Amino Acids (2002) 23: 301–308
DOI 10.1007/s00726-001-0142-2

Amino
Acids
Printed in Austria

Targeting of MPEG-protected polyamino acid carrier to human E-selectin *in vitro*

H. W. Kang, R. Weissleder, and **A. Bogdanov, Jr.**

Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Maryland, U.S.A.

Received June 29, 2001
Accepted August 8, 2001
Published online August 9, 2002;

Summary. Targeted diagnostic agents are expected to have a significant impact in molecular imaging of cell-surface associated markers of proliferation, inflammation and angiogenesis. In this communication, we describe a new class of targeted polyamino acid-based protected graft copolymers (PGC) of poly-(L-lysine) and methyl poly-(ethylene glycol) (PGC) covalently conjugated with a monoclonal antibody fragment, $F(ab')_2$. We utilized targeted PGC conjugates as carriers of near-infrared indocyanine fluorophores (Cy5.5) for optical imaging of endothelial cell populations expressing IL-1β inducible proinflammatory marker E-selectin. We compared two conjugation chemistries, involving either introduction of sulfhydryl group to $F(ab')_2$, or via direct attachment of the antibody fragment directly to the chemically activated PGC. Both PGC-based targeted agents demonstrated high binding specificity (20–30 fold over non-specific uptake) and were utilized for imaging E-selectin expression on human endothelial cells activated with IL-1β.

Keywords: E-selectin – Endothelium – IL-1β – Graft copolymer – Indocyanine – Antibody – Optical imaging

Introduction

Non-invasive imaging of endothelial cell surface markers holds promise in identifying early signs of angiogenesis (Brasch et al., 1997), inflammation (Zinn et al., 1999), or atherosclerosis in vivo. For example, recent studies have shown that endothelial cells expressing the proliferation marker $\alpha v\beta 3$ integrin can be visualized in vivo using targeted ^{18}F-labeled peptides and positron emission tomography (Haubner et al., 2001), whereas magnetic resonance imaging can be utilized for the same purpose in conjunction with integrin-targeted paramagnetic liposomes (Anderson et al., 2000; Sipkins et al., 1998).

Several other endothelial cell surface markers or adhesion-promoting molecules including endoglin (Bredow et al., 2000) and fibronectin isoform B-FN (Neri et al., 1997) have been investigated for imaging angiogenesis in tumors. There exist a mounting evidence suggesting that the expression of another endothelial cell-surface receptor, E-selectin (CD62E) (Bevilacqua et al., 1987), is upregulated in proliferating endothelial cells (Bischoff et al., 1997) and co-localizes with dividing microvascular endothelial cells in tissues with active angiogenesis. (Kraling et al., 1996). E-selectin is a proinflammatory marker involved in the rolling of leukocytes on endothelium in inflammation, as well as in angiogenesis. In tumors, E-selectin positive vessels were preferentially found in vascular-rich areas (Mayer et al., 1998). Apart from E-selectin-specific diagnostic probes for angiogenesis, another potentially important application of E-selectin targeted imaging lies in the detection of early changes in endothelial phenotype linked to atherosclerotic plaque formation (Gimbrone et al., 2000).

It is known that endothelial cells forming an endotube during in vitro induced angiogenesis undergo changes of endosomal compartment with the formation of mature large vesicles resembling vacuoles (Gamble et al., 1999). Therefore, we hypothesized that proliferating endothelial cells can be targeted via cell-surface E-selectin using polyamino acid-based agents that will be cleaved in large endosomal vesicles due to proteolytic activity associated with lysosomal/endosomal compartment. We previously demonstrated that polylysine-methoxy (polyethylene oxide) graft co-polymer (protected graft copolymer, PGC) can be labeled with indocyanine

dyes at self-quenching densities (Mahmood et al., 1999; Weissleder et al., 1999). After enzymatic cleavage of peptide bonds in PGC, these probes were spatially separated and near-infrared fluorescence of these probes was de-quenched. Here we report an attempt to target PGC probes to endothelial cells using a well-characterized high-affinity anti-human E-selectin antibody (H18/7 (Bevilacqua et al., 1987)). We further explored the utility of this conjugate in detection of inducible expression of E-selectin on the surface of human endothelial cells (HUVECs).

Materials and methods

Polyaminoacid-based graft co-polymers

Two types of graft copolymer of O-methoxy poly(ethylene glycol) and poly(L-lysine) were used in this study: 1) containing sulfhydryl-reactive terminal vinylsulfonate group (MPEG-PL-VS) and 2) terminal carboxyl group (MPEG-PL-COOH). The graft co-polymers were obtained as described in detail in (Bogdanov et al., 1996; Marecos et al., 1998) and modified with N-hydroxysuccinimidyl-PEG3000-vinylsulfonate (I) or bis-carboxy-PEG3000 (II) (Shearwater Polymers, Birmingham AL). The resultant PGC were purified using hollow-fiber ultrafiltration (UFP-100 cartridges; A/G Technologies Inc., Framingham MA) and contained 15% total N-ε-amino groups of poly-l-lysine modified with O-methoxy-PEG5000-O′-succinate and approximately 10% of total amino groups modified with hetero-bifunctional PEG analogs I or II.

The resultant conjugates, terminal vinylsulfonate-bearing (PGC1) and terminal carboxy-bearing (PGC2) were lyophilized and used in further experiments.

Labeling of PGC with Cy5.5NHS. To label copolymers with fluorophores, 10 mg of PGC1 or PGC2 (35 nmol) were dissolved in 1 ml of H_2O and total volume was adjusted to 2 ml with 0.1 M sodium bicarbonate buffer (pH 8.4). Twenty μl of monofunctional near-infrared dye, Cy 5.5-NHS (MW 1128, 0.20 μmol, Amersham Pharmacia, Piscataway, NJ) were added to the polymer solution and incubated with gentle shaking and being protected light overnight. 5 mg of acetic acid N-hydroxysuccinimide ester (AcNHS, MW 157.1, 31.8 μmol, Pierce Chemical Co., IL, USA) in 100 μL of DMSO was added to the solution twice with 30 min interval in between. The solution was dialyzed using dialysis tube (molecular weight cut off = 12,000–14,000) against 2 L PBS followed by water. Cy5.5-PGC solutions were concentrated on Microcon YM-50 (molecular weight cut off = 50,000, Millipore, USA).

F(ab′)$_2$ fragment of monoclonal anti-human E selectin antibody (H18/7, Vascular Research Division, Department of Pathology, Brigham and Women's Hospital) (91 nmol) was radioiodinated with ^{125}I using IodoGen (Pierce Chemical Co) as described by the manufacturer. Labeled antibody fragment was purified using P-6 gel Minicolumn (Bio-Rad, Hercules CA) equilibrated with PBS pH 7.2. Final specific radioactivity was 0.6–0.8 μCi/μg.

Modification of F(ab′)$_2$ fragment with N-succinimidyl S-acetylthioacetate. Conjugation of N-succinimidyl S-acetylthioacetate (SATA, Molecular Biosciences, Boulder, CO) to the antibody fragment was accomplished by adding 27 nmol SATA (0.5 mg/ml DMF) to 200 μg F(ab′)$_2$ solution (3.5 mg/ml in 0.05 M sodium bicarbonate pH 8.5) containing ^{125}I-F(ab′)$_2$ final specific activity $1 \cdot 10^5$ cpm/μg) and incubated with agitation for 2 hr. The 15 : 1 mol : mol ratio of SATA to antibody was optimal as established earlier (Duncan et al., 1983). The SATA-modified F(ab′)$_2$ fragment was de-acetylated by adding an equal volume of 25 mM hydroxylamine, 10 mM EDTA in PBS (pH 7.4) to F(ab′)$_2$-SATA. The excess of free thioacetic acid was removed by passing modified F(ab′)$_2$ through a P-6 gel centrifugation Minicolumn equilibrated with bicarbonate buffer (pH 8.4). Purified thioacetyl-F(ab′)$_2$ was then immediately used for conjugation to Cy5.5-PGC1.

Conjugation of F(ab′)$_2$-SATA to PGCI. A F(ab′)$_2$ fragment of H18/7 antibody was radioiodinated using Iodogen (Pierce) and working antibody solution was prepared by mixing cold and ^{125}I-labeled Fab$_2$′ (7.3 : 1, wt : wt) followed by an additional purification on a Mini-spin P-6 gel column equilibrated with 50 mM bicarbonate buffer (pH 8.7). S-acetyl thioacetate, N-hydroxysucciminidyl ester was added to the antibody at the ratio of SATA to antibody (15 : 1 mol/mol) (Duncan et al., 1983). Following the adding the mixture of hydroxyl amine and EDTA, the SATA was de-acetylated. The excess SATA was removed by passing through a BioGel P-6 column equilibrated with bicarbonate buffer (pH 8.7). The antibody-SATA was added to Cy5.5-PGCI (10 : 1, mol : mol) and stirred at 4°C overnight. Then, final product was obtained by application of the mixture on a column of and eluted at 0.25 ml PBS/min.

Conjugation of F(ab′)$_2$-SATA to PGC2. A 5-fold excess of acetic acid N-hydroxysuccinimide ester (AcNHS) in DMSO was added to the Cy5.5-PGC2 solution in 50 mM sodium bicarbonate (pH 8.7) and the product was purified by dialysis against water. Hydroxy-sulfosuccinimde and ethyl diaminopropyl carbodiimide were added to PGC2 solution at final concentration of 15 mM each and incubated for 10 min before purification on P-6 gel column. The eluate was antibody and further procedures were same as the polymer modified by SATA.

Conjugate purification and analysis. Cy5.5-PGC-F(ab′)$_2$ conjugates were purified using size-exclusion chromatography on a Novarose SE-1000/40 column (1 × 50 cm, Inovata, Bromma, Sweden) eluted with PBS at 0.25 ml/min. Each fraction (0.5 ml) was characterized ^{125}I radioactivity counting (Compugamma, Wallac, Sweden) and absorption at 675 nm (for Cy5.5 determination). The peaks were collected and pooled before polyacrylamide electrophoresis in 10% gels. Gels were stained with Coomassie stain, dried and subjected to autoradiography using PhosphoImager (Amersham-Pharmacia Biotech). Size-exclusion HPLC analysis was performed by using a SEC-5 column (Varian/Rainin Instruments Inc.) eluted with 0.05 sodium phosphate, pH 6.8. Column was calibrated by using Bio-Rad size exclusion standards.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were provided by Vascular Research Division, Department of Pathology, Brigham and Women's Hospital. HUVEC cells were plated in a gelatin-coated plastic flask containing endothelial cell growth basal medium (EBM) with endothelial growth supplements (Clonetics, BioWhittaker Inc.). Homogeneity of HUVEC culture was analyzed by flow cytometry using rat anti-human CD-31 antibody (BD Bioscience) staining followed by anti-rat fluorescein-labeled antibody.

Cell culture experiments. Cells were plated in 24 well-plates or 6-well transparent chamber plates (LabTek II. Nalge Nunc Intl., Naperville, IL) with gelatin-coated surfaces. Human rIL-1β solution was added to EBM (final concentration −250 pg/ml). Control cells were left untreated. Cells were incubated for 2 h at 37C, washed twice with Endothelial Basal medium (EBM, Clonetics). Aliquots of Cy5.5-PGC-F(ab′)$_2$ were diluted in 10% FBS/Hanks/25 mM Hepes (pH 7.2) and added to cells (5–20 μg antibody equivalents/well). Cells were incubated for 1 h at room temperature with slow agitation and then washed three times with sterile 10% FBS/Hanks/25 mM Hepes (pH 7.2) Cells were fixed with 2% formaldehyde in PBS. Fluorescence microscopy was performed using fluorescence

microscopy was performed using an inverted microscope (Zeiss Axiovert 100 TV, Wetzlar, Germany) fitted with fluorescein and far-red filter sets (XF100 and XF48; Omega Optical, Brattleboro, VT). Images were acquired using a Photometrics CH250 cooled CCD (Photometrics, Tucson, AZ) with image acquisition and storage controlled by IP LabSpectrum software (Signal Analytics, Vienna, VA).

Results

Synthesis of functionalized protected graft co-polymers

Synthesis of protected graft co-polymer precursors bearing reactive groups was accomplished by using a sequential attachment of the heterobifunctional PEG analogues to free N-e-aminogroups of poly-l-lysine and modification of the remaining free amino group fraction with methoxy-PEG chains so that the resultant modification degree would be 25% of total amino groups. By using N-hydroxysuccinimide esters of PEGvinylsulfone in a reaction with poly-l-lysine, we imparted reactivity towards free sulfhydryl groups (co-polymer PGC1, Fig. 1), whereas a single-point attachment of PEG-dicarboxyl derivative through one of the available carboxyls resulted in a co-polymer that can be converted into N-hydroxysuccinimide and reacted with free aminogroups of the targeting antibody (co-polymer PGC2, Fig. 2). The test conjugation of l-cysteine to PGC1 showed that approximately 28 vinylsulfonate groups per PGC molecule were covalently attached to cysteine via free sulfhydryl group. The free aminogroups of poly-l-lysine backbone were sequentially modified with Cy5.5 and the remaining free aminogroups were covalently modified with succinimidyl acetate to block positively charged lysines. By measuring specific absorption of Cy5.5 at 650 nm ($e = 250{,}000\ M\ cm^{-1}$) we found that each PGC molecule was carrying approximately between 8 (PGC2) and 24 (PGC1) Cy5.5 groups on the average.

Coupling of antibody F(ab′)$_2$ fragment to PGC

Modification F(ab′)$_2$ of with SATA at optimized SATA/protein ratio (15 : 1 mol/mol) resulted in a protein that was bearing protected S-acetyl-thioacetyl groups. These groups were de-protected using a mild treatment with a nucleophilic hydroxylamine and the resultant thioacetyl-F(ab′)$_2$ was immediately brought in a reaction with functionalized PGC1. Reaction with vinylsulfonate included 24 h incubation required for addition of SH bond to vinyl group on PEG terminus.

Table 1. Average molecular mass and Cy5.5 content in PGC conjugates

	Corresponding globular protein molecular mass, kD [A]	Concentration of Cy 5.5 (mol/L) [B]
Cy5.5-PGC1	403	ND
Cy5.5-PGC2	441	ND
Cy5.5-PGC1-F(ab′)$_2$	550	1.56×10^{-6}
Cy5.5-PGC2-F(ab′)$_2$	510	5.13×10^{-7}

ND, not determined, [A] The apparent molecular masses were calculated from the retention times (Fig. 2) using gel chromatography standards (Bio-Rad) as a reference. [B] $\varepsilon_{675\,nm} = 250{,}000\ M^{-1}\ cm^{-1}$

The resultant conjugate was purified from non-reacted thioacetyl-F(ab′)$_2$ using macroporous gel-permeation chromatography. The HPLC analysis of purified conjugate (elution time 6.4 min) demonstrated a complete separation from the free F(ab′)$_2$ peak (elution time 7.8 min) (Fig. 3).

A different strategy of antibody fragment was utilized in the case of PGC2. The graft co-polymer-associated carboxyls on PEG termini were treated with water-soluble carbodiimide in the presence of sulfosuccinimide. This reaction yields sulfosuccimidyl-PEG derivative that can be reacted with amino groups of antibody fragment at pH 7.5. As in the case of PGC1, Cy5.5-PGC-F(ab′)$_2$ was successfully purified from non-bound F(ab′)$_2$ fragment. The apparent molecular masses of resultant PGC-F(ab′)$_2$ conjugates determined by using globular protein standards were similar (550 and 510 kD, Table 1).

Electrophoretic analysis of PGC1- and PGC2-based conjugates showed Coomassie staining of large molecules that were excluded from separating 10% polyacrylamide gel (Fig. 4 lanes 2 and 3). The autoradiography of the gels showed that both conjugates contained ^{125}I-labeled antibody fragment and that both preparations were free of any admixture of the free fragment or the products of fragment degradation (Fig. 4).

Cell culture binding experiments were conducted in human umbilical vein endothelial cells (HUVEC) grown on the surface of multiwell plastic plates for ^{125}I-labeled fragment binding studies (Fig. 5) as well as in transparent chambers to investigate morphology of conjugate binding to the cell surface using Cy5.5 near-infrared fluorescence (Fig. 6). Preliminary binding experiments in cell culture demonstrated that native H18/7 F(ab′)$_2$ specifically binds to IL-1β-treated cells

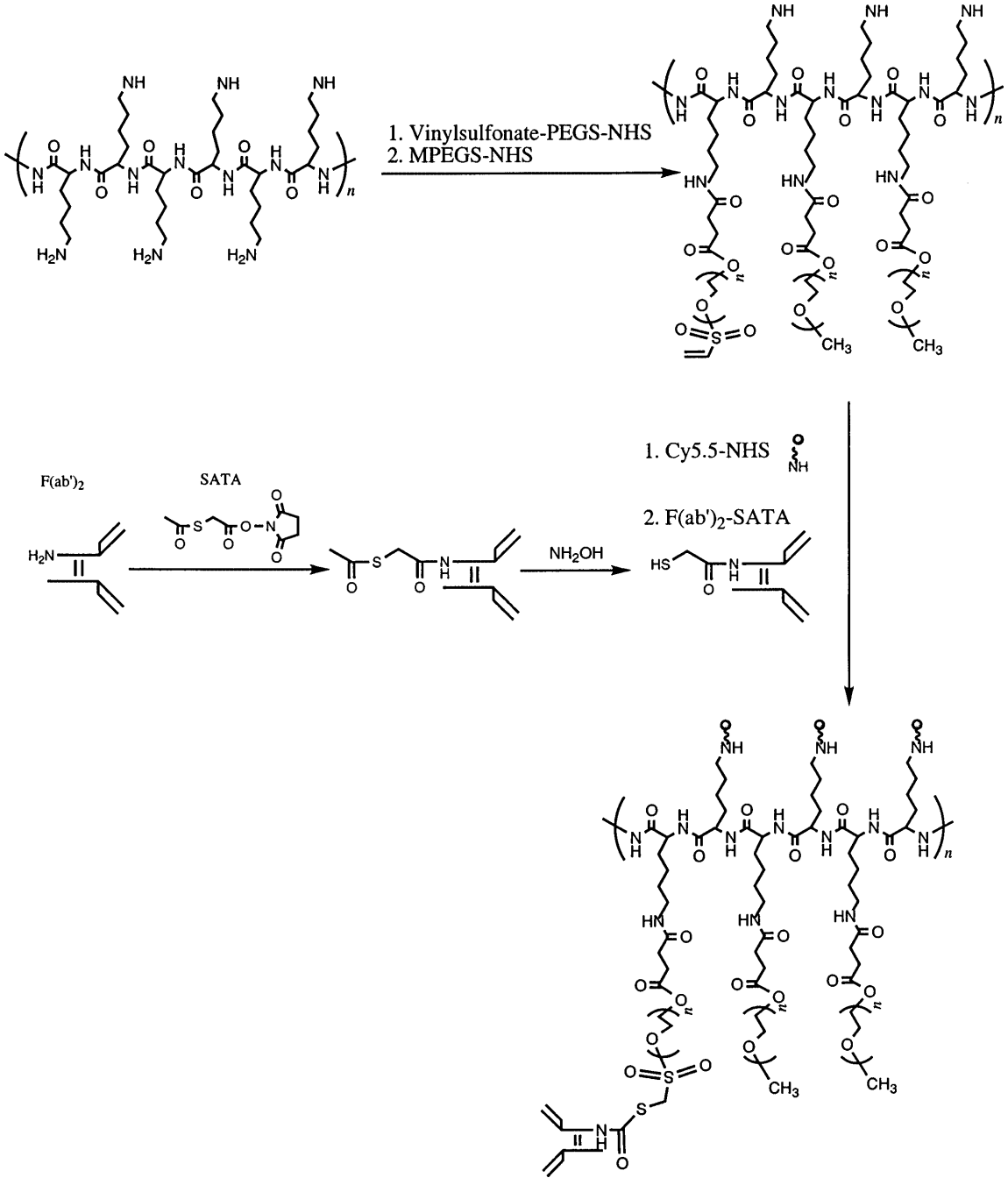

Fig. 1. A scheme of Cy5.5-PGC1-F(ab′)$_2$ synthesis. The covalent attachment of S-acetyl-thioacetylated antibody fragment is accomplished as a result of addition of the de-protected sulfhydryl group to vinylsulfonate group at the PEG terminus

with a high affinity (not shown). At the highest tested antibody/cell ratio (100 ng F(ab′)$_2$/10^4 cells), specific binding of antibody fragment was 100-times higher than the non-specific uptake. Scatchard analysis of binding curves was utilized to estimate the apparent dissociation constant, K_D. In the case of IL-1β-treated HUVECs K_D for H18/7 ^{125}I-F(ab′)$_2$ was 8.5 nM. Experiments with ^{125}I-F(ab′)$_2$ covalently bound to Cy5.5-PGC1 or Cy5.5-PGC2 showed that specific binding to IL-1β-treated HUVECs was 25 to 30 times higher than the non-specific uptake by control cells (Fig. 5). In general, non-specific binding of Cy5.5-PGC2-F(ab′)$_2$ was 3–4 times higher than PGC1-based conjugate. If compared to the free ^{125}I-F(ab′)$_2$, the binding yield of

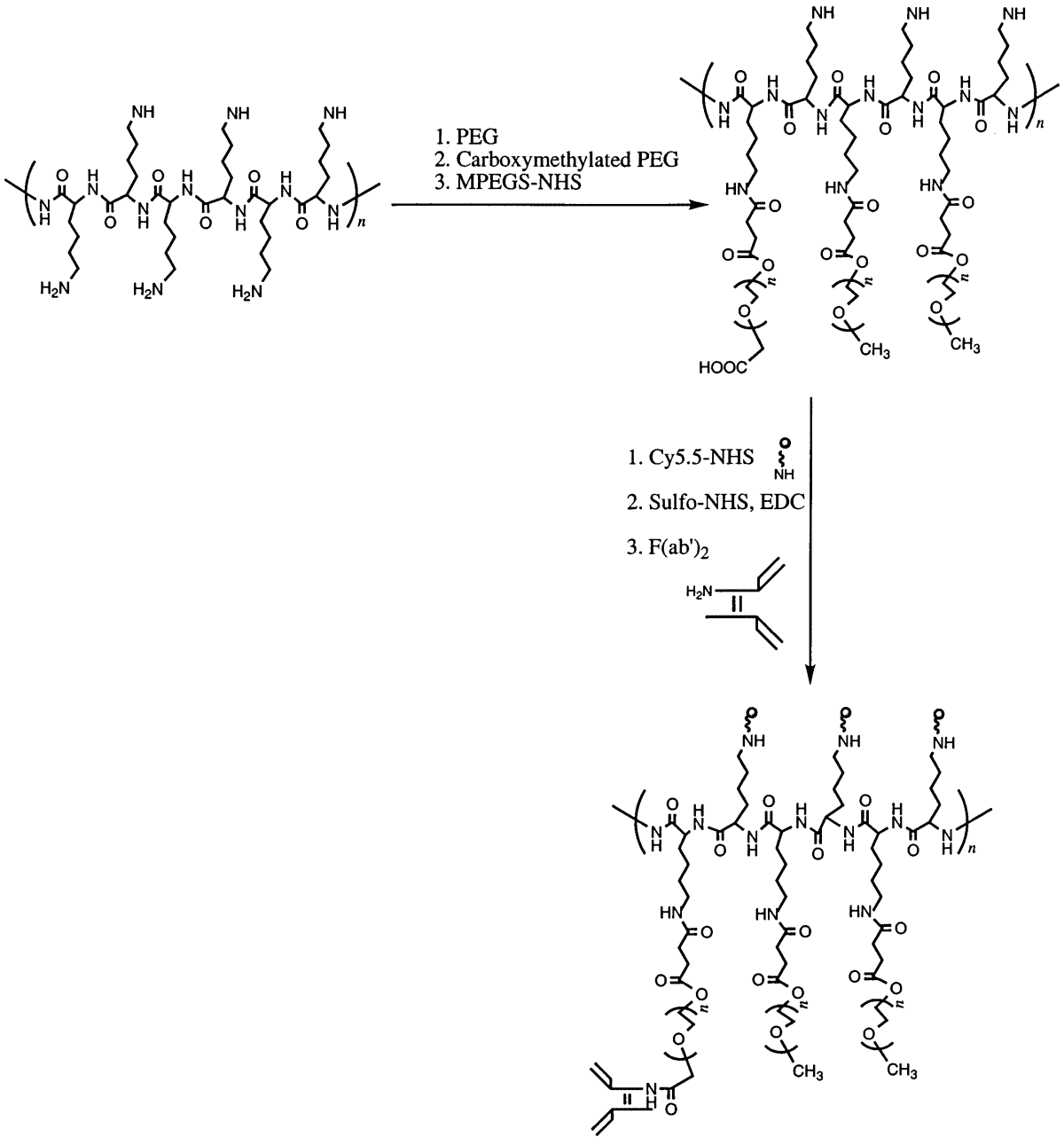

Fig. 2. A scheme of Cy5.5-PGC2-F(ab′)$_2$ synthesis. The covalent binding of antibody fragment is accomplished via activation of terminal PEG carboxyls in the presence of water-soluble carbodiimide and N-hydroxysulfosuccinimide

PGC-based conjugates was generally higher at both 5 μg and 20 μg antibody fragment added to the cells.

Fluorescence microscopy performed using IL-1β treated and control cells showed that the morphology of the distribution of Cy5.5-fluorescence in the case of the conjugates was very similar to that observed with the free Cy5.5-labeled F(ab′)$_2$ H18/7 antibody fragment (Fig. 6A). After 1 h incubation at 37°C we observed a characteristic staining of perinuclear intracellular vesicles only in IL-1β treated, but not in control cells. The morphology of cells incubated with Cy5.5-PGC1-F(ab′)$_2$ was nearly identical to that prepared using PGC2 graft co-polymer. The fluorescence of cells incubated with PGC2-based conjugate was generally lower than that of PGC1-based conjugate (Fig. 6 E,F). Control cells incubated with Cy5.5-F(ab′)$_2$ and fluorescent-labeled conjugates were non-fluorescent.

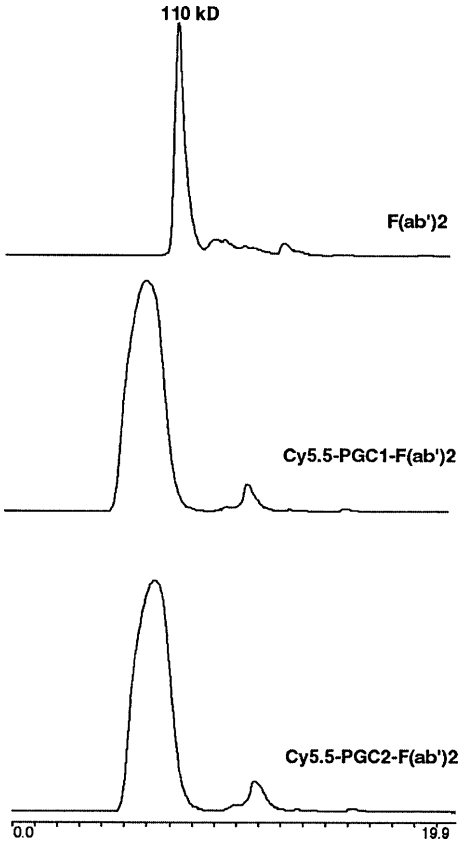

Fig. 3. Size-exclusion HPLC of F(ab′)$_2$; Cy5.5-PGC1-F(ab′)$_2$ and Cy5.5-PGC2-F(ab′)$_2$. The chromatography was performed by using SEC-5 column (Varian/Rainin Instruments Inc.) eluted with 0.05 sodium phosphate, pH 6.8. The peak F(ab′)$_2$corresponds to a 110 kD protein elution

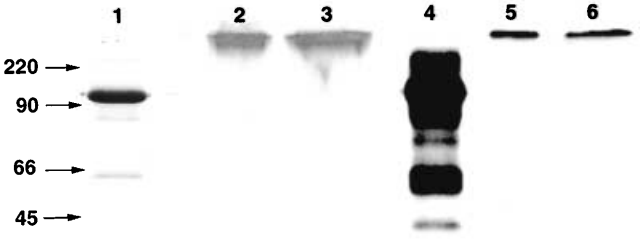

Fig. 4. Electrophoretic analysis of PGC-based conjugates prepared using ^{125}I-labeled F(ab′)$_2$. Coomassie staining – lanes 1–3; autoradiography – lanes 4–6. Lanes 1 and 4 – purified ^{125}I-labeled F(ab′)$_2$; Lanes 2 and 5 – Cy5.5-PGC1-F(ab′)$_2$; Lanes 3 and 6 – Cy5.5-PGC2-F(ab′)$_2$. Molecular mass marker migration is indicated on the left

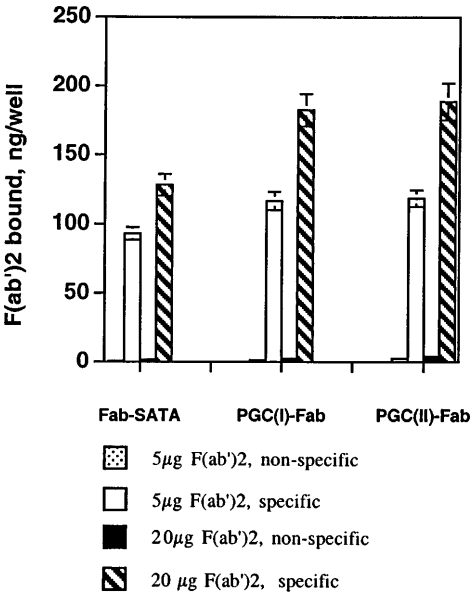

Fig. 5. Binding of ^{125}I-labeled F(ab′)$_2$ and the corresponding PGC-based conjugates to endothelial cell monolayers either non-treated (dotted and solid bars) or IL-1β-treated (open and hatched bars). The amount of added antibody fragment is indicated in the figure

Discussion

In vivo delivery of imaging agents to tumor endothelial cells is an enticing task due to the fact that non-invasive identification of proliferating vasculature is generally lacking. Among many endothelial markers associated with tumor angiogenesis, E-selectin, a proinflammatory marker (CD62E) is especially important since this endothelial cell surface marker expression is known to be upregulated in many tumors. E-selectin frequently co-localizes with dividing microvascular endothelial cells (Bischoff et al., 1997) as well as in tissues with active angiogenesis (Kraling et al., 1996; Nguyen, 1997). Previous research demonstrated that a monoclonal antibody to human E-selectin (H18/7) detects inducible E-selectin expression in human endothelial cells with high specificity (Bevilacqua et al., 1987). E-selectin targeted immunoliposomes and anti-E-selectin-hirudin conjugate bind to activated human endothelial cells in vitro at the levels exceeding 200-fold the levels measured in control cell culture (Kiely et al., 1995; Spragg et al., 1997). These findings suggest that H18/7 antibody or its fragments can potentially serve as targeting ligands for designing imaging probes targeted to cells expressing E-selectin. In current research we set forth to characterize and investigate cell-binding properties of covalent conjugates of H18/7 F(ab′)$_2$ fragments and co-polymers of polyamino acids and methoxy poly(ethylene glycol), MPEG. We anticipated that antibody-mediated targeted delivery would benefit from the following advantages of graft co-polymers protected by MPEG chains (protected graft co-polymers, PGC): 1) long circulation in the bloodstream (Bogdanov et al., 1993;

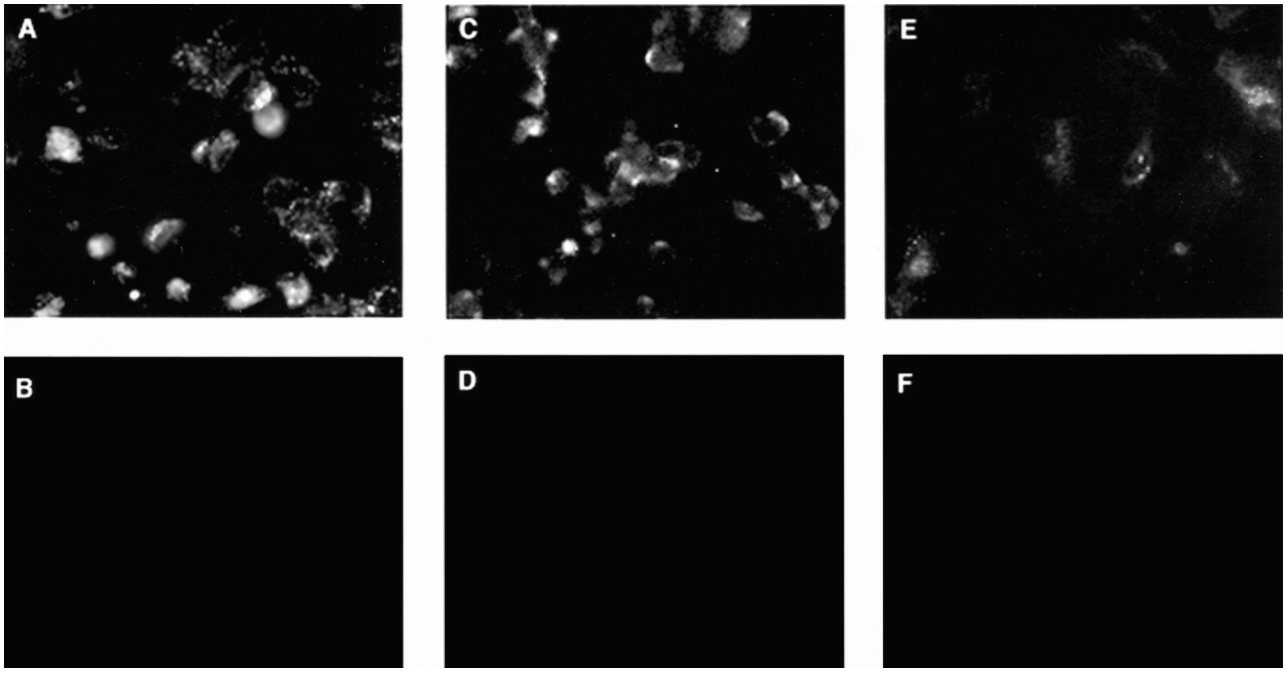

Fig. 6. Fluorescence microscopy of $F(ab')_2$ and corresponding PGC-based conjugate binding to the cells. **A,C,E**-cells activated with IL-1b; **B,D, F**-control cells. **A**,**B**-$F(ab')_2$; **C**,**D**-Cy5.5-PGC1-$F(ab')_2$; **E**,**F**-Cy5.5-PGC2-$F(ab')$

Weissleder et al., 1998); 2) high probe loading of graft co-polymer molecule (Weissleder et al., 1999); 3) the lack of immunogenicity and toxicity (Bogdanov et al., 1993); 4) the ability to cross "leaky" endothelial barriers in tumors (Marecos et al., 1998). We previously demonstrated that PGCs can be used as carriers of paramagnetic MR imaging labels (Bogdanov et al., 1993), chelated isotopes (Callahan et al., 1998) and fluorophores for optical imaging in vivo (Weissleder et al., 1999). In the latter case polyamino acid backbone of PGC can be used to provide molecular specificity to hydrolases associated with endocytic pathway. The specific hydrolysis of the polyaminoacid backbone results in a change of optical properties of PGC-associated fluorophore enabling imaging in vivo. We extended these studies the in current research by targeting PGC to E-selectin expressed specifically on the surface of IL-1β activated endothelial cells as a potential marker for tumor angiogenesis. The comparison of two chemical strategies of PGC conjugation to antibodies showed that at least in the case of H18/7 antibody fragment, there was no substantial difference in results obtained in cell culture. Both polycarboxylic PGC2 and vinylsulfonate-bearing PGC1 resulted in conjugates with a similar antibody/PGC ratios and apparent hydrodynamic radii (Table 1 and Fig. 3). However, it should be noted that the carbodiimide/sulfosuccinimide approach is mostly useful only if the drug or diagnostic label attached to PGC does not contain free carboxylic or amino groups. In experiments with stimulated cells the conjugates exhibited similar specificity towards E-selectin. For both PGC1 and PGC2-based conjugates the binding yield of the conjugate (expressed as a per cent of the total added antibody fragment) was almost 25% higher than for free $F(ab')_2$ indicating that conjugates with the higher $F(ab')_2$ content were preferentially bound to the cell surface due to the increased avidity effect. This effect is frequently observed for targeted drug carriers bearing several targeting molecules. Remarkably, the binding of both conjugates allowed the delivery of plurality of near-infrared fluorophore molecules into the activated cells (Fig. 6). In the case of a PGC2-based conjugate overall cell fluorescence was lower due to lower content of Cy5.5 in the polymer. Overall, our results suggest that targeting of optical imaging agents can be achieved in mixed cell culture populations and that these studies can be potentially performed in vivo. Imaging of activated endothelium would be instrumental in detecting cells expressing pre-inflammatory phenotype linked to tumor angiogenesis and atherosclesrosis.

In conclusion, both conjugation strategies used in our research can be potentially employed in the future

attempts to target E-selectin expression in vivo. The development of robust approach to optical imaging of individual cell populations would provide an alternative for diagnosis of disease manifested in early changes of vascular wall-lining cells.

Acknowledgement

The authors acknowledge the input made by Jeanne-Marie Kiely who provided H18/7antibody fragment, Kay Case for assistance with endothelial cell culture (Vascular Research Division, Department of Pathology, Brigham and Women's Hospital Boston MA) and Dr. Michael Gimbrone, Jr. for useful comments. This work has been supported in part by 1P50CA86355-01 (RW) and 5RO1 CA74424-01 (AB).

References

Anderson SA, Rader RK, Westlin WF, Null C, Jackson D, Lanza GM, Wickline SA, Kotyk JJ (2000) Magnetic resonance contrast enhancement of neovasculature with alpha(v)beta(3)-targeted nanoparticles. Magn Reson Med 44: 433–439

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA, Jr. (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 84: 9238–9242

Bischoff J, Brasel C, Kraling B, Vranovska K (1997) E-selectin is upregulated in proliferating endothelial cells in vitro. Microcirculation 4: 279–287

Bogdanov AA, Jr., Weissleder R, Frank HW, Bogdanova AV, Nossif N, Schaffer BK, Tsai E, Papisov MI, Brady TJ (1993) A new macromolecule as a contrast agent for MR angiography: preparation, properties, and animal studies. Radiology 187: 701–706

Bogdanov AA, Martin C, Bogdanova AV, Brady TJ, Weissleder R (1996) An adduct of cis-diamminedichloroplatinum(II) and poly(ethylene glycol)poly(L-lysine)-succinate: synthesis and cytotoxic properties. Bioconj Chem 7: 144–149

Brasch R, Pham C, Shames D, Roberts T, van Dijke K, van Bruggen N, Mann J, Ostrowitzki S, Melnyk O (1997) Assessing tumor angiogenesis using macromolecular MR imaging contrast media. J Magn Reson Imaging 7: 68–74

Bredow S, Lewin M, Hofmann B, Marecos E, Weissleder R (2000) Imaging of tumour neovasculature by targeting the TGF-b binding receptor endoglin. Eur J Cancer 36: 675–681

Callahan RJ, Bogdanov A, Jr., Fischman AJ, Brady TJ, Weissleder R (1998) Preclinical evaluation and phase I clinical trial of a 99mTc-labeled synthetic polymer used in blood pool imaging. Am J Roentgenol 171: 137–143

Duncan RJC, Weston P, Wrigglesworth R (1983) A new reagent which may be used to introduce sulfhydryl group into proteins, and its use in the preparation of counjugate for immunoasay. Analyt Biochem 132: 68–73

Gamble J, Meyer G, Noack L, Furze J, Matthias L, Kovach N, Harlant J, Vadas M (1999) B1 integrin activation inhibits in vitro tube formation: effects on cell migration, vacuole coalescence and lumen formation. Endothelium 7: 23–34

Gimbrone MA, Jr., Topper JN, Nagel T, Anderson KR, Garcia-Cardena G (2000) Endothelial dysfunction, hemodynamic forces, and atherogenesis. Ann NY Acad Sci 902: 230–239

Haubner R, Wester HJ, Weber WA, Mang C, Ziegler SI, Goodman SL, Senekowitsch-Schmidtke R, Kessler H, Schwaiger M (2001) Noninvasive imaging of alpha(v)beta3 integrin expression using 18F-labeled RGD-containing glycopeptide and positron emission tomography. Cancer Res 61: 1781–1785

Kiely JM, Cybulsky MI, Luscinskas FW, Gimbrone MA, Jr. (1995) Immunoselective targeting of an anti-thrombin agent to the surface of cytokine-activated vascular endothelial cells. Arteriosclerosis Thrombosis Vasc Biol 15: 1211–1218

Kraling BM, Razon MJ, Boon LM, Zurakowski D, Seachord C, Darveau RP, Mulliken JB, Corless CL, Bischoff J (1996) E-selectin is present in proliferating endothelial cells in human hemangiomas. Am J Pathol 148: 1181–1191

Mahmood U, Tung CH, Bogdanov A, Jr., Weissleder R (1999) Near-infrared optical imaging of protease activity for tumor detection. Radiology 213: 866–870

Marecos E, Weissleder R, Bogdanov A, Jr. (1998) Antibody-mediated versus nontargeted delivery in a human small cell lung carcinoma model. Bioconj Chem 9: 184–191

Mayer B, Spatz H, Funke I, Johnson JP, Schildberg FW (1998) De novo expression of the cell adhesion molecule E-selectin on gastric cancer endothelium. Langenbecks Arch Surg 383: 81–86

Neri D, Carnemolla B, Nissim A, Leprini A, Querze G, Balza E, Pini A, Tarli L, Halin C, Neri P, Zardi L, Winter G (1997) Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. Nature Biotechnol 15: 1271–1275

Nguyen M (1997) Angiogenic factors as tumor markers. Invest New Drugs 15: 29–37

Sipkins DA, Cheresh DA, Kazemi MR, Nevin LM, Bednarski MD, Li KC (1998) Detection of tumor angiogenesis in vivo by alphaVbeta3-targeted magnetic resonance imaging. Nature Med 4: 623–626

Spragg DD, Alford DR, Greferath R, Larsen CE, Lee KD, Gurtner GC, Cybulsky MI, Tosi PF, Nicolau C, Gimbrone MA, Jr. (1997) Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. Proc Natl Acad Sci USA 94: 8795–8800

Weissleder R, Cheng HC, Marecos E, Kwong K, Bogdanov A, Jr. (1998) Non-invasive in vivo mapping of tumour vascular and interstitial volume fractions. Eur J Cancer 34: 1448–1454

Weissleder R, Tung CH, Mahmood U, Bogdanov A, Jr. (1999) In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nature Biotechnol 17: 375–378

Zinn KR, Chaudhuri TR, Smyth CA, Wu Q, Liu HG, Fleck M, Mountz JD, Mountz JM (1999) Specific targeting of activated endothelium in rat adjuvant arthritis with a 99mTc-radiolabeled E-selectin-binding peptide. Arthritis Rheumatism 42: 641–649

Authors' address: Alexei Bogdanov, Jr, Center for Molecular Imaging Research, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Building 149, 13th Street, Charlestown, MA 02129, U.S.A., Fax: (617)726-5708, E-mail: abogdanov@helix.mgh.harvard.edu