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In-vivo and In-vitro Targeting of a Murine Sarcoma by Gelatin Microparticles Loaded with a Glycan (PS1)

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Abstract—PS1, a complex polysaccharide derived from *Mycobacterium bovis* (Bacillus Calmette-Guérin, BCG) with considerable antitumour activity in-vivo, was loaded onto gelatin microparticles (mean diam. 1·45 μm) at a level shown to not produce the burst effect often seen with drug-loaded microparticulate systems. In-vitro dissolution experiments had demonstrated a sustained-release behaviour, with a half-life of approximately 8 h for what is an extremely water-soluble material. These PS1/gelatin systems had no measurable cytotoxicity against an S180 murine sarcoma cell in-vitro although fibronectin-mediated targeting of the microparticles for the tumour cells could be demonstrated. Injection into mice, with the S180 cells, of PS1 solutions or suspensions of PS1-loaded gelatin microparticles resulted in almost identical dose-related suppression for the tumour cell growth. When injected at intervals following injection of the tumour cells, however, for a period of 24–48 h there was a relatively enhanced activity of the formulated PS1, compared with the aqueous solution, after which both formulated and unformulated material became progressively less effective.

Ideally, it is desirable to place a drug close to a target, be it a tissue, a population of cells, or cell receptors, thereby leaving the rest of the body relatively drug-free and minimizing side-effects of the drug. At present, drug targeting is achieved by one of two approaches. The first approach involves chemical modification of the parent compound to a derivative that is activated only at the target site. The second approach utilizes carriers such as liposomes, microspheres, nanoparticles, antibodies, cellular carriers (erythrocytes and lymphocytes), or macromolecules to direct the drug to its site of action.

Recently we have isolated an antineoplastic agent, PS1, from the Tice substrain of Bacillus Calmette-Guérin (BCG). This complex polysaccharide material is believed to be a bacterial glycan and has proved to be an extremely effective antitumour agent in-vivo but not in-vitro (Lou et al 1992). This suggests that the active compound behaves as an immunostimulant. PS1 has a molecular weight of approximately 22·4 kDa, is readily water-soluble, with only limited solubility in acetone or ethanol, and is remarkably heat-stable (Lou et al 1993, 1994). Although PS1 is freely water-soluble, adsorption of the compound onto gelatin, followed by a second lyophilization, produced a microparticulate system with a formulation that had a dissolution half-life of at least 8 h (Lou & Groves unpublished data). In addition, the system had the potential to act as a targeted immunotherapeutic drug delivery device and this was evaluated in the present work.

Fibronectin mediates the attachment of BCG cells to tumour surfaces in bladder carcinoma (Ratliff et al 1988 a,b; Ratliff 1989). Gelatin microparticles have been reported to interact with macrophages (Tabata & Ikada 1989) and were found to be capable of targeting surfaces enriched with fibronectin, commonly found on or near bladder tumours (Olson 1992; Lou et al 1993). Fibronectin, itself a family of homologous proteins, contains a gelatin-reacting domain (Carsons 1989). Thus, gelatin microparticles can be regarded as a delivery system that may target tumour surfaces with, or associated with, fibronectin, since human plasma fibronectin binds more strongly to denatured collagen (gelatin) than to native collagen (Nakamura 1992). We therefore attempted to determine if a sustained-release formulation of PS1, based on gelatin (Lou & Groves unpublished), could be demonstrated to target macrophages and fibronectin-bearing surfaces.

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Materials and Methods

Materials

S180 II murine sarcoma cells were purchased from the American Type Culture Collection, Rockville, MD. The cells were maintained in-vitro in Eagle's Minimal Essential Medium (MEM) with non-essential amino acids, Earle's basal salts, 5% calf serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (CMEM-E) in an atmosphere of 5% CO_2–95% air at 37°C. Buffering was with Dulbecco's phosphate-buffered saline (PBS, pH ~ 7·34) without Ca^{2+} and Mg^{2+}.

Eight-week-old female CFW Swiss-Webster mice were purchased from Charles River Laboratories, Portage, MI. They were maintained in VAF housing at the UIC Biologic Resource Laboratories and were used within 5–8 days after arrival according to VAF protocols and UIC animal care committee guidelines.

Human plasma fibronectins were obtained from both Serva Biochemicals (Westbury, NY) and Accurate Chemical and Scientific Corporation (Westbury, NY).

PS1 was isolated from Tice-substrain BCG in this institute (Lou et al 1994).

Gelatin was from bovine skin and lime cured (type B), Bloom strength of 225 (Sigma Chemical Co., St Louis, MO). Ethanol was anhydrous reagent grade denatured with 5%

Table 1. S180 tumour cell viability in-vitro three days after adding formulated PS1/gelatin microparticles.

	Control	(0·15/1·5 mg)/well	(15/150 μg)/well	(1·5/15 μg)/well
n	10	10	10	10
$\bar{x}$*	$9{\cdot}31 \times 10^5$	$8{\cdot}92 \times 10^5$	$9{\cdot}34 \times 10^5$	$8{\cdot}74 \times 10^5$
s.d. (±)	$4{\cdot}94 \times 10^4$	$6{\cdot}48 \times 10^4$	$5{\cdot}95 \times 10^4$	$7{\cdot}68 \times 10^4$
t		0·098	0·07	0·138
P		>0·10	>0·10	>0·10

* Mean number of viable cells/well.

2-propanol. Glutaraldehyde was 50% w/w in water. Sodium metabisulphite and all reagents other than gelatin were purchased from Fisher Scientific, Itasca, IL.

Preparation of gelatin microparticles

The method was developed in this institute by Öner & Groves (1993 a,b,c). Freeze-dried gelatin microparticles were prepared having a mean volume number diameter (d_{vn}) of 1·45 μm ± 0·82.

Formulation of PS1

Concentrations selected for evaluation were based on the adsorption isotherm data (Lou & Groves unpublished data). In-vitro dissolution experiments had previously demonstrated that the burst effect commonly seen with drug-loaded microparticulate systems did not occur at levels below the saturation concentration observed in an adsorption isotherm. The freeze-dried gelatin microparticle suspensions (0·1% w/v) were dispersed in $2{\cdot}45 \times 10^{-5}$ M PS1 in 1/15 M phosphate buffer, pH 7·4. The suspensions were stored in tightly sealed culture tubes and equilibrated at 25°C with continuous shaking overnight. After equilibration, the PS1-loaded gelatin microparticles were separated by centrifugation at 20 000 rev min^{-1} and again lyophilized. The final concentration of PS1/gelatin microparticles was $\sim 4{\cdot}41 \times 10^{-6}$ M. The products were stored at ~ 4°C before use.

In-vitro cytotoxicity

The cytotoxicity of the formulated PS1/gelatin microparticles was determined by distributing 0·9 mL samples of 1×10^4 viable S180 II murine sarcoma cells plus 0·1 mL PBS (as control) and different concentrations of PS1/gelatin microparticles into the centre eight wells of 24-well cluster plates (Costar, Cambridge, MA). At 72 h, wells were washed with PBS and cells removed with 0·25% trypsin in 1 mM EDTA. Numbers of viable cells were determined by counting trypan blue dye-excluding cells in a haemocytometer.

Targeting

Viable S180 cells (10^6/well) in MEM-E (Eagle's medium without serum) plus different concentrations of PS1/gelatin microparticles were distributed among the centre eight wells of cluster plates. After 18 h incubation at 37°C, nonadherent cells (found to be associated with PS1/gelatin microparticles) were resuspended by gentle aspiration three times with PBS from a Pasteur pipet, removed and counted. Adherent cells (most of which were not associated with PS1/gelatin microparticles) were harvested and counted in a haemocytometer.

Interaction with fibronectin

The same procedure was carried out in the presence of increasing concentrations of fibronectin (10–100 μg/well). After 18 h incubation at 37°C, cells were harvested and counted as described above.

In-vivo bioassays

The quantitative murine S180 sarcoma assay was performed as previously described (Klegerman et al 1991a, b). Eight-week-old female CFW Swiss Webster mice were inoculated subcutaneously in the right flank with 5 μg kg^{-1}–5 mg kg^{-1} doses of PS1 alone or the same amount incorporated into the gelatin microparticles in a volume of 0·1 mL PBS mixed with an equal volume of PBS containing 2×10^5 S180 sarcoma cells. Controls were eight-week-old female CFW Swiss-Webster mice inoculated subcutaneously with 2×10^5 S180 sarcoma cells. One day, two days and up to five days after injection, the animals were again injected with the PS1/gelatin (0·5 mg PS1 in 5 mg gelatin kg^{-1}) suspension or the PS1 (0·5 mg kg^{-1}) solution again at the same location. Fourteen days after the injection, all mice with palpable masses were killed and dissected to assess the incidence of granulomata and tumours. Differences in the tumour incidence at 14 days (TI_{14}) between the control and test groups were tested for significance by Fisher's exact test and by the chi-squared test for the total of PS1 alone or the PS1-gelatin microparticles.

Results

In-vitro toxicity

When PS1/gelatin microparticles were incubated with tumour cells for 72 h, the cell morphology was changed but the viability was unaffected, relative to the control group (Table 1), suggesting that PS1/gelatin microparticles had no direct cytotoxicity.

Table 2. The inhibitory effect of fibronectin on the interaction in-vitro of PS1-loaded gelatin microparticles on murine S180 sarcoma cells.

Fibronectin (mg/well)	Adherent viable cells (% control)
0	10·53 ± 5·50
10	47·77 ± 7·82
100	81·99 ± 8·77

Table 3. The variability of tumour incidence (%) in four PS1 batches.

PS1 batch	Control	Dose			
		5 mg kg^{-1}	500 μg kg^{-1}	50 μg kg^{-1}	5 μg kg^{-1}
PS1-1	10/13 (76·9)	2/10 (20)*	3/10 (30)*	—	—
PS1-3	8/10 (80·0)	1/10 (10)*	3/10 (30)*	5/7 (71)	—
PS1-6	11/14 (78·6)	2/10 (20)*	3/10 (30)*	6/10 (60)	7/10 (70)
PS1-7	8/10 (80·0)	1/10 (10)*	2/10 (20)*	6/10 (60)	6/10 (60)
Total	37/47	6/40**	11/40**	17/27	13/20
Mean	78·9	15·0	27·5	63·0	65·0
s.d. (±)	1·5	5·8	5·0	6·4	—

* Significantly lower than control (Fisher's exact test). ** Significantly lower than control (chi-square).

In-vitro targeting

Most tumour cells associated with PS1/gelatin microparticles resulted in lower cell adherence on the well wall. Compared with control cultures, there was no loss of viability after 18 h, indicating that PS1/gelatin microparticles only interfere with S180 adherence.

Inhibition of the gelatin/tumour cell interaction by fibronectin

Incubation of PS1/gelatin microparticles with fibronectin significantly reduced their ability to inhibit S180 adherence (Table 2). These results indicate that interaction of gelatin microparticles with fibronectin mediates the antagonism of tumour cell proliferation in-vitro.

In-vivo antitumour activity

The variability of tumour-inhibitory activity of four different lots of PS1 is shown in Table 3. Both 5 and 0·5 mg kg^{-1} dose groups significantly inhibited tumour growth ($\chi^2 = 33{\cdot}36$ and 24·2 at df 1, $P < 0{\cdot}005$), whereas the 50 μg kg^{-1} dose of PS1 was not statistically different from the control group ($\chi^2 = 1{\cdot}678$ at df 1, $P > 0{\cdot}05$). The highest concentration of PS1/gelatin microparticles (5 mg/50 mg) kg^{-1} prevented a murine sarcoma from growing, but produced a granulomatous reaction. The optimal PS1/gelatin dose for the mouse sarcoma model is therefore (0·5 mg/5 mg) kg^{-1} which shows neither tumour nor granulomata formation.

The results of the sustained release experiment for in-vivo antineoplastic activities are shown in Table 4. These data indicate that the PS1/gelatin microparticles exhibit a sustained release antitumour activity in-vivo in the S180 mouse sarcoma assay. The sustained effects were observed after the tumour had been growing for 24–72 h with an optimum activity after 24–48 h, when there was a significant inhibition of tumour growth (Fisher's exact test = 0·02), compared with the control group. A local inflammation was also observed at some injection sites.

Discussion

The present in-vitro data confirm the earlier studies of Lou et al (unpublished data) that suggested gelatin microparticles were capable of targeting fibronectin-bearing surfaces. The reaction was progressively inhibited in the presence of increasing concentrations of fibronectin and the PS1-loaded gelatin microparticles were noncytotoxic to tumour cells. Although the gelatin microparticulate formulation produced almost the same antineoplastic activity as that of the unformulated material, the highest dose tested (5 mg PS1/50 mg kg^{-1}) produced a marked granulomatous reaction that was not seen with PS1 alone. This would suggest that the gelatin microparticles enhance the local immune response by inducing an inflammatory, phagocytotic response similar to that previously reported by us for high doses of BCG (Klegerman et al 1991a,b). In fact, phagocytosis of gelatin particles by macrophages has been observed (Tabata & Ikada 1987). The optimum dose for this formulation may therefore be approximately (0·5 mg/5 mg) kg^{-1} since, at this level, sarcoma growth was inhibited, but granulomata were not formed. In addition, the finding that the formulated and unformulated PS1 were virtually identical in activity would suggest that, when PS1 or the PS1/gelatin formulation are mixed with tumour cells, they could easily be intimately associated with the tumour cells. Specific and nonspecific immune responses (macrophage and lymphocytes) may be involved in both treatments since we have found that PS1 exhibits potent antineoplastic activity in-vivo but, as noted here, has no direct cytotoxicity in-vitro.

Table 4. The variability of tumour incidence (%) when used in formulated and unformulated PS1.

Group	Time (h)					
	0	24	48	72	96	120
Control	24/29 (83)					
S180 + gel	7/11 (64)					
S180 + PS1	3/20 (15)*					
S180 + PS1/gel	2/20 (10)*					
PS1		11/20 (55)	14/20 (70)	15/20 (75)	8/10 (80)	7/10 (70)
PS1/gel		6/20 (30)*	7/20 (35)*	11/20 (55)	7/10 (70)	8/10 (80)

* Significantly lower than control (Fisher's exact test).

PS1 is freely water-soluble and should readily disperse into the body fluid compartments. This effect would reduce the concentration of PS1 at or close to the tumour. This probably explains why unformulated PS1 had reduced antitumour activity when administered at various periods after the injection of S180 cells.

On the other hand, gelatin microparticles have a high affinity for fibronectin-bearing surfaces (Nakamura 1992) and could accelerate macrophage phagocytosis (Tabata & Ikada 1987). As a result, post-injection, the formulated PS1 could first be targeted to tumour cells, followed by local macrophage phagocytosis. The maximum antineoplastic effect was 24–48 h after the cells had been implanted. This may correlate with the in-vitro dissolution profile of the formulated PS1 which shows a sustained release pattern lasting for 16 h, with a half-life of approximately 8 h (Lou & Groves unpublished). The antineoplastic effect may also be related to the tumour cell growth cycle. Klegerman et al (1993) found that the growth of S180 sarcoma cells obeyed exponential (log-linear) kinetics in in-vitro culture, exhibiting doubling times of 21 h. Formulated PS1 may, therefore, exert a particularly distinct antitumour activity before the individual tumour cells associate to form a solid mass.

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