In Vitro Cytotoxicity, Chemotactic Effect, and Cellular Uptake of Branched Polypeptides with Poly[L-Lys] Backbone by J774 Murine Macrophage Cell Line

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Branched polypeptides with polylysine backbone are promising candidates for selective delivery of drugs, epitopes. or reporter molecules. We reported earlier that polylysine-based polypeptides with polyanionic character were internalized by murine bone marrow derived macrophages via class A scavenger receptor. In the present studies, our investigations were extended to seven polypeptides with different amino acid composition and charge properties. We report on our findings on the concentration-dependent influence of these compounds on survival and chemotaxis of the murine macrophage-like cell line J774 and internalization properties of the polypeptides by J774 cells. Our observations indicate that the polypeptides regardless of their charge properties were essentially nontoxic and did not alter significantly the chemotaxis of J774 cells; therefore, the polypeptides suit the requirements for nontoxic and "neutral" carrier molecules. We also demonstrated that the polypeptides were internalized efficiently by J774 cells, depending on their chemical structure and charge properties. Using the scavenger receptor–ligand fucoidan as inhibitor, we established that the scavenger receptor played a role–in accordance with findings on murine bone marrow derived macrophages in the internalization only of the polyanionic polypeptides.

INTRODUCTION

Most of the compounds recently applied therapeutically for tumors and infectious diseases are small molecules that enter the cells via diffusion. Coupling of drugs to macromolecular carriers would not only improve their solubility but also could decrease side effects and offers a new possibility of cell- or tissue-specific introduction (*1*). The conjugation of these agents alters the mechanism of their internalization, because the larger conjugates cannot penetrate the cell membrane by diffusion but are internalized by a more specific active transport mechanism, e.g., macropinocytosis or receptor-mediated endocytosis, depending on the properties of the carrier (*2, 3*). Synthetic polymers have been widely used as carriers of bioactive compounds, epitopes, or reporter molecules. Most of the polymeric carriers like the nonbiodegradable HPMA (*N*-(2-hydroxypropyl)methacrylamide copolymer) (*4, 5*) and DIVEMA (divinyl ether−maleic anhydride copolymer) (*6*) or biodegradable polymeric carriers like poly(α-amino acids), poly[Lys], poly[Glu], or poly[Tyr]) (*7–9*), do not possess a specific targeting moiety. In our laboratory, several groups of poly(L-lysine)-based branched polypeptides were developed and applied as macromolecular carriers of drugs and epitopes (*3*). The first group of the polymeric polypeptides studied in the present work can be described with the general formula of poly[Lys(X_i)][1]; X_iK, possesses a single amino acid coupled to the ϵ-amino groups of the lysine residues. The other group of polypeptides, poly[Lys(DL-Ala$_m$)] (*10*), contain a short oligo[DL-alanine] side chain or an additional amino acid coupled to the α-amino group of the terminal alanines with the general formula poly[Lys(X_i-DL-Ala$_m$)], XAK, where $i \cong 1$ and $m \cong 4$ (*11*). *In vitro* and *in vivo* physiological properties of poly[L-lysine]-based polymeric polypeptides like *in vitro* and *in vivo* cytotoxicity (*12, 13*), biodistribution (*14, 15*), blood clearance (*13*), immunogenicity (*16*), and immunomodulatory effect (*17, 18*); chemotaxis (*19*) and class-A scavenger receptor (SR-A)-mediated cellular uptake have been investigated systematically over the past few years (*20*). Physicochemical properties of the polypeptides as interaction with phosphoplipid bilayers were also examined recently (*21, 22*). Our previous data confirmed that amino acid composition and length of the side chains and charge of the terminal amino acid greatly influence the biological properties of the polypeptides and their drug conjugates, e.g., with daunomycin (*23, 24*) and methotrexate (*25*). We have also demonstrated that polylysine-based polypeptides with polyanionic character (SuccEAK and

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[1] Abbreviations: Abbreviations of amino acids and their derivatives follow the revised recommendation of the IUPAC-IUB Committee on Biochemical Nomenclature entitled "Nomenclature and Symbolism for Amino Acids and Peptides" (recommendations of 1983). Nomenclature of branched chain polypeptides is used in accordance with the recommended nomenclature of graft polypeptides (*26*). For the sake of brevity codes of branched chain polypeptides were constructed by us using the one-letter codes of amino acids. All amino acids are L configuration unless otherwise stated. The other abbreviations in this paper are the following: Pcp, pentachlorophenyl; HOBt, 1-hydroxybezotriazole; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethyl-formamide; TEA, triethylamine; PBS, phosphate-buffered saline; Ac, acetyl; Succ, succinyl; Mal, maleyl; CF, 5(6)-carboxyfluorescein.

10.1021/bc7004544 CCC: $40.75
Published on Web 04/23/2008

MalEAK) enter murine bone marrow derived macrophages via SR-A-mediated endocytosis, and not only the charge density but also the chemical structure of the anionic end group have an influence on the efficacy of the internalization (*20*). In this article, we describe our findings on the effect of seven structurally related polypeptides with different characteristics according to their amino acid composition and charge properties on survival and chemotaxis of the J774 murine macrophage cell line. In order to determine the correlation between efficient cellular uptake and chemical structure of polypeptide carriers, we investigated the internalization of the polypeptides in several aspects. In order to find the carrier that can be most efficiently internalized by macrophages, we carried out the comparative study of the polypeptides with different charge properties on the macrophage cell line J774. We also wanted to clarify whether the scavenger receptor is involved in the uptake of the polyanionic polypeptides, using the scavenger receptor–ligand fucoidan as inhibitor. Our results indicate that the polypeptides elicited only moderate toxicity on J774 cells except the control polypeptide poly(L-lysine). They did not alter significantly the chemotaxis of the cells either. We demonstrated that the polypeptides were internalized efficiently by J774 cells depending on their chemical structure and charge properties. Taken together, the polypeptides suit the requirements for nontoxic and nonchemotactic macromolecular carriers. We established that the scavenger receptor played a role only in the internalization of two polyanionic polypeptides, poly[Lys(Succ-Glu$_{1.0}$-DL-Ala$_{3.8}$)], SuccEAK, and poly[Lys(Mal-Glu$_{1.0}$-DL-Ala$_{3.8}$)], MalEAK; thus, these polypeptides can be promising candidates as macromolecular carriers in drug conjugates applied for cell-specific delivery into macrophages.

MATERIALS AND METHODS

Reagents. Amino acids used for this study, HCl, acetic anhydride, succinic anhydride, and DMF were purchased from REANAL (Budapest, Hungary). Pentachlorophenol, 1-hydroxybenzotriazole, triethylamine, and DMSO were from Fluka (Buchs, Switzerland). Acetonitrile was from Merck (Darmstadt, Germany). Fucoidan from *Fucus vesiculosus* and 5(6)-carboxyfluorescein succinimide ester were obtained from Sigma-Aldrich Hungary Ltd. (Budapest, Hungary).

Synthesis of Poly[Lys(X$_i$)], X$_i$K and Poly[Lys(X$_i$-DL-Ala$_m$)], XAK-Type Polypeptides. Poly[Lys(X$_i$)] (X$_i$K) where X = Pro and XAK-type polypeptides where X = Ser or Glu were prepared according to Mezö et al. (*27*). Briefly, poly[Lys(DL-Ala$_m$)], AK, was synthesized by grafting short oligomeric DL-Ala chains to the $^{\epsilon}NH_2$ group of lysine residues using *N*-carboxy-DL-Ala-anhydride. Polymerization was continued at room temperature for 2 days. Terminal amino acids (X) were coupled to the $^{\alpha}NH_2$ groups of the terminal amino acid of the oligoalanine chains, where X = Glu or Ser. In the case of X$_i$K polypeptides, terminal amino acid was coupled to the $^{\epsilon}NH_2$-groups of polylysine as Z-X-OPcp, where X = Pro. The coupling reaction was carried out by *in situ* active ester method using an equivalent amount of 1-hydroxybenzotriazole (HOBt) dissolved in DMF as activating reagent (*28*). The polypeptides were purified by dialysis against distilled water for two days using a Visking tube (cutoff = 10000 Da) and isolated by freeze-drying.

Synthesis of Polyanionic Derivatives of Poly[Lys(Glu$_i$-DL-Ala$_m$)], (EAK). Synthesis of polypeptides of polyanionic character was performed via modification of EAK polypeptide as described previously (*20, 29*). Briefly, acetylation was carried out in DMF/water = 9:1 solvent mixture using 5 equiv freshly prepared imidazolyl acetate (*30*). Succinylation and maleylation were performed in 0.1 M carbonate buffer (pH 9.2) using 0.022 g (0.22 mmol) succinic anhydride or 0.02 g (0.2 mmol) maleic anhydride dissolved in DMSO (c = 100 mg/mL). The reaction was continued for 4 h at RT, followed by dialysis against distilled water for 2 days and then freeze−drying.

Amino Acid Analysis. Amino acid composition of the polymeric polypeptides was determined by amino acid analysis using a Beckman 6300 analyzer (Fullerton, USA) after hydrolysis in 6 M HCl in sealed and evacuated tubes at 105 °C for 24 h.

Labeling of Polymeric Polypeptides with 5(6)-Carboxyfluorescein. Ten milligrams (6×10^{-7} to 1.9×10^{-7} mol) polypeptides was dissolved in 2 mL sodium carbonate buffer (0.1 M; pH 9.4). 5(6)-Carboxyfluorescein succinimide ester was dissolved in DMF (c = 10 mg/mL, 0.21 mM) and was added to the polypeptide solution in 20 μL aliquots. The reaction was continued for 1 h at room temperature with continuous stirring. The product was purified by gel filtration on a PD10 column filled with Sephadex G25 using distilled water as eluent. The samples were then freeze-dried, and they were resolved in PBS (0.1 M, pH 7.4) before use. Carboxyfluorescein content of the samples was determined by RP-HPLC as described earlier (*20*).

Cell Culturing. J774 murine macrophage cell line of Balb/c origin was maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, and 0.16 mg/mL streptomycin at 37 °C in 5% CO_2 atmosphere. Cells were harvested in the logarithmic phase of growth.

Cytotoxicity of the Polypeptides on J774 Cells. Cells were divided into 96 well tissue-culture plates in 200 μL culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, and 0.16 mg/mL streptomycin) with initial cell number of 10^5 cells/well. The polypeptides were dissolved in fresh culture medium and were added to the cells at final concentrations of 0.2, 2.0, 20, and 200 μg/mL. After 1 and 24 h incubation at 37 °C, cell viability was determined by MTT assay using 0.37 mg/mL final concentration of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in each well. After 3 h incubation, the absorbance was measured with an ELISA reader (Labsystems MS Reader) at 540 and 620 nm as reference wavelengths. Statistical analysis of data was performed by ANOVA of *Origin 7.5*.

Chemotaxis of J774 Cells. Chemotactic ability of compounds was analyzed in a 96 well NeuroProbe chamber (96 well Boyden chamber) in the 0.02–20 μg/mL concentration range. A polycarbonate filter with 5 μm pore diameter was placed between the inner chambers containing the polypeptide solution and the outer chambers, in which J774 cells were placed (10^5 cells/well). The chamber was incubated at 37 °C for 3 h. The amount of the viable cells was determined by MTT assay using 24 h incubation with MTT. Statistical analysis was performed by ANOVA of *Origin 7.5*.

Uptake of Polypeptides by Nonattached Cells. Cells were plated on 24 well tissue culture plate (10^5 cells/well) 24 h prior to the experiment. Cells were incubated with 5(6)-CF-labeled polypeptides at 0.02, 0.2, 2, and 20 μg/mL for 60 min concentration or at 20 μg/mL for 1, 15, 30, 45, and 60 min. Extracellular fluorescence was quenched using 0.5% (m/V) trypane blue solution. Cells were washed twice with PBS (0.1 M; pH 7.4) then fixed with 4% (m/V) formaldehyde dissolved in PBS (0.1 M; pH 7.4). Change of fluorescence of the cells was determined by flow cytometry (FACSCalibur, Becton Dickinson, or BD LSR II) measuring 5000 or 10 000 cells. Data were analyzed with *CellQuest Pro* or *FACS DiVa* software. The mean fluorescence of untreated control cells was subtracted from the mean fluorescence of treated samples under all assay conditions applied. Statistical analysis of data was performed using Student's t test at the 95% confidence level.

Uptake of Polypeptides by Adherent Cells. Cells were plated into 24 well tissue culture plate (10^5 cells/well) 24 h

Table 1. Characteristics of Polylysine-Based Branched Chain Polymeric Polypeptides with Poly[Lys(X_i-DL-Ala_m)] Formula

		amino acid composition[b]					
polypeptide	code[a]	Lys	Ala (m)	X (i)	DP_n[c]	M_w[d] ± 5%	CF [%][e]
poly[Lys]	–	1.00	–	–	130	16700	–
poly[Lys(Pro_i)]	P_iK	1.00	–	0.95	90	24800	18.6
poly[Lys(DL-Ala_m)]	AK	1.00	3.80	–	60	23800	1.55
poly[Lys(Ser_i-DL-Ala_m)]	SAK	1.00	3.80	0.90	60	27400	2.4
poly[Lys(Glu_i-DL-Ala_m)]	EAK	1.00	3.80	1.00	60	31600	1.4
poly[Lys(Ac-Glu_i-DL-Ala_m)]	AcEAK	1.00	3.80	1.00	60	34500	2.6
poly[Lys(Succ-Glu_i-DL-Ala_m)]	SuccEAK	1.00	3.80	1.00	60	37900	1.6
poly[Lys(Succ-Glu_i-DL-Ala_m)]	MalEAK	1.00	3.80	1.00	60	37800	0.72

[a] Code of branched chain polymeric polypeptides, based on one-letter symbol of amino acids. K represents lysine, A represents alanine, and X is a proteinogenic amino acid in the side chain; m characterizes the average length of the oligo-DL-alanine side chains ($m \cong 4$), whereas i describes the average degree of substitution of amino acid X ($i \cong 1$) (*26*). [b] Amino acid composition was determined by amino acid analysis after hydrolysis in 6 M HCl at 105 °C for 24 h. [c] Average degree of polymerization determined by sedimentation equilibrium measurements. [d] Average molecular mass of polymers; calculated from the average degree of polymerization (DP_n) of poly[Lys] and of the side chain composition. [e] Average degree of substitution for 5(6)-carboxyfluorescein (CF) was determined by reverse-phase HPLC after hydrolysis of the samples in 6 M HCl at 105 °C for 24 h.

before treating with the polypeptides. Before the addition of CF-labeled polypeptides, cells were washed with serum-free medium and then incubated with the solution of the CF-labeled polypeptides in serum-free RPMI 1640 for 1 h at 37 or 4 °C. In case of inhibition, cells were pretreated with fucoidan dissolved in RPMI 1640 at 20 μg/mL concentration for 30 min. The presence of the inhibitor was maintained throughout the experiment (*30*). After 1 h incubation with the polypeptides at $c = 20$ μg/mL, cells were washed twice with RPMI. Cells were washed 2 times with PBS (0.1 M; pH 7.4) and then fixed with 4% (m/V) formaldehyde/PBS (0.1 M; pH 7.4). Cells were examined by flow cytometry (FACSCalibur and LSR II, Becton Dickinson). Data were recorded measuring 5000–10 000 cells, and fluorescence mean values were calculated. Statistical analysis of data was performed using Student's t test at the 95% confidence level.

Study of Uptake by Confocal Microscopy. For the microscopic studies, cells were mounted onto microscopic coverslips and placed into a 24 well tissue culture plate and allowed to attach for 24 h. Cells were treated with the CF-labeled polypeptides in serum-free RPMI 1640 medium for 1 h. Then, cells were fixed with 4% (m/V) formaldehyde/PBS (0.1 M; pH 7.4) and the coverslips were placed onto microscopic slides. The mounted cells were analyzed in a BIO RAD MRC 1024 confocal scanning laser microscope equipped with a krypton/argon mixed-gas laser as a light source. Excitation was carried out at $\lambda = 480$ nm.

RESULTS

Branched Chain Polypeptides. Branched polypeptides with the general formula poly[Lys(X_i)] or poly[Lys(X_i-DL-Ala_m)], ($m \cong 4$, $i \cong 1$) were used for these studies. Poly[Lys($Pro_{0.95}$)] (P_iK) and poly[Lys(DL-$Ala_{3.8}$)] (AK) and XAK type polypeptide poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)] (SAK) possessed polycationic character. Poly[Lys($Glu_{1.0}$-DL-$Ala_{3.8}$)] (EAK), an amphoteric polypeptide carrying free α-amino and γ-carboxyl groups, and its three polyanionic derivatives, in which the $^{\alpha}NH_2$ groups of the terminal glutamic acid residues were modified by acetyl, succinyl, and maleyl groups, were also examined. The three polyanionic polypeptides possess slightly different charge properties. Poly[Lys(Ac-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (AcEAK) carries one negative charge per monomer unit, whereas poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (SuccEAK) and (poly[Lys-(Mal-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (MalEAK) polypeptides contain two negative charges in their each monomer unit. The polypeptides were characterized with their amino acid composition and their relative average molar mass, determined by sedimentation equilibrium analysis. Chemical characteristics of the polypeptides are shown in Table 1.

Cytotoxicity of Polylysine-Based Polypeptides on J774 Cells. Cytotoxicity of the polypeptides was tested on J774 cells at the concentration range 0.2−200 μg/mL by MTT assay using polylysine as a control. Time dependence of the cytotoxic effect was also examined. Results are expressed as a percentage of optical density measured in the case of the untreated cells. Cytotoxicity proved to be dependent on the charge of the *N*-terminal amino acid of the side chains and on the distance of the charged group from the polylysine backbone. We found that after a 1 h incubation only the control polypeptide (poly[Lys]) was toxic (cytotoxicity = 88 ± 0.14% at 200 μg/mL, $LC_{50} = 65.7$ μg/mL). After the 24 h treatment, two other polycationic polypeptides like poly[Lys($Pro_{0.95}$)] (P_iK) (cytotoxicity = 54.6 ± 12% at c = 200 μg/mL, LC_{50} = 153.65 μg/mL), poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)] (SAK) (cytotoxicity = 15.2 ± 9% at c = 200 μg/mL) and also polyanionic poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (SuccEAK) exhibited a moderate cytotoxicity (38.4 ± 1.4% at c = 200 μg/mL) at the highest concentration studied (Figure 1).

Chemotaxis of J774 Cells. Chemotaxis of the polypeptides was studied using a 96 well NeuroProbe chamber at 0.02, 0.2, 2.0, and 20 μg/mL concentration. The amount of the viable migrated cells was determined by MTT assay. Two polycationic polypeptides, poly[Lys($Pro_{0.95}$)] (P_iK) and poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)] (SAK) induced a slight but significant chemotactic response on J774 cells, in both in an optimal concentration, which is characteristic of each polypeptide. Poly[Lys($Pro_{0.95}$)] (P_iK) elicited a repellent effect at $c = 20$ μg/mL, whereas SAK proved to be slightly chemoattractant at $c = 0.2$ μg/mL and 2.0 μg/mL (Table 2).

Uptake of Carboxyfluorescein-Labeled Polypeptides by J774 Cells. The internalization properties of polylysine-based polypeptides were studied on J774 cells under different conditions. The influence of polypeptide concentration, incubation time, and temperature was investigated on the uptake of CF-labeled polypeptides. We also studied the involvement of the scavenger receptor in the internalization process in inhibition assays.

Uptake of the Polypeptides by Nonattached Cells. The uptake of the polypeptides by nonattached J774 cells was examined using flow cytometry and confocal microscopy as well. The polycationic polypeptide (poly[Lys($Pro_{0.95}$)] (P_iK) possessing short side chains was taken up already at 2 μg/mL concentration. The uptake of poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)] (SAK) with polycationic character was significant only at the highest concentration studied, whereas the presence of amphoteric EAK (poly[Lys-($Glu_{1.0}$-DL-$Ala_{3.8}$)]) was not detectable in the cells. The polycationic AK (poly[Lys(DL-$Ala_{3.8}$)]) and P_iK ((poly[Lys($Pro_{0.95}$)]) were taken up the most efficiently. As poly[Lys(DL-$Ala_{3.8}$)] (AK)

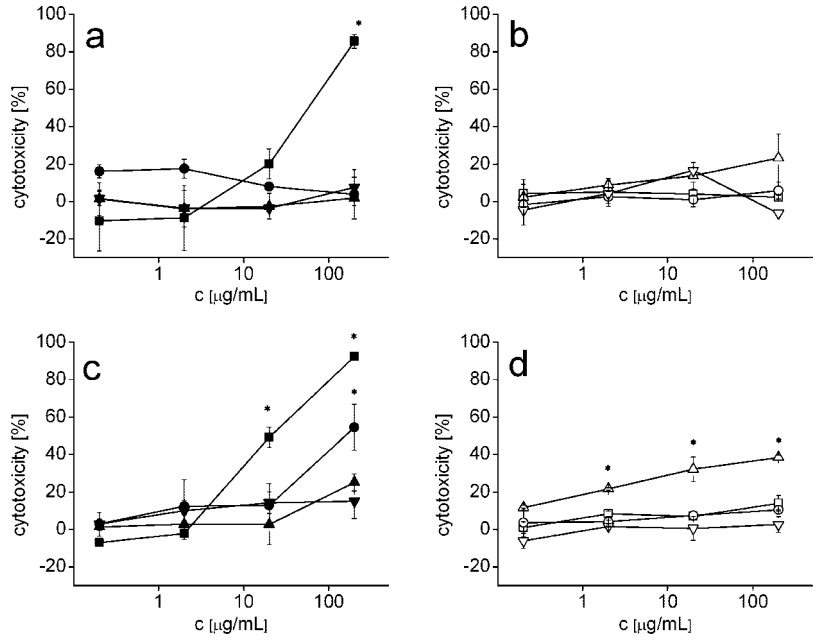

Figure 1. The effect of the branched CF polypeptides on the viability of J774 cells. Graphs a and b represent the 1 h treatment; c and d represent the 24 h treatment. Diagrams represent the average cytotoxicity of the polypeptides as a percentage of untreated control ± SD. Poly[L-Lys] ($\bar{DP}_n$ = 130, $\bar{M}_w$ = 16 800) was used as control. Statistical analysis of data was performed by Student's *t* test. (a) Polycationic polypeptides (■ poly[Lys], ● P_iK, ▲ AK, ▼ SAK); (b) amphoteric (□ EAK) and polyanionic polypeptides (O AcEAK, Δ SuccEAK, ∇ MalEAK).

Table 2. Chemotactic Effect of Polylysine Based Polypeptides on J774 Cells[a]

concn [μg/mL]	P_iK	AK	SAK	EAK	AcEAK	SuccEAK	MalEAK
0.02	108 ± 5	106 ± 1	131 ± 27	104 ± 4	101 ± 8	116 ± 7	82 ± 11
0.2	95 ± 1	98 ± 1	*113 ± 6	110 ± 4	101 ± 1	105 ± 6	85 ± 8
2	91 ± 8	101 ± 6	*115 ± 2	114 ± 9	109 ± 11	105 ± 9	105 ± 11
20	*86 ± 4	104 ± 15	118 ± 9	102 ± 4	99 ± 5	97 ± 15	85 ± 18

[a] Chemotactic ability of the cells was analyzed in a 96 well NeuroProbe chamber. The number of migrated cells was determined by MTT assay. Chemotactic index was expressed as percentage of control ± SD. Data represent the mean of 8 parallels. Statistical analysis of data was performed by one-way ANOVA of *Origin5* (*$p < 0.05$).

was one of the polypeptides that could be detected the best in the cells, time dependence of the uptake of this polypeptide was also examined by confocal microscopy (Figure 2). In the microscopic images, the binding of the CF-labeled AK (poly[Lys-(DL-$Ala_{3.8}$)]) polypeptide to the cell surface could be observed even after 1 min incubation. The vesicles containing the labeled polypeptide appear within the cells after 30 min incubation, and the number of the vesicles increased after 60 min incubation time (Figure 2).

Uptake of the Polypeptides by Adherent Cells. The rate of the uptake was influenced by the charge and the polypeptide concentration. Uptake of polycationic polypeptides and poly[Lys-(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (SuccEAK) was dependent on the polypeptide concentration, whereas SAK (poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)]), EAK (poly[Lys($Glu_{1.0}$-DL-$Ala_{3.8}$)]), and AcEAK (poly-[Lys(Ac-$Glu_{1.0}$-DL-$Ala_{3.8}$)]) were ingested only at the highest concentration applied (c = 20 μg/mL) (Figure 3). The kinetics of the uptake was examined by flow cytometry after 0, 5, 15, 30, 45, and 60 min incubation with the polypeptides. The internalization of the polyanionic polypeptides by J774 cells was in direct proportion to the incubation period, except (poly[Lys($Pro_{0.95}$)] (P_iK), where the kinetics of the uptake was exponential, and we could observe a saturation kinetics in the case of poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)] (SAK) polypeptide (Figure 4). An increased uptake of the polycationic AK, poly[Lys(DL-$Ala_{3.8}$)], the polyanionic SuccEAK (poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)]), and MalEAK (poly[Lys(Mal-$Glu_{1.0}$-DL-$Ala_{3.8}$)]) could be observed after attachment of the cells to the surface (Figure 5). Images taken by confocal microscopy also confirmed that CF-SuccEAK, poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] was ingested by the adherent cells at a higher level compared to nonadherent cells (Figure 5). By contrast, polycationic polypeptides like P_iK poly[Lys($Pro_{0.95}$)]), SAK (poly[Lys($Ser_{0.9}$-DL-$Ala_{3.8}$)]), AK (poly-[Lys(DL-$Ala_{3.8}$)]) were ingested at a higher level by the nonadherent J774 cells (Figure 6). The uptake of the polypeptides proved to be dependent also on temperature. By analyzing data obtained for two different temperatures, we detected a significant decrease in the internalization at 4 °C compared to 37 °C (Figure 7).

Effect of SR-Antagonist Fucoidan on the Uptake of the Polypeptides. We examined the role of the scavenger receptor in the internalization of the polypeptides; therefore, the uptake was inhibited with a SR ligand, the sulfated polysaccharide fucoidan. We found that 30 min pretreatment ($c_{fucoidan}$ = 20 μg/mL) significantly inhibited the uptake of polyanionic polypeptides, SuccEAK (poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)]) and MalEAK (poly[Lys(Mal-$Glu_{1.0}$-DL-$Ala_{3.8}$)]) but did not alter the uptake of polyanionic AcEAK (poly[Lys(Ac-$Glu_{1.0}$-DL-$Ala_{3.8}$)]) and polycationic AK (poly[Lys(DL-$Ala_{3.8}$)]). These results indicate that scavenger receptors play a role in the internalization of the two polyanionic polypeptides that possess two negative charges per monomer unit (Figure 8).

DISCUSSION

Recently, studies on cell-specific introduction of drugs is one of the most actively developing areas of drug delivery research. Numerous antitumor and antiparasitic agents as methotrexate or anthracyclines entering the cell membrane by diffusion are toxic not only to the target cells but also to the other cell types

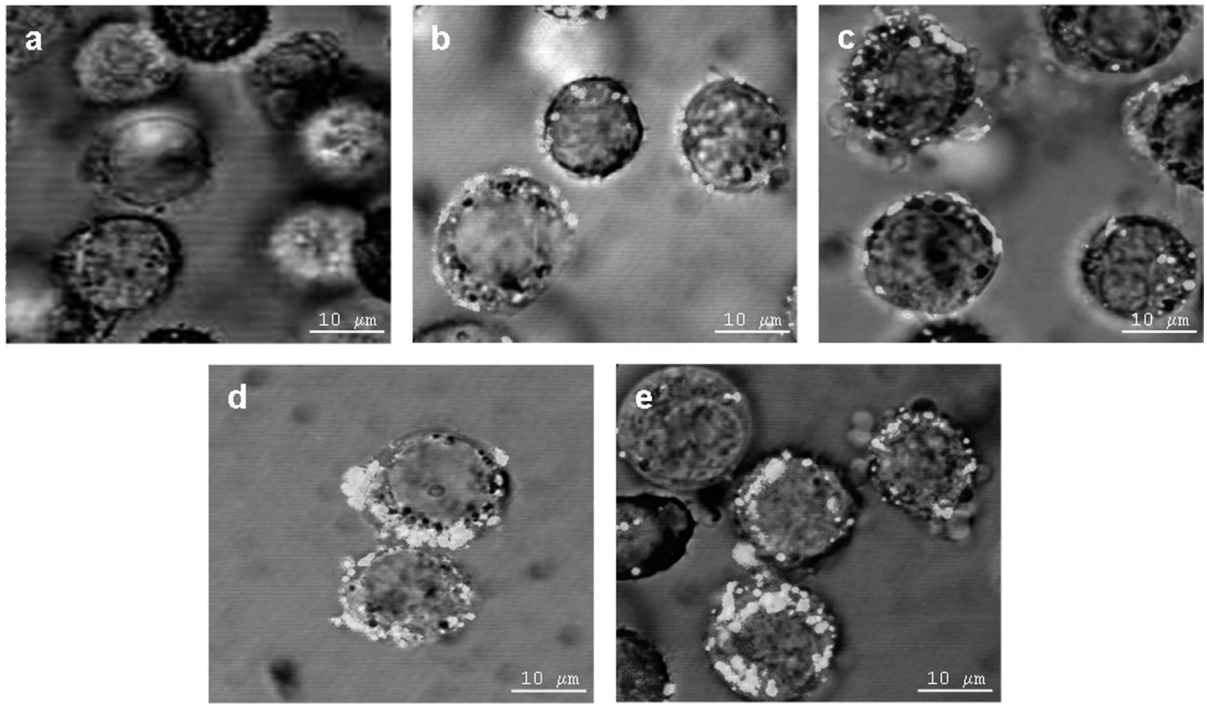

Figure 2. Time dependence of the endocytosis of CF-poly[Lys(DL-$Ala_{3,8}$)] (AK) polypeptide by nonattached J774 cells. The cells were analyzed by confocal microscopy (λ_{ex} = 488 nm): (a) untreated control, (b) CF-AK 1 min, (c) CF-AK 15 min, (d) CF-AK 30 min, (e) CF-AK 60 min.

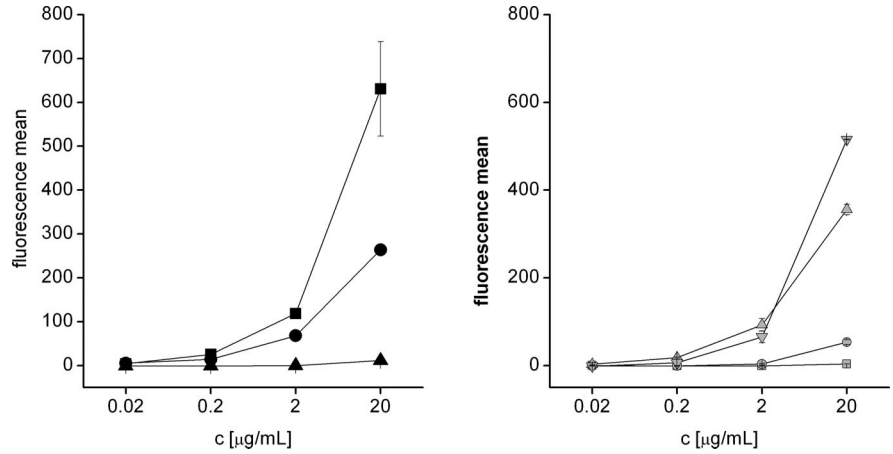

Figure 3. Concentration dependence of the uptake of the CF-polypeptides by J774 cells after 60 min incubation with the polypeptides. Symbols represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. (a) Polycationic polypeptides (■ P_iK, ● AK, ▲ SAK); (b) amphoteric (□ EAK) and polyanionic polypeptides (○ AcEAK, Δ SuccEAK, ∇ MalEAK).

of the organism (*31*). On the other hand, small hydrophilic molecules like peptides, oligonucleotides, and small proteins with low membrane permeability cannot reach the site of action (*32*). These problems can be solved by applying macromolecular carriers that can decrease the toxicity and improve the specificity of drugs or can deliver hydrophilic compounds through the cell membrane. The use of macromolecular carriers can also break multidrug resistance (*33*) and enhance the immunogenicity of the original compound (*34*). There are some important requirements for the ideal macromolecular carrier. It must not be toxic or immunogenic; it should possess reactive groups that can be modified by a simple chemical reaction; the carrier has to retain its specificity after conjugation and must not alter the efficacy of the active compound coupled to it (*35*).

J774 cells have been used for several cell physiological studies related to macrophages. Their inflammatory response (*36*), signal transduction (*37*), killing activity, and phagocytosis (*37–39*) and chemotactic activity (*40*) have been investigated in detail. Claus et al. characterized the activity of their endosomal enzymes (*41*). Maturation of phagosomes (*42*) and the effect of antimycobacterial agents (*43*) were also studied on *Mycobacterium*-infected J774 cells. J774 cells were used for modeling macrophages in several investigations on cell-specific delivery of active compounds via scavenger receptors (*44–46*).

The aim of our studies was to examine the effect of a group of systematically developed chemically related polypeptides on the macrophage like cell line J774. Our previous studies indicated that the chemical structure of the polypeptides, especially the charge and length of the side chains, influenced markedly the biological effects and physicochemical characteristics of the polypeptides (*12, 17*). Our present results show that charge and structure of the side chains both have a marked influence on *in vitro* toxicity and uptake of polylysine based

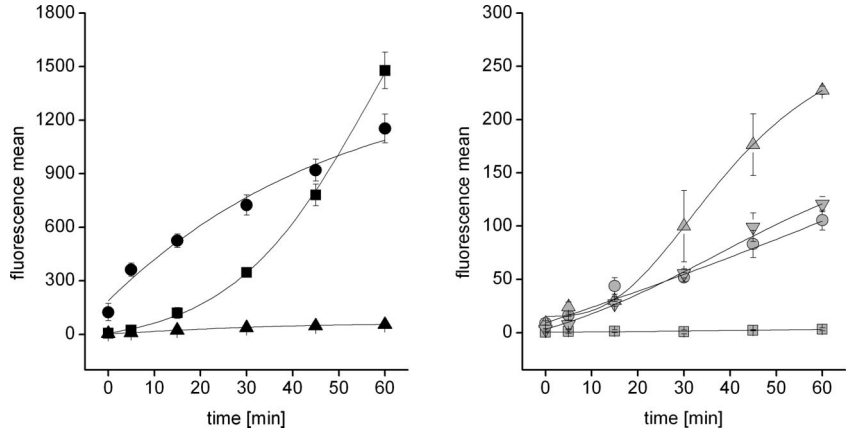

Figure 4. Time dependence of the endocytosis of polypeptides at $c = 20\ \mu$g/mL. Symbols represent the average of mean fluorescence values of a representative experiment of three independent assays ± SD after subtracting the control. (a) Polycationic polypeptides (■ P_iK, ● AK, ▲ SAK); (b) amphoteric (□ EAK) and polyanionic polypeptides (○ AcEAK, Δ SuccEAK, ∇ MalEAK).

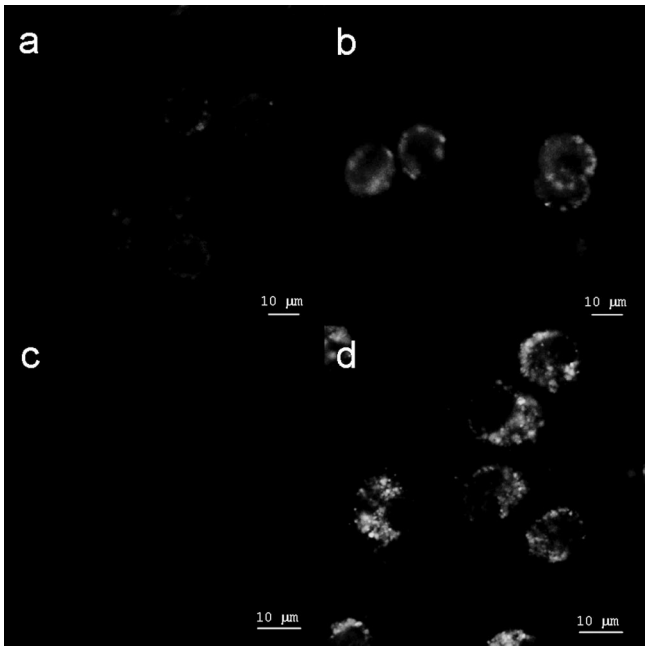

Figure 5. Internalization of CF-poly[Lys(Succ-Glu$_{1.0}$-DL-Ala$_{3.8}$)] (CF-SuccEAK) polypeptide at $c = 20\ \mu$g/mL by nonadherent (a) untreated, (b) CF-SuccEAK treated, and adherent (c) untreated, (d) CF-SuccEAK treated J774 cells at 60 min. Cells were analyzed by confocal microscopy with $\lambda_{ex} = 488$ nm.

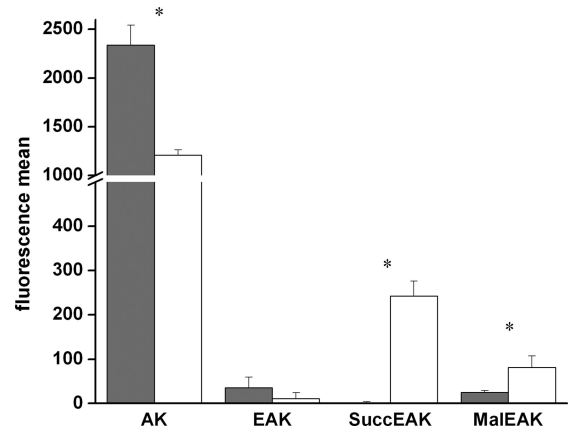

Figure 6. Uptake of polypeptides by nonattached and adherent J774 cells. Columns represent the average of mean fluorescence values of a representative of two independent assays ± SD after subtracting the control. ■ nonattached cells; □ adherent cells. Statistical analysis of data was performed by Student's *t* test; $*p < 0.05$.

polypeptides, whereas the structure of the terminal amino acid derivative could determine the chemotactic character of the molecule.

We determined the cytotoxicity of the branched-chain polypeptides after 1 and 24 h. We found that the branched chain polypeptides elicited moderate cytotoxicity at the highest concentration in contrast with control poly[Lys] containing free ε-amino groups that induced a marked cell death after 1 and 24 h incubation as well. We also observed a difference between the polyanionic polypeptides. AcEAK (poly[Lys(Ac-Glu$_{1.0}$-DL-Ala$_{3.8}$)]) and MalEAK poly[Lys(Mal-Glu$_{1.0}$-DL-Ala$_{3.8}$)] elicited a markedly lower toxicity on J774 cells than SuccEAK poly-[Lys(Succ-Glu$_{0.9}$-DL-Ala$_{3.8}$)]. Taken together, cytotoxicity of the polypeptides proved to be dependent on the charge of the *N*-terminal amino acid of the side chains, and in the case of polyanionic polypeptides, on the structure of the anionic end group.

Various molecules like bacterial peptides (e.g., fMLF) (*47*), chemokines, nucleotides (ADP), and molecules of the complement system (C5a) (*40*) can activate the chemotaxis of monocytes and macrophage-like cells. We would apply carriers that are able to elicit a chemotactic response and draw the cells to the target cell population, but these molecules usually induce an immune response as well. To avoid immunogenicity, it is advisable to choose a neutral carrier that does not induce chemotaxis but rather contains a nonchemotactic targeting moiety. Our previous studies on *Tetrahymena pyriformis* (*19*) demonstrated that the character of the terminal amino acid of the branches determined the chemotactic activity of the model organism. Compared to *Tetrahymena*, chemotactic activity of J774 cells was markedly lower. In summary, the polypeptides did not influence significantly the chemotaxis of the J774 cells, so they can be applied as neutral, nonchemotactic macromolecular carriers of drug targeting with respect to chemotactic drug targeting, a special type of drug delivery described recently (*48*).

Results of the internalization studies obtained by flow cytometry and confocal laser scanning microscopy illustrated that J774 cells ingested polypeptides depending on the polypeptide concentration, the incubation time, and the temperature. The charge of the side chains influenced markedly the rate and kinetics of the uptake. We observed a difference between the polypeptide internalization of nonadherent and adherent cells. Floating J774 cells internalized polycationic polypeptides more effectively than polyanionic ones, whereas the uptake of polyanionic polypeptides was significantly increased by the adherent cells. As adherent cells express certain receptors that

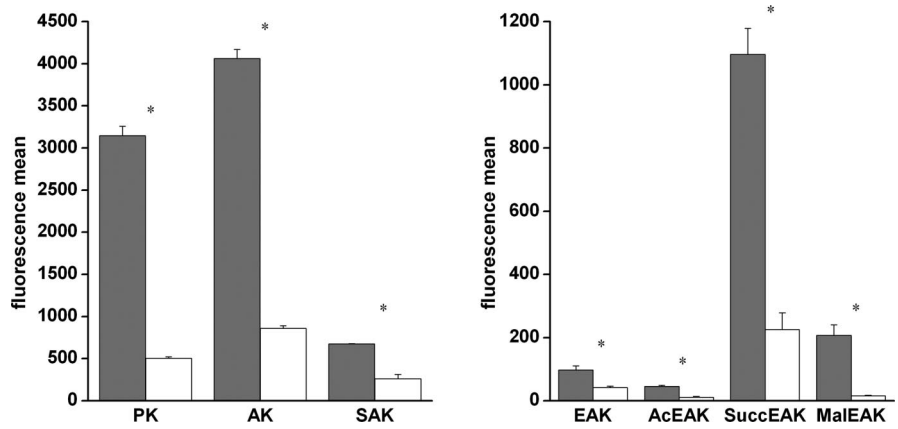

Figure 7. Uptake of polypeptides at 4 and 37 °C at $c = 20$ μg/mL after 1 h. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. ■ 37 °C; □ 4 °C. Statistical analysis of data was performed by Student's t test; *$p < 0.05$.

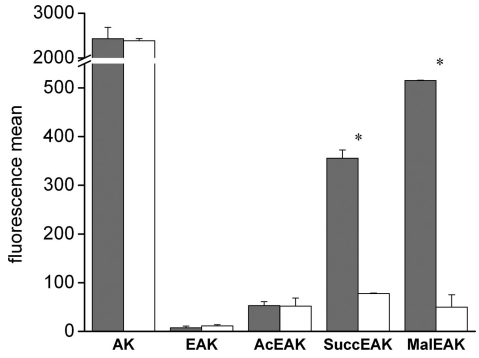

Figure 8. Uptake of the polypeptides in the presence of SR ligand fucoidan applied as inhibitor. Columns represent the average of mean fluorescence values of a representative experiment of two independent assays ± SD after subtracting the control. ■, Uptake of the polypeptides ($c = 20$ μg/mL) without pretreatment; □, uptake of polypeptide after pretreatment with fucoidan ($c = 20$ μg/mL). Statistical analysis of data was performed by Student's t test; *$p < 0.05$.

play a role in the internalization process (e.g., scavenger receptors) to a greater extent than nonadherent cells (*49*), the more effective uptake of polyanionic polypeptides (SuccEAK and MalEAK) imply a receptor-dependent uptake mechanism. Examination of the internalization by confocal microscopy seems to confirm that uptake of the polypeptides occurs by endocytosis: the polypeptides were ingested by the cells in vesicles can be observed in the microscopic images well.

In order to clarify whether a scavenger receptor is involved in the internalization of the polypeptides, we carried out inhibition experiments using a class A scavenger receptor ligand, the sulfated polysaccharide fucoidan as inhibitor (*30*). We observed that pretreatment with fucoidan reduced the uptake of both polyanionic polypeptides that possessed two anionic groups per monomer unit, but the internalization of AcEAK bearing only one negatively charged group on each monomer, and polycationic AK used as negative control was taken up at the same level after the inhibition. We can presume that the charge density (e.g., the number of negative charges per unit in the polyanionic polypeptides) influences the internalization of the polypeptides. These observations indicate that MalEAK and SuccEAK are taken up by J774 cells via scavenger receptor but AcEAK could be recognized by the same receptor with a much lower affinity, or AcEAK and polycationic polypeptides as well are ingested by a different process.

In conclusion, on the basis of results presented and discussed above we could select an appropriate polypeptide with the chemical characteristics that are essential for intracellular delivery. These polypeptides are essentially nontoxic, neutral macromolecular carriers for drugs that are to be introduced to specific cell types. We can presume that polyanionic polypeptides will be able to transport the drugs coupled to them into the macrophage cells via scavenger receptor. On the other hand, by further investigation of the polycationic polypeptides we can find an application that requires a non-cell-specific but effective delivery of reporter molecules, nucleotides, or small proteins into the cell.

ACKNOWLEDGMENT

This work was supported by Öveges József Programme (OMFB-00180/2007), GVOP-3.2.1-2004-04-0352/3.0 and GVOP-3.2.1-2004-04-0005/3.0 from National Office for Research and Technology, Hungary. The authors thank Dr. Hedvig Medzyhradszky-Schweiger for the amino acid analyses.

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BC7004544