Reduced Phagocytosis of Colloidal Carriers Using Soluble CD47

Yu-Chih Hsu,[1] Marcela Acuña,[2] Stanley M. Tahara,[2] and Ching-An Peng[1,3,4]

Received March 29, 2003; accepted June 12, 2003

Purpose. This study was designed to illustrate the feasibility of using soluble CD47 protein to antagonize phagocytosis of colloidal drug carriers by macrophages.

Methods. Expression of CD47-streptavidin (CD47-SA) fusion protein was achieved in B21CodonPlus host cells following IPTG induction. Murine macrophage cell line J774A.1, expressing high levels of SIRPα, was selected as the biologic model system for phagocytosis. FITC-labeled perfluorocarbon (PFC) emulsions were used as the colloidal carriers to trigger phagocytosis. Microscopy (inverted light and UV-fluorescence) and flow cytometry were used to qualitatively and quantitatively determine the degree of phagocytosis, respectively.

Results. The bacterially expressed, purified CD47-SA had neither cytotoxic nor cytostatic effects when incubated with J774A.1 cells up to a concentration of 400 nM for 24 h. Phagocytosis of FITC-labeled PFC emulsions was significantly diminished when macrophages were pretreated with 100 nM CD47-SA for 1 h.

Conclusions. We demonstrated that soluble CD47-SA antagonized phagocytosis of colloidal carriers to a significant degree by interaction with macrophage SIRPα.

KEY WORDS: CD47; colloidal drug carrier; perfluorocarbon emulsion; phagocytosis; SIRPα.

INTRODUCTION

Rapid clearance of intravenously administrated colloidal drug carriers (e.g., liposomes, polyplexes, and emulsions) by the mononuclear phagocyte system (MPS, including liver Kupffer cells and splenic red pulp macrophages) is a major obstacle to drug delivery (1). As a result, there have been several attempts to improve the circulatory persistence of colloidal drug carriers as therapeutic agents. Among these, PEGylation (coating with hydrophilic polyethylene glycol polymer) appears to be the most popular approach for modifying particulate surfaces to prolong circulatory lifetimes (2–5). Nevertheless, a better strategy for designing compatible colloidal carriers with increased circulatory persistence is to use a more physiologic basis for modification (1). Recently, integrin-associated protein (IAP or CD47) was demonstrated to function as a marker of self on murine erythrocytes (6). The absence of CD47 on these cells results in their rapid clearance from the bloodstream by splenic red pulp macrophages. This cell surface marker protects red cells from elimination by binding to a cognate receptor, SIRPα, on macrophage membranes. Based on this interesting finding, we hypothesized that CD47/SIRPα interaction may represent a feasible biomimetic approach for the design of persistent colloidal drug carriers. As a first step, we purified recombinant, soluble human CD47 and examined its potency in reducing ingestion of colloidal perfluorocarbon (PFC) emulsions by murine J774A.1 macrophages. Our results demonstrated that soluble CD47 consisting of only the extracellular domain with a conserved immunoglobulin (Ig) fold was able to significantly antagonize phagocytosis of PFC emulsions. It is conjectured that the antiphagocytic properties of CD47 will be enhanced if it is conjugated directly to colloidal drug carriers.

MATERIALS AND METHODS

Materials

Polyethylene glycol (avg. MW 1450; PEG_{1450}) was a generous gift from Union Carbide (Danbury, CT). Pentadecafluorooctanoyl chloride ($C_7F_{15}COCl$) was purchased from Aldrich (Milwaukee, WI), perfluorooctyl bromide (PFOB; perflubron) from Chem-surf (Sebastopol, CA), Talon resin (immobilized Co^{2+}) from Invitrogen (Palo Alto, CA), bacterial media components from Becton-Dickinson (Sparks, MD), Taq polymerase from Fisher Scientific, and restriction enzymes from New England Biolabs (Beverly, MA). All other chemicals and solvents not listed here were purchased from either Aldrich or Sigma (St. Louis, MO). All materials were used as received without further purification.

Methods

Synthesis of Perfluoroalkylated PEG Surfactant

PEG_{1450} was converted to diamino-PEG_{1450} as described (7,8), precipitated from diethyl ether, dried *in vacuo,* and reacted with pentadecafluorooctanoyl chloride to obtain the R_F-functionalized PEG surfactant (9). The final product, R_F-PEG_{1450}-NH_2 surfactant with amide-linked pentadecafluorooctanoyl (R_F) moiety, was precipitated from diethyl ether and dried *in vacuo.*

Fluorescein Isothiocyanate Labeling of R_F-PEG_{1450}-NH_2

Fluorescein isothiocyanate (FITC) was reacted with the amine-containing R_F-PEG_{1450}-NH_2 surfactant to create a stable isothiourea linkage. The procedure was based on a standard approach for protein labeling that involves reaction between the isothiocyanate group of FITC and the ε-amino groups of protein (10). The FITC-labeled fluorosurfactant was purified from unreacted FITC by repeated precipitation from hexane.

Emulsification of PFC

The FITC-labeled fluorosurfactant concentration used for making perflubron emulsions was 4% (w/v) in water. Both perflubron and surfactant solutions were filter-sterilized through 0.2-μm membrane filters before emulsification under aseptic conditions. Emulsification was achieved by homogenizing 1 mL of perflubron and 2 mL of FITC-labeled fluorosurfactant solution using a sonicator (550 W, 20 kHz; Misonix, Farmingdale, NY) for 3 min under a programmed cycle mode of 8 s on and 4 s off. Size distribution of PFC emulsion

[1] Department of Chemical Engineering, University of Southern California, Los Angeles, California 90089.
[2] Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California 90089.
[3] Department of Materials Science, University of Southern California, Los Angeles, California 90089.
[4] To whom correspondence should be addressed. (email: capeng@usc.edu)

was determined by laser light scattering (Mastersizer X-4700, Malvern Instruments, UK). Mean diameters were calculated from the volume-moment average of the particle size distribution.

Expression of Recombinant Soluble Human CD47-SA

Human CD47 cDNA (pIAP323) was obtained from Dr. Eric Brown (UCSF). The extracellular domain gene sequence corresponding to amino acids 17–142 (Ser^{17} to Thr replacement) was amplified with the following PCR primers: sense, GGCTCATGACAGCTCAACTACTGTTTAGT; antisense, GCGGGATCCTTTTCATTTGGAG. Core streptavidin (386 nucleotides, 129 amino acids) was PCR amplified from pSTE2-215 (yol) (11) and inserted between BamHI and XhoI sites of pET20b to give pMA005. The CD47 PCR product (378 nucleotides) was inserted between the NcoI and BamHI sites of pMA005 to yield pMA006. Expression of CD47-streptavidin (SA) fusion protein was achieved in BL21CodonPlus host cells following induction with 1 mM isopropylthiogalactoside (IPTG) for 1 h. The recombinant protein was purified from bacterial lysate by standard methods, and biotinyl agarose was used in the last step of purification.

Phagocytosis Assay

Murine macrophage cell line J774A.1 (ATCC) was used for *in vitro* phagocytosis assays. Cells (10^6) were inoculated into six-well culture dishes (BD Biosciences) with 4 mL of Dulbecco's modified Eagle's medium (DMEM, Irvine Scientific, Santa Ana, CA) supplemented with 7.5% fetal bovine serum (FBS, Gibco BRL, Rockville, MD), grown overnight at 37°C, and refed with 1 mL of complete medium before addition of recombinant soluble human CD47-SA fusion protein, as indicated. After incubation at 37°C for 1 h, 3 mL of unsupplemented medium containing 3 μL of FITC-labeled PFC emulsions was added. Cell incubation was continued at 37°C for 2 h as indicated. Emulsion phagocytosis by J774A.1 cells was determined by inverted visible light and fluorescence microscopy. The changes in cell morphology and presence of intracellular FITC-labeled PFC emulsion particles were documented by digital photography.

Assay of Phagocytosis by Flow Cytometry

The FACStar Plus flow cytometer (Dako-Cytomation Inc., Fort Collins, CO) was used to quantify phagocytic activity of J774A.1 cells following ingestion of FITC-labeled emulsion particles. Cells were harvested after FITC-labeled PFC emulsion challenge by gentle washing of the attached cells three times with 1 mL of phosphate-buffered saline (PBS) and detaching them by scraping them directly into 1 mL of ice-cold PBS. Cells were kept at 4°C until flow cytometry analysis. Cells were analyzed for intracellular fluorescence, and the fluorescent signal was gated through forward and 90-degree light-scattering detectors to eliminate autofluorescence signals from non–emulsion-containing macrophages and debris. A total of 5000 fluorescent particles (cells) of each gated population were analyzed. Fluorescence data were analyzed using the instrument manufacturer's software.

RESULTS AND DISCUSSION

Expression of CD47-Streptavidin Fusion Protein

The recombinant protein preparation was estimated to be >98% pure and had an observed molecular weight of 26,000 (see Fig. 1). As reported earlier, avoidance of phagocytosis can be attributed to ligation of CD47 to SIRPα receptors on macrophages (6), thus cells used for this study must have sufficient levels of the SIRPα receptor protein. Murine macrophage cell line J774A.1 was selected as the biologic model system for phagocytosis, and like most cell types of myeloid origin, this cell line is reported to express high levels of SIRPα (12). CD47-SA was incubated with J774A.1 cells for determination of any cytotoxic or cytostatic effects. We observed no deleterious effects of CD47-SA on J774A.1 viability or morphology up to the highest concentration tested (400 nM), based on the trypan blue exclusion method, 6, 12, and 24 h postaddition (data not shown).

FITC-Labeled PFC Emulsion

Colloidal particles used to assay phagocytosis by J774A.1 cells were perflubron-based oxygen carriers (so-called blood substitutes) that are currently in clinical trials (13). The surfactant used to prepare these perfluorocarbon (PFC) emulsions was a synthetic PEG-associated fluorosurfactant: $C_7F_{15}CONH\text{-}(CH_2CH_2O)_n\text{-}NH_2$ (designated as $R_F\text{-}PEG_{1450}\text{-}NH_2$). PFC emulsions prepared with this fluorosurfactant containing a low-molecular-weight PEG (MW 1450) triggered a higher ingestion rate in J774A.1 cells than another prepared with high-molecular-weight (MW 8000) PEG substitutions (9). The $R_F\text{-}PEG_{1450}\text{-}NH_2$ fluorosurfactant was further labeled with FITC for the purpose of quantifying phagocytosis

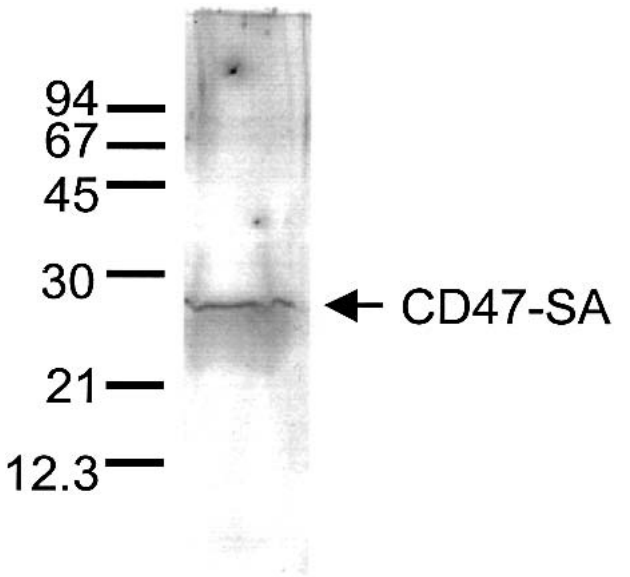

Fig. 1. Bacterially expressed CD47-streptavidin (SA) fusion protein. BL21CodonPlus bacterial strain transformed with pMA006 was used as source of CD47-SA. The protein was purified from bacterial lysate by biotinyl-agarose affinity chromatography. Approximately 100 ng of purified protein was analyzed by SDS-PAGE; the silver-stained gel is shown. Positions of molecular weight standards are indicated on left; position of CD47-SA is indicated by the arrow.

by J774A.1 cells. The size of FITC-labeled PFC emulsions ranged from 0.2 to 0.5 μm in diameter with a mean size of 0.34 ± 0.15 μm (mean ± SD; n = 3). The stability of PFC emulsion was verified by examining the average particle size in aqueous suspension over a period of 1 month. The effect of culture medium on the stability of PFC emulsions was also examined and determined to have no significant effect on particle size within the experimental period.

In control experiments, we determined that FITC covalently bound to fluorosurfactant did not significantly alter the rate or extent of phagocytosis of PFC emulsions by J774A.1 cells. Furthermore, photomicroscopic examination of J774A.1 cells containing ingested FITC-labeled PFC emulsions showed no notable changes in cell morphology compared to cells that had ingested non–FITC-labeled PFC emulsions (data not shown). Hence, the FITC-labeling technique developed here provides an alternative quantitative assay for phagocytosis in addition to the ^{19}F-NMR method described previously (9).

Antiphagocytic CD47-Streptavidin

In vitro phagocytosis of colloidal PFC microdroplets was examined in the presence and absence of soluble human CD47-SA. As shown in Fig. 2B, cells treated with PFC emulsions readily phagocytosed the extracellular emulsion particles. Brightfield microscopy showed extensive formation of PFC-containing vacuoles in the cytoplasm. This was confirmed by colocalization of green fluorescence to vacuoles containing FITC-labeled PFC emulsions (compare brightfield and UV views of Fig. 2B). In contrast, addition of soluble CD47-SA to cells (Fig. 2C) before challenge with PFC emulsions resulted in a visible reduction of PFC-laden vacuoles compared to untreated cells (Fig. 2B). To make sure the observed inhibition of phagocytosis was not the result of trace contamination by unreacted reagents or solvents, emulsified PFC particles were premixed with trace amount of chemicals used for CD47-SA purification before incubation with macrophages. In these control experiments, J774A.1 cells ingested PFC emulsions without any indication that phagocytosis was inhibited by the trace chemicals. Antagonism of phagocytosis was further examined by flow cytometry. As shown in Fig. 3, the mean fluorescence intensity of untreated cells vs. CD47-SA treated cells after FITC-labeled PFC emulsion challenge was reduced from 162.3 to 92.82; for comparison, background mean fluorescence intensity of untreated cells was 13.52. These quantitative results from flow cytometry corroborated the qualitative observations presented in Fig. 2.

The role of CD47/SIRPα interaction in the recognition of self and inhibition of phagocytosis has been illustrated by the observation that primary, wild-type mouse macrophages rapidly phagocytose CD47$^{-/-}$ RBCs (obtained from CD47$^{-/-}$ gene knockout mouse) but not wild-type (CD47$^{+/+}$) RBCs (6). It has also been reported that through its receptor, SIRPα, CD47 inhibits both $F_c\gamma$ and complement receptor-mediated phagocytosis (14). It has been observed that ligation of SIRPα by CD47 on macrophages results in cytoplasmic domain phosphorylation of tyrosine and recruitment of Src homology (SH) 2 domain-containing protein tyrosine phosphatase (SHP)-1, which is the major regulator of phagocytic responses (15). To be precise, the regulation of macrophage phagocytosis depends on the relative strength of phagocytic and CD47/SIRPα-generated signals. If the inhibitory signals generated by CD47/SIRPα interaction via the SHP-1 phosphatase are sufficient to counteract phagocytic signals mediated through $F_c\gamma$ and/or complement receptors, the process of ingesting colloidal particles will be reduced or inhibited. These experiments demonstrate that ligation of SIRPα by recombinant, soluble CD47 is sufficient to inhibit phagocytosis of colloidal PFC emulsions. We can only speculate as to the molecular events leading to interference of signal transduction events responsible for phagocytosis in this system.

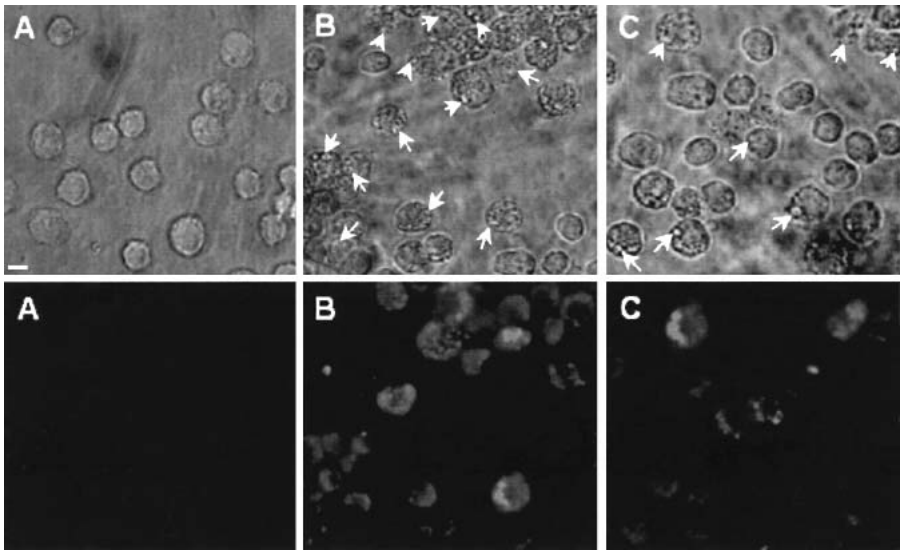

Fig. 2. Effect of CD47-SA on colloidal PFC particles taken up by J774A.1 cells. Examination of J774A.1 cell morphology and FITC-fluorescence after incubation with PFC emulsions (prepared with R_F-PEG_{1450}-NH_2 conjugated with FITC). (A) untreated cells; (B) cells incubated with 3 μl of PFC emulsions for 2 h; (C) cells pre-treated with 100 nM soluble human CD47-SA for 1 h then treated with PFC emulsions for 2 h. PFC-laden vacuoles are pointed out by the arrows. Bar = 10 μm.

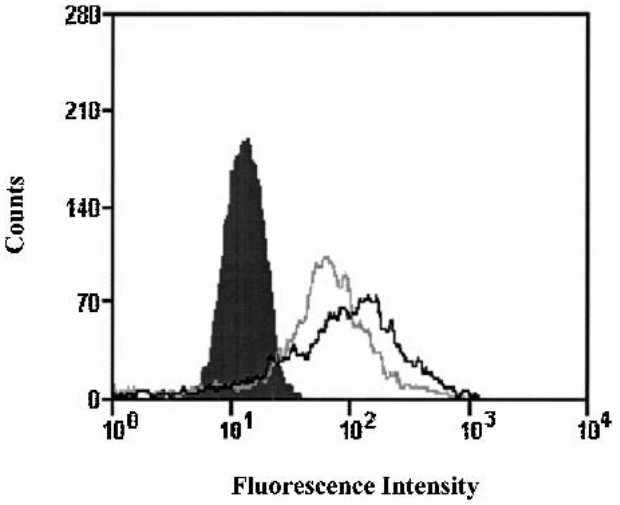

Fig. 3. FACS analysis of J774A.1 cell phagocytosis of FITC-labeled PFC emulsions. Cells were treated with PFC emulsions as described in legend to Fig. 2. The filled curve represents untreated cells with mean fluorescence intensity (MFI) of 13.52. The light-colored curve represents cells pretreated with CD47-SA for 1 h before cultivation with PFC emulsions; the MFI = 92.82. The dark-colored curve represents cells without CD47-SA pretreatment for 1 h before culturing with PFC emulsions; the MFI = 162.3. This set of overlaid FITC histograms represents one of the triplicate data sets.

Nonetheless, this result is important because synthetic materials containing this biomimetic ligand may impart a similar antiphagocytic property.

We observed that the activity of soluble CD47-SA is short-lived in this *in vitro* system. The efficacy of soluble CD47-SA in deterring phagocytosis via the inhibitory signals triggered by the CD47/SIRPα interaction appeared to last for only 2 h. After this period, cells regained their phagocytic capability. Why are the predominant inhibitory signals diminished after 2 h? We reasoned that this outcome could be attributed to (a) instability of CD47-SA in the culture medium or (b) cobinding of CD47-SA to $\alpha_v\beta_3$ integrins on J774A.1 cells. The latter would have the effect of decreasing the pharmacologic concentration of the protein by depleting the protein from the culture medium. Furthermore, we have no information regarding the rate of receptor turnover and *de novo* synthesis as a route for SIRPα (or $\alpha_v\beta_3$) replenishment at the cell membrane. From this discussion, we expect that the biologic activity of CD47 may be prolonged if it is immobilized on the surface of colloidal carriers. This can be done simply by binding CD47-SA to biotinylated PFC emulsions (via biotinylated R_F-PEG_{1450}-NH_2) or other chemical approaches to crosslink CD47 to the surface of colloidal particles.

In conclusion, we demonstrated that soluble CD47-SA antagonized phagocytosis of colloidal carriers to a significant degree by interaction with macrophage SIRPα. We surmise that the methods described here offer alternative ways to design and fabricate stable parenteral, colloidal drug carriers endowed with low phagocytic uptake.

ACKNOWLEDGMENTS

The authors thank Jeff Louie for assistance in the flow cytometry-based experiments. This work was supported by grants from the National Science Foundation (BES 0003006) and the Charles Lee Powell Foundation to C.A.P, and the Kahrs Foundation to S.M.T.

REFERENCES

1. S. M. Moghimi, A. C. Hunter, and J. C. Murray. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* **53**:283–318 (2001).
2. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS* **268**:235–237 (1990).
3. G. Storm, S. O. Belliot, T. Daemen, and D. D. Lasic. Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system. *Adv. Drug Deliv. Rev.* **17**:31–48 (1995).
4. W. T. Collard, Y. Yang, K. Y. Kwok, Y. Park, and K. G. Rice. Biodistribution, metabolism, and *in vivo* gene expression of low molecular weight glycopeptide polyethylene glycol peptide DNA co-condensates. *J. Pharm. Sci.* **89**:499–512 (2000).
5. F. Liu and D. Liu. Long-circulating emulsions (oil-in-water) as carriers for lipophilic drugs. *Pharm. Res.* **12**:1060–1064 (1995).
6. P.-A. Oldenborg, A. Zheleznyak, Y.-F. Fang, C. F. Lagenaur, H. D. Gresham, and F. P. Lindberg. Role of CD47 as a marker of self on red blood cells. *Science* **288**:2051–2054 (2000).
7. A. F. Bückmann, M. Morr, and G. Johansson. Functionalization of poly(ethylene glycol) and monomethoxy-poly(ethylene glycol). *Makromol. Chem.* **182**:1379–1384 (1981).
8. S. Zalipsky, C. Gilon, and A. Zilkha. Attachment of drugs to polyethylene glycols. *Eur. Polym. J.* **19**:1177–1183 (1983).
9. Y.-C. Hsu and C.-A. Peng. Diminution of phagocytosed perfluorocarbon emulsions using perfluoroalkylated polyethylene glycol surfactant. *Biochem. Biophys. Res. Commun.* **283**:776–781 (2001).
10. A. Jobbagy and K. Kiraly. Chemical characterization of fluorescein isothiocyanate-protein conjugates. *Biochim. Biophys. Acta* **124**:166–175 (1966).
11. S. Dubel, F. Breitling, R. Kontermann, T. Schmidt, A. Skerra, and M. Little. Bifunctional and multimeric complexes of streptavidin fused to single chain antibodies (scFv). *J. Imunol. Methods* **178**:201–209 (1995).
12. A. Veillette, E. Thibaudeau, and S. Latour. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. *J. Biol. Chem.* **273**: 22719–22728 (1998).
13. R. M. Winslow. Blood substitutes. *Curr. Opin. Hematol.* **9**:146–151 (2002).
14. P.-A. Oldenborg, H. D. Gresham, and F. P. Lindberg. CD47-signal regulatory protein α (SIRPα) regulates Fcγ and complement receptor-mediated phagocytosis. *J. Exp. Med.* **193**:855–861 (2001).
15. H. D. Gresham, B. M. Dale, J. W. Potter, P. W. Chang, C. M. Vines, C. A. Lowell, C. F. Lagenaur, and C. L. Willman. Negative regulation of phagocytosis in murine macrophages by the Src kinase family member, Fgr. *J. Exp. Med* **191**:515–528 (2000).