Simultaneous Binding of the Anti-Cancer IgM Monoclonal Antibody PAT-SM6 to Low Density Lipoproteins and GRP78

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Abstract

The tumour-derived monoclonal IgM antibody PAT-SM6 specifically kills malignant cells by an apoptotic mechanism linked to the excessive uptake of plasma lipids. The mechanism is postulated to occur via the multi-point attachment of PAT-SM6 to the unfolded protein response regulator GRP78, located on the surface of tumour cells, coupled to the simultaneous binding of plasma low density lipoprotein (LDL). We prepared and characterised LDL and oxidized LDL using sedimentation velocity and small-angle X-ray scattering (SAXS) analysis. Enzyme-linked immunosorbent assay (ELISA) techniques indicated apparent dissociation constants of approximately 20 nM for the binding of LDL or oxidized LDL to PAT-SM6. ELISA experiments showed a cross competition with LDL inhibiting PAT-SM6 binding to immobilised GRP78. In contrast to the results of the ELISA experiments, sedimentation velocity experiments indicated relatively weak interactions between LDL and PAT-SM6, suggesting an immunoabsorbance to the microtiter plate is driven by an avidity-based binding mechanism. The importance of avidity and the multipoint attachment of antigens to PAT-SM6 was further investigated using antigen-coated polystyrene beads. Absorption of GRP78 or LDL to polystyrene microspheres led to an increase in the affinity of PAT-SM6 binding to polystyrene microspheres coated with GRP78 or LDL. These results support the hypothesis that the biological action of PAT-SM6 in tumour cell apoptosis depends on the multivalent nature of PAT-SM6 and its ability to interact simultaneously with LDL and multiple GRP78 molecules clustered on the tumour cell surface.

Introduction

The human IgM monoclonal antibody, PAT-SM6, derived from human tumour tissue [1], is a potential anti-cancer agent capable of inducing tumour cell apoptosis in pre-clinical models of human cancer [2]. While the precise mechanism of PAT-SM6 induced cell death is not known, the process is accompanied by intracellular lipid accumulation leading to the hypothesis that PAT-SM6 facilitates the uptake of plasma lipids. Consistent with this proposal is the observation that both low density lipoproteins (LDL) and oxidized LDL interact with PAT-SM6 and enhance PAT-SM6-induced apoptosis [2]. PAT-SM6 also binds to the unfolded protein response regulator GRP78, which is over-expressed externally on the cell surface of tumour cells [3]. GRP78, also known as BiP (immunoglobulin heavy-chain binding protein), is a member of the heat-shock protein 70 (HSP70) family that prevents stress-induced apoptosis. Enzyme-linked immunosorbent assays (ELISAs), using different concentrations of coating GRP78, have already established a high avidity interaction between PAT-SM6 and GRP78 mediated by the multi-point attachment of PAT-SM6 to GRP78 clustered on the surface of the microtiter tray [4]. In the present study, we investigated the avidity of the interactions of PAT-SM6 with LDL and oxidized LDL and the competitive nature of the binding of LDL and GRP78 to PAT-SM6. The role of multivalency in the interactions of PAT-SM6 with target antigens was investigated using antigen-coated polystyrene beads. The results demonstrate the importance of antigen clustering in generating the high avidities that characterize PAT-SM6 antigen interactions. The ability of PAT-SM6 to bind both GRP78 and LDL is consistent with the proposal that PAT-SM6...
kills cells by delivering excess lipid in the form of LDL into tumours by binding simultaneously to GRP78 present on the surface of tumour cells [2].

Experimental Procedures

Materials

The human monoclonal antibody PAT-SM6 were expressed and purified from stable suspension cultures of a human cell line in serum-free media [5,6]. Isotype control IgM was obtained from Jackson ImmunoResearch Labs, Inc, West Grove, PA. PAT-SM6 was labelled with fluorescein using fluorescein isothiocyanate (Sigma-Aldrich) according to the manufacturer’s instructions. Mature human GRP78 containing a C-terminal 6 x His-tag was expressed and purified from E. coli as described previously [4].

Ethics Statement

Fresh human blood samples were obtained from St Vincent’s Hospital, Melbourne using protocols approved by the St Vincent’s Hospital Melbourne Human Research Ethics Committee. Written informed consent from participants was obtained for the original human work that produced the blood samples for the isolation of LDL. LDL was isolated by density fractionation preparative ultracentrifugation using KBr [7]. Oxidized LDL was prepared by the addition of 20 μM CuSO4 to 200 μg/mL LDL and incubation at room temperature for 16 hours [8]. Oxidation was monitored by measuring the absorbance at 234 nm and by thioldavin T (ThT) fluorescence. ThT was added to LDL to a final concentration of 8 μM and the fluorescence measured using a fluorimeter plate reader (Molecular Devices, Sunnyvale, CA) equipped with 444/485 nm excitation/emission filters. Oxidation was stopped by the addition of EDTA to a final concentration of 1 mM. LDL and oxidized LDL were dialysed into phosphate buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 1 mM EDTA and 0.1% NaN3 and stored at 4°C.

Microspheres

Mature human GRP78 containing a C-terminal 6 x His tag was expressed and purified from E. coli [8]. The human monoclonal antibody PAT-SM6 were expressed and purified from E. coli [9]. Isotype control IgM was obtained from Jackson ImmunoResearch Labs, Inc, West Grove, PA. PAT-SM6 was labelled with fluorescein using fluorescein isothiocyanate (Sigma-Aldrich) according to the manufacturer’s instructions. Mature human GRP78 containing a C-terminal 6 x His-tag was expressed and purified from E. coli as described previously [4].

Sedimentation Velocity Analysis

Sedimentation velocity experiments using absorbance optics were conducted using an XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with an An-60 Ti rotor at 20°C. Protein samples were added to double-sector epon-filled centrepieces with PBS in the reference compartment. Radial absorbance data were acquired at a rotor speed of 28,000 rpm, using a wavelength of 280 nm, and with radial increments of 0.003 cm in continuous scanning mode. Sedimentation velocity experiments were also performed using an XL-A analytical ultracentrifuge equipped with a fluorescence detection system (FDS; Aviv Biomedical) to monitor the sedimentation of fluorescein-labelled PAT-SM6 and KBPA-101. The sedimentation boundaries were fitted to a model assuming a distribution of sedimentation coefficients for non-interacting species, c(S), using the program SEDFIT [12]. Data were fitted using maximum entropy regularization, a P-value of 0.95 and a frictional ratio of 1.9 [4]. Sedimentation coefficients were corrected for the effects of LDL on solution density and viscosity, assuming values of 0.967 mL/g and 3.4 mL/g for the partial specific volume and intrinsic viscosity of LDL, respectively [10].

Antigen-coating of Polystyrene Microspheres

Suspensions of 1 μm diameter polystyrene microspheres (Polysciences, Inc) at a concentration of 2.5% (w/v) were washed by repeated pelleting (×3) in a microfuge and resuspended in PBS, PBS-0.1% Tween 20, and then a final wash with PBS. The washed microspheres were then pelleted and resuspended in 2 mg/mL of antigen (LDL or GRP78) or 5% (w/v) BSA (blocking solution) to a final microsphere concentration of 2.5% (w/v). Tubes of microsphere/antigen mixture were incubated overnight on a rotating platform at 4°C. To determine the degree of antigen binding, the microspheres were pelleted and the absorbance in the supernatant measured at 280 nm and compared to the
absorbance of the original antigen solution. The coated micro-spheres were then washed as above and then blocked using 5% BSA and stored at 4°C until ready for use.

**Results**

**Oxidation of LDL**

The Cu^{2+}-induced oxidation of LDL was monitored by measuring the light absorbance at 234 nm (Figure 1A). The increase in absorbance at 234 nm approaches a maximum after 24 hours and is attributable to the formation of conjugated dienes [13]. LDL oxidation was also monitored by a continuous ThT assay [8]. The results show that ThT fluorescence of LDL incubated with 20 mM CuSO_4 increases over the same time period reaching a plateau after 16 hours with no corresponding changes observed for control incubations of LDL or ThT alone. Further characterisation of oxidized LDL was carried out using sedimentation velocity analysis. Sedimentation coefficient distributions (Figure 1C) showed a single symmetrical peak for LDL with a modal sedimentation coefficient of approximately 4.5 S, while the distribution obtained for oxidized LDL was bi-modal with the major peak sedimenting with a modal sedimentation coefficient of approximately 8 S. This increase in sedimentation rate is attributable to a change in particle density due to a significant loss of lipid following oxidation [8].

**Small-angle X-ray Scattering (SAXS) Analysis of LDL and Oxidized LDL**

SAXS analysis has been used extensively to characterise LDL and Cu^{2+}-treated oxidized LDL [14,15,16]. This technique provides information on the overall size and shape of the lipoprotein particles from parameters such as the radius of gyration (R_g) and the maximum dimension (D_{max}), as well as information on the internal structure of the particles from the pair distance distribution function P(r). Here, LDL and oxidized LDL were subjected to synchrotron size-exclusion chromatography SAXS (SEC-SAXS) [4]. This method improves the quality of SAXS data by delivering the size fractionated the sample directly into the beam, and thus separating the scattering from the particle of interest from that of any aggregates, but also providing a near perfect buffer match for subtraction in the data reduction process.

The elution of LDL and oxidized LDL from the column was monitored by absorbance at 280 nm and SAXS intensity at zero-angle (I(0)) as a function of volume. The SAXS profiles of the peak species are shown in Figure 2A. For each species, R_g was calculated from the slope of their respective Guinier plots (ln[I(q)] vs. q^2) (Figure 2B). For LDL the Guinier plot was linear over a qR_g range of 0.8–2.4, giving an R_g value of 134±1 Å. The R_g of the peak LDL species measured here is in good agreement with the published mean value of 139 Å from the range 121–160 Å observed for 17 LDL preparations from 12 donors [16]. For oxidized LDL the Guinier plot was linear over a qR_g range of 0.8–1.6 giving an R_g value of 112±3 Å. Previous X-ray and neutron scattering studies of LDL oxidation employed static samples, which showed time-dependent and Cu^{2+}-dependent increases in apparent R_g up to ~160 Å, due to the formation of aggregates [16,17]. The SEC-SAXS derived R_g value reported here is the first measurement for isolated oxidized LDL particles in the absence of aggregates. For each SAXS profile the P(r) function was estimated by indirect Fourier transform (Figure 2C). The P(r) analysis indicated that LDL and oxidized LDL had similar D_{max} values of ~250 Å, which is consistent with previous measurements [16]. The R_g value for LDL from P(r) analysis was 131±2 Å and for oxidized LDL was 109±5 Å, which are in good agreement with the values from the Guinier analyses. Cu^{2+}-mediated oxidation causes characteristic changes in the internal organisation of the LDL particle, with has been attributed to a loss of ordered cholesterol ester and triacylglycerol structure [16]. P(r) analysis is diagnostic of this change, as the P(r) curve of LDL exhibits a strong negative peak at r=135 Å, which is completely lost upon oxidation (Figure 2C). It has been proposed that this negative peak is due to negative contrast of the ordered hydrocarbon chains in the outer monolayer of LDL, which have lower electron density.
than the surrounding solvent. Oxidation is thought to result in the addition of oxygen to the unsaturated acyl chains, making them more electron dense than water and resulting in positive contrast [16,17].

Enzyme-linked Immunosorbent Assays of PAT-SM6 Interactions with LDL and Oxidized LDL

ELISA experiments were performed using a range of PAT-SM6 concentrations and plates coated with different concentrations of either LDL or oxidized LDL (Figure 3). The results show a systematic increase in the ELISA signal as a function of PAT-SM6 concentration with more extensive binding observed at higher antigen coating concentrations. The data was fitted to a simple Langmuir binding isotherm to obtain the apparent binding constants ($K_a$) as a function of LDL coating concentration (Figure 3C). The results show that the interaction of PAT-SM6 with LDL or oxidized LDL is similar and relatively independent of coating concentrations with apparent $K_a$ values approximately 50 $\mu$M$^{-1}$, corresponding to dissociation constants of 20 nM. These values indicate that the interaction between PAT-SM6 and LDL is weaker than the interaction between PAT-SM6 and GRP78, where ELISA studies yielded apparent dissociation constants of approximately 4 nM [4].

The Simultaneous Binding of LDL and GRP78 to PAT-SM6

The ability of PAT-SM6 to interact simultaneously with GRP78 and LDL was examined using competition ELISA experiments. The results were performed using low plating concentrations of GRP78 or LDL to allow more efficient inhibition of PAT-SM6 binding by the addition of soluble antigen. Results using soluble LDL as a competitor indicated that significant inhibition of PAT-SM6 binding was observed for plates coated with either GRP78 or LDL. Conversely, results using GRP78 as a competitor revealed significant inhibition of PAT-SM6 binding to immobilised GRP78 or LDL, confirming the ability of GRP78 and LDL to compete with each other for the binding to PAT-SM6. A further observation from Figure 4 is that higher concentrations of LDL are required for half maximal inhibition, compared with GRP78, consistent with the higher affinity of GRP78 for PAT-SM6 revealed from direct ELISA experiments (Figure 3 and [4]).

Sedimentation Velocity Analysis of PAT-SM6 and LDL

The results in Figure 3 showed that the apparent $K_a$ values for the interaction of PAT-SM6 with immobilized LDL or oxidized LDL was independent of the antigen coating concentration. These findings are in contrast with the results obtained with GRP78 [4] where the strong dependence of PAT-SM6 binding on the coating concentration of GRP78 was taken as evidence for antigen clustering as a major determinant of the strength of PAT-SM6 binding interactions. The results observed for LDL suggested that LDL may be clustered on the microtiter plate, even at low plating concentrations, or that PAT-SM6 may bind directly to individual LDL particles. To examine the latter possibility, sedimentation velocity experiments were carried out to characterize the interaction between soluble LDL and PAT-SM6. The results in Figure 5A show sedimentation coefficient distributions for a mixture of LDL and PAT-SM6. Two major peaks are observed with modal sedimentation coefficients close to the values observed for PAT-SM6 alone. Analysis of the average sedimentation coefficient of the faster peak, correcting for the small effect of LDL on solution density and viscosity, revealed no significant difference compared to the average sedimentation coefficient for PAT-SM6 alone. The absence of any detectable change in the rate of sedimentation of PAT-SM6 in the presence of LDL indicates very little interaction between these species under these conditions and is at variance with the strong interactions observed in ELISA experiments (Figures 3 and 4). One possibility considered was that the use of 50 mg/mL BSA as a blocking agent...
in the ELISA experiments may act as a macromolecular crowding agent and increase the binding affinity [18]. To test this possibility sedimentation velocity experiments were performed using fluorescently labelled PAT-SM6 and the fluorescence detection system in the analytical ultracentrifuge. This allowed the rate of sedimentation of labelled PAT-SM6 to be monitored in the presence and absence of high concentrations of BSA. The results in Figure 5 show that LDL has very little effect on the rate of sedimentation of fluorescently labelled PAT-SM6 either in the presence or absence of 50 mg/mL BSA. The sedimentation velocity results in Figure 5 exclude a high affinity interaction between soluble LDL and individual sites on PAT-SM6 and support the proposal that the binding of PAT-SM6 to LDL immobilised on microtiter plates is driven by an avidity-based binding mechanism of otherwise weak interactions (at least high μM range).

The Interactions of PAT-SM6 with Antigen-coated Polystyrene Microspheres

The evidence the PAT-SM6 interacts by multi-point attachment to clustered antigens suggested that the absorption of GRP78 or LDL to polystyrene microspheres may increase the ability of these antigens to inhibit PAT-SM6 binding interactions. Antigen-coated polystyrene microbeads were prepared by incubation of polystyrene microbeads with antigens and monitoring the extent of binding using a centrifuge pelleting assay. The results in Figure 6 show that GRP78 and LDL bind rapidly to the polystyrene beads.
The results also show the extent of binding of GRP78 increases as a function of GRP78 concentration and that BSA binds to the beads in a saturable manner (Figure 6).

ELISA experiments to test whether GRP78 or LDL coated microbeads inhibit the binding of PAT-SM6 to antigen-coated microtiter plates were performed using high concentrations of coating antigen to maximise the extent of clustering on the plate. The results in Figure 7 show that GRP78 coated microspheres inhibit PAT-SM6 binding to microtiter plates coated with either GRP78 while, over the same concentration range and conditions, soluble GRP78 or BSA-coated control microspheres did not inhibit. These results indicate strong binding of PAT-SM6 to GRP78-coated beads via multivalent attachment that sequesters PAT-SM6 and prevents the binding of PAT-SM6 to GRP78 coated microtiter plates. Similar results were obtained using LDL-coated microtiter plates, although in this case both GRP78-coated beads and soluble GRP78 inhibited PAT-SM6 binding to the plates. This result is consistent with the higher apparent avidity of PAT-SM6 for GRP78 compared to LDL.

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Figure 5. Sedimentation velocity analysis of the PAT-SM6 interaction with LDL. (A) Sedimentation coefficient distributions obtained using absorption optics at 280 nm for PAT-SM6 (0.3 μM, black), LDL (1.8 μM, red) and a mixture of PAT-SM6 and LDL (0.3 μM and 1.8 μM, respectively (green). (B) Sedimentation coefficient distributions obtained using fluorescence optics for FITC-labelled PAT-SM6 (40 nM) in the presence (solid line) and absence (broken line) of LDL (1.8 μM). (C) Sedimentation coefficient distributions obtained in the presence of BSA (50 mg/mL) using fluorescence optics for FITC-labelled PAT-SM6 (40 nM) in the presence (solid line) and absence (broken line) of LDL (1.8 μM). doi:10.1371/journal.pone.0061239.g005

Figure 6. Binding of GRP78 to polystyrene microspheres. (A) Amount of protein bound per gram of microspheres as a function of GRP78 (closed circles) or BSA concentration (open circles). (B) Time-course of GRP78 (solid circles) and LDL (open triangles) binding to polystyrene microspheres. doi:10.1371/journal.pone.0061239.g006
Discussion

Natural IgM antibodies form part of the innate immune response where they are involved in the recognition of foreign particles including oxidized and chemically modified proteins and transformed cells [19]. This class of antibody typically show low antigen specificity with the ability to bind more than one type of antigen [20]. This ability to bind more than one type of antigen is evident in the case of PAT-SM6 where ELISA data indicates strong interactions with the structurally unrelated cell membrane-associated GRP78 in tumour cells [3] and with both native and oxidized plasma low density lipoproteins [2]. Previous studies show that the interaction of PAT-SM6 with GRP78 is mediated through an avidity-based mechanism based on the multivalency of the antibody. The results presented in the present study suggest a similar avidity-based mechanism operates for the binding of PAT-SM6 with LDL. Several observations support this conclusion. The interaction of PAT-SM6 with LDL analysed by sedimentation velocity revealed no significant change in the rate of sedimentation of PAT-SM6 in the presence of LDL. In contrast, strong interactions between immobilized GRP78 and PAT-SM6 were observed in ELISA experiments. These studies suggest relatively weak interactions (in the micromolar concentration range) when PAT-SM6 and LDL are free in solution, but much stronger interactions with LDL clustered on the microtiter plate.

Support for the role of clustering is also provided by the results from experiments employing antigen-coated polystyrene microspheres, which showed enhanced inhibition of the binding of PAT-SM6 to antigen coated microtiter plates, compared to equivalent concentrations of soluble forms of the antigen. A marked difference with PAT-SM6 interactions with LDL compared to the interactions with GRP78 is that the apparent avidity for PAT-SM6 interactions with LDL did not reveal a dependence on the coating concentration of LDL. In the case of GRP78 the apparent avidity for PAT-SM6 increases with coating concentration, consistent with an antigen clustering effect [4]. A possible explanation for this difference is the large size of LDL and stronger binding to the microtiter plate leading to clustering, even at low coating concentrations.

Analysis of the binding of PAT-SM6 to LDL and oxidized LDL indicated similar apparent avidities ($K_d$ approximately 20 nM) and overall weaker binding compared with the interaction with GRP78. Previous studies of the interaction of PAT-SM6 with LDL and oxidized LDL, performed using a single antigen coating and PAT-SM6 concentration yielded a lower ELISA signal (65%) for the binding to LDL compared with oxidized LDL [2] in contrast to the results presented in Figure 3, where the binding curves for PAT-SM6 to LDL and oxidized LDL were very similar. Possible explanations for this discrepancy are variations in the methods used for preparing oxidized LDL. The more significant observation from both studies is the ability of PAT-SM6 to...
recognise both native LDL and oxidized LDL. The current hypothesis for the killing action of PAT-SM6 on tumour cells is that cell death occurs via an apoptotic pathway accompanied by intracellular lipid accumulation [2]. The present study demonstrates the ability of LDL and GRP78 to compete for binding sites on PAT-SM6 data, providing strong evidence that these sites share binding sites on the antibody. The multivalent nature of PAT-SM6 would therefore permit this antibody to bind LDL and GRP78 simultaneously, consistent with a mechanism where PAT-SM6 binds plasma LDL and targets tumour cells by binding to GRP78 which is over-expressed externally on the cell surface of tumour cells [3].

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Deposition of data

The LDL and oxLDL small angle X-ray scattering data have been deposited with BIOISIS (bioisis.net) which is an open access database dedicated to the study of biological macromolecules by SAXS.

Author Contributions

Conceived and designed the experiments: ZR YM MG TM DH GH. Performed the experiments: ZR YM SY. Analyzed the data: ZR YM SY MG TM DH GH. Contributed reagents/materials/analysis tools: FH ZR YM. Wrote the paper: TM DH GH.

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