Protein Precoating of Polylactide Microspheres Containing a Lipophilic Immunopotentiator for Enhancement of Macrophage Phagocytosis and Activation

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Biodegradable microspheres containing a lipophilic muramyl dipeptide, MDP-B30, were prepared from a 1-lactic acid-glycolic acid copolymer. The effect of precoating the microspheres with watersoluble polymers including proteins on the antitumor activity of mouse peritoneal macrophages (Mφ) was investigated. Macrophages activated by phagocytosis of the microspheres exhibited growth inhibitory activity toward Meth-A tumor cells. The activity correlated with the extent of Mφ phagocytosis of the microspheres. Mφ phagocytosis was greatly augmented by gelatin precoating of the microspheres, resulting in a significant increase of in vitro antitumor activity of Mφ by the microspheres. However, potentiation of Mφ activity by gelatin precoating was minimal after intraperitoneal injection of the microspheres, but cross-linking of the coated gelatin with glutaraldehyde afforded potentiation of the antitumor activity in vivo.

KEY WORDS: polylactide microspheres; macrophage; phagocytosis; antitumor activation; gelatin; immunopotentiator.

INTRODUCTION

Macrophages (Mφ) activated by various immunopotentiators are able to recognize and destroy neoplastic cells, while leaving nonneoplastic cells unharmed (1,2). However, one of the major problems associated with therapeutic trials using immunopotentiators is serious side effects of the high-dosage regimens required for significant therapeutic efficacy. Drug delivery systems of the immunopotentiators for targeting to the site of action may overcome this limitation. We have been studying biodegradable polylactide microspheres as a sustained-release vesicle for delivering a lipophilic immunopotentiator, MDP-B30, to Mφ. After being phagocytosed by Mφ, the microspheres are degraded in the Mφ interior, resulting in the slow release of MDP-B30 in the cells. MDP-B30 incorporated in the microspheres is more efficient in enhancing the inhibitory activity of Mφ against tumor-cell growth than free MDP-B30 under in vitro and in vivo conditions (3).

A preliminary study has revealed that Mφ phagocytosis of polymer microspheres is enhanced by surface precoating with some opsonic proteins such as immunoglobulin, fibronectin, and gelatin, in contrast to the reduction of phagocytosis by surface coating with various water-soluble polymers without opsonic ability (4).

The present work was undertaken to study in more detail the effect of surface precoating of microspheres with various polymers on Mφ phagocytosis and the resulting tumor growth inhibitory activity of Mφ. We also describe the in situ Mφ activation by intraperitoneal injection of the precoated microspheres.

MATERIALS AND METHODS

Culture Media and Reagents

Culture medium (RPMI-FCS) was prepared by supplementing RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Japan) with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, Md.), 5 mM L-glutamine, and penicillin (100 units/ml) and buffered with 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and NaHCO₃ at pH 7.0. Phosphate-buffered saline (PBS) solution was obtained from Nissui Seiyaku Co., Ltd., Tokyo. Lipopolysaccharide (LPS; Escherichia coli 0111:B4) was obtained from Difco Laboratories, Detroit, Mich. The immunopotentiator used here, 6,6-o-(2-tetradecyl-hexadecanoyl)-MDP (MDP-B30), was kindly supplied by Daiichi Seiyaku Co., Ltd., Tokyo, and the preparations were free of endotoxins as determined by the Limulus amoebocyte lysate assay. Proteins employed here were bovine serum albumin (BSA) (Seikagaku Kogyo Co., Ltd., Tokyo), bovine immunoglobulin (IgG) (Cohn fraction II, Sigma Chemical Co., St. Louis, Mo.), tuftsin (Cambridge Research Biochemicals, Ltd., Harston, England), gelatin (Nitta Gelatine Co., Ltd., Osaka, Japan), and human plasma fibronectin (FN) isolated from frozen human plasma by affinity chromatography with a gelatin-Sepharose column (5).

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Water-soluble polymers are poly(vinyl alcohol) (PVA; degree of polymerization, 1000; saponification value, 87.69%; Unitika Kasei Ltd., Osaka, Japan), carboxymethyl cellulose (CMC; F-SH, 1%; viscosity, 3500 cps; degree of saponification, 0.6; Daiichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan), poly(acrylamide) (PAAm; Daiichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan), dextran (weight-average molecular weight, 200,000; Nakarai Chemicals, Ltd., Kyoto, Japan), and poly(vinylpyrrolidone) (PVP; weight-average molecular weight, 24,500; Nakarai Chemicals, Ltd., Kyoto, Japan). L-lactic acid (90% aqueous solution) and glycolic acid (70% aqueous solution) were purchased from C.V. Chemie Combinatie, Amsterdam, The Netherlands, and Dupont Chemical Co., Ltd., respectively, and used as obtained. Other reagents of guaranteed grade were purchased from Nakarai Chemicals, Ltd., Kyoto, Japan.

Preparation of Microspheres Containing MDP-B30

The L-lactic acid and glycolic acid copolymer (PGLA) used here was synthesized by copolycondensation of L-lactic acid and glycolic acid at an equivalent weight ratio for 5 hr at 180°C under reduced pressure from 760 to 100 mm Hg. No catalyst and diluent were employed for copolymerization. The polymerization product was purified by repeated precipitation into methanol from the chloroform solution for removal of the monomers. The monomer conversion to PGLA copolymer was 50%. The weight-average molecular weight and the molar composition of the copolymer were 2900 and 43/57 (L-lactic acid/glycolic acid), respectively (3). Repeatability of the polymer synthesis was satisfactory.

Microspheres were prepared by the solvent evaporation method (3). One milliliter of methylene chloride solution containing 20 mg of PGLA and 0.4 mg of MDP-B30 was added to 10 ml of 2% PVA aqueous solution, followed by ultrasonic emulsification at 64 W for 2 min. The resulting emulsion was agitated continuously at 30°C until the complete evaporation of the methylene chloride. The microspheres were then washed with cold distilled water four times by centrifugation at 5000 rpm for 5 min. No appreciable dissolution of the copolymer in water was observed during washing of the microspheres with water. The washed microspheres were lyophilized and stored at 4°C until use. The yield of the microspheres was 23%, and the amount of MDP-B30 included was 1.8 ± 0.2 µg/mg microsphere as determined by the modified Levvy and McAllan method (6). The low content of the drug may be due to partial solution of the drug into the PVA solution during emulsification of the microspheres.

The surface of microspheres containing MDP-B30 was modified by physical adsorption of proteins or other water-soluble polymers, such as BSA, IgG, transferrin, gelatin, and FN, or PVA, CMC, PAAm, dextran, and PVP. Five-tenths milligram of the microspheres was placed in 1 ml of PBS solution, containing the polymers to be coated, for 1 hr at 37°C, and then 0.1 ml of the resulting suspension was added to 0.9 ml of culture medium for in vitro assays. The concentrations of polymer solutions were in the range of 1.5 × 10^-10 to 1.5 µg/ml. Cross-linking of gelatin adsorbed onto the microsphere surface to reduce the water solubility was conducted with 0.23% glutaraldehyde in PBS for several minutes up to 60 min at 4°C.

Phagocytosis Assay

Mφ preparation and phagocytosis assay were performed according to the method reported previously (4). The adherent cells collected from the peritoneal cavity of BALB/c mice 4 days after intraperitoneal injection of thioglycollate broth were used as mouse peritoneal Mφ. More than 98% of the cells had morphologic and phagocytic properties of Mφ.

For Mφ phagocytosis, 1 ml of RPMI-FCS containing 50 µg of microspheres precoated with 1.5 mg/ml of polymer solutions was added to each 16-mm dish of 24-well multidish culture plates (A/S Nunc, Kampstrup, Roskilde, Denmark) with a round cover-glass slip on which 2 × 10^9 Mφ had been adhered. After incubation for 6 hr, the slips were washed with RPMI-1640 medium, fixed with 2.5% glutaraldehyde in the medium, and embedded with glycerin jelly. The average number of the microspheres taken up by one Mφ was estimated by phase-contrast microscopy for 400 cells. Experiments were independently performed three times for each microsphere.

In vitro Mφ Activation by PGLA Microspheres

In vitro Mφ activation was estimated according to the method reported previously (3). Mφ (2 × 10^5) in 1 ml of RPMI-FCS per dish were pretreated for 24 hr at 37°C with PGLA microspheres precoated with 1.5 mg/ml of various proteins or non precoated. In all cases, the dose of the microspheres was 50 µg/2 × 10^5 cells, which was below the toxic level to Mφ. Mφ cultures were run thoroughly with RPMI-1640 medium to remove non phagocytosed microspheres before the addition of tumor cells.

Inhibitory Activity of Mφ on Tumor-Cell Growth

Meth-A cells, methylcholangthrene-induced fibrosarcoma cells of BALB/c mice, originally established by Dr. Uno (7) were used for assessing the effect of Mφ on tumor-cell growth. Assays were always performed on cells in the exponential growth phase. Tumor cells (1 × 10^6) in 1 ml of RPMI-FCS were added to the Mφ monolayers prepared as described above. Under these conditions, untreated Mφ exerted no inhibitory effect on tumor growth. The number of viable tumor cells was counted after culture for 48 hr at 37°C in a 5% CO2-95% air atmosphere. The growth inhibitory activity of Mφ toward Meth-A cells was evaluated according to the following formula (7): percentage growth inhibition = [(No. of tumor cells cultured with untreated Mφ) - (No. of tumor cells cultured with activated Mφ)]/(No. of tumor cells cultured with untreated Mφ) × 100.

In Situ Mφ Activation by PGLA Microspheres

Mice were intraperitoneally injected with 0.5 ml of PBS containing 200 µg of free MDP-B30, 1.4 mg of empty microspheres, or 1.4 mg of microspheres containing 2.5 µg of MDP-B30. Gelatin-coated microspheres with or without cross-linking were also injected to estimate the effect of gelatin coating on in situ Mφ activation. Six, 24, and 48 hr after injection, Mφ were harvested from peritoneal cavities to assess the inhibition activity on tumor-cell growth as described above.