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Functionally Active VEGF Fusion Proteins

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Angiogenesis is stimulated by vascular endothelial growth factor (VEGF) acting via endothelial cell-specific receptors, such as VEGFR-2, that are overexpressed at the sites of angiogenesis. If VEGF retains activity as a fusion protein with a large N-terminal extension, it would facilitate development of VEGF-based vehicles for receptor-mediated delivery of therapeutic and diagnostic agents to the sites of angiogenesis. We have constructed, expressed in *Escherichia coli*, and purified VEGF fusion proteins containing a 158-amino acid N-terminal extension fused to human $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. We report here that VEGF fusion proteins induce tyrosine autophosphorylation of VEGFR-2 and its downstream targets, as well as cell contraction in cells overexpressing VEGFR-2. Although N-terminal extensions decrease the affinity of VEGF fusion proteins to VEGFR-2, at saturating concentrations these proteins are as efficient as correct size $VEGF_{165}$. We hypothesize that VEGF fusion proteins may be employed for targeting endothelial cells at the sites of angiogenesis.

Angiogenesis is a tightly controlled process of growing new blood vessels. In adult organisms, under normal circumstances, it takes place only during muscle or weight gains, development of the corpus luteum, and wound healing (reviewed in 1, 2). However, angiogenesis occurs in a large number of pathologies, such as solid tumor growth, various eye diseases, chronic inflammatory states, and ischemic injuries (reviewed in 3). It is widely believed that inhibitors and stimulators of angiogenesis may be effective therapeutics for these pathologies, and a number of compounds targeting angiogenesis are currently at various stages of development (reviewed in 4). The crucial positive regulator of angiogenesis is an endothelial cell-specific vascular endothelial growth factor (VEGF), also known as vascular permeability factor (reviewed in 5). VEGF is a secreted homodimeric N-glycosylated protein that exists in four major isoforms containing 121, 165, 189, or 206 amino acids as a result of alternative splicing. VEGF isoforms also differ in their ability to bind to heparin, and therefore, to circulate in blood or be stored in the extracellular matrix associated with heparan sulfate proteoglycans.

The action of VEGF on endothelial cells is mediated by endothelial cell specific tyrosine kinase Flt-1 and KDR/Flk-1 receptors, now known as VEGFR-1 and VEGFR-2, with the latter being overexpressed at the sites of angiogenesis and playing the dominant role in angiogenesis (reviewed in 6, 7). Since VEGF binds specifically to endothelial cells, this growth factor provides a unique vehicle for delivery of therapeutic and diagnostic agents to sites of angiogenesis. One possibility that has already been explored is to fuse cytotoxic moieties such as diphtheria toxin to VEGF (8). The other possibility is to fuse a peptide or a protein that may serve as a platform for loading therapeutic or diagnostic agents. The success of either approach is critically dependent upon maintaining the functional activity of the VEGF moiety in fusion proteins with N-terminal extensions. Therefore, in this investigation we have fused $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ with a 158 aa long N-terminal extension and have established its effects on VEGF functional activities. We report here that VEGF fusion proteins based on $VEGF_{121}$ isoform retain the highest ability to induce signalling via VEGFR-2 in cells overexpressing this receptor.

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MATERIALS AND METHODS

Plasmids. pLen-121, pLen-165, and pLen-189 plasmids containing the precursors for the 121-, 165-, and 189-residue forms of human VEGF were kindly provided by Dr. J. Abraham (Scios Nova, Inc., Mountain View, CA). pBal/Pst/pur-KDR mammalian expression plasmid containing the full-length human VEGFR-2 and puromycin resistance gene was kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY).

Construction of VEGF expression plasmids. $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ coding sequences were amplified by PCR from pLen-121, pLen-165, and pLen-189 plasmids respectively, using a sense oligonucleotide primer (5′-TAAGGCCTATGGCAGAAGGAGGAGGG) containing a *Stu*I restriction site (underlined) and an antisense primer (5′-TACTCGAGTCACCGCCTCGGCTTGTCAC) containing a *Xho*I restriction site (underlined). Gel purified PCR fragments were digested with *Stu*I and *Xho*I to yield fragments that did not encode the N-terminal alanine residue of VEGF. The codon for this residue was supplied by cleaving the pET32a expression vector (Novagen, Madison, WI) with *Nco*I restriction endonuclease, extending the recessed strands by one deoxynucleotide with Klenow enzyme in the presence of dCTP, and removal of the single-stranded overhangs with Mung bean nuclease. The 5′-ligation site in the vector was created by digestion of the modified vector with *Xho*I nuclease.

Expression and purification of VEGF fusion proteins. VEGF fusion proteins were expressed in BL21(DE3)LysS *Escherichia coli*. The expression of VEGF fusion proteins was induced by the addition of 1 mM of isopropylthiogalactoside (Gibco, USA) to the cultures grown to an optical density of 0.5 unit at 600 nm. The induced cultures were grown for two additional hours and then harvested by centrifugation for 25 min at 5000*g*. To purify each of the VEGF fusion proteins, a corresponding cell culture pellet was resuspended in ice cold buffer containing 50 mM Tris–HCl pH 7,5, 0.1 M $MgCl_2$, 0.1 mM DTT, 200 mg/l PMSF, 25 mg/l antitripsin, 50 mg/l leupeptin, 25 mg/l aprotinin. After five cycles of freezing and thawing, DNAse was added to the cell suspension, 50 U per ml. The suspension was incubated for 20 min at room temperature, then centrifuged at 5000*g* for 30 min at 4°C. The inclusion bodies pellet was solubilized in 10 ml of 8 M urea, followed by sonication for 5–10 min in an ice-cold water sonicator (FC 14, Fisher Sci., U.S.A.). The protein solution was clarified by centrifugation at 14,000*g* for 10 min at 4°C, and the supernatant was dialyzed against 10 mM Tris–HCl, pH 8.0, 150 mM NaCl for 16 h at 4°C. The concentrations of fusion proteins were determined with an S-tag Rapid Assay kit (Novagen, Madison, WI) according to the manufacturer's protocol. Interestingly, VEGF fusion proteins were active in VEGFR-2 tyrosine phosphorylation assay only when solubilization and refolding of the proteins were carried out in the absence of reducing agents. Refolding after treatment with 10 mM dithiothreitol (37°C, 30 min) yielded functionally inactive VEGF fusion proteins (data not shown). ^{125}I-Labeled $txVEGF_{121}$ protein with a specific activity of $\sim 1.5 \times 10^4$ cpm/ng was a gift from Dr. V. Sidorov (SibTech, Inc., Newington, CT).

Cell lines. Transformed human primary embryonic kidney cells, 293 (ATCC CRL-1573) were from the American Type Culture Collection (Rockville, MD). Porcine aortic endothelial cells overexpressing the full-length human VEGFR-2 (PAE/KDR) and 293 cells overexpressing a soluble secreted extracellular domain of the VEGFR-2 fused to the Fc portion of human IgG (293/KDR-Fc) were gifts from Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY). The 293 cells overexpressing the full-length human VEGFR-2 were developed by transfecting 293 cells with pBal/Pst/pur-KDR using Mirus *Trans IT*-LT1 Polyamine Transfection Reagent (Pan Vera, Madison, WI), followed by selection in the presence of 0.375 μg/ml puromycin. The clone with the highest level of $VEGF_{165}$-induced VEGFR-2 tyrosine autophosphorylation was designated 293/KDR and used for further experiments. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics at 37°C, 5% CO_2.

VEGF-induced tyrosine phosphorylation. VEGF-induced tyrosine phosphorylation of VEGFR-2 in 293/KDR, and tyrosine phosphorylation in PAE/KDR cells, were assayed as described (9). Briefly, subconfluent cells were incubated in serum-free DMEM at 37°C for 4 h, then shifted to serum-free DMEM containing 0.1 mM orthovanadate, 100 ng/ml bovine serum albumin, 25 mM Hepes, pH 7.2, for 20 min at 37°C, followed by a 20-min incubation at 4°C. After addition of VEGF fusion proteins, cells were incubated for 1 h at 4°C, then for 10 min at 37°C, lysed, and analyzed by SDS–PAGE. VEGF-induced tyrosine phosphorylation of PLCγ in 293/KDR cells was assayed as described in (10).

Western blot analysis. Cell lysates were separated by SDS–PAGE on 7.5% gels and transferred to nitrocellulose membranes (Life Technologies, Baltimore MD). After incubation with RC20H anti-phosphotyrosine antibody, (PharMingen, San Diego, CA), or rabbit polyclonal anti-VEGFR-2 serum (kindly provided by Dr. B. Terman, Albert Einstein School of Medicine, Bronx, NY), immune complexes were visualized by the *femto*-Lucent Chemiluminescence System (Geno Technology, St. Louis, MO). PLCγ was immunoprecipitated from 293/KDR cells (10^6 cells/immunoprecipitation) with

anti-PLCγ antibody (Transduction Laboratories, Cincinnati, OH) according to manufacturer's instructions. Immunoprecipitates were processed for Western blot analysis as described above.

Radioligand binding to soluble and cellular VEGFR-2. Soluble VEGFR-2 fused to the Fc portion of human IgG was purified from conditioned medium of 293/KDR-Fc cells as described (9). Radioligand binding to soluble and cellular VEGF receptors was performed as described (9).

Microscopy and image processing. Cells were observed with an Optonics DEI 750 Cooled CCD Camera attached to a Zeiss IM35 microscope. Images were processed using Adobe Photoshop software through the following procedure: same size areas were selected and binarized to black and white with a common threshold level (Adobe command, Image: Adjust: Threshold). The space between cells appeared as white and was quantitated by a histogram analysis (Adobe command, Image: Histogram).

RESULTS

Expression of VEGF fusion proteins. Coding sequences for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ isoforms were cloned into pET32a vector and expressed in *E. coli* as fusion proteins with a 158 aa long N-terminal extension provided by the vector. The extention MSDKI IHLTDDSFDTDVLKADGAILVDFWAEWCGPCK MIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKY GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFL DANLAGSGSGHM*HHHHHH*SSGLVPRGSGMKETA AAKFERQHMDSPDLGTDDDDK contains *E. coli* thioredoxin (bolded), and two purification tags: His-tag (italisized), and S-tag (underlined). VEGF fusion proteins, named $txVEGF_{121}$, $txVEGF_{165}$, and $txVEGF_{189}$, were found in inclusion bodies and their sizes, as judged by SDS–PAGE under reducing conditions, corresponded to monomeric proteins (Fig. 1A). All proteins were solubilized from inclusion bodies and refolded as described under Materials and Methods. VEGF fusion proteins recovered from inclusion bodies were 80–95% pure and were used without further purification. Under nonreducing denaturing conditions a significant proportion of $txVEGF_{121}$ was detected at a position corresponding to dimers, while $txVEGF_{165}$, and $txVEGF_{189}$ formed more oligomers with higher molecular weights than dimers (Fig. 1B). In a separate experiment we found that approximately 50% of the ^{35}S-labeled $txVEGF_{121}$ binds to soluble VEGFR-2 (data not shown). Since only VEGF dimers bind to VEGFR-2 with high affinity we concluded that our refolding process yielded ~50% of properly folded $txVEGF_{121}$.

Induction of VEGFR-2 tyrosine autophosphorylation by VEGF fusion proteins. Potential targets for VEGF

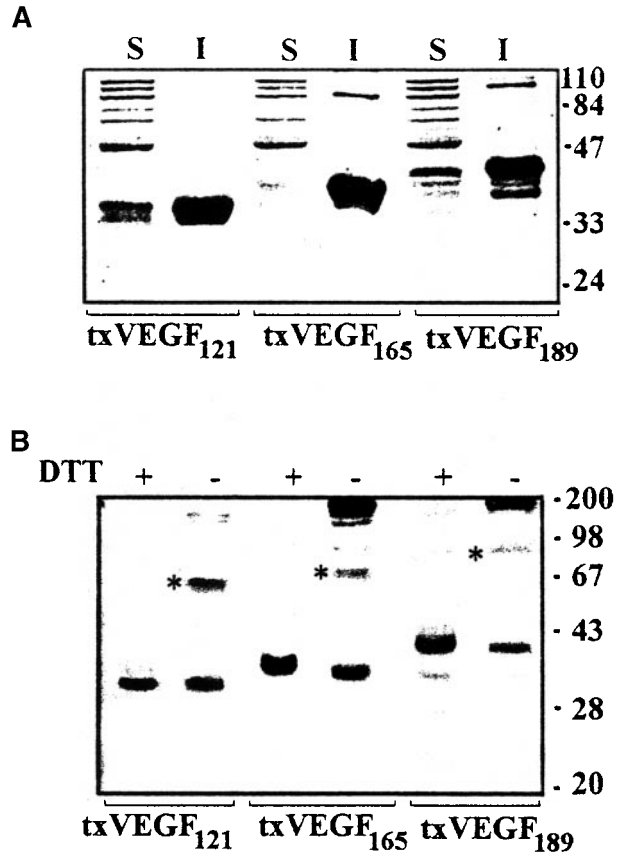

FIG. 1. SDS–PAGE analysis of VEGF fusion proteins. The proteins were expressed in *E. coli* strain BL21(DE3)pLysS and purified as described under Materials and Methods. (A) Soluble (S) and insoluble (I) parts of bacterial lysates were separated on a 15% gel followed by Coomassie staining. (B) Purified proteins were separated on a 15% gel in the presence (+) or absence (−) of DTT. Asterisks indicate dimers of corresponding VEGF fusion proteins.

fusion proteins are VEGFR-2 overexpressing endothelial cells at the sites of angiogenesis. Therefore, in order to test the functional activities of VEGF fusion proteins in cell-based assays, we constructed a derivative of 293 transformed human primary embryonic kidney cells overexpressing VEGFR-2 receptors (293/KDR cell line). Scatchard's plot analysis of ^{125}I-$VEGF_{165}$ binding revealed that these cells express 2.4×10^6 VEGFR-2/cell that bind $VEGF_{165}$ with a K_d of 0.3 nM (Fig. 2A). We have also used porcine aortic endothelial cells overexpressing VEGFR-2 (PAE/KDR) for functional testing of fusion proteins. Western blot analysis indicated that the latter cells express approximately 10-fold less VEGFR-2 than 293/KDR cells (Fig. 2B). VEGF fusion proteins induced VEGFR-2 tyrosine autophosphorylation in 293/KDR cells with $txVEGF_{121}$ being the most active fusion protein in this assay (Fig. 2C). $txVEGF_{121}$, $txVEGF_{165}$, and $txVEGF_{189}$ induced comparable levels of VEGFR-2 tyrosine autophosphorylation at concentrations 10- to 100-fold higher than the correct size

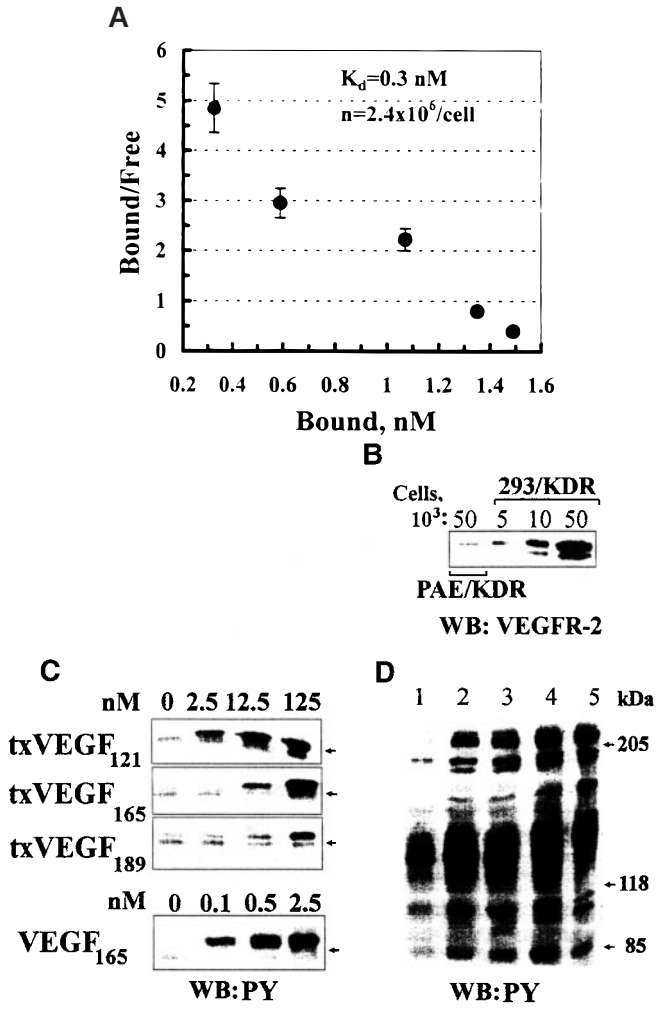

FIG. 2. Activation of cellular VEGFR-2 by VEGF fusion proteins. (A) Scatchard's analysis of ^{125}I-VEGF$_{165}$ binding to VEGFR-2 in 293/KDR cells. Cells were grown on 24-well plates. (B) Western blot analysis of VEGFR-2 in PAE/KDR and 293/KDR cells. (C) VEGFR-2 tyrosine phosphorylation in 293/KDR cells. Arrows indicate positions of 205 kDa markers. (D) Tyrosine phosphorylation in nonstimulated PAE/KDR cells (lane 1) and PAE/KDR cells stimulated with 2.5 nM correct size VEGF$_{165}$ (lane 2) or 125 nM VEGF fusion proteins txVEGF$_{189}$ (lane 3), txVEGF$_{165}$ (lane 4), txVEGF$_{121}$ (lane 5).

VEGF$_{165}$ (Fig. 2C). Since only dimeric VEGF is functionally active (5), the lower activities of txVEGF$_{165}$ and txVEGF$_{189}$ are most likely due to the lower yield of dimeric forms in the preparations of these fusion proteins as compared to txVEGF$_{121}$ (see Fig. 1B). However, it appears that N-terminal extension did not affect the intrinsic ability of VEGF to activate VEGFR-2. Indeed, at a concentration of 125 nM, VEGF fusion proteins were as efficient as 2.5 nM of the correct size VEGF$_{165}$ in inducing VEGFR-2 tyrosine phosphorylation in 293/KDR cells and in inducing a distinctive pattern of tyrosine phosphorylation in PAE/KDR cells (Fig. 2D).

Decreased affinities of VEGF fusion proteins for VEGFR-2. VEGF fusion proteins competed with ^{125}I-VEGF$_{165}$ for binding to a soluble extracellular domain of VEGFR-2 receptor with different efficiencies (Fig. 3A). In this assay, txVEGF$_{121}$ was approximately as efficient as VEGF$_{165}$, while txVEGF$_{165}$ and txVEGF$_{189}$ were 50- to 100-fold less efficient. As with VEGFR-2 phosphorylation, these variations can be attributed to a difference in the proportion of properly folded VEGF fusion proteins.

Scatchard's plot analysis of ^{125}I-txVEGF$_{121}$ binding to cellular VEGFR-2 in 293/KDR cells revealed that txVEGF$_{121}$ binds to the same number of VEGFR-2 as

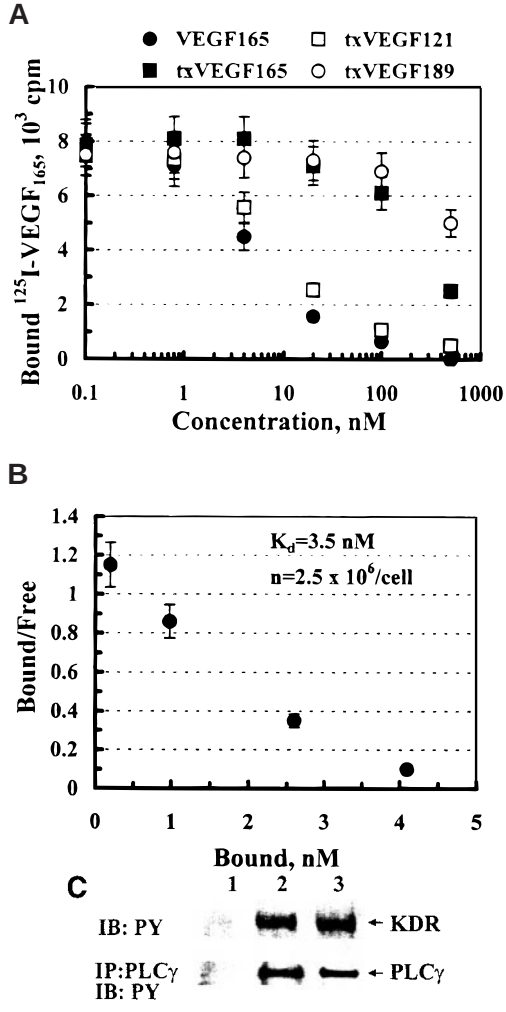

FIG. 3. Decreased affinity of VEGF fusion proteins to VEGFR-2. (A) Binding of ^{125}I-VEGF$_{165}$ to soluble VEGFR-2 in the presence of increasing amounts of correct size VEGF$_{165}$ (closed circles), txVEGF$_{121}$ (open squares), txVEGF$_{165}$ (closed squares), or txVEGF$_{189}$ (open circles). (B) Scatchard's analysis ^{125}I-txVEGF$_{121}$ binding to VEGFR-2 in 293/KDR cells. Cells were grown on 6-well plates. (C) Tyrosine phosphorylation of VEGFR-2 and PLCγ in untreated 293/KDR cells (lane 1) or cells stimulated with 2.5 nM VEGF$_{165}$ (lane 2) and 25 nM txVEGF$_{121}$ (lane 3).

the correct size $VEGF_{165}$, but with a 10-fold lower affinity characterized by a K_d of 3.5 nM (Fig. 3B, compare to Fig. 2A). Interestingly, the 10-fold difference in affinity was readily compensated by a corresponding increase in the concentration of $txVEGF_{121}$ in biochemical assays. Indeed, when 293/KDR cells were treated with 10-fold different concentrations of $VEGF_{165}$ and $txVEGF_{121}$, we found similar levels of tyrosine phosphorylation of VEGFR-2 and PLCγ, a downstream target of VEGFR-2 (10, 11) (Fig. 3C).

Induction of 293/KDR cell contraction by txVEGF$_{121}$. VEGF was originally discovered as vascular permeability factor (6, 7), and it displays the ability to induce permeability of endothelial cell monolayers in several *in vitro* assays (12–14). Recent evidence indicates that VEGF-enhanced permeability may be mediated by alterations in phosphorylation of the components of intercellular junctions (15–18) and activation of $p125^{FAK}$/paxillin, p38 MAP, and Akt kinases pathways (11, 19–21) that lead to cytoskeleton rearrangement and changes in cell shape. Importantly, morphological alterations could be detected faster than effects of VEGF on thymidine incorporation or tube formation in collagen. The latter assays are significantly longer (days) and performed under stressful conditions, such as extended periods of serum starvation (10).

We therefore tested whether $txVEGF_{121}$ can induce shape alterations in cells overexpressing VEGFR-2. We found that 293/KDR cells are particularly suited for these experiments because they form colonies of flat, tightly packed cells (Fig. 4A for 293/KDR cells). A 3-h exposure of 293/KDR cells maintained in complete medium to 50 nM $VEGF_{165}$ or $txVEGF_{121}$ induced cell contraction that led to the appearance of clear areas between treated cells (Fig. 4B for $txVEGF_{121}$). This effect was mediated by VEGFR-2 because exposure of parental 293 cells to VEGF proteins did not alter cell morphology (data not shown). The morphological alterations induced by $txVEGF_{121}$ depended on its continuous presence in the medium as cells rapidly reverted to the original phenotype in fresh medium (data not shown).

The proportion of the clear intercellular areas was quantitated with Adobe Photoshop software through a procedure similar to that described by Wild *et al.* (22). Specifically, four same size fields per image were selected, binarized to black and white with a common threshold level, and the amount of white space in each field was quantitated as described in Material and Methods (see Figs. 4C and 4D, which shows processed images of Figs. 4A and 4B). We found that treatment with $txVEGF_{121}$ increased clear intercellular space from $5.5 \pm 2\%$ to $22.8 \pm 4\%$ of the total image. The simplicity of 293/KDR cell contraction assay (a 3-h exposure, quantitation with a widespread software) suggests that it may offer advantages over longer (days) traditional assays, such as thymidine incorporation or tube formation in collagen.

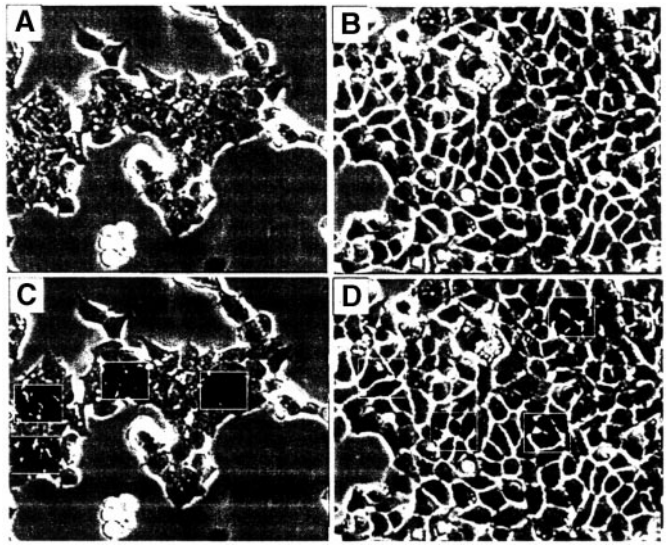

FIG. 4. Contraction of $txVEGF_{121}$-stimulated 293/KDR cells. Twenty hours after plating, near-confluent 293/KDR cells were shifted to fresh medium (A) or to fresh medium containing 50 nM $txVEGF_{121}$ (B) and incubated for 3 h at 37°C. C and D show images A and B, respectively, with same size areas binarized into black and white fields with a common threshold level.

DISCUSSION

We report here that VEGF fusion proteins with a 158-aa long N-terminal extension induce VEGFR-2 tyrosine autophosphorylation. Relative activities of VEGF fusion proteins in this assay ($txVEGF_{121} > txVEGF_{165} > txVEGF_{189}$) most likely reflect a declining content of functionally active dimers in preparations of $txVEGF_{121}$, $txVEGF_{165}$, and $txVEGF_{189}$. The binding experiments indicate that VEGF fusion proteins have lower affinity to VEGFR-2 than the correct size $VEGF_{165}$. However, at saturating concentrations, VEGF fusion proteins induce VEGFR-2 tyrosine autophosphorylation as efficient as correct size $VEGF_{165}$. The latter results suggest that N-terminal extensions in VEGF fusion proteins would not significantly affect interactions of the intracellular VEGFR-2 domains.

There are indications that growing endothelial cells at the sites of angiogenesis express significantly more VEGFR-2 than quiescent endothelial cells (23–28). The following arguments suggest that the lower affinity of VEGF fusion proteins may be exploited for targeting growing endothelial cells at the sites of angiogenesis. When VEGF binds to VEGFR-2, it induces dimerization of VEGFR-2, and tyrosine autophosphorylation proceeds in VEGF/$(VEGFR\text{-}2)_2$ complexes (5, 7, 29). Thus,

the efficiency of VEGFR-2 tyrosine autophosphorylation depends on the relative rates of VEGF/VEGFR-2 complex dissociation and dimerization with the second VEGFR-2 molecule. Since dimerization is a bimolecular reaction, its rate is proportional to $[VEGFR\text{-}2]^2$ and should be enhanced in cells overexpressing VEGFR-2. Thus, for growing endothelial cells at the sites of angiogenesis, the enhanced rate of dimerization of VEGFR-2 may compensate for the lower affinity of VEGF fusion proteins. In contrast, quiescent endothelial cells with a relatively low number of VEGFR-2, may be less susceptible to VEGF fusion proteins because dissociation of relatively weak VEGF/VEGFR-2 complexes would proceed faster than dimerization. This difference between growing and quiescent endothelial cells, opens the possibility of using fusion VEGF proteins for selective delivery of therapeutic and diagnostic agents to the sites of angiogenesis.

Our results suggest that $VEGF_{121}$ may be the best platform to construct fusion proteins because its preparations contain the highest proportion of dimeric VEGF molecules, and it is the least affected by addition of a 158 aa long N-terminal extension. In addition, $VEGF_{121}$, unlike $VEGF_{165}$ and $VEGF_{189}$ isoforms, does not have heparin-binding domains and displays a selective affinity for VEGFR-2 (30). We therefore recently constructed $VEGF_{121}$ fusion protein containing a 293 aa N-terminal extension encoding A-subunit of Shiga-like toxin (31). This protein selectively inhibits growth of endothelial cells overexpressing VEGFR-2 with IC_{50} of 0.1 nM. Furthermore, we have recently demonstrated that S-tag in $VEGF_{121}$ fusion protein can be employed as a platform for assembling DNA delivery vehicles for VEGFR-2-mediated DNA delivery (32). Experiments are now in progress to test the feasibility of using $VEGF_{121}$ fusion proteins for delivery of therapeutic and diagnostic agents.

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