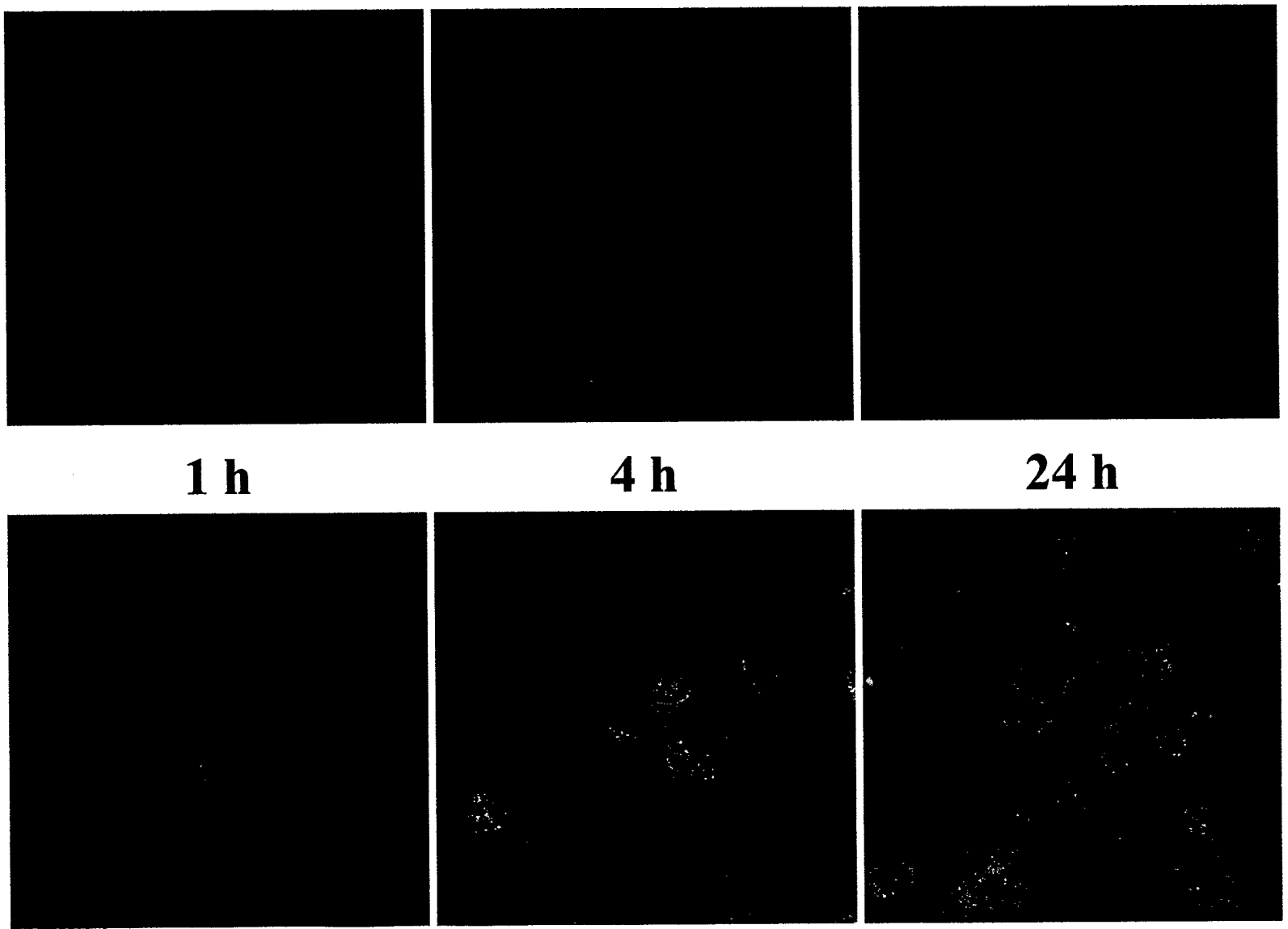


Figure 6

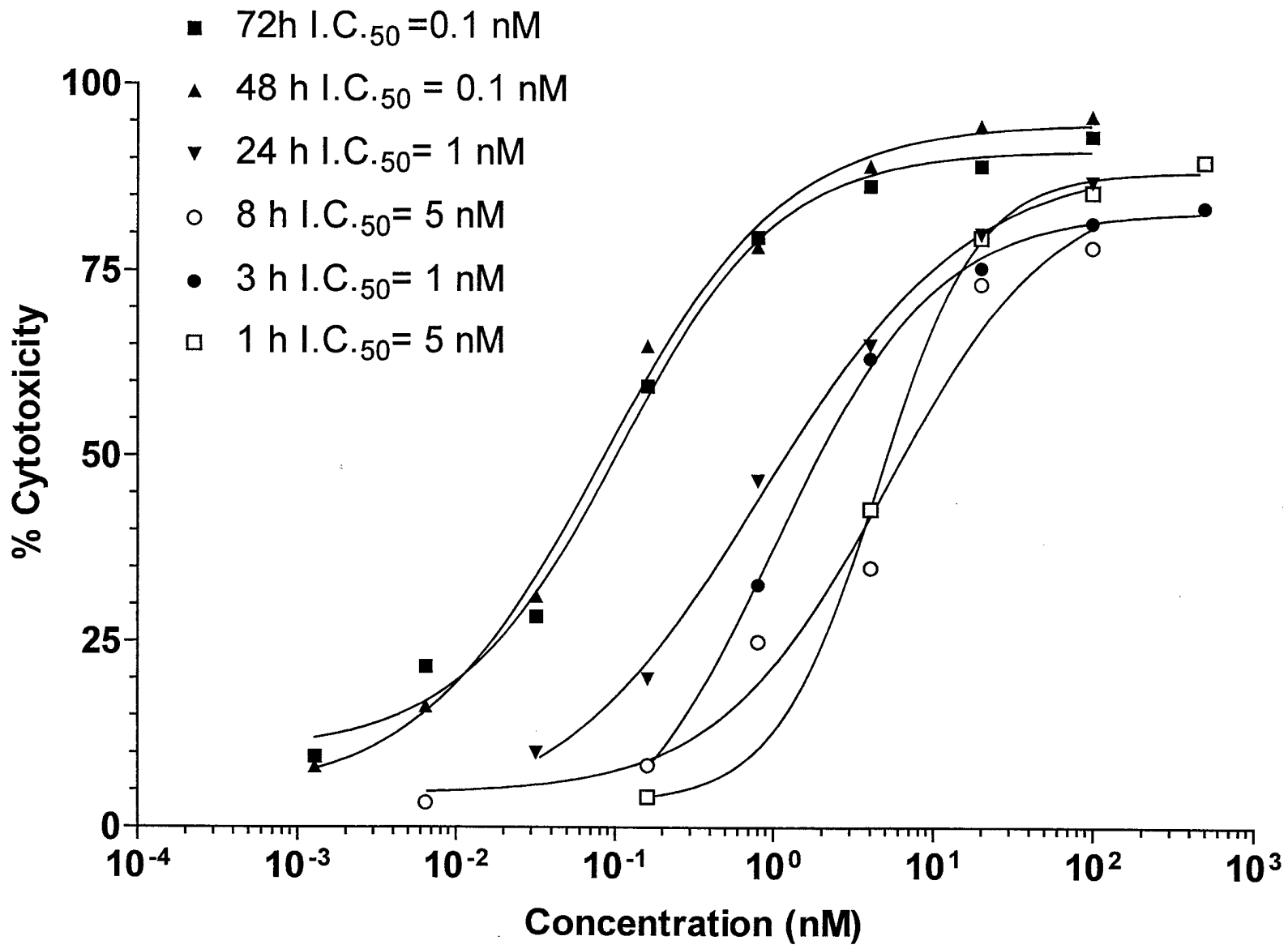

72h I.C.50 =0.1 nM
48 h I.C.50 = 0.1 nM
24 h I.C.50= 1 nM
8 h I.C.50= 5 nM
3 h I.C.50= 1 nM
1 h I.C.50= 5 nM
% Cytotoxicity
100
75
50
25
0
10-4
10-3
10-2
10-1
100
101
102
103
Concentration (nM)

Figure 7

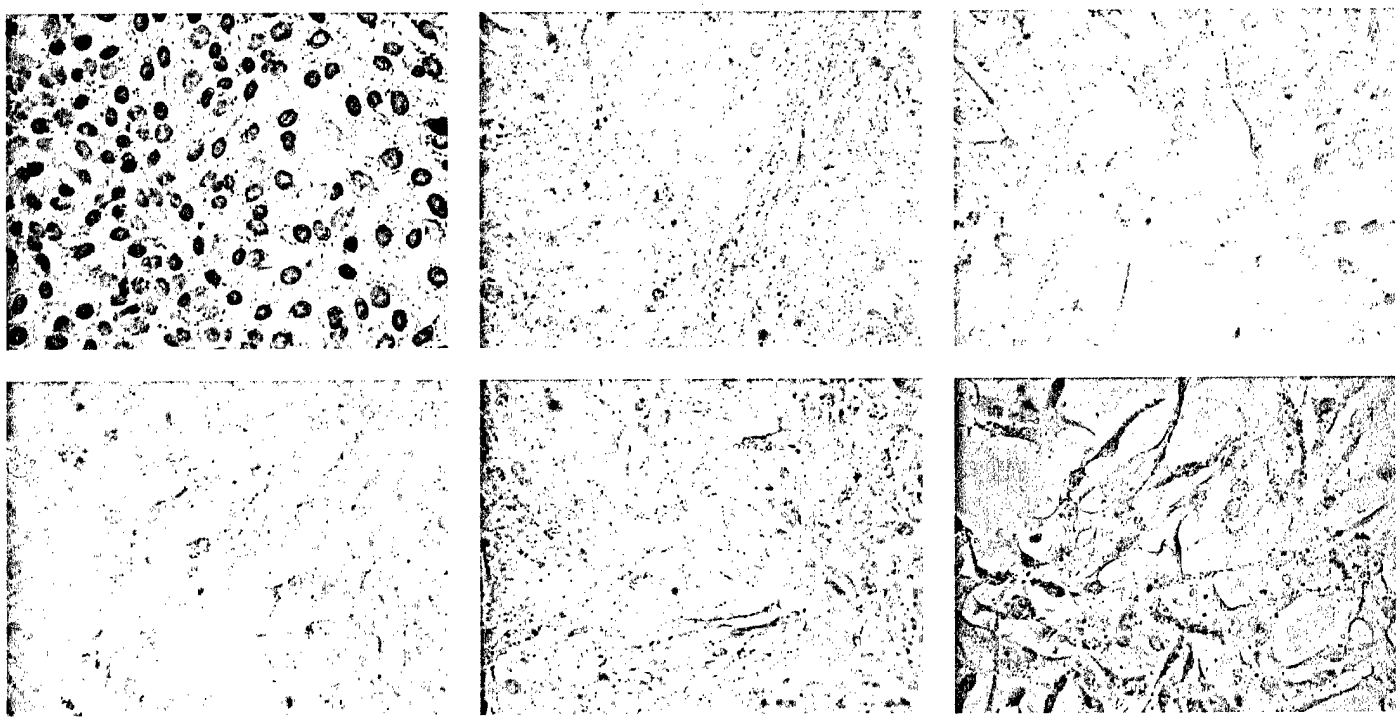


Figure 8

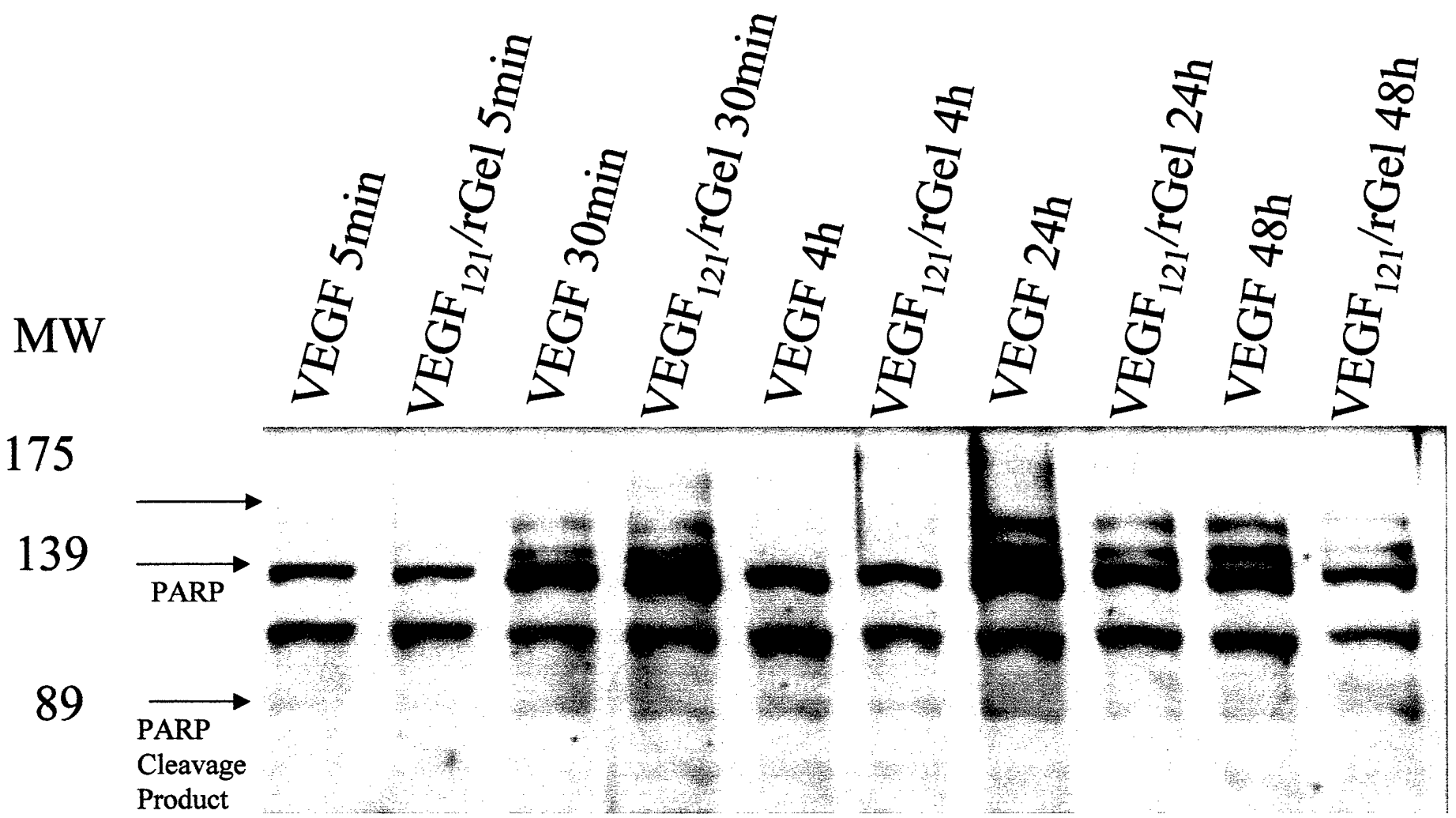

MW
VEGF 5min
VEGF121/rGel 5min
VEGF 30min
VEGF121/rGel 30min
VEGF 4h
VEGF121/rGel 4h
VEGF 24h
VEGF121/rGel 24h
VEGF 48h
VEGF121/rGel 48h
175
139
PARP
89
PARP
Cleavage
Product

Figure 9

The Vascular-Ablative Agent, $VEGF_{121}$/rGel, Inhibits Pulmonary Metastases of MDA-MB-231 Breast Tumors

Running title: $VEGF_{121}$/rGel Inhibits Pulmonary Breast Metastases

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Key Words: VEGF, gelonin, fusion toxin, vascular targeting, metastatic breast tumors

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ABSTRACT

We evaluated the effect of $VEGF_{121}$/rGel fusion toxin treatment on the growth of metastatic MDA-MB-231 tumor cells in SCID mice. Tumor cells ($0.5x10^6$ per mouse) were injected i.v. and 8 days after innoculation, mice (6 per group) were treated 6 times either with $VEGF_{121}$/rGel (100 ug/dose) or free gelonin. Three weeks after treatment, mice were sacrificed and the lungs were harvested and examined. $VEGF_{121}$/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 also 50% smaller than control mice (210 ± 37 um^2 versus 415 ± 10 um^2 for $VEGF_{121}$/rGel and control, respectively; $p< 0.01$). In addition, the vascularity of metastatic foci as assessed by the mean number of blood vessels per mm^2 in metastatic foci was significantly reduced (198 ± 37 versus 388 ± 21 for treated and control, respectively). Approximately 62% of metastatic colonies from the $VEGF_{121}$/rGel-treated group had fewer than 10 vessels per colony as compared to 23% in the control group. The VEGF receptor (flk-1) was intensely detected on the metastatic vessels in the control but not on the vessels in the $VEGF_{121}$/rGel-treated group. Metastatic foci present in lung had a 3-fold lower Ki-67 labeling index compared to control tumors. These data strongly suggest that the anti-tumor vascular-ablative effect of $VEGF_{121}$/rGel may be utilized not only for treating primary tumors but also for inhibiting metastatic spread and vascularization of metastases.

INTRODUCTION

Biological studies examining the development of the vascular tree in normal development and in disease states have identified numerous cytokines and their receptors responsible for triggering and maintaining this process (1-7). Tumor neovascularization is central not only to the growth and development of the primary lesion but appears to be a critical factor in the development and maintenance of metastases (8-12). Clinical studies in bladder cancer (9) have demonstrated a correlation between micro-vessel density and metastases. In addition, studies of breast cancer metastases by Fox et al. and Aranda et al. (11-12) have demonstrated that microvessel count in primary tumors appears to be related to the presence of metastases in lymph nodes and micrometastases in bone marrow.

The cytokine vascular endothelial growth factor-A (VEGF-A) and its receptors Flt-1 and KDR have been implicated as one of the central mediators of normal angiogenesis and tumor neovascularization (13-20). Up-regulation or over-expression of the KDR receptors or the VEGF-A ligand itself has been implicated as poor prognostic markers in various clinical studies of colon, breast and pituitary cancers (21-23). Recently, Padro et al. (24) have suggested that both VEGF-A and KDR may play a role in the neovascularization observed in bone marrow during AML tumor progression and may provide evidence that the VEGF/KDR pathway is important in leukemic growth.

For these reasons, there have been several groups interested in developing therapeutic agents and approaches targeting the VEGF-A pathway. Agents which prevent VEGF-A binding to its receptor, antibodies which directly block the KDR receptor itself and small molecules which block the kinase activity of the KDR and thereby block growth factor signaling are all under development (25-37). Recently, our laboratory reported the development of a growth factor

fusion construct of $VEGF_{121}$ and the recombinant toxin gelonin (rGel) (38). The rGel toxin is a single chain n-glycosidase similar in its action to ricin A chain (39). Immunotoxins and fusion toxins containing rGel have been shown to specifically kill tumor cells in vitro and in vivo (40-43). In clinical studies currently ongoing, gelonin does not appear to generate capillary leak syndrome that limits use of other toxins (44). Our studies demonstrated that this agent was specifically cytotoxic only to cells expressing the KDR receptor and was not cytotoxic to cells over-expressing the Flt-1 receptor. In addition, this agent was shown to localize within tumor vasculature and caused a significant damage to vascular endothelium in both PC-3 prostate and A375 orthotopic xenograft tumor models.

The current study seeks to extend our original observations describing the in vitro biological effects of this novel fusion construct and we examined the effects of this agent in both orthotopic and metastatic tumor models.

MATERIALS AND METHODS

Materials. Bacterial strains, pET bacterial expression plasmids and recombinant enterokinase were obtained from Novagen (Madison, WI). All other chemicals were from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). TALON metal affinity resin was obtained from Clontech laboratories (Palo Alto, CA). Other chromatography resin and materials were from Pharmacia Biotech (Piscataway, NJ). Endothelial cell growth supplement (ECGS) from bovine neural tissue was obtained from Sigma Chemical Company. Murine brain endothelioma (bEnd.3) cells were provided by Professor Werner Risau (Max Plank Institute, Munich, Germany). Tissue culture reagents were from Gibco BRL (Gaithersburg, MD) or Mediatech Cellgro (Herndon, VA).

Antibodies. Rat anti-mouse CD31 antibody was from PharMingen (San Diego, CA). Rabbit anti-gelonin antibody was produced in the Veterinary Medicine Core Facility at MDACC. Hybridoma producing the mouse monoclonal 6w/32 antibody directed against human HLA antigen was purchased from ATCC. The 6w/32 antibody was purified from hybridoma supernatant using Protein A resin. MECA 32, a pan mouse endothelial cell antibody, was kindly provided by Dr. E. Butcher (Stanford University, CA) and served as a positive control for immunohistochemical studies. The Ki-67 antibody was from Abcam, Inc. (Cambridge, UK). Goat anti-rat, anti-mouse and anti-rabbit secondary antibodies conjugated to HRP were purchased from Daco (Carpinteria, CA).

Cell Culture. Porcine aortic endothelial cells transfected with the KDR receptor (PAE/KDR) or the Flt-1 receptor (PAE/Flt-1) were a generous gift from Dr. J. Waltenberger. MDA-MB-231 cells were a generous gift from Dr. Janet Price. Cells were maintained as a monolayer in F12 Nutrient Media (HAM) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum. Cells were harvested by treatment with Versene (0.02%EDTA). Tumor cells intended for injection into mice were washed once and resuspended in serum-free medium without supplements. Cell number and viability were determined by staining with 0.2% trypan blue dye diluted in saline. Only single-cell suspensions of greater than 90% viability were used for in vivo studies.

Expression and Purification of $VEGF_{121}$/rGel. The construction, expression and purification of $VEGF_{121}$/rGel has been previously described (38). The fusion toxin was stored in sterile PBS at -20°C.

Cytoxicity of $VEGF_{121}$/rGel and rGel. Cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase PAE/KDR and PAE/Flt-1 cells have been previously described (38). Here, we assessed

the cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase MDA-MB-231 cells and compared their cytotoxicity to PAE/KDR cells. Cells were grown in 96-well flat-bottom tissue culture plates. Purified $VEGF_{121}$/rGel and rGel were diluted in culture media and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 M sodium citrate, pH 4.2 in 50% ethanol). Absorbance was measured at 630 nm.

Western Blot Analysis. Whole cell lysates of PAE/KDR and PAE/Flt-1 cells were obtained by lysing cells in Cell Lysis buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol) supplemented with protease inhibitors (leupeptin (0.5%), aprotinin (0.5%) and PMSF (0.1%). Protein samples were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF memberane overnight at 4°C in transfer buffer (25 mM Tris-HCl, pH 7.6, 190 mM glycine, 20% HPLC-grade methanol). The samples were analyzed for KDR with rabbit anti-flk-1 polyclonal antibody (Santa Cruz Biotechnology, Inc.) and Flt-1 using an anti-flt-1 polyclonal antibody (Santa Cruz Biotechnology, Inc.). The membranes were then incubated with goat-anti-rabbit IgG horseradish peroxidase (HRP), developed using the Amersham ECL detection system and exposed to X-ray film.

Immunoprecipitation. Cells were lysed as described (see Western protocol). 500 μg MDA-MB-231 cell lysate was mixed with 2 μg anti-flk-1 antibody in a final volume of 250 μl and incubated for two hours at 4°C. 100 μg PAE/KDR and PAE/Flt-1 cell lysates were immunoprecipitated as controls. The mixtures were then incubated overnight with 20 μl Protein A beads that had been blocked with 5% BSA. The beads were washed 4 times in lysis buffer and the samples, along with 30 μg PAE/KDR cell lysate, were run on a gel, transferred overnight onto a PVDF membrane and probed using an anti-flk-1 polyclonal antibody.

Localization of $VEGF_{121}$/rGel to Blood Vessels of MDA-MB-231 Tumors. All animal experiments were carried out in accordance with institutional guidelines and protocols. SCID mice (3 mice per group) bearing MDA-MB-231 metastatic tumors were intravenously injected with 100 ug of the fusion protein or equivalent amount of free gelonin. Four hours later mice were sacrificed and exsanguinated. All major organs and tumor were harvested and snap-frozen for preparation of cryosections. Frozen sections were double stained with anti-CD-31 (5 ug/ml) followed by detection of the localized fusion protein using rabbit anti-gelonin antibody (10 ug/ml). CD-31 rat IgG was visualized by goat anti-rat IgG conjugated to FITC (green fluorescence). Rabbit anti-gelonin antibody was detected by goat anti-rabbit IgG conjugated to Cy-3 (red fluorescence). Co-localization of both markers was indicated by yellow color. Anti-gelonin antibody had no reactivity with tissues sections derived from mice injected with saline or with $VEGF_{121}$.

Metastatic Model of MDA-MB-231 Tumors. Female SCID mice, aged 4-5 weeks, were injected in a tail vein with 0.1 ml of MDA-MB-231 cell suspension ($5x10^5$ cells). The mice were randomly separated into two groups (6 mice per group) and were treated with either $VEGF_{121}$/rGel or gelonin alone (100 μg intraperitoneally, 6 times total with the interval of 3 days) starting the 8^{th} day after the injection of cells. Intraperitoneal rather than intravenous injection was chosen solely to prevent necrosis of the tail vein due to repeated injections. Animal weight was monitored. Three weeks after termination of the treatment, the animals were sacrificed and their lungs were removed. One lobe was fixed in Bouin's fixative and the other lobe was snap-frozen. After fixation in Bouin's fixative, the tumor colonies on the lung surface appear white, whereas the normal lung tissue appears brown. The number of tumor colonies on

the surface of each lung was counted and the weight of each lung was measured. The values obtained from individual mice in the $VEGF_{121}$/rGel and rGel groups were averaged per group.

Determination of Number, Size and Vascular Density of Lung Metastatic Foci. Frozen samples of lung tissue was cut to produce sections of 6 μm. Blood vessels were visualized by MECA 32 antibody and metastatic lesions were identified by morphology and by 6w/32 antibody, directed against human HLA antigens. Each section was also double stained by MECA 32 and 6w/32 antibodies to ensure that the analyzed blood vessels are located within a metastatic lesion. Slides were first viewed at low magnification (x 2 objective) to determine total number of foci per a cross-section. Six slides derived from individual mice in each group were analyzed and the number was averaged. Images of each colony were taken using digital camera (CoolSnap) at magnifications of x 40 and x 100 and analyzed using Metaview software that allows measurements of smallest and largest diameter, perimeter (μm) and area (mm^2). The vascular endothelial structures identified within a lesion were counted and number of vessels per each lesion was determined and normalized per mm^2. The mean number of vessels per mm^2 was calculated per each slide and averaged per $VEGF_{121}$/rGel and rGel groups (6 slides per group). The results are expressed ± SEM. The same method applied to determine the mean number of vessels in non-malignant tissues.

Immunohistochemical Analysis of Proliferation of Tumor Cells in the Lung Colonies. Frozen sections of mouse normal organs and metastatic lungs were fixed with acetone for 5 min and rehydrated with PBST for 10 min. All dilutions of antibodies were prepared in PBST containing 0.2% BSA. Primary antibodies were detected by appropriate anti-mouse, anti-rat or anti-rabbit HRP conjugates. HRP activity was detected by developing with DAB substrate (Research Genetics). To determine number of cycling cells sections were

stained with the Ki-67 antibody followed by anti-mouse IgG HRP conjugate. Sections were analyzed at magnification of x100. Number of cells positive for Ki-67 was normalized per mm^2. The mean number ± SD per $VEGF_{121}$/rGel and control group is presented. The average numbers derived from analysis of each slide were combined per either $VEGF_{121}$/rGel or rGel group and analyzed for statistical differences.

Expression of KDR in metastatic Lung Tumors. The expression of VEGF receptor-2 on the vasculature of breast tumors metastatic to lung was also assessed using the RAF-1 antibody as described by Sophia Ran et al., (in press). Frozen sections of lungs from mice treated with $VEGF_{121}$/rGel or free gelonin stained with monoclonal rat anti-mouse VEGFR2 antibody RAFL-1 (10 μg/ml). RAFL-1 antibody was detected by goat anti-rat IgG-HRP

Statistical Analysis. Results are expressed as mean ± SEM unless otherwise indicated. Statistical significance was determined by one way analysis of variance followed by the Student's t-test.

RESULTS

Cytotoxicity of $VEGF_{121}$/rGel on MDA-MB-231 Cells. We have previously demonstrated that $VEGF_{121}$/rGel is cytotoxic to endothelial cells expressing KDR but not Flt-1. As assessed by Western blot, MDA-MB-231 cells do not appear to express VEGFR-1 or VEGFR-2, the receptors which bind $VEGF_{121}$. We additionally examined the cytotoxicity of $VEGF_{121}$/rGel on MDA-MB-231cells in culture and showed an $I.C._{50}$ slightly higher than that observed for recombinant gelonin (Fig. 1), indicating that $VEGF_{121}$/rGel does not have a specific target on MBA-MB-231 cells. The IC_{50} is similar to that of the untargeted gelonin toxin towards PAE/KDR cells.

Localization of $VEGF_{121}$/rGel to Vasculature of MDA-MB-231 Tumors. Mice bearing metastatic MDA-MB-231 tumors were injected intravenously with either $VEGF_{121}$/rGel (50 ug/mouse) or free gelonin (20 ug/mouse) and, four hours later, the mice were exsanguinated. Frozen sections were prepared from the tumor and normal organs and examined immunohistochemically to determine the location of the free gelonin and the gelonin fusion construct. $VEGF_{121}$/rGel was primarily detected on endothelium of tumor (Fig. 2). In average, sixty percent of vessels positive for MECA 32 were also positive for gelonin in the group of $VEGF_{121}$/rGel – injected mice. In the tumor regions of increased vascularity (hot spots), up to 90% of tumor vessels were labeled by anti-gelonin IgG. Vessels with bound $VEGF_{121}$/rGel were homogeneously distributed within the tumor vasculature. Vessels in normal organs were unstained with the exception of the kidney where weak and diffuse staining was detected in the glomeruli. Free gelonin did not localize to tumor or normal vessels in any of the mice, indicating that only targeted gelonin was able to bind to the tumor endothelium. These results indicate that $VEGF_{121}$/rGel specifically localizes to tumor vessels, which demonstrate high density and favorable distribution of the $VEGF_{121}$/rGel – binding sites.

MDA-MB-231 Model of Experimental Pulmonary Metastases and Rationale for Therapeutic Regime. Human breast carcinoma MDA-MB-231 cells consistently lodge in lungs following intravenous injection into the tail vein of athymic or SCID mice. Micrometastases are first detected 3 to 7 days after injection of $5x10^5$ cells and macroscopic colonies develop in 100% of the injected mice within 4 to 7 weeks. Mortality occurs in all mice within 10-15 weeks. This model of experimental breast cancer metastasis examines the ability of tumor cells to survive in the blood circulation, extravasate through the pulmonary vasculature and establish growing colonies in the lung parenchyma.

We evaluated the effect of $VEGF_{121}$/rGel on the growth and survival of the established micrometastases. We, therefore, started the treatment 8 days after injection of the tumor cells. By that time, based on our prior observations, tumor cells that were able to survive in the circulation and transverse the lung endothelial barrier are localized within the lung parenchyma and initiate tumor angiogenesis. Treatment with $VEGF_{121}$/rGel was given for the following 2 weeks as described under Methods, allowing the mice to receive the maximal tolerated accumulative dose of the drug (600 μg per mouse). Prior studies established that $VEGF_{121}$/rGel given at such dose did not cause histopathological changes in normal organs. The accumulative dose of 640-800 μg of total $VEGF_{121}$/rGel fusion protein, given i. p. over period of 4 weeks, did not induce significant toxicity as judged by animal behavior morphological evaluation of normal organs. Transient loss of weight (~10%) was observed 24 hours after most of the treatments with complete weight recovery thereafter. Colonies were allowed to expand in the absence of treatment for the three following weeks in order to evaluate long-term effect of $VEGF_{121}$/rGel on size of the colonies, proliferation index of tumor cells and their ability to induce new blood vessel formation.

Effect of $VEGF_{121}$/rGel on Number and Size of MDA-MB-231 Tumor Lesions in Lungs. Treatment with $VEGF_{121}$/rGel but not with free gelonin significantly reduced by between 42-58% both the number of colonies per lung and the size of the metastatic foci present in lung as shown in Fig. 3 and Table 1.

Effect of $VEGF_{121}$/rGel on Vascularity of the MDA-MB-231 Pulmonary Metastatic Foci. The overall mean vascular density of lung colonies was reduced by 51% compared to the rGel treated controls (Table 2 and Fig. 4), however; the observed effect was non-uniformly distributed by tumor colony size. The greatest impact on vascularization was observed on mid-size and

extremely small tumors (62 and 69% inhibition respectively) while large tumors demonstrated the lease effect (10% inhibition). The majority of lesions in the $VEGF_{121}$/rGel-treated mice (~70%) were avascular whereas only 40% of lesions from the control group did not have vessels within the metastatic lung foci.

Effect of $VEGF_{121}$/rGel on Number of Cycling Cells in the Metastatic Foci. The number of cycling tumor cells in lesions from the $VEGF_{121}$/rGel group was also reduced by ~60% as compared to controls (Fig. 5). This finding suggests that vascularity of metastases directly affects tumor cell proliferation.

Effect of $VEGF_{121}$/rGel on flk-1 Expression in Tumor Vessel Endotheluim. The expression of KDR on the remaining few vessels present in lung metastatic foci demonstrated a significant decline compared to that of lung foci present in control tumors (Fig. 6). This suggests that the $VEGF_{121}$/rGel agent is able to significantly down-regulate the receptor or prevent the outgrowth of highly receptor-positive endothelial cells.

DISCUSSION

Neovascularization is a particularly important hallmark of breast tumor growth and metastatic spread (45-49). The growth factor VEGF-A and the receptor KDR have both been implicated in highly metastatic breast cancers (50-52). We have previously demonstrated that the $VEGF_{121}$/rGel growth factor fusion toxin specifically targets KDR-expressing tumor vascular endothelial cells and inhibits growth of subcutaneously implanted, human tumor xenografts (38). The current study was designed to evaluate its effect on development of breast cancer metastases in lungs following intravenous injection of MDA-MB-231 cells.

The salient findings of our study of the $VEGF_{121}$/rGel construct are that this fusion toxin is specifically cytotoxic to cells over-expressing the KDR receptor for VEGF. However, the human breast MDA-MB-231 cells employed for these studies do not express this receptor and, therefore, were not directly affected by this agent. The antitumor effects of $VEGF_{121}$/rGel observed from our in vivo studies appear to be solely the result of targeting tumor vasculature. Administration of the $VEGF_{121}$/rGel construct to mice previously injected (i.v.) with tumor cells dramatically reduced the number of tumor colonies found in lung, their size and their vascularity. In addition, the number of cycling breast tumor cells within lung metastatic foci was found to be reduced by an average of 60%. In addition to the reduced number of blood vessels present in lung metastases of treated mice, we also found that the few vessels present had a greatly reduced expression of VEGFR2. Therefore, this construct demonstrated an impressive, long-term impact on the growth and development of breast tumor metastatic foci found in lung.

Targeting tumor vasculature with a variety of technologies has been shown to inhibit the growth and development of primary tumors as well as metastases. Recently, Shaheen et.al. (53) demonstrated that small molecule tyrosine kinase inhibitors active against the receptors for VEGF, fibroblast growth factor and platelet-derived growth factors were also capable of inhibiting microvessel formation and metastases in tumor model systems. Previously, Seon et al. (54) demonstrated long-term anti-tumor effects of an anti-endoglin antibody conjugated with ricin A-chain (RTA) in a human breast tumor xenograft model.

Surprisingly, one finding from our study was that administration of $VEGF_{121}$/rGel resulted in a 3-fold decrease in the number of Ki-67 labeled (cycling) cells in the metastatic foci present in lung (Fig. 5). Clinical studies have suggested that tumor cell cycling may be an important prognostic marker for disease-free survival in metastatic breast cancer, but that Ki-67 labeling

index, tumor microvessel density (MVD) and neovascularization appear to be independently regulated processes (55-56). To our knowledge, this is the first report of a significant reduction in tumor labeling index produced by a vascular targeting agent.

Another critical finding from our studies is the observation that the vascular-ablative effects of the $VEGF_{121}$/rGel fusion construct alone were unable to completely eradicate lung metastases. Although the growth of larger pulmonary metastases was completely inhibited by this therapeutic approach, development of small, avascular, metastatic foci within lung tissue was observed. This data strongly suggests that combination of vascular targeting agents with chemotherapeutic agents or with radiotherapeutic agents which directly damage tumor cells themselves may provide for greater therapeutic effect. Studies of several vascular targeting agents in combination with chemotherapeutic agents have already demonstrated a distinct in vivo anti-tumor advantage of this combination modality against experimental tumors in mice (57). Studies by Pedley et al. (58) have also suggested that combination of vascular targeting and radioimmunotherapy may also present a potent antitumor combination. Finally, studies combining hyperthermia and radiotherapy with vascular targeting agents have demonstrated enhanced activity against mammary carcinoma tumors in mice (59). Studies in our laboratory combining $VEGF_{121}$/rGel and various chemotherapeutic agents, biological agents or therapeutic agents targeting tumor cells are currently ongoing.

The presented findings demonstrate that $VEGF_{121}$/rGel can clearly and specifically target KDR expressing tumor vasculature both in vitro and in vivo and that this agent can have an impressive inhibitory effect on tumor metastases. Studies are continuing in our laboratory to examine the activity of this agent alone and in combination against a variety of orthotopic and metastatic tumor models.

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FIGURE LEGENDS

Fig. 1. MDA-MB-231 cells are not targeted by $VEGF_{121}$/rGel due to the lack of expression of VEGFR-2/KDR.

Fig. 1A. Western analysis demonstrating the presence of VEGFR2 on endothelial cells transfected with the R2 receptor (PAE/KDR) but not on cells expressing the FLT-1 receptor (PAE/FLT-1, negative control). As shown, the MDA-MB-231 cells did not express detectable amounts of VEGFR-2.

Fig. 1B. Log-phase MDA-MB-231 and PAE/KDR cells were treated with various doses of $VEGF_{121}$/rGel or rGel for 72 hrs. $VEGF_{121}$/rGel was far more toxic than rGel towards PAE/KDR cells (IC50 of 1 nM versus 100 nM). In contrast, the cytotoxic effects of both agents were similar towards MDA-MB-231 cells (IC50 of 150 nM with $VEGF_{121}$/rGel versus 40 nM with rGel) demonstrating no specific cytotoxicity of the fusion construct compared to free toxin on these cells.

Fig. 2. $VEGF_{121}$/rGel localizes to vasculature of breast tumor foci in the lungs of mice.

Female SCID mice were injected i.v. with 0.1 ml of MDA-MB-231 cell suspension ($5x10^5$ cells) as described in Materials and Methods. Six weeks later, mice were administered one dose (i.v., tail vein) of 100 μg $VEGF_{121}$/rGel. Four hours later, the mice were sacrificed and the tumor-bearing lungs fixed. Tissue sections were stained for blood vessels using the anti-CD-31 antibody (red) and the section was counter-stained using an anti-gelonin antibody (green). Co-localization of the stains (yellow) demonstrate the presence of the $VEGF_{121}$/rGel fusion construct specifically in blood vessels and not on tumor cells.

Fig. 3. $VEGF_{121}$/rGel reduces number of large colonies in the metastatic lungs. The size of tumor colonies was analyzed on slides stained with 6w/32 antibody which specifically recognizes human HLA antigens. The antibody delineates colonies of human tumor cells and defines borders between metastatic lesions and mouse lung parenchyma. The largest size differences between $VEGF_{121}$/rGel and control groups were found in groups of colonies having diameter either less than 50 μm or more than 1000 μm. In the $VEGF_{121}$/rGel-treated mice more than 40% of total foci were extremely small (< 50 micron) as compared to 18% in the control group. The control mice had approximately 8% of the extremely large colonies (>1000 μm) whereas $VEGF_{121}$/rGel-treated mice did not colonies of this size.

Fig. 4. $VEGF_{121}$/rGel inhibits vascularization of MDA-MB-231 pulmonary metastases.

Fig. 4A. Lungs derived from $VEGF_{121}$/rGel and rGel - treated mice were stained with MECA 32 antibody and the vascular density within the metastatic foci was determined. The mean number of vessels per mm^2 in lung metastases of $VEGF_{121}$/rGel - treated mice was reduced by approximately 50% as compared to those in rGel - treated mice.

Fig. 4B. Representative images demonstrating reduction of vascular density in foci of comparable size in mice treated with rGel (left) and $VEGF_{121}$/rGel fusion protein (right).

Fig. 5. $VEGF_{121}$/rGel inhibits proliferation of metastatic MDA-MB-231 cells in the lungs.

Frozen sections of lungs derived from $VEGF_{121}$/rGel and rGel - treated mice were stained with Ki-67 antibody. Stained sections were examined under x 40 objective to determine a number of tumor cells with positive nuclei (cycling cells). Positive cells were enumerated in 10 colonies per

slide on six sections derived from individual mice per each treatment group. The mean number per group ± SEM is presented. $VEGF_{121}$/rGel treatment reduced the average number of cycling cells within the metastatic foci by approximately 60%.

Fig. 6. Detection of VEGFR2 on vasculature of metastatic lesions by anti-VEGFR2 antibody, RAFL-1. Frozen sections of lungs from mice treated with $VEGF_{121}$/rGel or free gelonin stained with monoclonal rat anti-mouse VEGFR2 antibody RAFL-1 (10 μg/ml). RAFL-1 antibody was detected by goat anti-rat IgG-HRP, as described under Materials and Methods. Sections were developed with DAB and counterstained with hematoxylin. Representative images of lung metastases of comparable size (700-800 μm in the largest diameter) from each treatment group are shown. Images were taken with an objective of X20. Note that the pulmonary metastases from the $VEGF_{121}$/rGel treated group show both reduced vessel density and decreased intensity of anti-VEGFR2 staining, as compared to control lesions.

Table 1 *Effect of $VEGF_{121}$/rGel on number and size of pulmonary metastases of MDA-MB- 231 human breast carcinoma cells*

Parameter	Treatment [a]		% inhibition *vs.* rGelonin treatment	P value [b]
	rGelonin	$VEGF_{121}$/rGel		
No. surface colonies per lung (range) [c]	53.3 ± 22 (33-80)	22.4 ± 9.2 (11-41)	58.0%	0.03
No. intraparenchymal colonies per cross-section (range) [d]	22 ± 7.5 (18-28)	12.8 ± 5.5 (5-18)	42.0%	0.02
Mean area of colonies (μm) [e]	415 ± 10	201 ± 37	51.9%	0.01
Mean % of colonies-occupied area per lung section [f]	57.3 ± 19	25.6 ± 10.5	55.4%	0.01

[a] Mice with MDA-MB-231 pulmonary micrometastases were treated i.p. with $VEGF_{121}$/rGel or free gelonin as described under Methods and Results.

[b] P value was calculated using t-Student test.

[c] Lungs were fixed with Bouin's fixative for 24 hours. Number of surface white colonies was determined for each sample and averaged among 6 mice from $VEGF_{121}$/rGel or rGel control group. Mean number per group ± SEM is shown. Numbers in parentheses represent range of colonies in each group.

[d] Frozen sections were prepared from metastatic lungs. Sections were stained with 6w/32 antibody recognizing human tumor cells. Number of intraparenchymal colonies identified by brown color was determined for each cross-section and averaged among 6 samples of individual mice from $VEGF_{121}$/rGel or rGel control group. Mean number per group ± SEM is shown. Numbers in parentheses represent range of colonies in each group.

[e] Area of foci identified by 6w/32 antibody was measured by using Metaview software. Total number of evaluated colonies was 101 and 79 for rGel and $VEGF_{121}$/rGel group, respectively. Six individual slides per each group were analyzed. The mean area of colony in each group ± SEM is shown.

[f] The sum of all regions occupied by tumor cells and the total area of each lung cross-section was determined and % of metastatic regions from total was calculated. The values obtained from each slide were averaged among 6 samples from $VEGF_{121}$/rGel or rGel control group. The mean % area occupied by metastases from total area per group ± SEM is shown.

Table 2 *Effect of $VEGF_{121}$/rGel on vascularity of pulmonary metastases of MDA-MB- 231 human breast carcinoma cells*

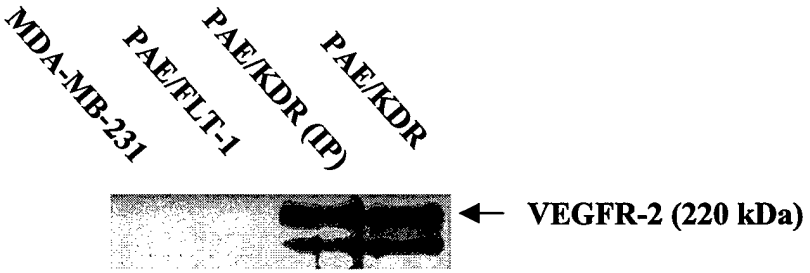
Size of colonies			No. vascularized colonies from total analyzed (%)[a]		Inhibition
Group[b]	Description	Largest diameter range (μm)	rGel	$VEGF_{121}$/rGel	% Inhibition vs. radiation treatment
A	Extremely small	<50	7/24 (29%)	3/32 (9.3%)	69
B	Small	50-200	19/48 (39.5%)	6/24 (25%)	37
C	Mid-size	200-500	25/30 (83.3%)	8/25 (32%)	62
D	Large	500-1000	17/17 (100%)	10/11 (90.0%)	10
E	Extremely large	>1000	8/8 (100%)	N/A	N/A
No. vascular foci/ total analyzed (%)[c]			76/127 (59.8%)	27/92 (29.3%)	51

[a] Frozen lung sections from $VEGF_{121}$/rGel and rGel treated mice were stained with MECA 32 antibody. A colony was defined as vascularized if at least one blood vessel branched out from the periphery and reached a center of the lesion. Six slides per each group derived from individual mice were analyzed and data were combined.

[b] Colonies identified on each slide of a metastatic lung were subdivided into 5 groups (A-E) according to their largest diameter.

[c] Total number of the analyzed colonies was 127 and 92 for rGel and $VEGF_{121}$/rGel treated groups, respectively. Seventy percent of foci in the $VEGF_{121}$/rGel-treated group were avascular whereas only 40% of lesions from the control group did not have vessels within the metastatic foci.

A.


MDA-MB-231
PAE/FLT-1
PAE/KDR (IP)
PAE/KDR
VEGFR-2 (220 kDa)

B.

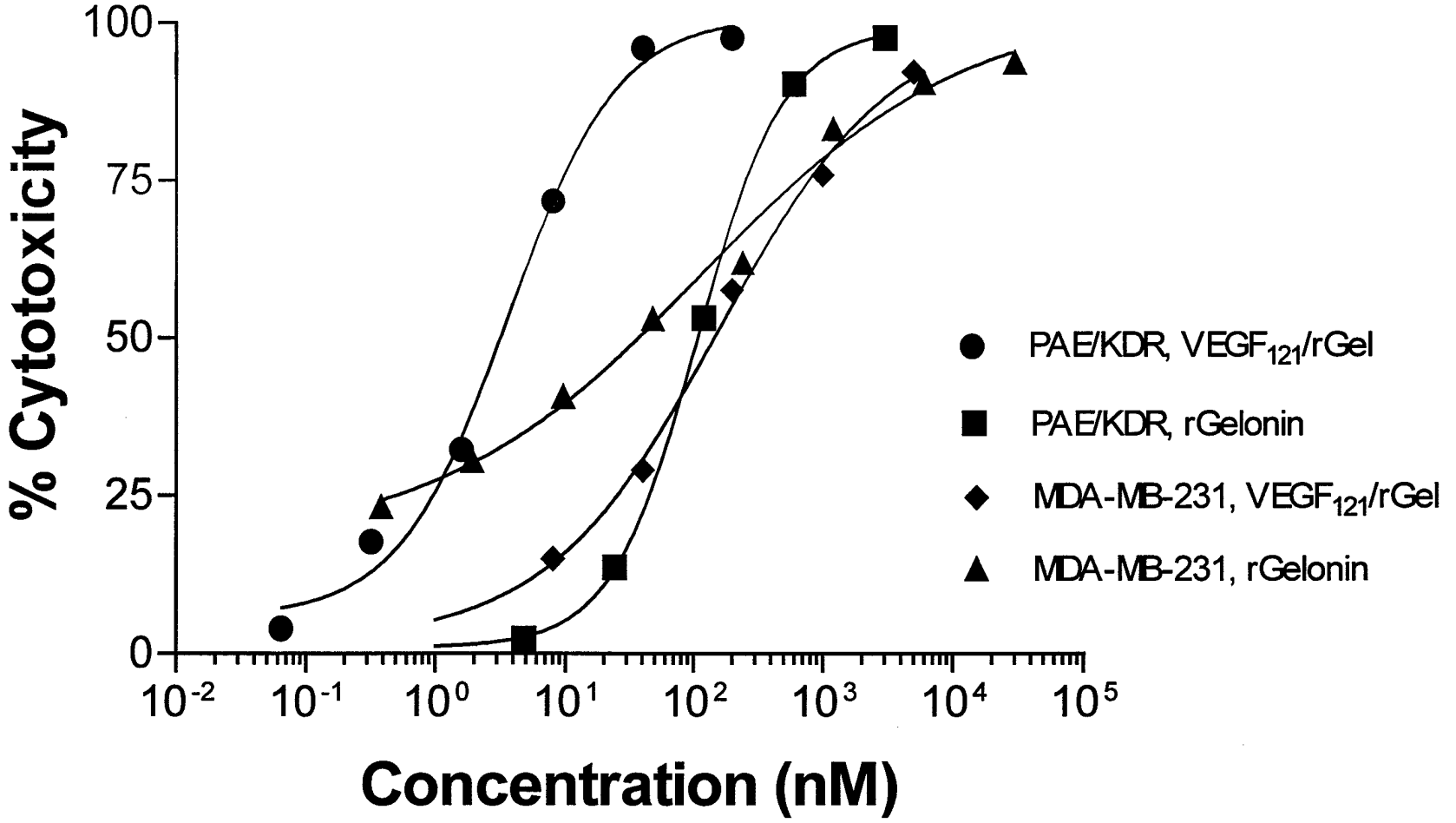

100
75
50
25
0
% Cytotoxicity
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
Concentration (nM)
PAE/KDR, $VEGF_{121}$/rGel
PAE/KDR, rGelonin
MDA-MB-231, $VEGF_{121}$/rGel
MDA-MB-231, rGelonin

Figure 1

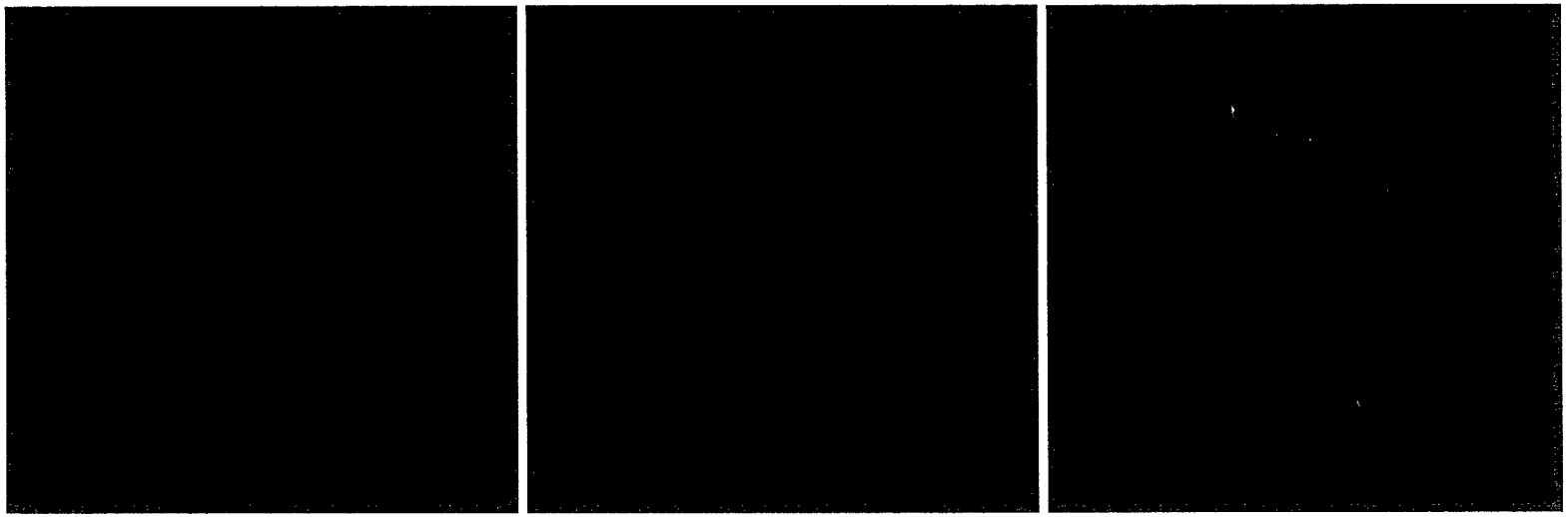


Figure 2

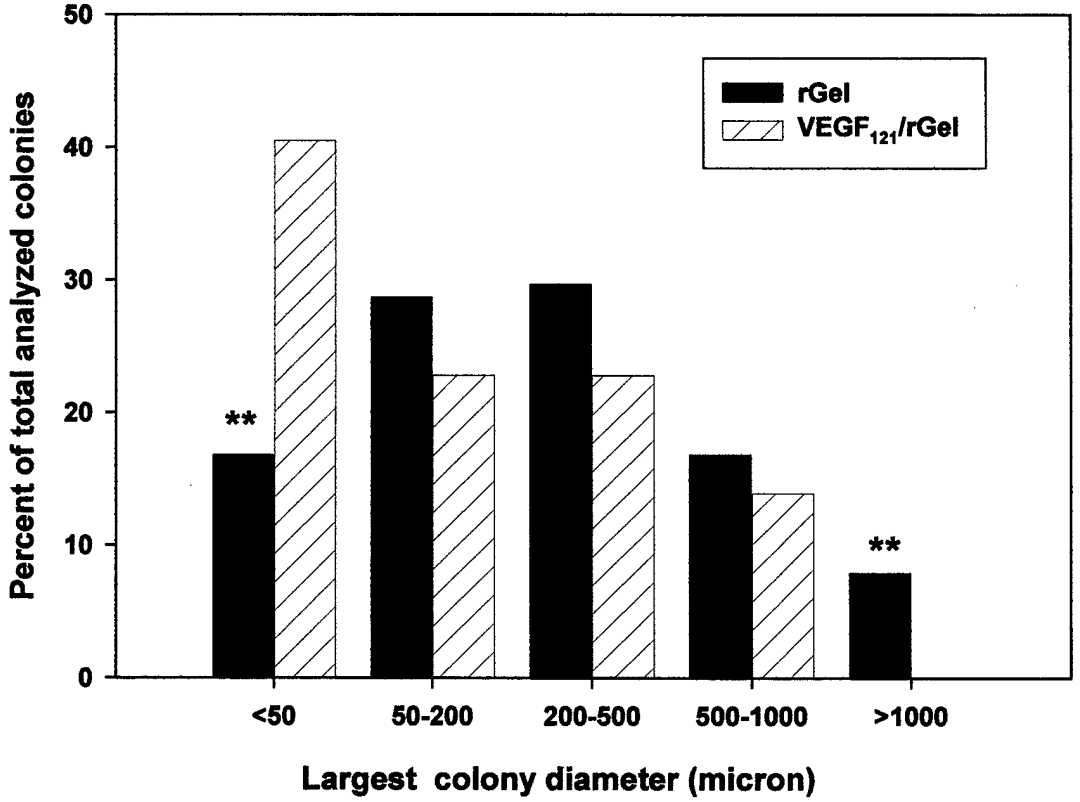

50
40
30
20
10
0
Percent of total analyzed colonies
rGel
VEGF121/rGel
**
**
<50
50-200
200-500
500-1000
>1000
Largest colony diameter (micron)

Figure 3

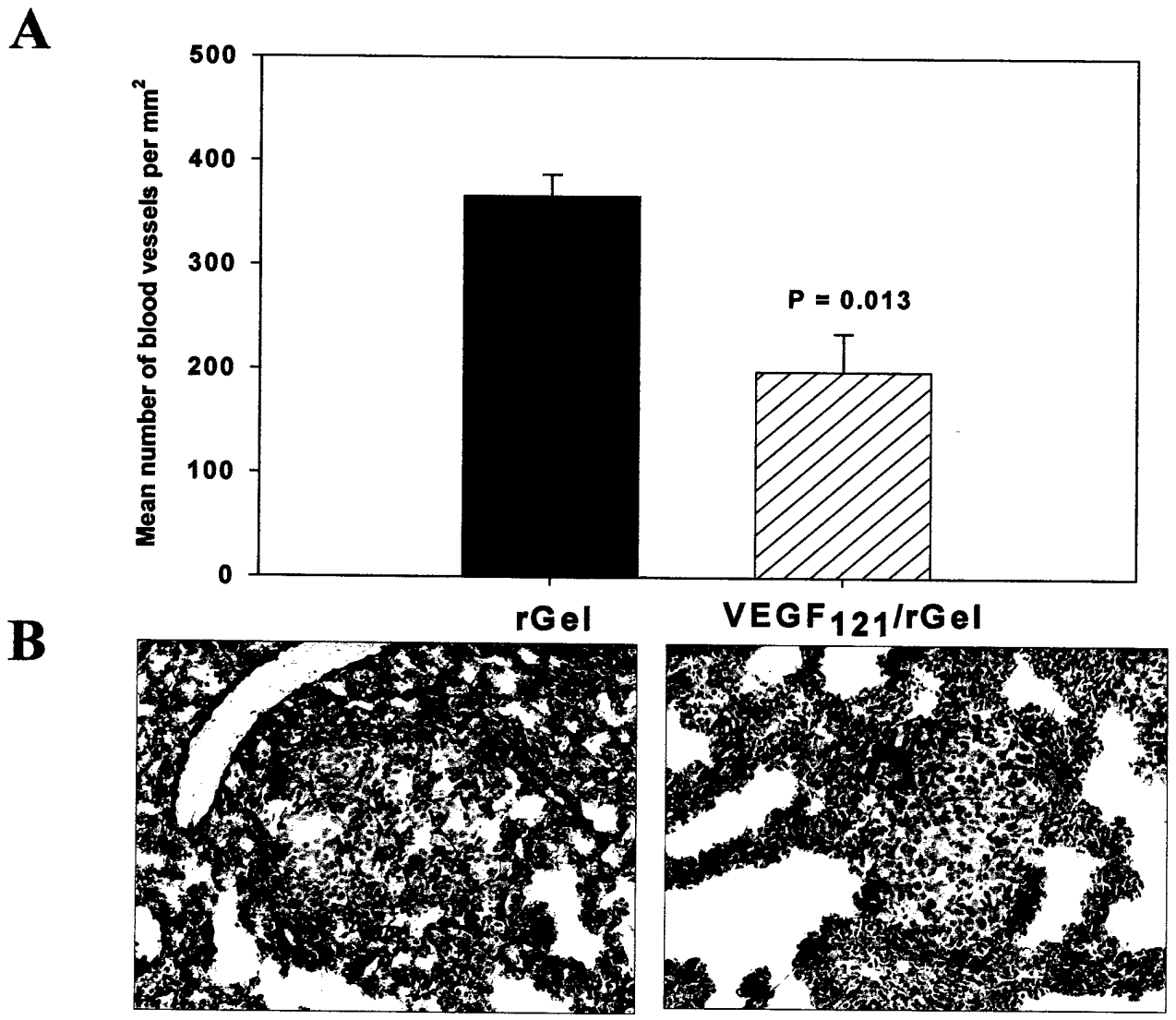

A
500
400
300
200
100
0
Mean number of blood vessels per mm2
P = 0.013
B
rGel
VEGF121/rGel

Figure 4

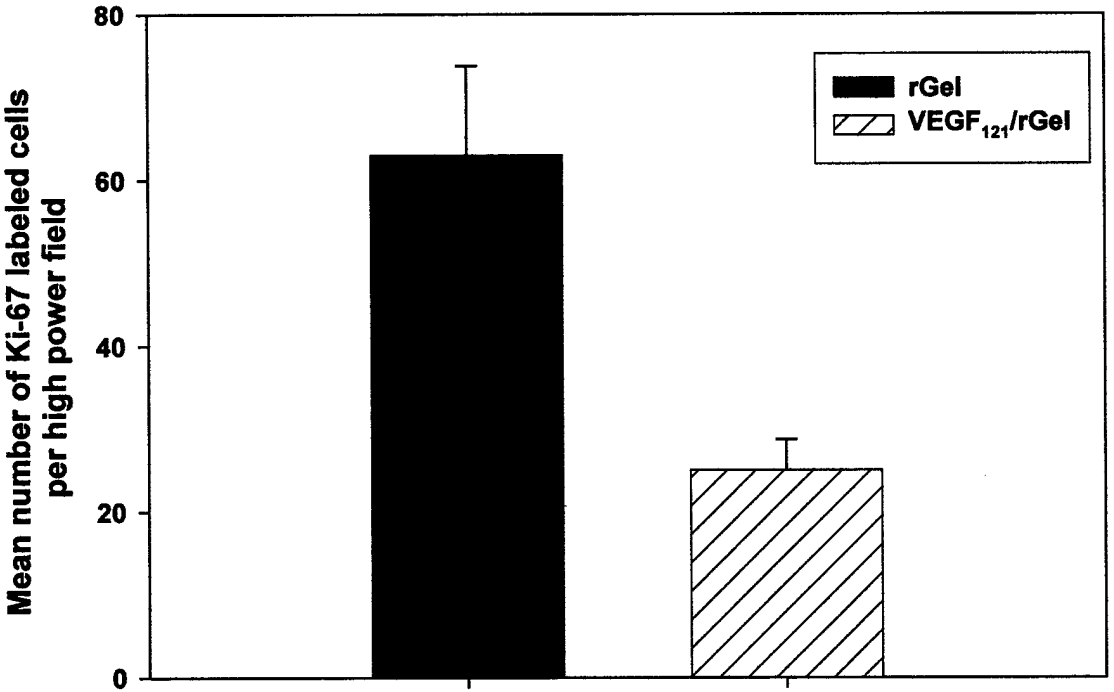

Mean number of Ki-67 labeled cells per high power field
80
60
40
20
0
rGel
VEGF121/rGel

Figure 5

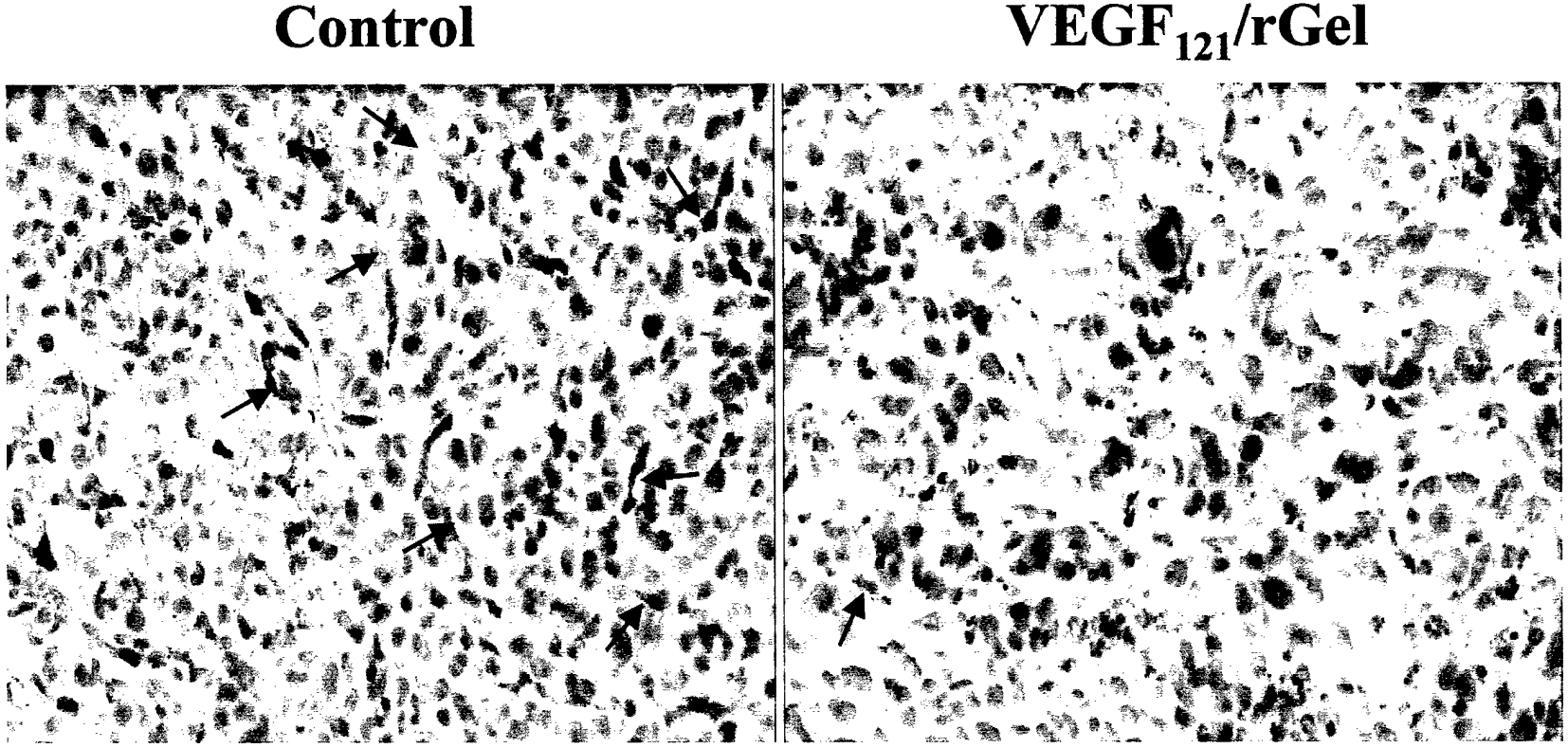


Figure 6

Manuscript submitted to The Journal of Biological Chemistry

Targeting the KDR Receptor with $VEGF_{121}$/rGel Fusion Toxin Inhibits Vascular Endothelial Growth: Specific Effects Assessed Using Microarray Analysis

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Running Title: Inhibition of Angiogenesis by $VEGF_{121}$/rGel

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Abstract

$VEGF_{121}$/rGel, a fusion protein composed of the growth factor $VEGF_{121}$ and the recombinant toxin gelonin targets the tumor neovasculature and exerts impressive cytotoxic effects on cells by inhibiting cellular protein synthesis. $VEGF_{121}$/rGel effects on angiogenesis were assessed using in vitro and in vivo models. A concentration as low as1 nM of $VEGF_{121}$/rGel was sufficient to inhibit tube formation by over 50% of endothelial cells over-expressing VEGFR-2 on matrigel-coated plates. Endothelial cells over-expressing VEGFR-1 were relatively insensitive to $VEGF_{121}$/rGel, requiring 100 nM to inhibit tube formation by 50%. Cells were similarly insensitive to unconjugated gelonin. In vascularization studies using chicken chorio-allantoic membranes, 1 nM $VEGF_{121}$/rGel completely inhibited bFGF-stimulated neovascular growth. $VEGF_{121}$/rGel treatment decreased newly-sprouting vessels but had no effect on mature vessels. As expected, treatment with gelonin alone, at equivalent concentrations, had no effect. Examination of the molecular mechanisms involved in the generation of $VEGF_{121}$/rGel-induced cytotoxicity against HUVE cells by microarray analysis revealed a total of 22 genes which were upregulated by $VEGF_{121}$/rGel treatment and included genes involved in the control of cell adhesion, apoptosis, transcription regulation, chemotaxis and inflammatory response. Upregulation of selected genes including E-Selectin, SCYA2, NF-κBIα and TNFAIP were validated by RT-PCR. Our data suggests that $VEGF_{121}$/rGel induces the expression of a unique "fingerprint" profile of genes which are involved in mediating the cytotoxic effects of this construct on tumor vascular endothelial cells. The observed cytotoxic effects of $VEGF_{121}$/rGel appear to be necrotic rather than apoptotic since no discernable TUNEL staining nor alterations in protein levels of Bax, Bcl-XL or caspase-3 was observed. These data represent for the first time an analysis of genes involved in intoxication of mammalian cells by a

toxin-based targeted therapeutic agent. Finally, these data confirm the selectivity of the fusion construct for KDR over-expressing endothelial cells and provide further support for use of this molecule in understanding the relationship of VEGFR-2 expression in the development of toxicity with VEGF-containing fusion toxin constructs.

Introduction

Angiogenesis has emerged as a critically-important developmental process in numerous pathological states and intervention in the neovascularization process may have therapeutic value in diseases including diabetic retinopathy(1-4), arthritis(5-8) and in tumor maturation and metastatic spread(9-12). The tumor neovascularization process is becoming increasingly complex as we identify more factors which can play a role in driving critical events, however, vascular endothelial growth factor-A (VEGF-A) and its receptors (Flt-1 and KDR) appear to be of exceptional importance to many aspects of this process(13-16).

With the understanding that tumor neovascularization provides an available target for therapeutic intervention, numerous groups have focused drug development strategies on the elements of this process. Strategies employing inhibitors of various growth factor receptor tyrosine kinases(17-19), blocking antibodies to interfere with receptor signal transduction(20-23) and strategies to trap growth factor ligands(24-26) have all been utilized in pre-clinical studies as well as in clinical trials with varying degrees of success.

In addition to these approaches, numerous laboratories have developed recombinant growth factor fusion constructs of VEGF-A and various toxins(27-31) to target cells bearing receptors for VEGF-A. We described a novel fusion toxin(32) composed of the 121 amino acid splice mutant of VEGF-A (designated $VEGF_{121}$) and containing the highly potent recombinant toxin gelonin (rGel) . Studies in our laboratory have demonstrated that this construct is highly cytotoxic at the nanomolar level to both log-phase and to confluent endothelial cells over expressing the KDR receptor and is not specifically cytotoxic to cells overexpressing the Flt-1 receptor. In addition, tumor xenograft studies demonstrate impressive tumor growth inhibitory effects of the fusion construct as a systemically-administered monotherapy against established

melanoma and prostate tumors. Ongoing studies in our laboratory are examining the effects of $VEGF_{121}$/rGel on numerous other orthotopic and metastatic models. Although toxin-based therapeutic agents have been studied for many years, the exact molecular mechanisms within the target cell which are impacted as part of the cytotoxic effect has never been clearly identified. Therefore, as a part of these studies, we characterize the ability of $VEGF_{121}$/rGel to inhibit tube formation in vitro and bFGF-mediated angiogenesis in vivo, and its effects on intracellular events of cells in culture using micro-array technology to delineate potential molecular mechanisms which describe a unique profile of the effects of this agent at the gene level.

Experimental Procedures

Materials: Bacterial strains, pET bacterial expression plasmids and recombinant enterokinase were obtained from Novagen (Madison, WI). All other chemicals were from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). bFGF was purchased from R&D Systems (Minneapolis, MN). TALON metal affinity resin was obtained from Clontech laboratories (Palo Alto, CA). Other chromatography resin and materials were from Pharmacia Biotech (Piscataway, NJ). Tissue culture reagents were from Gibco BRL (Gaithersburg, MD) or Mediatech Cellgro (Herndon, VA). Rabbit anti-gelonin antisera was obtained from the Veterinary Medicine Core Facility at MDACC. Antibodies against the following proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (catalog numbers in parentheses): KDR (sc-504), p-KDR (sc-16628-R), Bcl-2 (sc-7382), Bcl-XL (sc-7195), Bax (sc-493), cytochrome C (sc-8385), caspase-3 (sc-7148), caspase-6 (sc-1230), E-Selectin (14011) and actin (sc-1616), MKP-1 (sc-1199), ERK1/2 ().

Cell culture: Porcine aortic endothelial cells transfected with the KDR receptor (PAE/KDR) or the FLT-1 receptor (PAE/FLT-1) were a generous gift from Dr. J. Waltenberger. KDR and FLT-1 receptor sites per cell have been previously determined(33,34). Both cell lines have been used as in vitro models of the tumor neovasculature. Cells were maintained as a monolayer in F12 Nutrient Media (HAM) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal bovine serum. HUVECs were maintained in EBM media (Clonetics). Cells were harvested by treatment with Versene (0.02% EDTA/PBS).

Purification of $VEGF_{121}$/rGel: Construction and purification of $VEGF_{121}$/rGel was essentially as described, with minor modifications(35). $VEGF_{121}$/rGel was concentrated and stored in sterile PBS at –20°C.

Cytoxicity of $VEGF_{121}$/rGel and rGel: Cytotoxicity of $VEGF_{121}$/rGel and rGel against log phase PAE/KDR, PAE/FLT-1 and HUVE cells was performed as follows. Log phase cells (1×10^4 HUVE cells, 1×10^3 PAE/KDR and PAE/FLT-1 cells) were plated in 96 well flat-bottom tissue culture plates and allowed to attach overnight. Purified $VEGF_{121}$/rGel and rGel were diluted in culture media and added to the wells in 5-fold serial dilutions. Cells were incubated for 72 hours. The remaining adherent cells were stained with crystal violet (0.5% in 20% methanol) and solubilized with Sorenson's buffer (0.1 M sodium citrate, pH 4.2 in 50% ethanol). Absorbance was measured at 630 nm.

Effect of $VEGF_{121}$/rGel on phosphorylation of KDR: Whole cell extracts were prepared of HUVE and PAE/KDR cells, either untreated or treated with $VEGF_{121}$/rGel for up to 48 hours at their respective IC_{50} doses. Cells were lysed in 50 mM Tris (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 0.1 M KCl, 20% glycerol, 1% Triton-X100, 2 μg/ml leupeptin, 1.5 μg/ml aprotinin, 1 mM PMSF. 20 μg of cell lysate was run on a SDS-PAGE gel and the protein transferred to a PVDF/Imobilon membrane (Millipore). The membrane was blocked with 5% BSA followed by incubation for one hour with the following primary antibodies: anti-KDR, anti-p-KDR and actin. Appropriate secondary antibodies were used at 1: 2000 dilution for one hour and detected by the ECL detection kit (Amersham).

Effect of $VEGF_{121}$/rGel on E-Selectin protein levels: Whole cell extracts of HUVE and PAE/KDR cells treated for up to 48 hours with $VEGF_{121}$/rGel at their respective IC_{50} doses were analyzed for changes in levels of E-Selectin. Whole cell extracts and western blots were prepared as detailed above.

Endothelial Cell Tube Formation Assay: PAE/KDR and PAE/FLT-1 cells were grown to 80% confluence, detached by versene and plated at a concentration of 2×10^4 cells per well in a 96-well Matrigel-coated plate under reduced serum (2% FBS) conditions. Cells were treated with 100 nM, 10 nM, 1 nM, 0.1 nM and 0.01 nM $VEGF_{121}$/rGel or rGel, in triplicate, for 24 hours. Inhibition of tube formation was assessed by counting the number of tubes formed per well under bright field microscopy. The ability of $VEGF_{121}$/rGel to inhibit tube formation as a function of incubation time prior to plating on matrigel was studied by pre-treating PAE/KDR cells at the IC_{50} dose (1 nM) for different time points up to 24 hours. Cells were detached and plated in 96-well Matrigel-coated plates under the above conditions and the number of tubes per well was assessed.

Angiogenesis Assessment in Chick Chorioallantoic Membranes (CAM): Fertilized chicken eggs (SPAFAS; Charles River Lab., Wilmington, MA) were incubated at 37°C at 55% humidity for 9 days. An artificial air sac was created over a region containing small blood vessels in the chicken chorioallantoic membrane (CAM) as described(36). A small window was cut in the shell after removing 3 ml of albumen. Filter disks (6 mm in diameter) were coated with cortisone acetate in absolute ethanol (3 mg/mL). The CAM was locally treated with filter disks saturated

with a solution containing bFGF (50 ng/disk) and $VEGF_{121}$/rGel (at 1 or 10 nM), rGel (at 1 or 10 nM), or buffer (PBS). The filter was placed on the CAM in a region with the lowest density of blood vessels and in the vicinity, as reference, of a large vessel. Angiogenesis was monitored by photography 3 days after treatment. Images were captured using an Olympus stereomicroscope (SZ x12) and Spot™ Basic software (Diagnostic Instruments, Inc.). The relative vascular area was determined by measuring the area taken up by blood vessels(37). This analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The number of blood vessel branch points was quantified by two researchers, and compared to the treatment controls(36).

RNA extraction: HUVE and PAE/KDR cells were treated with their respective IC_{50} $VEGF_{121}$/rGel dose for various time periods up to 48 hours. Control cells were treated with PBS. Total RNA was extracted using the Rneasy mini-kit (Qiagen) and its integrity verified by electrophoresis on a denaturing formaldehyde-agarose gel and on a 2100 Bioanalyzer (Agilent).

Gene expression analysis: HUVEC RNA was amplified using the protocol previously described(38). The test and control samples (HUVECs treated with $VEGF_{121}$/rGel or saline, respectively, for 24 h) were labeled by using Cy3- and Cy5-dCTP in the reverse transcription reaction. Duplicate experiments were conducted by dye swapping. The labeled samples were hybridized to a cDNA array of 2304 sequence-verified clones in duplicate printed by the Cancer Genomics Core Laboratory, Department of Pathology, M.D. Anderson Cancer Center. The array includes genes involved in signal transduction, stress response, cell cycle control, hypoxia and

metastatic spread. Hybridization was performed overnight at 60°C in a humid incubator. After washing, the hybridized slides were scanned with a GeneTAC LS IV laser scanner (Genomic Solutions) and the signal intensities were quantified with ArrayVision™ (Imaging Research Inc., St. Catherines, Ontario, Canada). The local background subtracted spot intensities were used for further analysis which was performed by the MDACC in-house program for microarray analysis(39). Differentially expressed genes were identified based on a cutoff value of the T value. Generally a cut off value of |3| is considered to be statistically significant.

The data from the dye swapping experiments were designed to address concerns regarding dye bias in microarray experiments. The two factors addressed by this design are the dye incorporation differences and the gene-specific dye effects. Normalization of the data typically corrects for dye incorporation differences affecting all the genes. Dye-specific effects can be insignificant in magnitude compared to other sources of variation in the experiment(40). Hence the dye swapping experiments were treated as duplicates and genes which showed values greater than |2| in 3 out of 4 arrays were identified and the average fold change was determined.

RT-PCR Correlative Analysis: Microarray data was verified by performing RT-PCR analysis on genes which showed the highest level of induction, namely E-Selectin (SELE), cytokine A2 (SCYA2), tumor necrosis factor alpha induced protein 3 (TNFAIP3) and NF-κB inhibitor alpha (NF-κBIα). Primers were designed based on the accession numbers from the microarray and confirmation of homology using Blast (NCBI). Induction of E-Selectin in PAE/KDR cells was alos verified by RT-PCR. GAPDH primers were made as controls. The primers were as follows: SELE forward 5'GGTTTGGTGAGGTGTGCTC, SELE reverse 5' TGATCTTTCCCGGAACTGC, SCYA2 forward 5' TCTGTGCCTGCTGCTCATAG, SCYA2

reverse 5' TGGAATCCTGAACCCACTTC, TNFAIP3 forward 5' ATGCACCGATACACACTGGA, TNFAIP3 reverse 5' CGCCTTCCTCAGTACCAAGT, NF-κBIα forward 5' AACCTGCAGCAGACTCCACT, NF-κBIα reverse 5' GACACGTGTGGCCATTGTAG, PORESEL forward 5' GCCAACGTGTAAAGCTGTGA, PORESEL reverse 5' TCCTCACAGCTGAAGGCACA, GAPDH forward 5' GTCTTCACCACCATGGAG, GAPDH reverse 5' CCACCCTGTTGCTGTAGC. Isolated RNA was subjected to first-strand cDNA synthesis as described by the manufacturer of the Superscript First Strand Synthesis System (Invitrogen). RT-PCR was performed using a Minicycler PCR machine (MJ Research, Inc.).

Effect of $VEGF_{121}$/rGel on apoptotic markers: Whole cell extracts of HUVE and PAE/KDR cells treated for 24 hours with $VEGF_{121}$/rGel at their respective IC_{50} doses were analyzed for changes in levels of apoptotic markers caspase-3, caspase 6, cytochrome C, Bcl-XL, bcl-2, and Bax. Whole cell extracts and western blots were prepared as detailed above.

TUNEL assay: Log phase PAE/KDR and PAE/FLT-1 cells were diluted to 2000 cells/100 µl. Aliquots (100 µl) were added in 16-well chamber slides (Nalge Nunc International) and incubated overnight at 37°C with 5% CO_2. Purified $VEGF_{121}$/rGel was diluted in culture media and added at 72, 48 and 24-hour time points at a final concentration of 1 nM (twice the IC_{50}). The cells were then processed and analyzed for TUNEL as described by the manufacturer of the reagent. Positive control cells were incubated with 1mg/ml DNAse for 10 minutes at 37°C.

Results

$VEGF_{121}$/rGel is specifically cytotoxic to endothelial cells over-expressing KDR.

The cytotoxic effect of $VEGF_{121}$/rGel and rGel in vitro was examined employing both HUVEC and porcine endothelial(PAE) cells transfected with either human FLT-1(PAE/FLT) or KDR(PAE/KDR) receptors. Each cell type expressed varying levels of KDR receptor(41-43). Treatment of log-phase cells with $VEGF_{121}$/rGel for 72 hrs showed the greatest degree of cytotoxic effects against PAE/KDR cells, with an IC_{50} of 1 nM (Figure 1). In contrast, the IC_{50} of $VEGF_{121}$/rGel on HUVE and PAE/FLT-1 cells was approximately 300 nM. The cytotoxic effect of the untargeted rGel was similar for all three cell lines(IC_{50}~ 150 nM).

$VEGF_{121}$/rGel treatment results in activation of the KDR receptor.

We have previously shown that the cytotoxicity of $VEGF_{121}$/rGel is mediated via interaction with theVEGFR-2 (KDR) receptor and not the VEGFR-1 (FLT-1) receptor. However, the VEGF component of $VEGF_{121}$/rGel could also potentially stimulate cell growth through interaction with the receptors for VEGF. We investigated this by evaluating endogenous and phosporylated levels of KDR in endothelial cells that had been subjected to $VEGF_{121}$/rGel treatment. PAE/KDR cells expressed levels of phosphorylated KDR that increased within 2 hours of $VEGF_{121}$/rGel treatment. Phosphorylated KDR peaked at 4 hours and gradually reduced to endogenous levels by 24 hours (Figure 2). Endogenous levels of total KDR also increased by 4 hours and reduced to untreated levels by 24 hours (Figure 2A). In contrast, endogenous levels of total KDR in HUVECs decreased slightly 24 hours after treatment with $VEGF_{121}$/rGel, while phosphorylated KDR levels were markedly upregulated after 24 hours compared to untreated

cells (Figure 2B). Thus, the cytotoxic effect of rGel component of $VEGF_{121}$/rGel does not interfere with the stimulatory effect of the VEGF component.

The cytotoxic effects of $VEGF_{121}$/rGel on endothelial cells are not mediated via apoptotic mechanisms.

In order to investigate the mechanism of $VEGF_{121}$/rGel cytotoxic effect on endothelial cells, we performed a TUNEL assay on cells treated with the construct for 24, 48 and 72 hours. As shown in Figure 3, we found that no TUNEL staining was observed with PAE/KDR cells exposed to $VEGF_{121}$/rGel up to 72 hours. In contrast, nuclei of positive control cells showed intense staining. This study clearly indicates that the mechanism of cytotoxicity of $VEGF_{121}$/rGel appears to be necrotic rather than apoptotic. To confirm and validate these observations, we additionally examined various key apoptotic signaling events via western analysis (Figure 4). HUVE and PAE/KDR cells were treated with $VEGF_{121}$/rGel or saline for 24 hours and their whole cell extracts were harvested and analyzed. Levels of cytochrome C, Bcl-2 and caspase-6 were undetectable after treatment (data not shown). Caspase-3 (full length pre-cursor), Bax (a pro-apoptotic protein) and Bcl-XL (an apoptosis inhibitor) levels were not affected by $VEGF_{121}$/rGel treatment. In addition, the p11 and p20 subunits of activated/cleaved Caspase-3 were also not detected after treatment with the fusion construct.

Microarray analysis of HUVECs treated with $VEGF_{121}$/rGel.

To further elucidate biochemical mechanisms which account for the effects of $VEGF_{121}$/rGel on endothelial cells, we treated HUVECs with saline or the IC_{50} dose of $VEGF_{121}$/rGel for 24 hours. RNA was isolated, evaluated for integrity, and subjected to

microarray analysis. A dye-swap comparison was performed for reproducibility. The signal-to-ratio of the images were evaluated to determine the quality of the array in terms of how many spots had sufficient signal intensity above noise. Only those spots with a signal-to-noise ratio of ≥2 were evaluated (80%). Only those differentially expressed genes whose levels were elevated by ≥2-fold over the baseline value in repeated experiments were selected. On this basis, 22 different genes (out of 4800) were upregulated by $VEGF_{121}$/rGel at 24 hours (Table 1). In addition to upregulation of select genes known to be induced by VEGF alone, $VEGF_{121}$/rGel treatment was found to upregulate genes involved in inflammation, chemotaxis and transcription regulation. The highest-expressing genes from four gene classifications were validated by RT-PCR. Normalized for GAPDH, all four of the other PCR products showed an increase upon treatment with $VEGF_{121}$/rGel, validating the results observed in the original microarray (Figure 5A). However, induction of E-Selectin protein levels did not match the induction of mRNA (Figure 5B). Because PAE/KDR cells have been used as in vitro models for endothelial cells in the tumor neovasculature, we investigated the effect of $VEGF_{121}$/rGel on gene induction and protein expression on these cells. PAE/KDR cells were treated with saline or the IC_{50} dose of $VEGF_{121}$/rGel for up to 48 hours. As shown in Figure 6A, RT-PCR of E-Selectin confirmed the increase in message within 2 hrs after treatment of cells with $VEGF_{121}$/rGel. In addition, western analysis also demonstrated a slight increase in E-Selectin protein expression although the increase in cellular protein levels was very modest compared to the observed increase in message (Figure 6B). Western blots using anti-MKP-1 and anti-ERK1/2 antibodies also showed no change in protein expression (data not shown).

$VEGF_{121}$/rGel inhibits tube formation in KDR-expressing endothelial cells.

The anti-angiogenic effect of $VEGF_{121}$/rGel in vitro was investigated by examining inhibition on tube formation in receptor-transfected PAE cells. PAE/KDR and PAE/FLT-1 cells were placed on matrigel-coated plates and varying concentrations of either $VEGF_{121}$/rGel or rGel were then added and tube formation was assessed 24 hrs later. As shown in Figure 7A, addition of 1 nM $VEGF_{121}$/rGel significantly inhibited tube formation on KDR-transfected cells while rGel alone had little effect at this dose level. Doses of rGel itself caused ~42% inhibition only at the highest doses tested (100 nM). 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 or rGel to inhibit tube formation by 50% (Figures 7B). To investigate if pre-treatment of PAE/KDR cells with $VEGF_{121}$/rGel affected tube formation, cells were treated with the IC_{50} dose of $VEGF_{121}$/rGel for 4, 16 and 24 hours, washed with PBS, detached and added to matrigel-coated plates in $VEGF_{121}$/rGel-free media for 24 hours. Pre-incubation of cells with $VEGF_{121}$/rGel for at between 16 and 24 hrs virtually abolished tube formation (Figure 8).

$VEGF_{121}$/rGel inhibits angiogenesis in the CAM of chicken embryos.

We investigated the *in vivo* antiangiogenic effects of $VEGF_{121}$/rGel using a chicken CAM model. Angiogenesis in the CAMs of 9-day chicken embryos were stimulated by treatment with filter disks containing either bFGF, or bFGF in addition to $VEGF_{121}$/rGel (at the concentrations of 1 and 10nM), rGel (at 1 or 10 nM), or buffer (PBS) alone(45). CAMs were treated for 72 hrs and the effect of the different treatments was quantified by determining the relative vascularized area, which is based on the area taken up by blood vessels, normalized to that of CAMs treated with PBS (equal to 100%). CAMs treated with bFGF showed a significant increase in

vascularized area of about 35% ($P < 0.001$, t-test, double-sided) over PBS-treated CAMs (Figure 9A and Figure 10A). This observation was consistent with a significant increase of more than 60% in the number of newly-sprouted vessels with respect to the PBS-treated CAMs ($P < 0.001$, t-test, double-sided) (Figure 10B). Incubation of CAMs with bFGF with or without 10 nM rGel resulted in angiogenic activity and the formation of ordered neovasculature (Figures 9A andB). However, treatment with 1nM or 10 nM $VEGF_{121}$/rGel resulted in significant destruction of the neovasculature (Figure 9C). Treatment with $VEGF_{121}$/rGel completely inhibited bFGF-stimulated angiogenesis ($P < 0.001$, t-test, double sided) (Figure 10). Many CAMs also appeared devoid of vessel infiltration. Interestingly, the number of branching points in the $VEGF_{121}$/rGel-treated CAMs was similar to PBS-treated CAMs ($P > 0.5$, t-test, double-sided) (Figure 10B), suggesting that the $VEGF_{121}$/rGel mainly inhibits bFGF-mediated formation of newly sprouting branches from pre-exisiting vessels. As expected, the disks treated with bFGF in combination with rGel (at 1 or 10 nM), consistently showed extensive vascularization, that was comparable to the single treatment with bFGF ($P > 0.5$, t-test, double sided).

Discussion

Our study clearly demonstrates that the $VEGF_{121}$/rGel fusion construct is specifically cytotoxic to KDR over-expressing endothelial cells and that the cytotoxic effect observed appears to be due to necrosis rather than to an apoptotic effect. Previous studies with gelonin-based immunotoxins targeting tumor cells have also observed that intoxicated cells do not appear to display apoptotic characteristics(44). In this regard, the toxin gelonin appears to be distinct from other toxins such as engineered diptheria toxin (DT) (45,46) and pseudomonas exotoxin (PE), (47,48) which have both been demonstrated to generated an apoptotic damage to target cells. A closely-related toxin, ricin-A chain (RTA) also generates an apoptotic damage to target cells(49,50), however, Baluna et al(51) suggest that different portions of the RTA molecule may be responsible for generating apoptotic and necrotic effects.

VEGF-A has been shown to play a role in tube formation of endothelial cells in vitro(52,53) as well as angiogenesis(54-56). The effect of $VEGF_{121}$/rGel on tube formation of endothelial cells on matrigel-coated plates was striking in that KDR over-expressing cells but not cells over-expressing the FLT-1 receptor were affected. This result correlates with our findings that $VEGF_{121}$/rGel is cytotoxic only to KDR-expressing cells(57) and that $VEGF_{121}$/rGel is internalized only into cells that express KDR but not FLT-1 (manuscript submitted). The fact that the IC_{50} dose for cytotoxicity is identical to the IC_{50} dose preventing tube formation in PAE/KDR cells and suggests that $VEGF_{121}$/rGel action in vitro causes immediate disruption of angiogenic tube formation as a temporal prelude to its eventual cytotoxicity to rapidly dividing endothelial cells. Our preliminary results examining in vivo endothelialization of matrigel plugs appears to support the observation that the $VEGF_{121}$/rGel construct is capable of ablating neo-vascularization at several steps in this complex process.

Chorio-allantoic (CAM) membrane assays are frequently used to assess the anti-angiogenic potential of agents(58-61). The inhibition by $VEGF_{121}$/rGel of tube formation in vitro translates well to inhibition of vascular endothelial growth and of neovasculature in vivo. Treatment with doses as low as 1 nM of $VEGF_{121}$/rGel resulted in complete ablation of b-FGF induced neovasculature. Not surprisingly, the CAM assay also demonstrated that mature vessels did not appear to be affected by treatment with the construct. This is a critical finding which suggests that mature vessels in normal tissues as well as mature vessels in tumors do not appear to be affected by this agent. Therefore, treatment of small newly-vascularizing tumors and metastases may be the most susceptible to therapy with this agent.

To better understand the cytotoxic effects of $VEGF_{121}$/rGel at the molecular level, we subjected HUVECs to treatment with $VEGF_{121}$/rGel for 24 hours, and harvested the RNA for microarray analysis. The results suggest that treatment of HUVECs with $VEGF_{121}$/rGel results in the increase in the RNA levels of several genes that are involved in inflammation, chemotaxis, intermediary metabolism and apoptotis pathways (Table 1). Only two of these genes (MKP-1 and CXCR4) were also found to be upregulated by treatment with $VEGF_{165}$ for 24 hours(62). Therefore, most of the genes we found to be upregulated in our study appear to be attributed to the $VEGF_{121}$/rGel construct instead of the VEGF component itself. As far as we are aware, this is the first microarray analysis performed on cells treated with plant-derived protein toxins such as gelonin.

By far, the highest level of mRNA induction was observed with the cell adhesion molecule E-Selectin. In previous studies, treatment with VEGF has been shown to induce adhesion molecules (E-Selectin, VCAM-1 and ICAM-1) in HUVECs(63-65) via an NFκB-mediated process. E-Selectin has been shown to be upregulated by VEGF treatment(66) or in

response to inflammation(67,68), and plays an important role in tube formation as well as angiogenesis. E-Selectin also plays a major role in the adhesion of epithelial cancer cells to the endothelium(69). The ability of cancer cell clones to bind E-Selectin on endothelial cells appears to be directly proportional to their metastatic potential(70,71). Moreover, drugs such as cimetidine, which inhibits the expression of E-Selectin, blocks the adhesion of tumor cells to the endothelium and prevents metastasis(72). However, E-Selectin does not necessarily have a role in the adhesion of all cancer cells(73,74), nor do all cancer cells require expression of the same endothelial adhesive molecule(75,76). This study shows that $VEGF_{121}$/rGel falls into the class of molecules that can prevent E-Selectin-mediated metastasis because protein levels rise barely 2-fold following $VEGF_{121}$/rGel treatment in both PAE/KDR and HUVECs. This pattern of induction of RNA but not protein levels was observed with other genes as well. For example, although MKP-1 RNA levels were induced in HUVECs following $VEGF_{121}$/rGel treatment, a corresponding increase in protein levels was not observed in western blots of PAE/KDR and HUVEC whole cell extract (data not shown). In addition, levels of ERK1/2, shown to be upregulated by MKP-1 in HUVECs following endothelial cell injury(77), do not change over 48 hours following $VEGF_{121}$/rGel treatment. Taken together, we conclude that although mRNA levels of genes that are important in cell adhesion, migration and spreading are induced in response to $VEGF_{121}$/rGel, a concomitant increase in protein expression was generally not observed. Since the rGel component of the fusion construct operates by inhibition of protein synthesis, this agent could inhibit synthesis of critical proteins which are important for suppression of these specific genes. In addition, these data suggests that in addition to the cytotoxic effect of $VEGF_{121}$/rGel, this agent may additionally act through cellular mechanisms involved in inflammation and stress.

Several genes have been shown to be induced as a result of inflammation of HUVECs. For example, Early growth response factor 1 (EGR1), SCYA2 (MCP-1), E-Selectin and VCAM-1 are all up-regulated in HUVECs(78,79), and all of these genes are induced by $VEGF_{121}$/rGel treatment. Several members of the small inducible cytokine (SCYA) family of proteins were also over-expressed as a result of $VEGF_{121}$/rGel treatment. All respond to inflammation stimuli, and play a role in chemotaxis. SCYA2 plays a role in inflammation and wound healing(80-82); SCYA4 (MIP-1β) is involved in directional migration of cells during normal and inflammatory processes(83,84); SCYA7 (MCP-3) and SCYA11 (Eotaxin) share a 65% amino acid sequence identity and play major roles in the recruitment and activation of eosinophils in allergic disorders(85,86). However, they bind to different receptors and have different modes of action(87). Another molecule that plays a role in chemotaxis is CXCR4. Although CXCR4 shows a lower than 2 fold change (88), the array spot intensities and reproducibility indicate that the increase is significant.

One of the larger classes of genes to be upregulated by treatment with $VEGF_{121}$/rGel were transcription factors. Interestingly, two of them are from the NF-κB family, NF-κBIα (IκB-α) and NF-κB (p105 subunit). Since NF-κB and IκB-α interact in an autoregulatory mechanism, the up-regulation of IκB-α is most likely the result of NF-κB mediating activation of the IκB-α gene, resulting in replenishment of the cytoplasmic pool of its own inhibitor(89-92). NF-κB may play a role in the upregulation of several genes including SCYA2, SCYA7, SCYA11 and JunB(93,94). Another transcription factor, Kruppel-like factor (KLF4), has not previously been shown to be expressed in endothelial cells. However, it has been shown to be an important nuclear factor in upregulation of histidine decarboxylase, an enzyme that catalyzes the

conversion of histidine to histamine, a bioamine that plays an important role in allergic responses, inflammation, neurotransmission and gastric acid secretion(95).

In terms of apoptosis, TNFAIP3, a putative DNA binding protein that is part of the NF-κB signal transduction pathway, functions by inhibiting NF-κB activation and TNF-mediated apoptosis(96). BIRC3, another gene that is upregulated during $VEGF_{121}$/rGel treatment, has been shown to form a hetero-dimer with a caspase 9 monomer in vitro and prevents the latter's activation in apoptosis(97). It was surprising to note that several genes involved in the control of the apoptotic pathway were modulated in response to the fusion toxin even though the overall cytotoxic effect on target cells did not show an impact on the apoptotic pathway.

A critical finding of this study was the identification of a number of genes which are regulated in response to treatment with the $VEGF_{121}$/rGel fusion construct. Since many of these genes impact cell adhesion, chemotaxis and inflammatory responses, it is possible that the construct may have an impact on tumor development in addition to its direct cycotoxic effects on tumor neovasculature. Therefore, an important consideration for future study is the effect of $VEGF_{121}$/rGel cytotoxicity to tumor endothelial cells and its potential bystander effects on adjacent tumor cells. Current studies are underway on breast and prostate orthotopic and metastatic(lung and bone) tumor cells to further characterize the effects of this drug in vitro and in vivo.

Figure Legends

Figure 1. Cytotoxicity of $VEGF_{121}$/rGel on endothelial cells. Log phase HUVECs, PAE/KDR and PAE/FLT-1 cells were plated in 96-well plates and incubated with serial dilutions of $VEGF_{121}$/rGel and rGel for 72 hours as described in Experimental Procedures. The cytotoxicity experiment was performed in triplicate, and data points are represented as the mean.

Figure 2. PAE/KDR and HUVECs decrease KDR levels and increase phosphorylated KDR levels upon 24h treatment with $VEGF_{121}$/rGel at their IC_{50} doses. The effect of $VEGF_{121}$/rGel on KDR and activated KDR levels was studied. Samples were prepared as described in Materials and Methods and probed with anti-KDR and anti-p-KDR antibodies. Actin levels were determined as a loading control. (A) Total KDR and p-KDR levels in PAE/KDR cells show an increase within 2 hours of treatment and gradually level off by 48 hours. (B) KDR levels of HUVECs decrease slightly 24 hours after $VEGF_{121}$/rGel treatment but p-KDR levels increase.

Figure 3. TUNEL assay of endothelial cells treated with $VEGF_{121}$/rGel. Cytotoxicity of $VEGF_{121}$/rGel to PAE/KDR cells does not result in apoptosis. PAE/KDR cells were grown overnight and a dose of 1 nM $VEGF_{121}$/rGel was added and the cells were further incubated for 24, 48 and 72 hours. The cells were analyzed for TUNEL. Positive control cells were incubated with 1 mg/ml DNAse for 10 minutes at 37°C.

Figure 4. $VEGF_{121}$/rGel treatment does not affect apoptosis markers in endothelial cells. To understand the mechanism of cytotoxicity of $VEGF_{121}$/rGel, protein levels of key apoptosis markers were assessed 24 hours after treatment. Protein levels of Bax, Bcl-XL and Caspase-3 were unchanged, suggesting that the mechanism of cell death is not apoptotic. Levels of cytochrome C, Caspase-6 and Bcl-2 were not detectable (data not shown).

Figure 5. Analysis of HUVECs treated with $VEGF_{121}$/rGel. (A) Validation of the microarray by RT-PCR. Upregulation of genes for E-Selectin, TNFAIP3, NF-κBIα and SCYA2 were validated by RT-PCR. GAPDH levels were assessed as a control. (B) Protein levels of E-Selectin in HUVECs treated with $VEGF_{121}$/rGel.

Figure 6. Translation of upregulated E-Selectin RNA is inhibited $VEGF_{121}$/rGel. (A) RNA from PAE/KDR cells that were untreated or treated with $VEGF_{121}$/rGel for the time-points indicated were examined by RT-PCR for upregulation of E-Selectin, one of the genes identified by micro-array analysis. GAPDH primers were used as a control for loading. RNA levels of E-Selectin are all up-regulated in PAE/KDR cells as seen in HUVECs. (B) Protein levels of E-Selectin are also up-regulated.

Figure 7. $VEGF_{121}$/rGel inhibits tube formation of PAE/KDR cells. PAE/KDR and PAE/FLT-1 cells were added to matrigel-coated plates, treated with different concentrations of $VEGF_{121}$/rGel and rGel as indicated, and analyzed for tube formation after 24 hours. (A) A 1 nM dose of $VEGF_{121}$/rGel was sufficient to inhibit tube formation by 50%, whereas the same

degree of inhibition was seen with 100 nM rGel. (B) In contrast, up to 100 nM $VEGF_{121}$/rGel was needed to inhibit tube formation of PAE/FLT-1 cells, same as the untargeted gelonin toxin.

Figure 8. Inhibition of tube formation of PAE/KDR cells by $VEGF_{121}$/rGel is time-dependent. PAE/KDR cells were treated with 1 nM $VEGF_{121}$/rGel for the times indicated, detached, incubated on matrigel-coated plates for 24 hours, and assessed for tube formation. Incubation of PAE/KDR cells with $VEGF_{121}$/rGel for as little as 9 hours was sufficient to abolish the ability of these cells to form tubes by 50%.

Figure 9. $VEGF_{121}$/rGel inhibits angiogenesis in the CAM of chicken embryos. Shown are representative CAMs treated with bFGF (50ng), or bFGF in combination with $VEGF_{121}$/rGel (1nM) or rGel (10nM) (x 0.5 objective). Angiogenesis was induced on CAMs from 9-days chick embryos by filter disks saturated with bFGF. Disks were simultaneously treated with $VEGF_{121}$/rGel or rGel. At 72 hours, CAMs were harvested and analyzed under an Olympus stereomicroscope. Experiments were repeated twice per treatment, with 6 to 10 embryos per condition in every experiment. Panel A shows the vasculature of CAMs following stimulation with bFGF alone. Recombinant gelonin had no effect on angiogenic stimulation of bFGF at 1 nM or 10 nM rGel (Panel B) concentrations. Panel C depicts the inhibitory effect on angiogenesis of 1 nM $VEGF_{121}$/rGel in the presence of 50 ng bFGF.

Figure 10. $VEGF_{121}$/rGel reduces the vascular area and the number of vascular branches in the CAM assay. Quantitative evaluation of $VEGF_{121}$/rGel-mediated inhibition of the angiogenesis in the CAM model was determined, after the indicated treatments, by image

analyses and the results were normalized to CAMs treated with PBS (equal to 100%). (A) $VEGF_{121}$/rGel at 1 nM and 10 nM concentrations both suppressed vascular area. As expected, Gelonin had no effect. Data are represented as mean ± standard deviation from replicated experiments. *, $P < 0.001$, t-test, double-sided. (B) $VEGF_{121}$/rGel Decreases the Number of Newly Sprouting Vessels. 1 nM $VEGF_{121}$/rGel had a dramatic effect on the formation of neovasculature, completely inhibiting b-FGF mediated stimulation of neovasculature. As expected, rGel had no effect on the number of newly sprouting vessels. Data are represented as mean ± standard deviation from replicated experiments. *, $P < 0.001$, t-test, double-sided.

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Table 1

HUVEC genes that increase following treatment with $VEGF_{121}$/rGel for 24 hours, compared to untreated cells

Gene Classification	Accession Number	Symbol	Gene	Mean Fold Change
Cell adhesion	H39560	SELE	Selectin E (endothelial adhesion molecule 1)*	94.6
	H07071	VCAM	Vascular cell adhesion molecule 1	4.9
	AA284668	PLAU	Plasminogen activator, urokinase	2.3
Apoptosis	AA476272	TNFAIP3	Tumor necrosis factor alpha-induced protein 3*	13.5
	H48706	BIRC3	baculoviral IAP repeat-containing 3	3.3
Transcription regulation	T99236	JUNB	jun B proto-oncogene	4.9
	W55872	NF-κBIα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*	4.8
	AA451716	NF-κB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	2.3
	H45711	KLF4	Kruppel-like factor 4	2.3
Chemotaxis	AA425102	SCYA2	small inducible cytokine A2 (MCP-1)*	20.2
	H62985	SCYA4	small inducible cytokine A4 (MIP-1β)	5.8
	AA040170	SCYA7	small inducible cytokine A7 (MCP-3)	5.5
	T62491	CXCR4	chemokine (C-X-C motif), receptor 4 (fusin)	1.85
Structural Organization	NM004856	KNSL5	kinesin-like 5 (mitotic kinesin-like protein 1)	6.4
	AA479199	NID2	nidogen 2	3.1
	AA453105	H2AFL	H2A histone family, member L	2.5

Inflammatory Response	W69211	SCYA11	small inducible cytokine A11 (Cys-Cys) (eotaxin)	8.4
	NM_001964	EGR1	early growth response 1	3.9
	NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2 (COX-2)	3.3
	AA148736	SCD4	syndecan 4 (amphiglycan, ryudocan)	3.2
Signalling	W65461	DUSP5	dual specificity phosphatase 5 (MKP-1)	2.7
Metabolic	AA011215	SAT	spermidine/spermine N1-acetyltransferase	2.1

* Confirmed by RT-PCR at 4 and 24 hours post-treatment

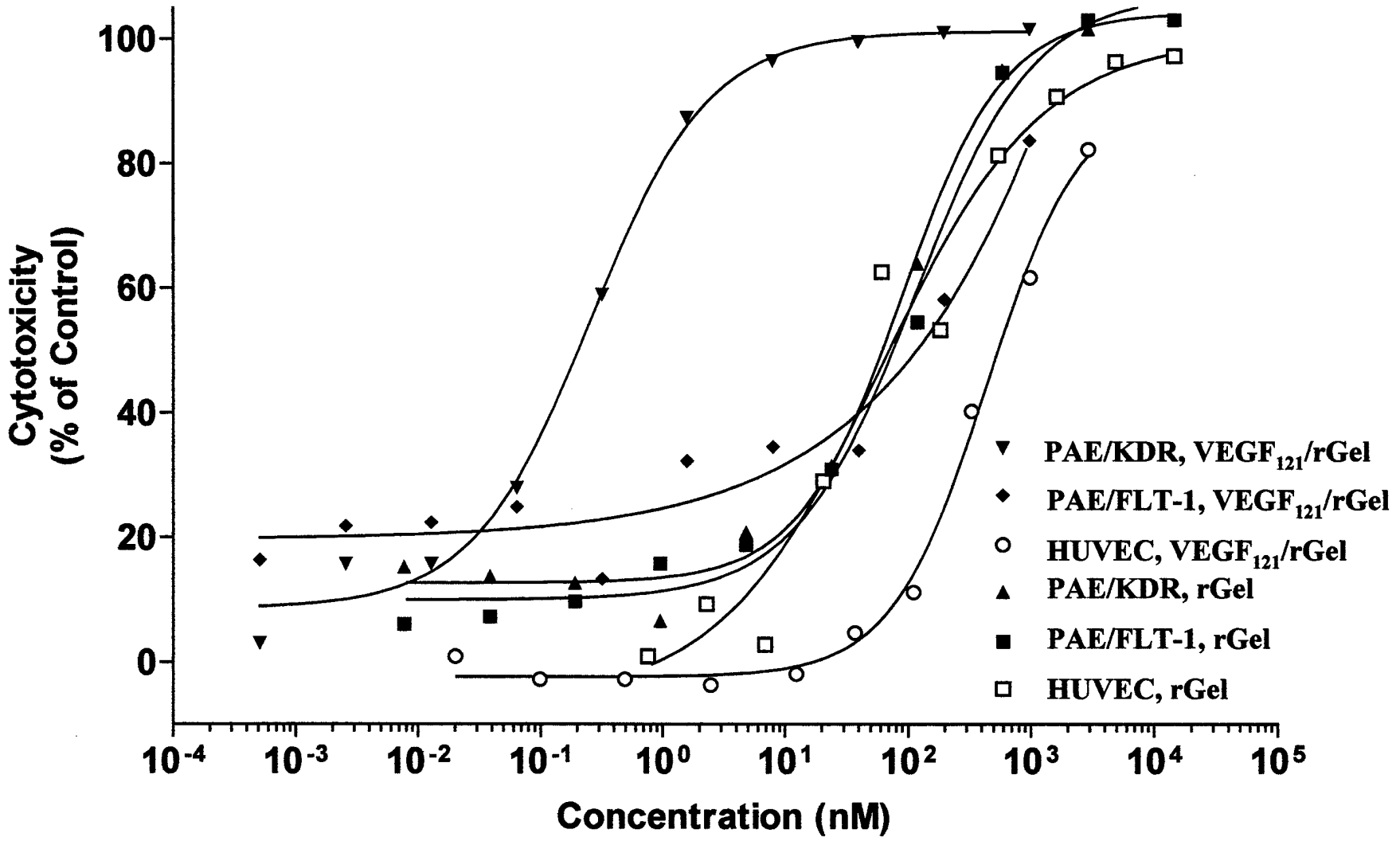

Cytotoxicity
(% of Control)
100
80
60
40
20
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
Concentration (nM)
PAE/KDR, $VEGF_{121}$/rGel
PAE/FLT-1, $VEGF_{121}$/rGel
HUVEC, $VEGF_{121}$/rGel
PAE/KDR, rGel
PAE/FLT-1, rGel
HUVEC, rGel

Figure 1

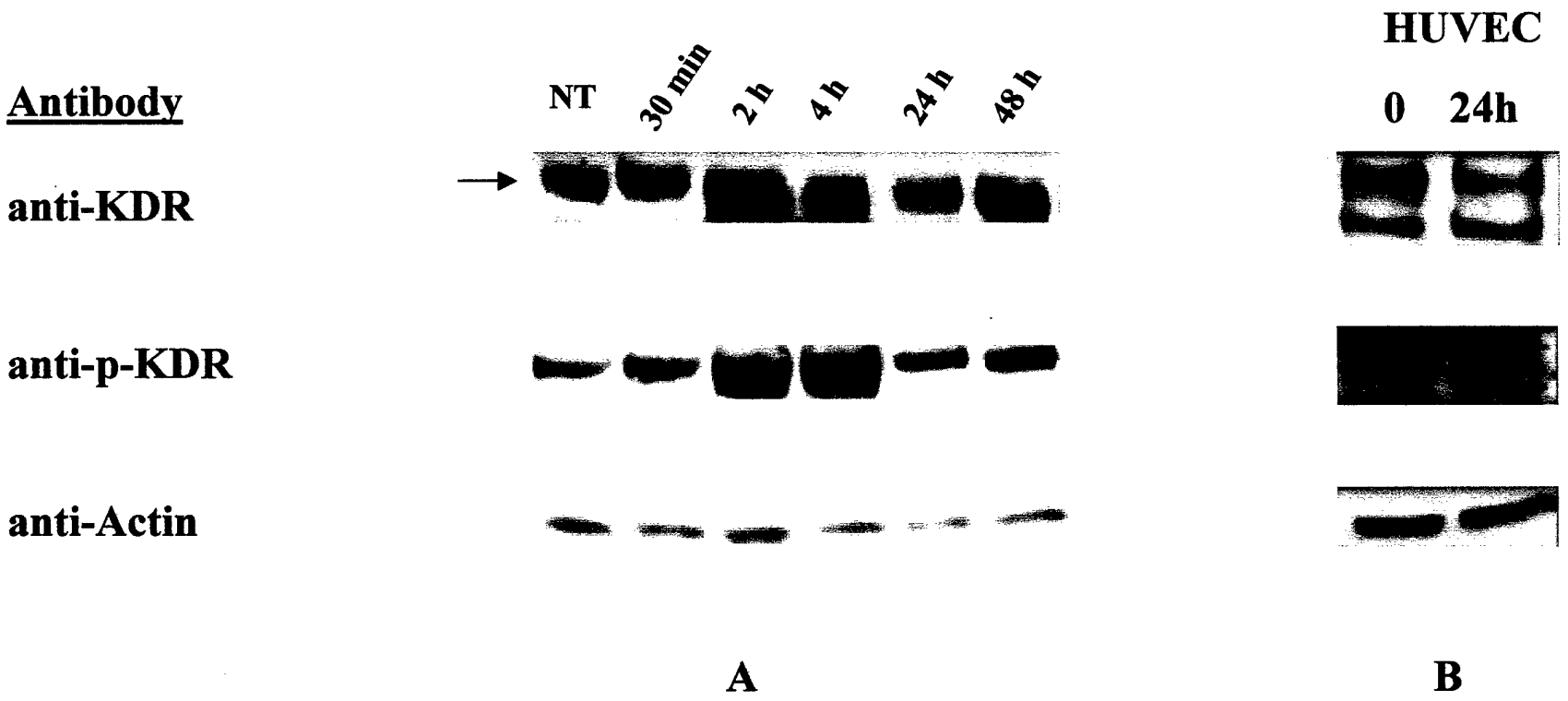

Antibody
NT
30 min
2 h
4 h
24 h
48 h
anti-KDR
anti-p-KDR
anti-Actin
A
HUVEC
0
24h
B

Figure 2

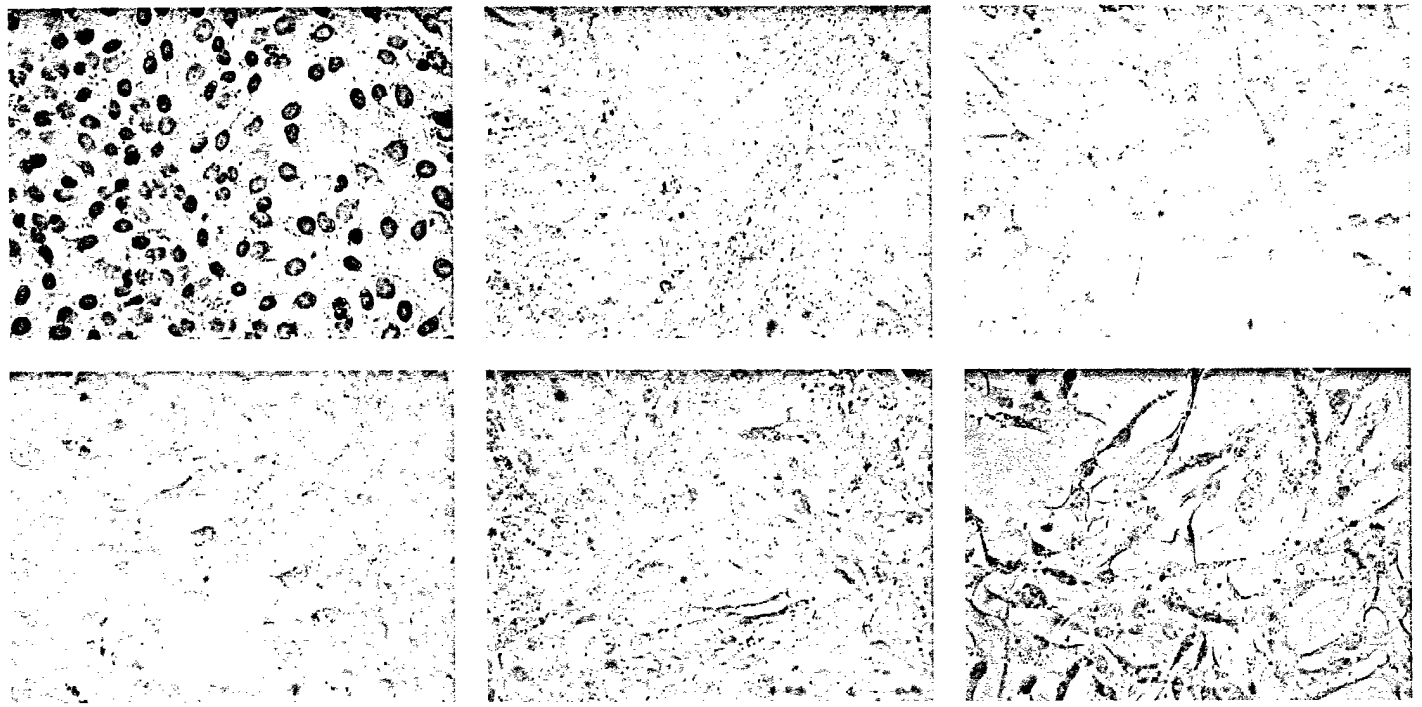


Figure 3

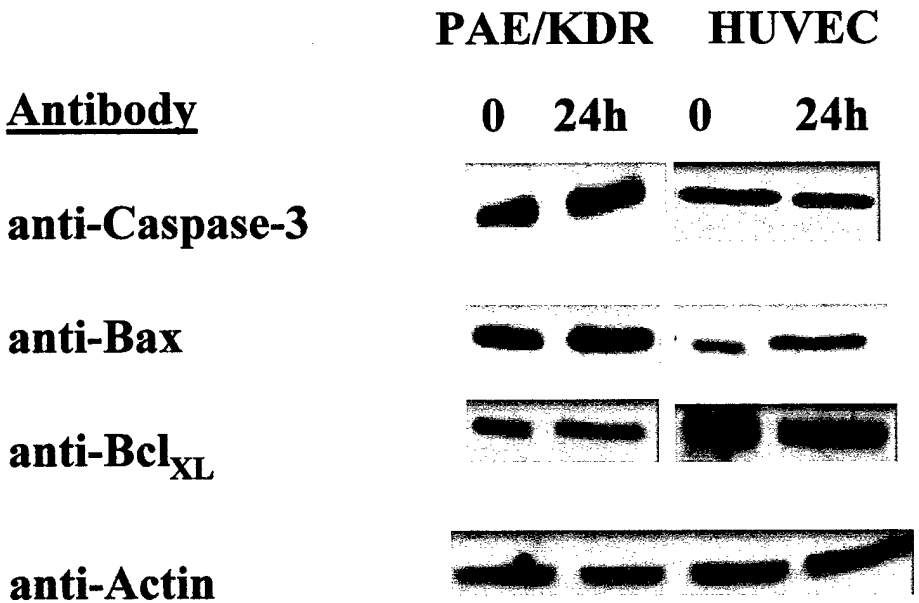
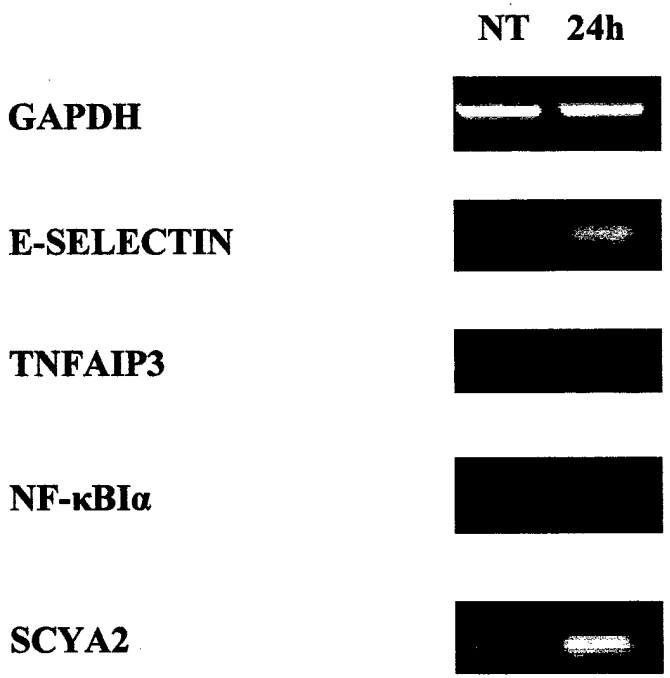

PAE/KDR
HUVEC
Antibody
0
24h
0
24h
anti-Caspase-3
anti-Bax
anti-Bcl$_{XL}$
anti-Actin

Figure 4


NT 24h
GAPDH
E-SELECTIN
TNFAIP3
NF-κBIα
SCYA2

A.

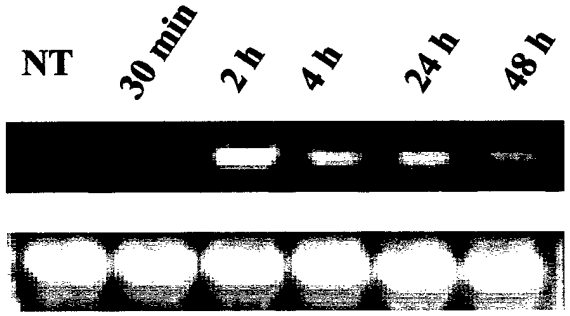

NT 24h
E-SELECTIN

B.

Figure 5

RT-PCR Analysis

A.



Western Analysis

B.

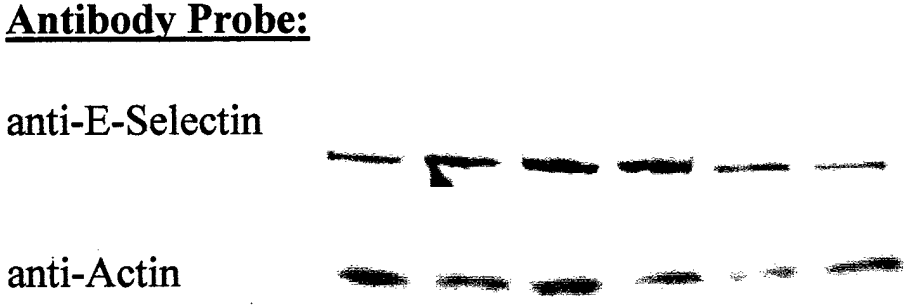
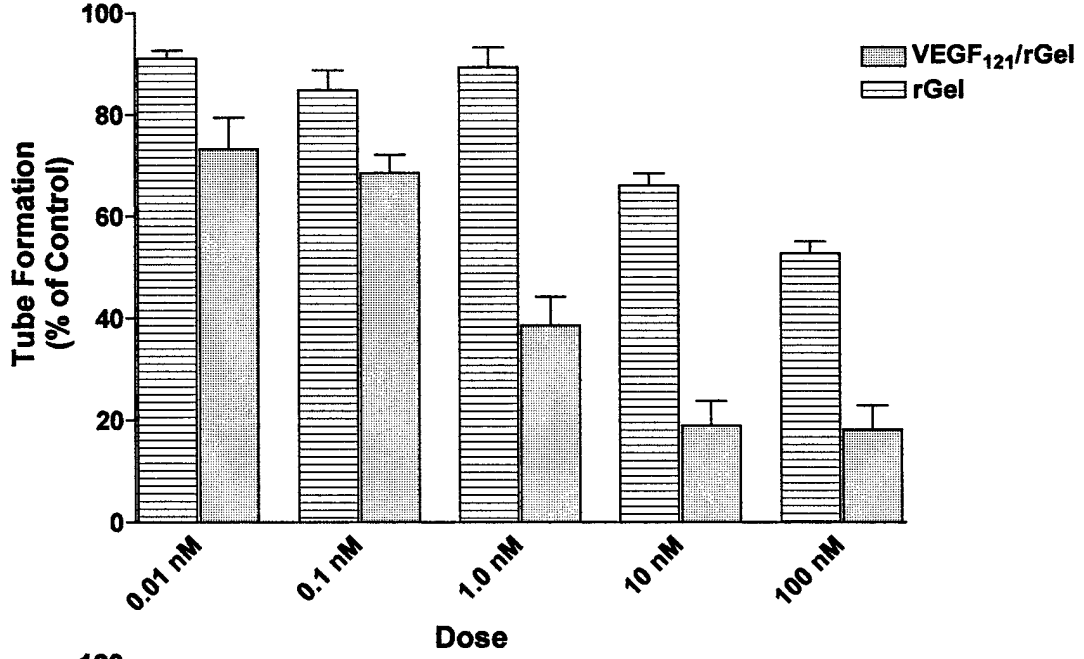


Figure 6

A.


100
80
60
40
20
0
Tube Formation
(% of Control)
VEGF121/rGel
rGel
0.01 nM
0.1 nM
1.0 nM
10 nM
100 nM
Dose

B.

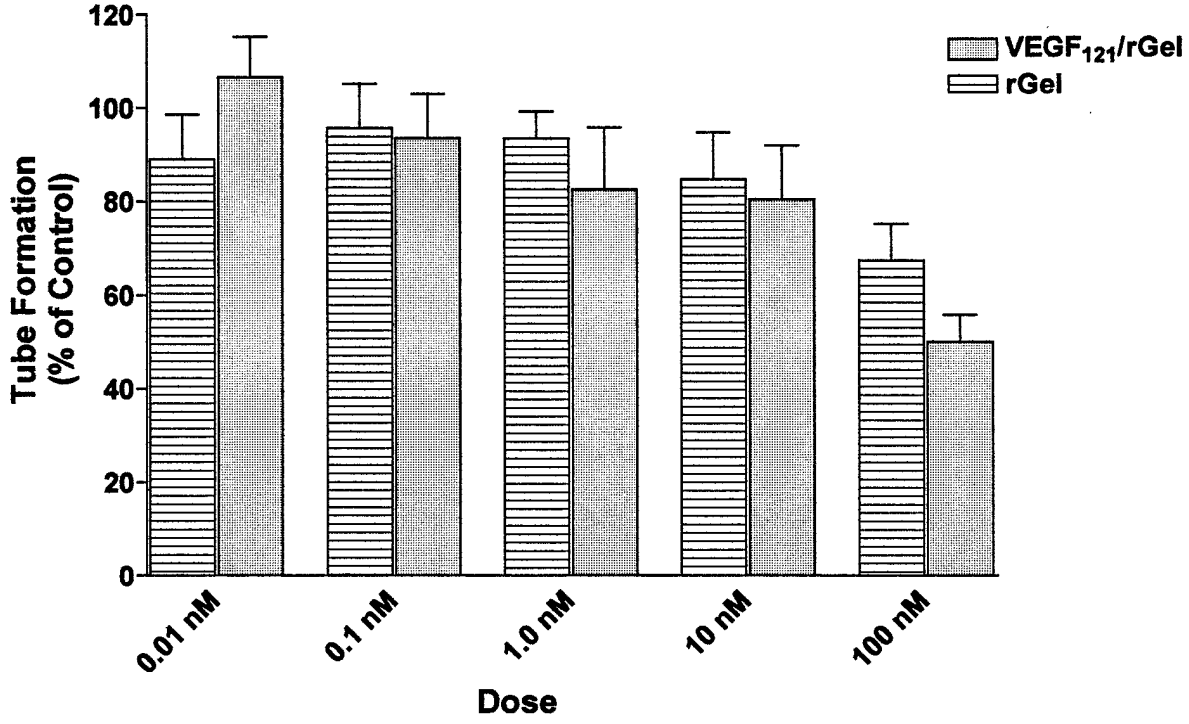

120
100
80
60
40
20
0
Tube Formation
(% of Control)
VEGF121/rGel
rGel
0.01 nM
0.1 nM
1.0 nM
10 nM
100 nM
Dose

Figure 7

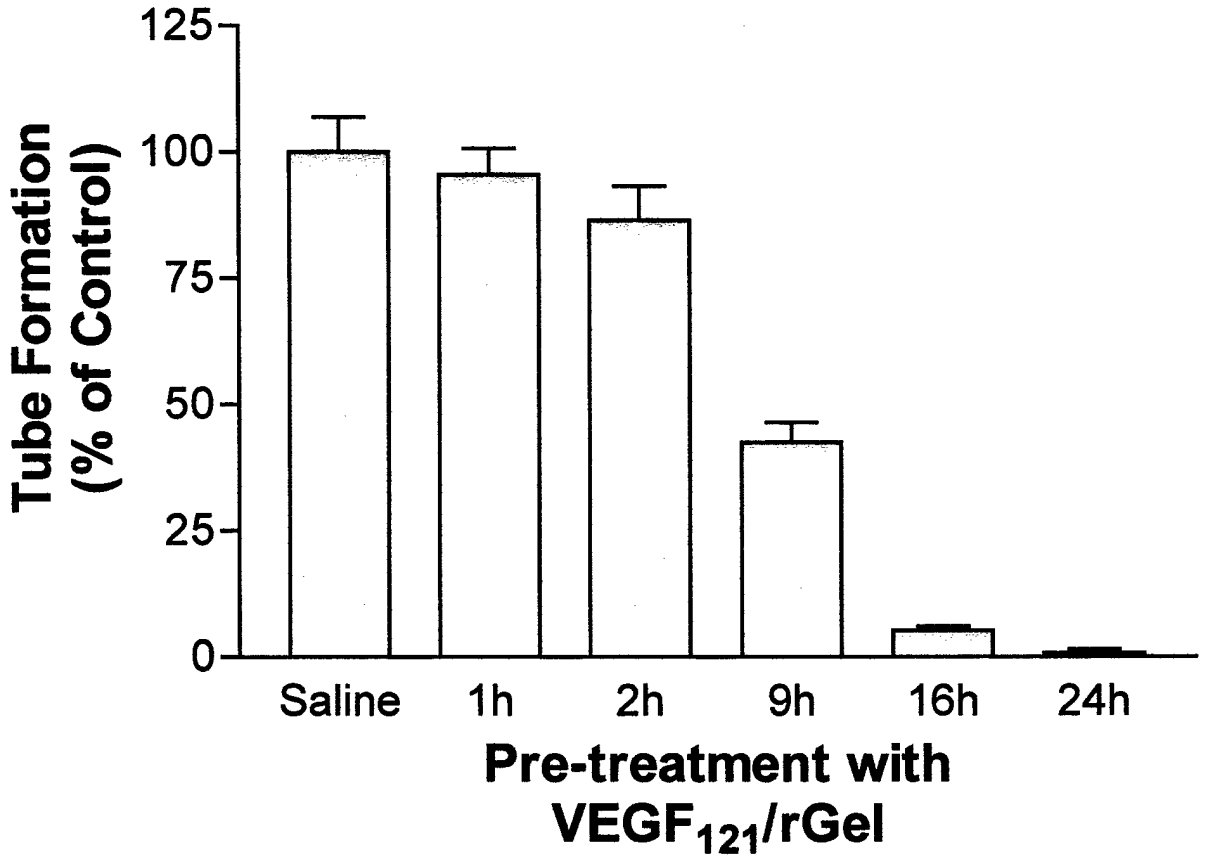
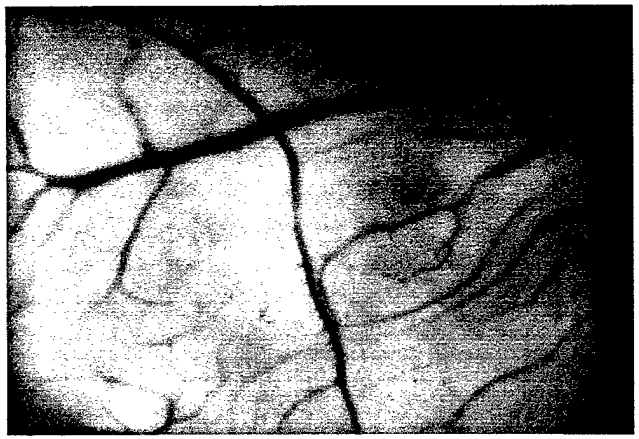
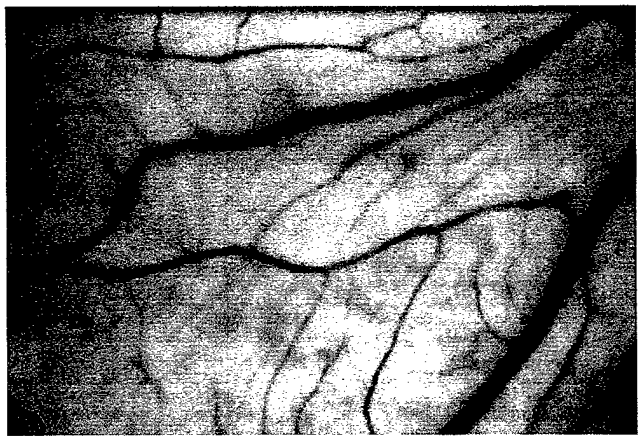

125
100
75
50
25
0
Tube Formation
(% of Control)
Saline
1h
2h
9h
16h
24h
Pre-treatment with
VEGF121/rGel

Figure 8

A.



B.



C.

Figure 9

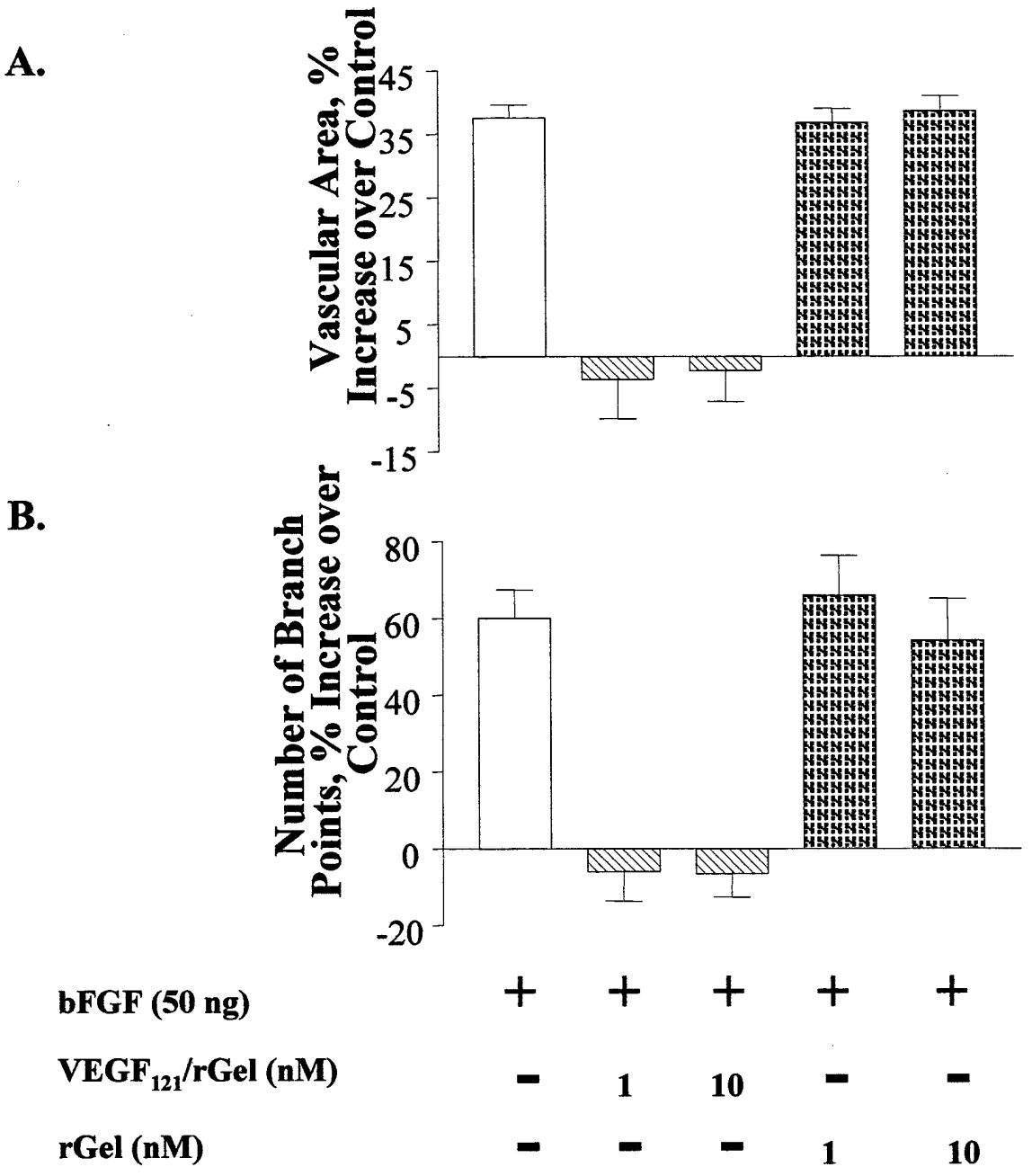

A.
Vascular Area, % Increase over Control
45
35
25
15
5
-5
-15
B.
Number of Branch Points, % Increase over Control
80
60
40
20
0
-20
bFGF (50 ng)
+ + + + +
VEGF121/rGel (nM)
- 1 10 - -
rGel (nM)
- - - 1 10

Figure 10