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E-selectin is an inducible cell adhesion molecule which mediates rolling of neutrophils as well as adhesion of carcinoma cells to endothelium. Inhibition of selectin-mediated interaction is a possible means for controlling inflammation induced diseases and metastatic spread. The purpose of these studies is to identify peptide forms mimicking Lewis X (LeX), sialyl-Lewis X (SA-LeX), sialyl-Le^a (SA-Le^a), and Lewis Y (LeY) carbohydrate structures to disrupt lectin-ligand interactions and evaluate their properties to block adenocarcinoma cell adhesion to endothelial cells and tumor metastasis in vitro and in vivo. We used recombinant peptide library to identify novel ligands for E-selectin. We have identified five dodeca peptides mimicking SA-Le^a carbohydrate, which is one of the E-selectin ligands, via means of random peptide library screening using SA-Le^a-specific monoclonal antibody (MAb) NS19-9. Peptides with the highest binding affinity for the MAb will be synthesized and characterized for its anti-metastatic activity using JC murine breast and H-59 lung carcinoma cells in metastatic model in vivo.

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INTRODUCTION

Aberrant glycosylation in tumors relative to their normal counterparts represents a phenotypic feature associated with different human malignancies. Carbohydrate tumor-associated structures may be instrumental in the diapedesis of metastatic cancer cells which have escaped from the primary lesion and are in the process of colonizing distant sites. Tumor-specific antigens such as LeX, SA-LeX, and SA-Le^a have been shown to bind to E-selectin expressed on vascular endothelium (1). In addition, Lewis Y (LeY) structure might be involved in adhesion of tumor cells to endothelial cells via non-selectin mediated pathways highlighting the heterogeneity of tumor/endothelial cells interactions. The inhibition of these interactions is a possible point of therapeutic intervention.

The long-term goal of the proposed studies is to identify small molecules conformationally mimicking carbohydrate ligands as anti-adhesive agents to combat human tumors. MAbs specific for Lewis antigens have shown utility for inhibiting tumor cell adhesion properties. The synthetic peptides identified in random peptide libraries which target carbohydrate receptors can lead to more useful agents to study adhesion properties of tumor cells, as well as agents that are potentially more useful in vivo (2). This approach may also be applied for inhibition of neutrophil adhesion to endothelium to control inflammation-mediated diseases such as rheumatoid arthritis or psoriasis.

Recently several methods to construct and screening peptide libraries were developed. We report here the use of recombinant peptide display technology to identify peptide ligands for selectins (2-4).The FliTrx™ peptide library which displays peptides on the surface of *E. coli* using the major bacterial flagella protein (FliC) and thioredoxin (TrxA) was applied in this project. Random dodeca peptides are inserted into the active site loop of thioredoxin, which is inserted into the dispensable region of flagella gene and expressed in bacteriophage as a fusin protein FliTrx on the cell surface allowing the display of peptides and panning.

BODY

Methods

Panning. A vial of the FliTrx^{TM} (Invitrogen) peptide library representing 1.8 x 10^8 of primary clones was grown in 50 ml of IMC medium containing 100 μg/ml ampicillin overnight at room temperature. Cells (1 x 10^{10}) are induced for the peptides expression by addition of 100 μg/ml tryptophan in 50 ml of IMC medium containing 100 μg/ml ampicillin for 6 hr at room temperature with vigorous aeration. A 60 mm tissue culture plate (Nunclon^R Delta) was coated with 20 μg/ml NS19-9 a MAb specific to a monosialoganglioside SA-Le^a (5) in 1 ml sterile water by gentle agitation for 1 hr. The plate is rinsed with 10 ml water and blocked by 10 ml blocking solution (1% milk, 150 mM NaCl and 1% α-methyl mannoside in IMC medium containing 100 μg/ml ampicillin) for 1 hr by gentle rocking at room temperature. Ten ml of induced peptide library culture was treated with 1% milk, 150 mM NaCl and 1% α-methyl mannoside and immediately poured onto the NS19-9 coated tissue culture plate and allowed to bind for 1 hr. Unbound cells were decanted off and the plate was gently washed five times with 10 ml of 1% α-methyl mannoside in IMC medium containing 100 mg/ml ampicillin for 5 min by gentle rocking. The bound peptide library containing bacterial cells were eluted into a small volume of residual wash solution by vortexing the plate for 30 seconds.

The selected cells were grown in 10 ml of IMC medium containing 100 μg/ml ampicillin for 15 hr at room temperature with vigorous aeration. Cells (1 x 10^{10}) are used for the induction of peptides expression by addition of 100 μg/ml tryptophan in 50 ml of IMC medium containing 100 μg/ml ampicillin for 6 hr and the second round of panning.

After the fifth panning and the overnight growth of the library, the culture was streaked onto RMG plates containing 100 μg/ml ampicillin and incubated overnight at 30°C to select for single colonies. Twenty single colonies were selected and grown in rich medium (RM) containing 100 μg/ml ampicillin overnight for DNA preparation and Western blotting.

Western blot. Forty μl cultures from each culture of 20 single colonies were taken and induced by 100 μg/ml tryptophan in 2 ml of IMC medium containing 100 μg/ml ampicillin at 37°C shaker until the cells reach mid-log phase (16 hr). One and a half (1.5) ml of induced cells were harvested and the pellets were resuspended with 100 μl of sample buffer and boiled for 5 min. 10-15 μl samples were run in 12% polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose paper in 20 mM Tris, 150 mM glycine, and 20% methanol for 2 hr at 4°C. The nitrocellulose membrane was blocked with 5% milk in 20 mM Tris HCl, pH 7.5, and 500 mM NaCl (TBS) overnight and incubated with 15 ml of 10 μg/ml NS19-9 in TBS. The nitrocellulose was washed with 0.2% Tween 20 in TBS three times for 10 min rocking at room temperature. ^{125}I-anti-mouse immunoglobulin (IgG) was added as a secondary antibody and allowed to bind for 1 hr by gentle rocking and washed three times. Dried nitrocellulose filter was exposed on a X-ray film (Kodak) and developed for the detection of 53 kDa fragments.

Plasmid DNA preparation. One and a half (1.5) ml cultures from the five-time panned, single colonies selected by Western blotting were harvested and the plasmid DNA was purified using QIA Prep Spin Plasmid Mini Preparation Kit (Qiagen). Pellets were resuspended in 250 μl of 50 mM Tris-HCl, pH 8.0; 10 mM EDTA containing 100 μg/ml RNase A. Cells were lysed by adding 250 μl lysis solution (200 mM NaOH, 1% SDS), mixing gently by inverting 7 times and neutralized with 350 μl of 3 M potassium acetate, pH 5.5 and centrifuged for 10 min at 14,000 x g at 4°C. Supernatants were applied to QIA prep spin columns (Qiagen) and centrifuged for 60 seconds. Columns were washed with 0.5 ml of 50 mM MOPS, pH 7.0 buffer containing 1 M NaCl and 15% ethanol, and centrifuged two times to ensure the removal of wash buffer. DNA was eluted with 50-100 μl of H_2O by incubating 1 min and centrifugation for 1 min. The expression of plasmid DNA was confirmed by the presence of 5 kb fragments generated by EcoRI restriction enzyme digestion followed by 1% agarose gel electrophoresis.

Radioimmunoassay (RIA) and fluoresceine activated cell sorter analysis (FACS). Cell monolayers were incubated with MAb at 10 μg/ml. After 1 hr of incubation at room temperature cells were washed and incubation was continued with ^{125}I labeled goat anti-mouse Ig fraction. The amount of bound radioactivity was counted in a γ-counter. Fluoresceine-labeled instead of iodinated antibody was used in FACS analysis of suspension cells.

Results

Screening of recombinant peptide library. The FliTrx™ peptide library displays peptides on the surface of *E. coli* using the major bacterial FliC and TrxA proteins. Random dodeca peptides are inserted into the active site loop of TrxA which is inserted into the dispensable region of flagella gene and expressed in bacteriophage λ under the control of P_L promoter. The DNA encoding the TrxA peptide fusion was cloned into the FliC gene replacing a large, soluble, exposed, non-essential domain. The FliTrx peptides are propagated in *E. coli* GI826 where cI repressor gene is under the control of Trp promoter. When the induction is initiated by the addition of tryptophan, the fusion protein FliTrx is exported and assembled into flagella on the surface of bacterial cell allowing the display of peptides.

Four independent peptide display library screenings were performed for the selection of peptides which interact with NS19-9, antibody specific for SA-Le^a, a major E-selectin ligand.

NS19-9 MAb recognition of isolated peptides. Twenty single colonies were selected via five rounds of panning with NS19-9 MAb from each library screening. The expression of peptides in bacterial cultures was determined by Western blotting. The positive clones were re-grown and again the expression of peptides was confirmed using Western blot with NS19-9 MAb. Two strongly and three weakly binding clones were identified in the random 12-mer library, suggesting that they indeed represent peptides mimicking SA-Le^a carbohydrate structure. The representative clones are shown in Fig. 1.

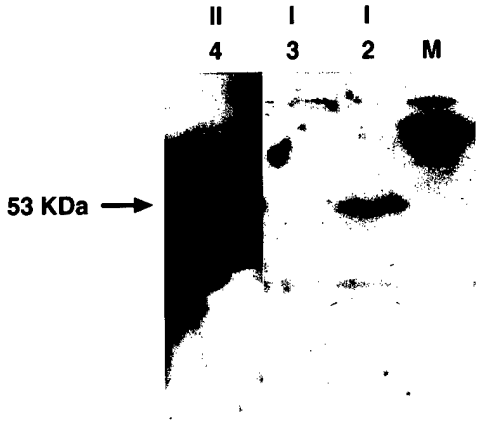

Fig. 1. Fusion protein containing dodeca peptide sequence as detected by SA-Le^a-specific MAb binding in Western blot. Roman numerals refer to the library screening experiment. Arabic numbers refer to isolated clone number.

Amino acid sequence of selected peptides. The bacteria from each positive clone were grown and harvested. The plasmid DNA was purified using specific cartridges and submitted for DNA sequence analysis. The purity and the size of the isolated plasmid DNA were determined using standard 1% agarose gel electrophoresis and DNA molecular weight markers (Fig. 2).

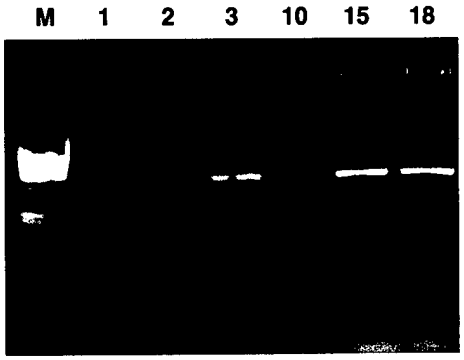

Fig. 2. Plasmids isolated from bacterial clones, which were identified by positive binding of NS19-9 MAb using Western blot technique. Isolated plasmids were separated on 1% agarose gel

electrophoresis and stained with ethidium bromide. M, λ DNA/HindIII fragments as molecular weight markers.

Pure plasmid DNA was submitted for sequencing at the oligonucleotide sequencing facility at The Wistar Institute. Sense and antisense oligonucleotide primers complementary to oligonucleotide sequences flanking DNA encoding peptides were used to ensure sequencing of both DNA strands. The results of DNA sequencing and deduced amino acid sequences are presented in Tables 1 and 2, respectively.

Table 1. Sequences encoding peptides as determined by dideoxynocleotide sequencing of isolated plasmids.

Sequence 1:	$5'$ACCCANCTTCTAGCACGTTATAGTACGCGTCAAAGTC$3'$
Sequence 2:	$5'$GTAGGGATCTGGAGCGTTGTGTCGGAAGGAAGTAGG$3'$
Sequence 3:	$5'$CAAGATGGCGTCTGGGAACATGTTTTGGAGGGCGGT$3'$
Sequence 15:	$5'$GTGGAACTCNGCNGTCGAGGGGGGGNNANTGCACATGG$3'$
Sequence 18:	$5'$ACAATCGAGCCCGTCCTGGCGGAGATGTTTATGGGC$3'$

Table 2. Amino acid sequence of isolated peptides inferred from the oligonucleotide sequences of isolated plasmid DNA containing the sequence encoding dodecapeptides.

1.	T H L L A R Y S T R Q K
2.	V **G** I **W** S V **V** S **E** **G** S R
3.	Q D **G** **V** **W** E H **V** L **E** **G** G
15.	**V** E L C*N R G **G** N N A H
18.	T I E P **V** L A E M F M **G**

Homologous amino acids are marked in bold.
* N represent not finally assigned sequences.

In vivo model to test inhibition of metastasis with carbohydrate mimicking peptides. To establish a model to test ability of isolated peptides to inhibit tumor metastasis in vivo we have gathered a panel of murine breast adenocarcinoma cell lines. Mouse mammary carcinoma cell lines were initially characterized for expression of carbohydrate ligands. The mammary adenocarcinoma cell lines JC (6), 410-4 (7), and 66-1 (7) (ATCC) are derived from spontaneous tumors and are devoid of mammary tumor viruses that commonly cause breast cancer in mice but are not thought to play a role in the etiology of human breast cancer. Cells were grown in Iscove's minimal essential medium (IMEM) supplemented with 10% fetal calf serum and glutamine. Human colon carcinoma cell line SW948 express on the cell surface ligand for E-selectin such as SA-Le^a (5) as well as LeY structure (8). The expression of carbohydrate adhesion ligands in murine breast carcinoma cells was determined using two types of assays, i.e., RIA and FACS. Out of three breast carcinoma cell lines tested the positive binding of NS19-9 antibody was observed only to JC cell line but not 410.4 and 66.1. Neither of the cell lines tested expressed LeY carbohydrate, which is postulated ligand involved in non-selectin mediated adhesion of the tumor cells. We are in a process of characterizing murine Lewis lung carcinoma sublines H-59 and M-27 (9) for expression of these ligands. These cell lines appear extremely valuable for our in vivo experiments. Recently published report demonstrated that the formation of metastasis was inhibited by administration of E-selectin specific MAb, which strongly suggests that metastatic process is E-selectin mediated. Therefore, they may be a suitable in vivo model to test peptides mimicking E-selectin ligands.

Discussion

Of more than 1 x 10^8 peptides 5 were selected so far. Two peptides (#2 and 3) contain 5 conserved residues, which appear to be required for high affinity binding, since they were the strongest binding peptides by the NS19-9 MAb in Western blot. The level of amino acid sequence homology with peptides #2 and 3 correlated with the binding affinity with NS19-9 antibody. Terminal G in peptide #18 was homologous with G in peptides #2, 3, and 15 which implies that G at this position is necessary for the Ab binding. In addition peptide #18 shares V at the same location with peptides #3 and 15. The oligonucleotide sequence of peptide #15 is not finally determined, but V and G at the same positions are shared with peptides #3 and 18. In addition peptide #15 shares two amino acids L and R at the same positions with peptide #1. We are in a process of identifying the sequence of 5 more peptides selected in 2 additional panning experiments.

In summary, the peptide family which was identified in these experiments retain conformation properties of carbohydrate ligand as determined by antibody binding. This family of peptides has clear structural relationship with respect to amino acid homology which suggests that the requirements for binding of NS19-9 and E-selectin are stringent.

The analysis of sequence homology revealed that the peptide mimicking LeY hexasaccharide structure (APWLYAGP) (10) shares some homology with peptides mimicking monosaccharides recognized by lectin proteins such as ConA (YYPY) and WGA (WRY) (11). All proteins which interact with carbohydrate ligands demonstrate the preference of aromatic groups separated by an intervening residue such as YPY, WRY, YRY. The central Planar residue-X-Planar residue tract is observed also in the sequence APWLY recognized by LeY specific MAb B3. Subsequently, it may turn out that planar and hydroxyl group containing peptides displayed in a variety of ways can lead to inhibition of cell adhesion. Sequences identified in these experiments do not display amino acid sequence homology with Planar-X-Planar motive found in a putative peptide identified by phage display panning with anti-Y antibody, B3. The sequence homology might be expected since the low energy-conformations of Lewis structures overlap in antigenic presentation, which might be mimicked by homologous peptides (12). On the other hand, mimics of structures containing highly charged moieties such as sialic acid in SA-Le^a molecule might not

follow structural tract displayed by peptides mimicking neutral sugars. We are planning to use the same methods to isolate peptides mimicking another sialylated E-selectin ligand, i.e., SA-LeX, which are expected to share similar conformational features.

Of more interest for this application is the extent to which peptides will be inhibitory of the metastasis formation. The development of synthetic peptide reagents identified in peptide libraries which target carbohydrate receptors can lead to more useful agents to study adhesion properties of tumor cells, as well as agents that are potentially more useful in vivo. This approach may also be useful for inhibition of neutrophil adhesion to endothelium to control inflammation-mediated diseases such as rheumatoid arthritis or psoriasis. Several potential therapeutics have been tested for their ability to inhibit the E-selectin adhesion, including carbohydrates, antibodies, soluble E-selectin and selectin-Ig chimeras. While these molecules have shown their utility to block selectin interaction for treating inflammation, each has significant drawbacks as a therapeutics including short half-life, potential immunogenicity and high cost. Peptides overcome these limitations and provide efficient means to improve the conformational and pharmaceutical properties of these molecules.

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