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Synthesis and Immune Response of Non-native Isomers of Vascular Endothelial Growth Factor[†]

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ABSTRACT: Native proteins often lack immunogenicity and thus limit vaccine and mAb development. We described here a unique method to enhance the immunogenicity of native proteins. This is achieved by creating non-native isomers of disulfide proteins (X-isomers) using the method of disulfide scrambling. X-isomers have the potential to be developed as vaccines and effective immunogens, as they are capable of breaking the immune tolerance and eliciting antibodies that cross-react with the native protein. In this report, we describe production of X-isomers of vascular endothelial growth factor (X-VEGF). The aim is to develop X-VEGF for cancer immunotherapy targeting reduction of VEGF. The production of mouse X-VEGF is achieved by expressing the short version of VEGF (1−110) commonly shared by all VEGF isoforms, with two Cys ⇒ Ala mutations at Cys^{51} and Cys^{60} to generate R-$VEGF_{110}$ (R stands for fully reduced). R-$VEGF_{110}$ was then allowed to undergo oxidative folding in the absence of denaturant to form N-$VEGF_{110}$ (N stands for native) or in the presence of denaturant to generate five fractions of X-$VEGF_{110}$ isomers. While N-$VEGF_{110}$ exhibits only marginal immunogenicity in mice, all five fractions of X-$VEGF_{110}$ isomers were shown to elicit high titers of antibodies that cross-react with N-$VEGF_{110}$. In sera of immunized mice, the amounts of anti-N-VEGF antibodies elicited by X-$VEGF_{110}$ isomers range from 54 to 186 μg/mL, which are compatible with or greater than the concentration required for effective therapy using anti-VEGF MAbs. The underlying mechanism of enhanced immunogenicity of X-$VEGF_{110}$ is investigated and elaborated. These data suggest that X-$VEGF_{110}$ isomers are potential compounds in developing active immunotherapy for treatment of VEGFR bearing tumors and the wet form of age-related macular degeneration.

Vascular endothelial growth factor (VEGF)[1] is an important regulator of angiogenesis required for blood vessel formation (*1*−*4*). VEGF is also a well-characterized target for cancer therapy (*5*, *6*). Passive immunotherapy using MAbs against VEGF (Bevacizumab, Avastin; Genentech) has been approved by FDA and has proven efficacy in treatment of colon, lung, renal, glioblastoma, and breast cancer (*7*−*10*). However, side effects of hypertension, proteinuria, bleeding, and thrombosis have been associated with the clinical application of bevacizumab (*11*−*13*). More importantly, the cost of passive immunotherapy is prohibitive, primarily due to the high expense of antibody production and high dose (5 mg/kg) required for effective therapy. There is a strong financial/clinical incentive to develop more cost-effective immunotherapeutic agents. The approach of active immunotherapy is a viable alternative to address these concerns. Active anti-VEGF therapy would stimulate the body's own immune system to block VEGF, suppress the angionenesis, and inhibit the growth of tumors. Compared with passive immunotherapy, active immunotherapy has two additional advantages: (1) ease of administering protein or peptide immunogens rather than the immunoglobulins used for passive immunotherapy and (2) the ability to induce long-term immunity. However, native VEGF (N-VEGF) is not immunogenic in syngeneic animal systems, which represents a challenge to vaccine development.

In order to develop active immunotherapy targeting VEGF reduction, it is essential to generate effective immunogens that are capable of eliciting high titers of anti-VEGF antibodies. Since endogenous proteins, such as native VEGF, are usually not immunogenic, we plan to produce X-isomers of VEGF (X-VEGF) as immunogen for evaluation of their immunological properties. X-isomers are fully oxidized, non-native isomers of disulfide containing proteins that are amenable to fractionation, isolation, characterization, and further clinical application. They can be systematically produced using the method of disulfide scrambling (*14*, *15*). Initial studies conducted in our laboratory have shown that X-isomers exhibit two distinct structural and immunological properties (*16*): (1) X-isomers exhibit increased aggregation and immunogenicity as compared to the native protein; (2) X-isomers break the immune tolerance and elicit high-titer antibodies that cross-react with the corresponding native protein as well as the X-isomers. These unique immunological properties suggest that X-isomers of disease-related proteins have potential as immunogens for developing therapeutic vaccines and antibodies.

In this report, we describe (1) design and production of a panel of X-VEGF isomers and (2) evaluation of immunogenicity of X-VEGF isomers in mice, with the ultimate goal of developing them as therapeutic vaccines for cancer treatment.

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[1]Abbreviations: VEGF, vascular endothelial growth factor; X-VEGF, scrambled VEGF; HPLC, high-performance liquid chromatography; GdmCl, guanidine hydrochloride; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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EXPERIMENTAL PROCEDURES

Expression and Purification of Reduced $VEGF_{110}$. The mouse VEGF gene has up to eight exons. Alternative exon splicing results in the production of four major VEGF isoforms with the length of 121, 165, 189, or 206 amino acid residues, respectively. All four isoforms share an identical N-terminal domain (residues 1−110). The truncated $VEGF_{110}$ protein, with two Cys ⇒ Ala mutations, has been expressed and used for production of X-$VEGF_{110}$. The PCR-amplified product was cloned into pET-3d plasmid via *Nco*I and *Bam*HI restriction sites. The sequences of the amplified product were confirmed by DNA sequencing. The interchain disulfide bond formation was blocked by mutagenesis. VEGF protein was expressed and purified by reverse-phase high-performance liquid chromatography (HPLC) on an Agilent 1100 HPLC system (Column ZORBAX 3000 SB-C18, 9.4 mm × 25 cm), with buffer A containing 0.088% trifluoroacetic acid (TFA) in water and buffer B containing 0.084% TFA in 90% acetonitrile. The molecular mass and sequence of the purified VEGF protein were verified by matrix-assisted laser desorption ionization−time of flight (MALDI-TOF) mass spectrometry. Purified protein was freeze-dried and stored at −20 °C.

Preparation of N-$VEGF_{110}$ and X-$VEGF_{110}$ from the Fully Reduced R-$VEGF_{110}$ Mutant by Oxidative Folding and Disulfide Scrambling. Reduced $VEGF_{110}$ (1 mg/mL) was incubated in Tris-HCl buffer (pH 8.4) containing β-mercaptoethanol (0.2 mM) and different concentrations of guanidine hydrochloride (GdmCl) (0−6 M). The reactions were carried out at 23 °C for 16 h. Folded samples were acidified with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by HPLC using the conditions described above. N-$VEGF_{110}$ and fractions of X-$VEGF_{110}$ isomers were purified by HPLC, lyophilized, and stored at −20 °C.

Unfolding of N-$VEGF_{110}$ Using the Method of Disulfide Scrambling. N-$VEGF_{110}$ (0.5 mg/mL) was incubated in Tris-HCl buffer (pH 8.4) containing β-mercaptoethanol (0.2 mM) and different concentrations of GdmCl (0−6 M). The reactions were carried out at 23 °C for 16 h. Unfolded samples were acidified with an equal volume of 4% TFA and analyzed by HPLC.

Unfolding of N-$VEGF_{110}$ Using the Method of Reductive Unfolding. N-$VEGF_{110}$ (0.5 mg/mL) was incubated in Tris-HCl buffer (pH 8.4) containing dithiothreitol (DTT) (150 mM) in the absence of denaturant. The reactions were carried out at 23 °C for up to 60 min. Unfolded samples were acidified with an equal volume of 4% TFA and analyzed by HPLC.

Characterization of Folded N-$VEGF_{110}$ and X-$VEGF_{110}$ Isomers. HPLC-purified N-$VEGF_{110}$ (15 μg) was digested with 1.5 μg of thermolysin (Sigma, P1512) in 30 μL of *N*-ethylmorpholine/acetate buffer (50 mM, pH 6.4) or with Glu-C in 30 μL of ammonium bicarbonate buffer (50 mM, pH 8.0). Peptides were then isolated by HPLC and analyzed by both MALDI-TOF mass spectrometry and Edman sequencing to identify peptide fragments containing disulfide bonds. The molecular masses of VEGF mutants and isomers, both unmodified and those modified with vinylpyridine and iodoacetic acid, were determined by a MALDI-TOF mass spectrometer. Far-UV circular dichroism (CD) spectra of N-$VEGF_{110}$ and X-$VEGF_{110}$ isomers were measured using a JASCO (J-715) spectropolarimeter.

Immunization of Male C57/BL6 Mice. Male C57/BL6 mice were purchased from Jackson Laboratories and divided randomly into eight groups (eight mice in each group). The mice were immunized with mouse N-$VEGF_{110}$, R-$VEGF_{110}$, or one of five isomers (denoted X-$VEGF_{110}$-a through X-$VEGF_{110}$-e) and isomer mixture (Mix) as well. The eight mice which received vehicle buffer served as negative control. The immunization protocol consisted first of an injection with $VEGF_{110}$ isomers (600 μg/mL, 100 μL) with complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO). Two weeks later, mice received another injection of VEGF (600 μg/mL, 100 μL) with incomplete Freund's adjuvant (IFA), followed by reinjection once a month (for the subsequent 3 months) with $VEGF_{110}$ (600 μg/mL, 100 μL) in phosphate-buffered saline (PBS). One week after each boost injection, plasma samples were collected for antibody response analysis by ELISA.

Immunogenicity Determination Using ELISA. Antibody levels in plasma were determined using conventional ELISA. A 96-well MaxiSorp plate (Nunc) was coated overnight at 4 °C with 100 μL/well purified native VEGF (50 ng/mL) expressed in *Escherichia coli*. The plate was blocked with 150 μL/well starting block (PBS) blocking buffer (Pierce) for 1 h, the diluted sera samples were applied to this 96-well plate in duplication, and the plate was incubated for 1 h. The unbound antibody was eliminated by three times PBS-T wash, whereas bound antibody was probed with 1 h incubation of goat anti-mouse IgG antibody coupled with horseradish peroxidase (catalog no. 6789; Abcam, Cambridge, MA) (1:5000) and then developed with TMB peroxidase substrate kit (Pierce). After the final wash, the color reaction was initiated by adding 100 μL/well TMB for 0.5 h and stopped by 2 N H_2SO_4. Samples were analyzed in triplicate, absorbance values at 450 nm were read, and the averages were subjected to statistical analysis. The amount of antibody in mice sera was determined by measuring the absorbance at 450 nm and compared to a standard curve. The standard curve was made in the same plate using a specific rabbit anti-VEGF polyclonal antibody from Abcam (ab9953-100, lot no. 324832), which was prepared with the same dilution of mouse serum.

Measurement of PIGF Levels in Mouse Sera after X-$VEGF_{110}$ Immunization. PIGF levels were measured in the sera of immunized mice (six mice/group) using ELISA as described above. A 96-well plate was coated with 100 μL/well goat anti-PIGF polyclonal antibody (Santa Cruz Biotechnology, K-20). The plate was then washed and blocked with blocking buffer (Pierce) for 1 h. Then 100 μL of 10-fold diluted sera was applied and incubated for 2 h followed by adding 100 μL/well detecting antibody (1 μg/mL). This detecting antibody was made by conjugating biotin to goat anti-PIGF polyclonal antibody using EZ-link sulfo-NHS-biotinylation kit (Pierce). The NeutrAvidin-HRP (Pierce) was added and incubated for 2 h. After the final wash, the color reaction was initiated by adding 100 μL/well TMB for 0.5 h and stopped by 2 N H_2SO_4. The levels of PIGF in sera were determined by measuring the absorbance at 450 nm.

RESULTS

Expression, Purification, and Characterization of R-$VEGF_{110}$. $VEGF_{110}$ comprises eight cysteines, of which six are engaged in three native disulfide bonds (Cys^{26}−Cys^{68}, Cys^{57}−Cys^{102}, and Cys^{61}−Cys^{104}), and two Cys (Cys^{51} and Cys^{60}) are involved in the dimer formation and irrelevant to the native conformation of N-$VEGF_{110}$. To avoid complication of generating an excess number of possible X-VEGF isomers, we have replaced both Cys^{51} and Cys^{60} with inert Ala. This would reduce the possible X-$VEGF_{110}$ isomers from 104 to 14, which are more amenable to HPLC analysis. Expressed $VEGF_{110}$ was purified

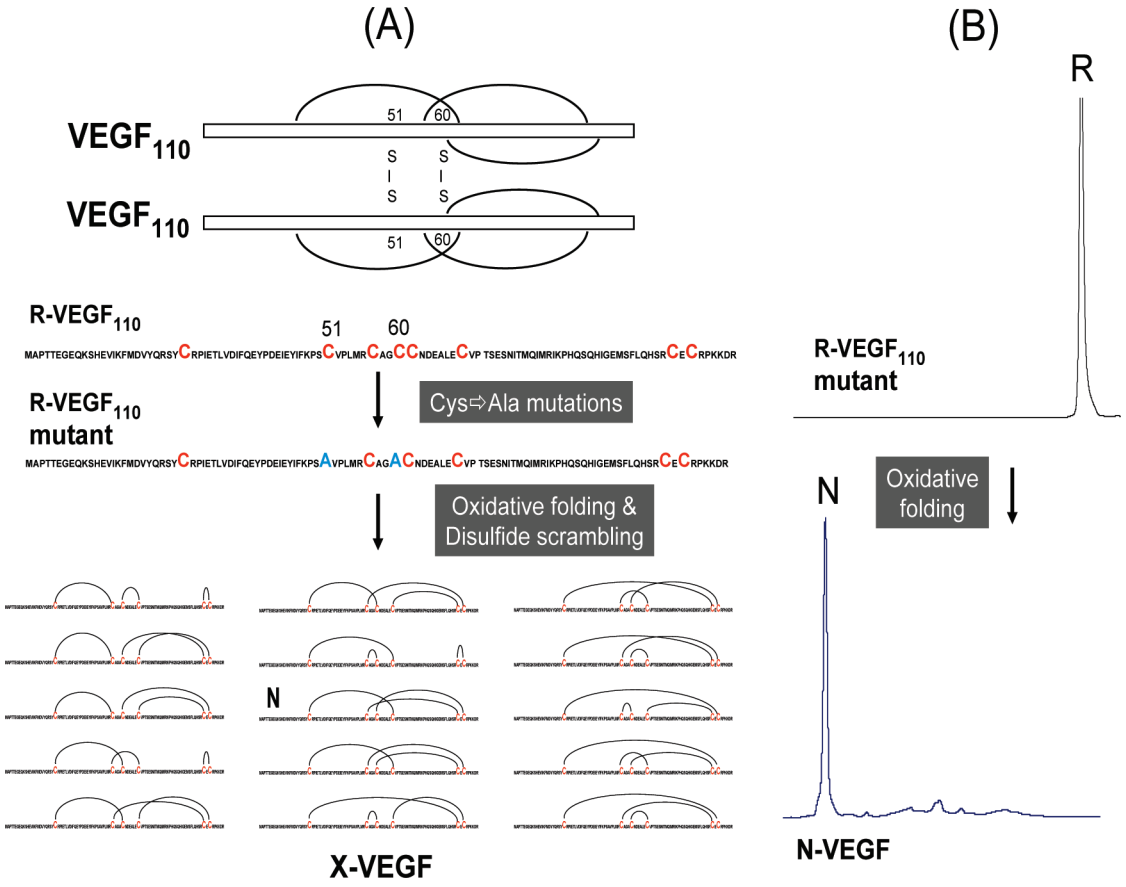

FIGURE 1: (A) Scheme for producing stabilized conformational isomers of X-VEGF. Natural occurring native $VEGF_{110}$ comprises two identical monomers linked by two interchain disulfide bonds, $Cys^{51}-Cys^{51}$ and $Cys^{60}-Cys^{60}$. The reduced form of $VEGF_{110}$ monomer with $Cys^{51} \Rightarrow Ala^{51}$ and $Cys^{60} \Rightarrow Ala^{60}$ mutations was expressed and isolated. Mutant R-$VEGF_{110}$ was then allowed to undergo proper oxidative folding to form N-VEGF, or undergo oxidative folding and disulfide scrambling in the presence of denaturant to form diverse isomers of X-$VEGF_{110}$. (B) Oxidative folding of R-$VEGF_{110}$ to form N-$VEGF_{110}$. The reactions were carried out at 22 °C for 20 h in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.2 mM). The end product comprises a predominant isomer containing three native disulfide bonds ($Cys^{26}-Cys^{68}$, $Cys^{57}-Cys^{102}$, and $Cys^{61}-Cys^{104}$).

and isolated by HPLC in fully reduced form (R-$VEGF_{110}$) with the purity of more than 97% (Figure 1A). The molecular mass of R-$VEGF_{110}$ was analyzed by MALDI-TOF MS and was found to be 12803.66, consistent with its theoretical value (12804). To confirm that R-$VEGF_{110}$ comprises six free cysteines, the protein was modified with vinylpyridine, purified by HPLC, and analyzed by MALDI-TOF. The results showed that vinylpyridine-modified R-$VEGF_{110}$ exhibited a molecular mass of 13414, an additional 616 Da, due to the coupling of six vinylpyridines. Purified R-$VEGF_{110}$ was subsequently used for production of N-$VEGF_{110}$ and X-$VEGF_{110}$ isomers (Figure 1A).

Oxidative Folding of R-$VEGF_{110}$ To Form N-$VEGF_{110}$. Fully reduced R-$VEGF_{110}$ was allowed to undergo oxidative folding in Tris-HCl buffer (pH 8.4) containing β-mercaptoethanol (0.2 mM), a standard condition that has been successfully used to refold fully reduced disulfide proteins. The reaction was performed overnight, and the final product was analyzed by HPLC (Figure 1B). R-$VEGF_{110}$ folded to form N-$VEGF_{110}$ with the recovery of 90%. The three native disulfide bonds ($Cys^{26}-Cys^{68}$, $Cys^{57}-Cys^{102}$, and $Cys^{61}-Cys^{104}$) of N-$VEGF_{110}$ were verified by mass and sequence analysis of thermolysin-digested peptides (data not shown).

N-$VEGF_{110}$ Is Unable To Undergo Disulfide Scrambling in the Presence of Denaturant and Thiol Catalyst To Generate X-$VEGF_{110}$ Isomers. X-isomers of denatured proteins are typically generated from native disulfide proteins (*14*, *15*). This is achieved by incubating the native protein in the alkaline buffer (pH 8.0−8.5) containing a selected denaturant (e.g., 1−6 M GdmCl or 1−8 M urea) and a thiol catalyst (e.g., 0.2 mM β-mercaptoethanol). Under these conditions, a native protein unfolds by shuffling its native disulfide bonds and converts to form a mixture of fully oxidized X-isomers (*14*−*19*). N-$VEGF_{110}$ has been expected to unfold and convert to a mixture of X-$VEGF_{110}$ isomers under similar conditions. Surprisingly, N-$VEGF_{110}$ remains totally intact, unable to shuffle its native disulfide bonds and convert to X-$VEGF_{110}$ isomers even at 6 M GdmCl (Figure 2A).

X-$VEGF_{110}$ Isomers Were Produced from Oxidative Folding of R-$VEGF_{110}$ in the Presence of Denaturant and Thiol Catalyst. As N-$VEGF_{110}$ is unable to undergo disulfide scrambling, an alternative strategy was used to produce X-$VEGF_{110}$ isomers. Fully reduced R-$VEGF_{110}$ was incubated overnight in Tris-HCl buffer (0.1 M, pH 8.4) containing β-mercaptoethanol (0.2 mM) and different concentrations of GdmCl (1−6 M). The folded proteins were analyzed by HPLC (Figure 2B). The products of oxidative folding of R-$VEGF_{110}$ were found to comprise a mixture of N-$VEGF_{110}$ and X-$VEGF_{110}$ isomers. Five fractions of X-$VEGF_{110}$ isomers (a to e) were identified. They are fully oxidized and all contain three disulfide bonds. The recovery of X-$VEGF_{110}$ isomers increases as the concentration of GdmCl rises (Figure 2B). At 1.7 M of GdmCl, the recoveries of N-$VEGF_{110}$/X-$VEGF_{110}$ isomers are 50:50. We have therefore selected 4 M GdmCl for the production of X-$VEGF_{110}$ isomers.

There are 14 possible species of X-VEGF$_{110}$ isomer which can be produced from oxidative folding of R-VEGF$_{110}$ in the presence of denaturant (Figure 1). It is likely that some of the five identified fractions of X-VEGF$_{110}$ (Figure 2B) may comprise more than one species of X-VEGF$_{110}$ isomer. As those isomers are intended to be used as immunogen, further purification has not been pursued.

Conformational Properties of X-VEGF$_{110}$ Isomers. We analyzed the molecular mass of each X-VEGF$_{110}$ isomer prior to immunization studies by both mass spectrometry and SDS−PAGE. The results (Figure 3A) show that even after 6 months of prolonged storage (in lyophilized form) the majority of X-VEGF$_{110}$ still exists in monomeric state. There are trace amounts of dimers detected with isomers X-a, X-b, and X-c. For isomers X-d and X-e and fully reduced R-VEGF$_{110}$, about 10−15% of the proteins are present in the aggregated state.

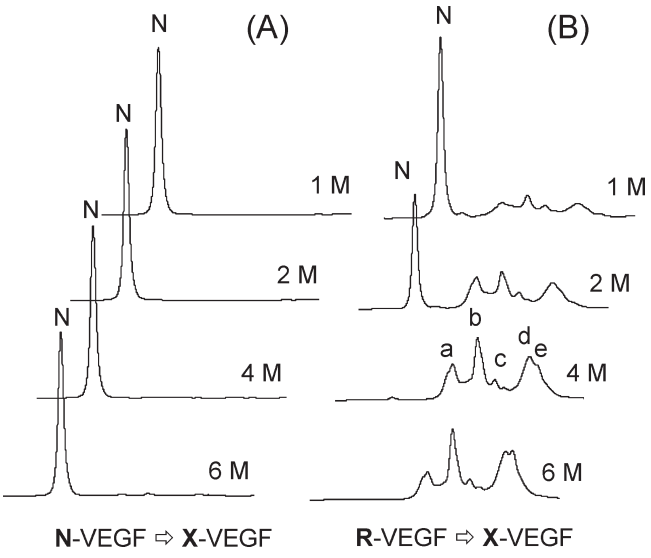

FIGURE 2: (A) Unfolding of N-VEGF$_{110}$ in the presence of thiol catalyst and denaturant. N-VEGF$_{110}$ (0.5 mg/mL) was incubated at 22 °C for 20 h in Tris-HCl buffer containing 2-mercaptoethanol (0.2 mM) and indicated concentrations of GdmCl. Under these conditions, N-VEGF$_{110}$ is expected to unfold via disulfide scrambling and convert to a mixture of X-VEGF$_{110}$. The results presented here show that N-VEGF$_{110}$ remained intact. (B) Oxidative folding of R-VEGF$_{110}$ in the presence of thiol catalyst and denaturant. R-VEGF$_{110}$ (0.5 mg/mL) was incubated at 22 °C for 20 h in Tris-HCl buffer containing 2-mercaptoethanol (0.2 mM) and indicated concentrations of GdmCl. Recovery of N-VEGF$_{110}$ decreases as the GdmCl increases. At high concentration of GdmCl, the end products were shown to comprise five major fractions of X-VEGF$_{110}$ isomers (designated from a to e). Samples were analyzed by HPLC using the conditions described in the Experimental Procedures.

The CD spectra of N-VEGF$_{110}$ and X-VEGF$_{110}$ isomers were also analyzed (Figure 3B). The receptor binding domain of VEGF (residues 8−109) comprises mainly β-sheet structure (*20*, *21*). Our analysis shows that N-VEGF$_{110}$ contains 59% of β-sheet and 10% of α-helix, consistent with the documented structure of VEGF (*20*). In contrast, the structure of X-VEGF$_{110}$ isomers comprises mainly random coil (∼35−40%) and decreased content of β-sheet (10−20%). The results indicate the significant structural difference between N-VEGF$_{110}$ and X-VEGF$_{110}$ and further reveal structural variations among X-VEGF$_{110}$ isomers.

Immunogenicity of X-VEGF$_{110}$ Isomers. Mice were immunized with N-VEGF$_{110}$ and five X-VEGF$_{110}$ isomers. The immunogenicity was evaluated by the quantities of serum antibody binding to the coated N-VEGF$_{110}$. All five X-VEGF$_{110}$ were shown to exhibit increased immunogenicity as compared with N-VEGF$_{110}$ at 1:100 and 1:1000 dilutions. The results are presented in Figure 4A. This experiment demonstrates that none of the mice immunized with N-VEGF$_{110}$ produced a meaningful titer of antibody. Mice immunized with X-VEGF$_{110}$ produced high titers of antibodies that cross-reacted with N-VEGF$_{110}$ and showed statistically higher immunogenicity ($P < 0.01-0.0001$). Significantly, all 48 mice immunized with X-VEGF$_{110}$ isomers generated antibody that recognized N-VEGF$_{110}$. An increased immune response was also observed with samples from fourth bleeding. Furthermore, antibodies elicited by X-VEGF$_{110}$ are specific against N-VEGF$_{110}$, as they do not bind to control proteins (mouse prion protein). The amounts of anti-N-VEGF antibody in sera of immunized mice were further determined. This was achieved by ELISA of serial diluted sera and comparing to a standard curve, obtained by using a commercially available anti-VEGF polyclonal antibody (Abcam). The results (Figure 4B) show that N-VEGF$_{110}$ immunized mice exhibited marginal amount of antibodies (∼5.8 μg/mL). In contrast, X-VEGF$_{110}$ immunized mice produce 10−35-fold higher concentration of anti-N-VEGF antibodies (54−186 μg/mL), with the highest immune response of 279 μg/mL generated by immunization using X-VEGF-e isomer.

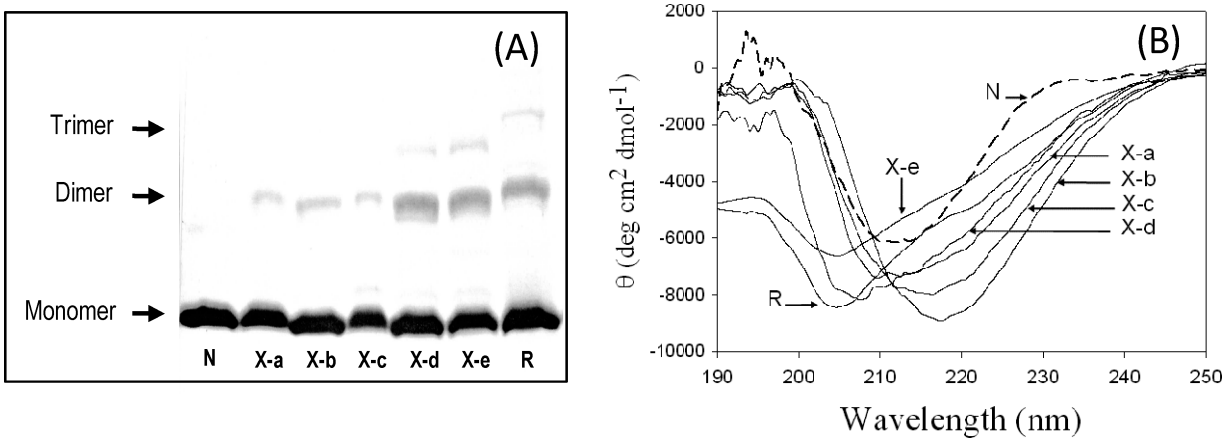

FIGURE 3: (A) SDS−PAGE of N-VEGF$_{110}$ and X-VEGF$_{110}$ isomers. The molecular masses corresponding to monomer, dimer, and trimer are indicated. Samples were analyzed using a nondenaturing PAGE (12%). (B) CD spectra of N-VEGF$_{110}$ and X-VEGF$_{110}$ isomers. All spectra were recorded on a Jasco J-715 spectropolarimeter at 23 °C. The proteins were dissolved in PBS (pH 7.4) with the concentration of 0.2 mg/mL.

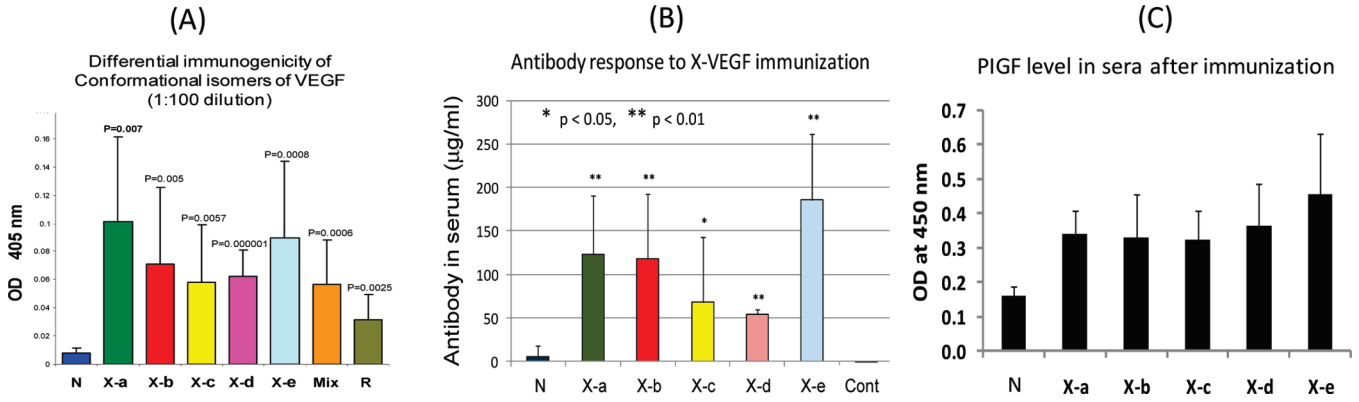

FIGURE 4: (A) Immunogenicity of N-VEGF$_{110}$ and X-VEGF$_{110}$ isomers. The immune response of immunized mice was evaluated by ELISA as described in the text. The *X*-axis denotes the groups of mice immunized with different immunogens. Each bar is an average antibody response of eight mice. The panel shows the results from the first bleeding (3 weeks after first immunization and 1 week after second immunization). The results obtained from the second, third, and fourth bleedings (5 months after first immunization) are similar to those presented here. (B) Amounts of anti-N-VEGF$_{110}$ antibodies generated in immunized mice. The amount of antibody was determined by ELISA of serial diluted sera and compared to a standard curve, obtained by using an anti-VEGF polyclonal antibody (Abcam). (C) PIGF levels in mouse sera after immunization with N-VEGF$_{110}$ and XVEGF$_{110}$. PIGF levels were measured in the sera of X-VEGF$_{110}$ and N-VEGF$_{110}$ immunized mice (six mice per group) using ELISA. Each bar represents an average PIGF level of six mice from the readings of duplicate assays of ELISA. The levels of PIGF in sera were determined by measuring the absorbance at 450 nm as described in the Experimental Procedures.

Measurement of Placental Growth Factor (PIGF) Level in Sera of X-VEGF$_{110}$ Immunized Mice. Placental growth factor (PlGF) is another member of the VEGF family of proteins that binds to VEGFR-1. It has been previously reported that increased serum G-CSF, PIGF, and SDF1 levels occurred following anti-VEGF therapy (*22*, *23*). Therefore, we have also determined the levels of PIGF in mouse serum after X-VEGF$_{110}$ immunization to evaluate the effectiveness of X-VEGF$_{110}$ active immunization. We observed that PIGF levels in all five X-VEGF$_{110}$ immunized mice are almost 2−3-fold higher than those in N-VEGF$_{110}$ immunized mice, as shown in Figure 4C. The exact biologic significance of this increase is unknown. The data suggest that the increased levels of PIGF might be a consequence of lower VEGF levels after X-VEGF$_{110}$ immunization because the induction of greater levels of hypoxia in normal tissues due to lowered VEGF levels will induce the release of more PIGF to compensate this hypoxic effect.

DISCUSSION

Non-Native Protein Isomers (X-Isomers) Are Opulent Resources of Biomolecules. Non-native conformational isomers of unfolded proteins are opulent resources of biomolecules that have remained untapped for their potential use in disease treatment and diagnosis. One of the major obstacles in exploiting this untapped potential is that it is inherently difficult to fractionate, isolate, and characterize structurally defined conformational isomers. The method of disulfide scrambling (*14*, *15*) allows production of non-native conformational isomers of disulfide proteins (X-isomers) that are amenable to fractionation, isolation, and further structural and functional studies (*16*).

X-isomers comprise diverse and constrained conformational epitopes that render them a new class of immunogens with immense versatility and broad applications. In the case of α-lactalbumin (αLA), X-αLA isomers were able to break the immune tolerance and elicit anti-N-αLA antibodies. While N-αLA is not immunogenic, three X-αLA isomers were shown to elicit high titers of antibodies that cross-react with N-αLA (*16*). Since X-isomers of relevant proteins can be systematically produced by the method of disulfide scrambling, they can be routinely prepared to test their immunogenicity. X-isomers are thus potentially a vast resource of compounds for development as effective immunogens and therapeutic vaccines.

Technical Challenge in the Preparation of X-VEGF$_{110}$ Isomers. X-isomers of disulfide proteins are typically generated from the native proteins using the method of disulfide scrambling. This is accomplished by incubating native proteins in the alkaline solution containing selected denaturant and a thiol catalyst (*14*, *15*). This established method has been successfully applied to produce X-isomers of numerous disulfide proteins, including hirudin (*17*), leech carboxypeptidase inhibitor (LCI) (*18*), and human proinsulin (*19*) and 15 other proteins (*16*). The fact that N-VEGF$_{110}$ is unable to undergo disulfide scrambling in the presence of denaturant and thiol catalyst is therefore perplexing. However, N-VEGF$_{110}$ is not alone in displaying this unusual property. A native domain (LA5) of the ligand binding module 5 of the low-density lipoprotein receptor also behaves in a similar manner (*24*).

The most likely cause is the extraordinary covalent stability of the disulfide bonds of N-VEGF$_{110}$, endowed by surrounding noncovalent structures. This is manifested by their resistance against dithiothreitol (DTT) reduction in both the absence and presence of denaturant (Figure 5). Similar to many disulfide proteins (*25*), the three native disulfide bonds of N-VEGF$_{110}$ are reduced by DTT in the absence of denaturant (reductive unfolding) in a collective manner (Figure 5). However, the concentration of DTT required to reduce N-VEGF$_{110}$ is extraordinarily high (Figure 5A,B). Even in the presence of denaturant, reduction of disulfide bonds of N-VEGF$_{110}$ is notably slow. Most disulfide proteins become fully reduced using the standard denaturation/reduction conditions, which comprise incubation of native proteins in the buffer (pH 8.0−8.5) containing 30 mM DTT and 6 M GdmCl for 90 min at room temperature. However, only 60% of N-VEGF$_{110}$ is reduced under the same conditions (Figure 5C), a remarkable stability of disulfide bonds that has not been observed previously in our laboratory. Another possible underlying cause is the unique disulfide pattern of N-VEGF$_{110}$, which consists of three highly intertwined disulfide bridges, known as cystine knot motif (*26*). This structural motif has been found in small protease inhibitor, toxins, cyclotides, and various

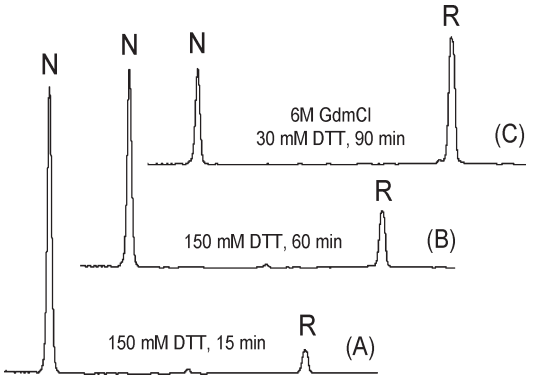

FIGURE 5: (A) Reductive unfolding of N-VEGF$_{110}$. N-VEGF$_{110}$ (0.5 mg/mL) was incubated at 22 °C for 15 min in Tris-HCl buffer containing 150 mM DTT. (B) Reductive unfolding of N-VEGF$_{110}$. N-VEGF$_{110}$ (0.5 mg/mL) was incubated at 22 °C for 60 min in Tris-HCl buffer containing 150 mM DTT. (C) Reduction of N-VEGF$_{110}$. N-VEGF$_{110}$ (0.5 mg/mL) was incubated at 22 °C for 90 min in Tris-HCl buffer containing DTT (30 mM) and GdmCl (6 M). Samples were acidified and analyzed by HPLC using the conditions described in the Experimental Procedures. "R" indicates R-VEGF$_{110}$.

growth factors (*27*). It was thought to be a major determinant for the thermodynamic stability of these proteins (*28*). However, the presence of cysteine knot motif alone may not be able to satisfactorily account for the inability of N-VEGF$_{110}$ to undergo disulfide scrambling. Studies in our laboratory have shown that transforming growth factor-β (TGF-β), also a cystine knot protein, can readily undergo disulfide scrambling in the presence of denaturant and thiol catalyst (unpublished data).

As X-VEGF$_{110}$ is unable to be generated from N-VEGF$_{110}$ using the standard method of disulfide scrambling (*14*, *15*), we have adopted an alternative strategy to achieve our goal. In this case, we are able to successfully generate X-VEGF$_{110}$ isomers using R-VEGF$_{110}$ as the starting material (Figure 2B). This strategy is based on our previous observations that the equilibrium between N- and X-isomers and their final ratio are governed by free energy state and independent of the starting isomers (*17*). For instance, when hirudin (a three-disulfide protein) is incubated in the alkaline buffer containing β-mercaptoethanol (0.25 mM) and GdmCl (6 M), the equilibrated end products always comprise 40% of N-hirudin and 60% of X-hirudin isomers, regardless of whether the stating material is native hirudin (N-hirudin), fully reduced hirudin (R-hirudin), partially reduced hirudin, or any of purified X-hirudin isomers (see Figure 3 in ref *17*).

Tumor Active Immunotherapy Using X-VEGF$_{110}$ Isomers. We have demonstrated in this report that all five X-VEGF$_{110}$ isomers or isomer mixtures exhibit significantly increased immunogenicity than that of N-VEGF$_{110}$. They are capable of eliciting high titers of antibodies that cross-react with N-VEGF$_{110}$. The generated antibodies are VEGF specific, as they did not react with a control protein (mouse prion protein) which was produced by using the same *E. coli* expression system. Our future aim will be to investigate the therapeutic effects of X-VEGF$_{110}$ immunization on tumor growth and metastasis in tumor mouse models. A notable result is the serum concentration of anti-N-VEGF antibodies generated by X-VEGF$_{110}$ immunization (Figure 4B). While N-VEGF$_{110}$ immunization elicits marginal immune response (~5.8 μg/mL), those immunized with X-VEGF$_{110}$ isomers produce the amounts of antibody in the range (54−186 μg/mL) that are compatible with or greater than the concentration needed for therapeutic effectiveness. For instance, the FDA recommended dose of bevacizumab treatment is 5 mg/kg for every 2−3 weeks. This would translate into about 50−70 μg/mL antibody concentration in treated patients. The neutralizing capacity of elicited antibody is also supported by the 2−3-fold increase of placental growth factor (PlGF) (Figure 4C), an observation noted in mice and human following anti-VEGF therapy (*22*, *23*). It remains however to be demonstrated whether X-isomers of human VEGF will exhibit comparable immunogenicity. The toxicity of X-VEGF also needs to be addressed.

Mechanism of Enhanced Immunogenicity of X-VEGF$_{110}$ Isomers. The underlying mechanism of enhanced immunogenicity of X-VEGF$_{110}$ has yet to be established. We have shown that all X-VEGF$_{110}$ isomers exist predominantly in monomeric form prior to immunization (Figure 3A). Although a minor amount of aggregated X-VEGF$_{110}$ isomers (Figure 3A) may also contribute to the observed immunogenicity, the extent is probably limited, as the most aggregated isomer X-d exhibited the lowest immunogenicity. It is likely that the increased immue response of X-VEGF$_{110}$ is mainly attributed to the diverse and constrained epitopes that are better recognized by the immune system. Whether the enhanced immunogenicity is elicited by linear (continuous) or conformational (discontinuous) epitopes of X-VEGF isomers has to be delineated via extensive analysis of epitope mapping. It is most likely that structures and epitopes constrained by diverse non-native disulfide bonds contribute significantly to the observed immunogenicity as fully reduced R-VEGF$_{110}$ exhibits nuch lower immunogenicity than that of R-VEGF$_{110}$ isomers. A fully reduced protein, such as R-VEGF$_{110}$, also has tendency to undergo oxidative folding in serum to form X-isomers as folding intermediates (*29*). This may partly explain the observed immunogenicity of R-VEGF$_{110}$.

Concluding Remarks. Data obtained in this report demonstrate the potential of X-VEGF$_{110}$ isomers as therapeutic vaccines for treatment of VEGF-bearing tumors and as effective immunogens for production of anti-VEGF monoclonal antibodies. Our ensuing task is to investigate the therapeutic effect of X-VEGF$_{110}$ immunization on primary tumor growth and tumor metastasis in tumor mouse models. It is relevant to mention that during the final stage of this study immunized mice were implanted with colorectal tumor CMT-93 cells in order to evaluate the preventive effect of X-VEGF$_{110}$ immunization. This preliminary study showed varied yet promising results. While some X-VEGF$_{110}$ isomers exhibit only marginal effect on inhibition of tumor growth, those immunized with certain X-VEGF$_{110}$ isomers showed no tumor growth. However, this preliminary result needs to be further confirmed in a study comprising a larger number of animals.

Finally, the demonstration that X-VEGF$_{110}$ and X-αLA (*16*) are capable of breaking immune tolerance and elicit high titers of anti-native protein antibodies suggests that X-isomers of other proteins may behave in a similar manner. These observations are significant because X-isomers of disulfide proteins may occur in nature. The most compelling evidence is the presence of complex thiol oxidoreductase machinery in both prokaryotic and eukaryotic cells (*30*−*32*), which catalyze disulfide shuffling of misfolded disulfide proteins, including X-isomers. In theory, proteins are unable to fold 100% into one single native structure. Under physiological conditions and in the milieu of thiol reagents (*33*), a native disulfide protein may exist in equilibrium with minute concentrations of X-isomers. Indeed, a systematic *in vitro* study

conducted in our laboratory has shown that under physiological conditions about 0.1−1.5% of the unfolded X-isomers are present in equilibrium with the native protein (*34*). The concentration of X-isomers is dependent upon the conformational stability of the native protein. It may increase if the protein is destabilized due to mutations or under stressful conditions (e.g., high temperature). In the case of human epidermal growth factor (EGF), the concentration of X-EGF increases from 0.03% to 0.2% and 3.1% as the temperature rises from 22 to 37 and 50 °C, respectively. If such diminutive amounts of unfolded X-isomers are able to induce immune response, they may play roles in the development of autoimmune diseases (*35*). In the cases of protein drugs containing disulfide bonds, the undesirable side effects of immunological response are likely also due to the byproduct of trace amounts of X-isomers (*36*).

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