Targeting of endothelial KDR receptors with 3G2 immunoliposomes in vitro

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Abstract

Immunoliposomes (IL) containing anti-angiogenic drugs directed selectively to the easily accessible kinase insert domain containing receptor (KDR) vascular endothelial growth factor (VEGF), which is predominantly expressed on tumour vessels are a promising tool to inhibit tumour angiogenesis. To explore this strategy, we have prepared fluorescent-labelled IL presenting antibodies against the KDR receptor (3G2) on their surface. 3G2-IL were composed of egg phosphatidylcholine and cholesterol (6:4), containing 2 mol% of the new thiol reactive linker lipid \( O-(3\text{-cholesteryloxycarbonyl})propionyl-O'\text{-m}\text{-maleimido-benzoyl} \text{tetraethylene glycol} \). Specific binding of 3G2-IL to immobilised recombinant KDR was used to show the maintenance of sufficient immunoreactivity of 3G2 antibodies upon the coupling procedure. 3G2-IL bound to Chinese hamster ovarian (CHO) cells stably transfected to overexpress KDR to a five times higher amount as compared to mock-transfected CHO cells. Subsequently, specific binding of 3G2-IL to KDR could also be demonstrated on KDR expressing cells, human umbilical vein endothelial cells and human microvascular endothelial cells, whereas only low binding of 3G2-IL to NIH-3T3 mouse fibroblast cells, which do not express KDR, was found. The binding of 3G2-IL to KDR receptors could not be blocked by VEGF, suggesting that the binding site for VEGF is not identical with the epitope recognised by 3G2. We could demonstrate that 3G2-IL is able to bind in vitro even in the presence of high levels of VEGF. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Treatment of cancer with chemotherapeutic agents is causing a wide range of undesired effects limiting their use and dosage. Liposomes are widely investigated for their properties as site-specific drug carriers allowing higher drug doses due to fewer systemic side effects [1,2]. Though conventional liposomes allow passive tumour site targeting to some degree, the idea of conjugation of cell-specific antibodies to liposomes (IL, immunoliposomes) has been studied for...
selective drug delivery [3–5]. While the use of these IL to target tumour cells appears to be promising in vitro, their use in vivo is very limited due to insufficient extravasation and tumour tissue penetration and consequently IL do not reach their target [6–8]. Tumour-related target sites easily accessible from the bloodstream appeared to be attractive alternatives for targeting IL.

Angiogenesis is recognised as an important factor determining tumour growth but also playing a key role in inflammatory diseases such as rheumatoid arthritis or diabetic retinopathy as well as in physiological processes such as the female cycle [9]. Targeting angiogenesis is under investigation for cancer therapy [10]. Structures on the surface of endothelial cells that are upregulated during the process of angiogenesis have been investigated as targets for IL. Spragg et al. used IL binding to the extracellular domain of E-selectin to achieve endothelial cell selective binding [11]. Nicolau et al. reported accumulation of IL in atherosclerotic plaques of rabbits targeting vascular cell adhesion molecule (VCAM 1) [12]. Sikins et al. investigated the integrin αvβ3 which is essential during vessel growth for targeting tumour endothelium. They report accumulation of IL in tumours targeting αvβ3 [13]. These reports show the feasibility of vessel targeting with IL but all targets chosen so far are only moderately specific for tumour angiogenesis since they are also upregulated in inflammatory tissue.

Tumour angiogenesis is mediated by growth factors such as vascular endothelial growth factor (VEGF) [14]. Kinase insert domain containing receptor (KDR) is a specific receptor for VEGF [15]. KDR receptors are tyrosine kinase receptors characterised by a splitted tyrosine kinase catalytic domain, a single short membrane-spanning sequence and an extracellular domain containing seven immunoglobulin (Ig)-like loops. Upon VEGF binding, auto- or transphosphorylation occurs [16]. So far, KDR has been recognised predominantly on endothelial cells. Endothelial cells lining vessels in tumour vasculature are known for upregulated expression of this receptor [17]. Encapsulation of anti-angiogenic drugs in IL which are selectively targeted to the easily accessible receptor KDR seem to be a promising tool to inhibit tumour angiogenesis specifically [18].

The aim of this study was to investigate whether binding of an antibody targeting KDR (3G2) to liposomes containing the fluorescent dye calcein allows specific targeting of these IL to endothelial cells. Furthermore, we describe the first study using O-(3-cholesteryloxy carbonyl)propionyl-O′-m-maleimido-benzoyl tetraethyleneglycol (MCS-4) [19], a novel coupling lipid, to achieve covalent coupling of antibodies to the liposomal surface.

2. Materials and methods

2.1. Materials

Triton X-100 and 4-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES) were purchased from Merck (Darmstadt, Germany). 2-Iminothiolane, DTNB, bovine Ig were from Sigma (Deisenhofen, Germany). Sephadex G25 superfine, Sephadex G75 superfine, Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). BCA protein assay was purchased from Pierce (Rockford, USA). Egg phosphatidylycholine (EPC) was from Lipoid (Ludwigshafen, Germany). Cholesterol, N-ethylmaleimide and calcein were from Fluka (Buchs, Switzerland). Trypsin–ethylenediaminetetraacetic acid (EDTA) was from Gibco BRL (Paisly, UK).

2.2. Antibody thiolation

3G2 antibodies (from mice) were incubated with a 20-fold molar excess of 2-iminothiolane (2 mg in 0.4 ml phosphate-buffered saline (PBS)) at room temperature (RT). The reaction was stopped after 1 h by passing the solution over a column (10 cm × 1 cm) packed with swollen Sephadex G25 superfine and flushed with phosphate buffer (0.1 M Na2POH, pH 7.4). The number of thiol groups per antibody was calculated by determining the protein content with the BCA protein assay and thiol group content by using modifications of an assay based on 5,5′-dithiobi-(2-nitrobenzoic acid) (DTNB) [20]. In brief, following chromatography, 45 µl of thiocapped antibodies in solution were mixed with 45 µl DTNB solution (0.1 mg per ml phosphate buffer, pH 8.0). The concentration of thiol groups was calculated from determination of the resulting yellow colour at 405 nm using a standard curve obtained from
cysteine. A 20-fold excess of 2-iminothiolane resulted in 2–3 thiol groups per protein molecule.

2.3. Liposome preparation

MCS-4 was synthesised as previously described by Kley et al. [19]. Cholesterol was further purified by recrystallisation from ethanol. The lipid composition of the liposomes consisted of EPC/cholesterol/MCS-4 at a molar ratio of 58.8:39.2:2. The lipid mixture was dissolved in chloroform/methanol 2:1 (v/v) and dried to a thin film using a rotary evaporator at 40°C under reduced pressure. The lipid film was rehydrated with calcine solution (100 mM calcine, 20 mM HEPES, pH 6.0). The resulting dispersion of large multilamellar vesicles was extruded 15 times through a 400 nm polycarbonate membrane using Lipofast Extruder (Avestin, Ottawa, Ont., Canada). The temperature during extrusion was kept at about 40°C. This procedure was repeated with a 100 nm polycarbonate membrane. After extrusion, liposomes were purified using size-exclusion column chromatography. For each purification, a 40 cm column with a diameter of 1 cm was packed with swollen Sephadex G75 superfine and flushed with HEPES buffer (20 mM HEPES, 130 mM NaCl, pH 6.0). During all parts of the described process, liposomes were protected from light to minimise calcine degradation.

2.4. Coupling of antibodies to liposomes

Freshly prepared liposomes were incubated with freshly thiolated antibodies. The pH was adjusted to 7.6 using 0.01 N NaOH. The coupling reaction was carried out at 4°C for 12 h under protection from light and constant rotation. By adding a 100-fold molar excess of N-ethylmaleimide (with respect to the protein content), the reaction was stopped. After 1 h of blocking excess thiol groups with N-ethylmaleimide IL were purified by size exclusion column chromatography under protection from light. For this purpose, a 40 cm column with a diameter of 1 cm was packed with Sepharose CL-4B and flushed with HEPES buffer (20 mM HEPES, 130 mM NaCl, pH 7.4). The liposome dispersion was kept at 4°C for a maximum of 7 days.

2.5. IL characterisation

Total lipid was quantified according to the method of Eibl and Lands [21]. Proteins were separated from lipids according to Wessel and Flügge [22]. Protein content was determined by dot blot with bovine Ig as standard. The amount of coupled protein to liposomes is expressed as μg protein to μmol total lipid. The liposome size was determined using a Nicomp 370 photon correlation spectroscopy (PCS) system (Nicomp, USA).

2.6. Binding of IL to recombinant receptors

In this study, we used recombinant KDR receptor protein consisting of the entire extracellular domain of human KDR receptors fused on the C-terminal end with the Fc portion of human IgG1 [23]. This fusion protein was expressed in baculo-virus-infected insect cells (Martiny-Baron, unpublished). Prior to these binding experiments with 3G2-IL, 3G2 had been demonstrated to bind to this recombinant receptor with high affinity (Reusch, unpublished observations). One μg recombinant Fc fusion proteins dissolved in 100 μl PBS was incubated per well of a 96-well MaxiSorp plate overnight at 4°C. Unspecific binding was blocked adding 100 μl of PBS containing 0.1% Tween-20 and 1% bovine serum albumin (BSA). Each well was subsequently washed three times with PBS. In each well, an appropriate amount of liposome dispersion was added according to the desired final liposome concentration and diluted up to 100 μl per well with 0.5% BSA in PBS. Incubation was carried out at RT for 2 h under protection from light. Incubation was followed by washing with PBS. Bound liposomes were lysed by adding 0.1 ml lysis buffer (5% Triton X-100, 50 mM HEPES, pH 7.5) and warming up to 60°C for 30 min followed by centrifugation (100×g, 5 min).

2.7. Cell culture

NIH-3T3 cells were maintained in Dulbecco’s MOD Eagle medium/10% foetal calf serum (FCS)/1% streptomycin/1% penicillin and Chinese hamster ovarian cells (CHO cells) were kept in MEM Alpha Medium/10% FCS/1% streptomycin/1% penicillin/
2.5% geneticin at 37°C/10% CO₂ in a humified atmosphere. Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells 1 (HMEC-1) were maintained in endothelial cell growth medium/5% FCS at 37°C/5% CO₂ in a humified atmosphere.

2.8. Binding of IL to cells

CHO cells have been transfected with pBKCMV modified with human KDR sequence and demonstrated to express KDR stably (F. Totzke, unpublished results). CHO-mock cells have been transfected with unmodified pBKCMV. Cells cultured as monolayers were brought into suspension with trypsin–EDTA and washed with medium. Cells (2 x 10⁶ cells/ml) were kept in suspension with medium and incubated with liposomes over 3 h at 37°C under constant rotation. Unbound liposomes were removed by washing three times with PBS. Subsequently bound liposomes and cells were lysed by adding 0.25 ml of lysis buffer (5% Triton X-100, 50 mM HEPES, pH 7.5) and warming up to 60°C for 30 min followed by centrifugation (100 x g, 5 min).

Incubation in the presence of VEGF 165 was performed as follows: 100 µg recombinant VEGF [33] was incubated with CHO-KDR, CHO-mock cells and HUVEC in the presence of liposomes. The number of cells was determined using a Coulter Counter 71 (Coulter Eurodiagnostics, Germany). Specificity of 3G2-IL binding to KDR was demonstrated by competition of binding with free 3G2 antibody: 50 µg 3G2 was added to cell suspensions 30 min prior to addition of liposomes and incubated at 37°C. Subsequently, liposomes were added and incubation performed as described above.

2.9. Measurement of liposome binding

The amount of liposomes bound to cells or recombinant receptors was measured by fluorescence intensity. Using lysis buffer (5% Triton X-100, 50 mM HEPES, pH 7.5), encapsulated calcein was released and due to dilution dequenched. Fluorescence intensity was detected using a fluorescence spectrometer LS 50 B (Perkin-Elmer, Germany) at excitation wavelength 480 nm and emission wavelength 520 nm.

3. Results and discussion

3.1. Preparation of calcein containing liposomes

In this study, a new coupling lipid, MCS-4, recently described by Kley et al. [19] was used for covalent binding of antibodies to the liposomal surface. Incorporation of MCS-4 into liposome bilayers was easy and showed no effect on the liposomal size. 10 µg thiolated 3G2 was incubated with 10 µmol lipid, resulting in a coupling efficiency of 38%. The mean size of liposomes resulting was in the range of 90-200 nm as determined by PCS.

MCS-4 is a member of a new family of coupling lipids, based on a cholesterol anchor, a chain of polyethylene glycol (PEG) of variable length and maleimide as reactive group. Thus it is possible to covalently couple thiolated proteins to the distal end of PEG chains. PEG on the surface of liposomes is known to enhance circulation time [24–27]. To allow coupled antibodies to interact with their antigen coupling at the distal end of these chains has been demonstrated to be suitable [28–31]. This is the first report of successful application of a member of this class of new coupling lipids.

With a tetraethylene glycol spacer containing coupling lipid as we used in this study, we do not expect to enhance circulation time for liposomes in vivo. MCS-4 serves as a model for applications of this new kind of coupling lipid. Further studies using longer spacer chains are under way.

3.2. Binding of IL to immobilised KDR receptors

To demonstrate binding of 3G2-IL to KDR receptors, we initially chose a cell free approach. Recombinant KDR receptor fragments were immobilised and subsequently incubated with 3G2-IL and respective liposomes bearing no antibodies (unconjugated liposomes). As shown in Fig. 1, the binding of 3G2-IL to immobilised recombinant KDR exceeded the binding of unconjugated liposomes at both liposome concentrations (0.1 mM and 0.5 mM). At both concentrations, binding of unconjugated liposomes was less than 10% of the binding observed with 3G2-IL. A 5-fold increase of the liposome concentration (0.1–
0.5 mM) did not lead to similar enhancement of 3G2-IL binding.

The advantage of this cell-free approach we are presenting in this study lies in the chance to study IL-antigen interactions not influenced by cell-dependent factors such as antigen density, surface accessibility or endocytosis. As demonstrated in this experiment, 3G2 antibodies coupled to liposomes are able to bind to KDR receptors despite manipulation during coupling procedures. Apparently, coupling of 3G2 to the liposomal surface seemed to orientate sufficient amount of the antibody in such a manner that binding to the antigen was possible.

### 3.3. Binding of 3G2-IL to CHO cells

CHO cells were transfected either with an empty vector (CHO-mock) or transfected to stably express KDR (CHO-KDR). Using these cells, we were able to investigate unspecific interactions of 3G2-IL with cells by comparing binding of 3G2-IL on CHO-KDR cells with binding on CHO-mock cells. Unconjugated liposomes were used in further control experiments demonstrating liposome-cell interactions independent of antibody ligand-mediated binding.

CHO-mock/CHO-KDR cells in suspension were incubated with increasing concentrations of either 3G2-IL or unconjugated liposomes. As shown in Fig. 2, unconjugated liposomes showed only minimal binding to both cell types in all three concentrations. On the other hand, 3G2-IL could bind to the antigen for 3G2 antibodies, KDR receptors, on CHO-KDR cells which is reflected by high fluorescence intensity. Unlike with unconjugated liposomes, experiments with 3G2-IL on CHO-mock cells showed increased fluorescence intensity. The interactions of 3G2-IL with CHO-mock cells have to be considered as unspecific and not based on antibody-antigen recognition. Because unconjugated liposomes did not show significant liposome binding, liposomes in themselves did not carry characteristics responsible for unspecific interactions. Hence, unspecific interactions were dependent on the presence of antibodies on the liposomal surface. Näsander et al. [7] reported changes in the zeta potential during the process of Fab' coupling to negatively charged liposomes. The zeta potential was reported to become more positive (−4 mV to −12 mV). We assume that the neutral liposomes used in this study underwent similar alterations resulting in positively charged liposomes which can undergo electrostatic interactions with negatively charged cell membranes.

Unspecific binding doubled when increasing the liposome concentration from 0.05 mM to 0.1 mM but increased by seven times this value when the
liposome concentration was further increased to 0.2 mM. Taking the above made conclusions into consideration, the values obtained for the binding of 3G2-IL to CHO-KDR cells must be corrected for this portion which is independent of antigen presence. These values represent antigen-specific 3G2-IL binding solely due to binding of 3G2 to KDR receptors. For the concentrations of 0.05 and 0.1 mM, the antigen-specific binding amounted to more than three quarters of total binding. For the concentration of 0.2 mM, the antigen-specific binding decreased to about half of total binding mainly as a consequence of growing antigen-independent binding.

3.4. Binding of 3G2-IL to endothelial cells

To investigate 3G2-IL binding to cells naturally expressing KDR, we performed binding experiments with HUVEC and HMEC-1 and compared results for these cells with mouse fibroblasts (NIH-3T3).

Taking results of binding experiments with CHO-mock/CHO-KDR cells into account, we chose a liposome concentration of 0.1 mM for 3G2-IL respectively unconjugated liposomes for following experiments. At this concentration, the ratio between unspecific effects and specific binding of 3G2-IL was expected to be minimal.

3G2-IL showed high binding values on both endothelial cell lines with only low binding of unconjugated liposomes (Fig. 3A). The highest binding of 3G2-IL could be observed on HUVEC. On HMEC-1 measured fluorescence intensity for incubation with 3G2-IL was still in the range of 3G2-IL binding to CHO-KDR cells. We speculate that differences in fluorescence intensity might be due to differences in receptor density or accessibility of the receptor on these cells. NIH-3T3 cells do not express.

![Fig. 3. A: Binding of 3G2-IL and unconjugated liposomes to endothelial cells compared to the binding to receptor negative cells. CHO-mock and CHO-KDR cells as well as two endothelial cell lines (HUVEC and HMEC-1) and a fibroblast cell line (NIH-3T3) were brought into suspension at a concentration of 2 x 10⁶ cells/ml and incubated with 0.1 mM of either 3G2-IL or unconjugated liposomes over 3 h at 37°C (both liposomes containing equal amount of calcein); following two washing steps, bound liposomes were lysed and calcein fluorescence was determined. B: Influence of 3G2 pre-incubation on binding of 3G2-IL. CHO-mock and CHO-KDR cells as well as HUVEC cells were incubated with 50 μg 3G2 antibodies each prior to incubation with 3G2-IL; blocking KDR receptors with free 3G2 antibodies specificity of 3G2-IL binding to receptor expressing cells was determined. C: Influence of VEGF on 3G2-IL binding. CHO and CHO-KDR cells were incubated with 3G2-IL in the presence of 100 μg VEGF in order to determine interactions of VEGF binding to KDR receptors and 3G2-IL binding to KDR expressing cells.](attachment:image)
KDR. Consequently, the binding of both types of liposomes was in the same range as the binding of these liposomes to CHO-mock cells.

To investigate the amount of unspecific binding of 3G2-IL to endothelial cells, blocking experiments were performed. Prior to incubation with 3G2-IL CHO, CHO-KDR and HUVEC were incubated with soluble 3G2 antibodies to block specific binding. Subsequently, we added 3G2-IL. Results obtained (Fig. 3B) were compared to incubation of 3G2-IL with respective cells without 3G2 antibody pre-incubation. As shown in Fig. 3B, no specific binding of 3G2-IL to any cell type could be observed after pre-incubation of cells with soluble 3G2 antibodies. Thus HUVEC did not only prove to have a high total fluorescence intensity but considering only small values of unspecific binding, as seen with incubation after blocking of KDR receptors, it could be shown that specific binding is by far the main mechanism of 3G2-IL binding. This result is similar to results found for CHO-mock and CHO-KDR cells (0.1 mM).

These results taken together not only demonstrate KDR receptor selective binding of 3G2-IL but also a possible influence of antigen density on IL binding as previously described for other target ligands. Influence of KDR receptor density on binding could favour our model of IL utilisation for endothelial cell targeting selective for tumour endothelium. We may expect only neglectable binding of 3G2-IL to endothelium outside tumours with only traces of KDR receptor expression but accumulation in areas with high KDR receptor density, i.e. tumour vessels.

3.5. 3G2-IL binding in the presence of VEGF

VEGF is found in high levels in many tumours and systemic circulation [32]. Thus prior to in vivo experiments, it appeared to be necessary to investigate the influence of the natural ligand VEGF on 3G2-IL binding to KDR receptors. For this experiment, we pre-incubated CHO-mock and CHO-KDR cells with an excess amount of recombinant VEGF [33]. Subsequently, these cells were incubated with 3G2-IL.

As shown in Fig. 3C, no significant change in binding characteristics could be observed. Taking these results, we could conclude that 3G2 recognises structures on KDR which are not responsible for VEGF binding. Nor is this epitope altered or made inaccessible for 3G2 following VEGF binding and phosphorylation of the receptor. Thus, 3G2-IL are generally able to bind to KDR receptors expressed in tumour vessels where increased amount of VEGF is expected, regardless of whether the receptor is activated or not.

4. Conclusions

In this study, we were using a new coupling lipid (MCS-4) to covalently bind antibodies to the liposomal surface. Using 3G2 antibodies recognising KDR receptors coupled to liposomes (3G2-IL), we could demonstrate binding of these 3G2-IL to immobilised recombinant KDR receptors. Furthermore, experiments with CHO cells expressing KDR after stable transfection (CHO-KDR cells) confirmed binding of 3G2-IL to KDR receptor expressing cells. Finally, we could confirm these results with human endothelial cells HUVEC and HMEC-1 while the low degree of binding of 3G2-IL to the fibroblast cell line NIH-3T3 and CHO-mock determined specificity of 3G2-IL binding to KDR receptors. Blocking KDR receptors prior to incubation with free 3G2 antibodies demonstrated specificity of 3G2-IL for KDR expressing cells. The presence of the natural ligand for KDR receptors, VEGF, did not interfere with 3G2-IL binding.

In a further step, in vivo studies will investigate the ability of 3G2-IL to accumulate in tumour blood vessels. IL are expected to serve as optimised vehicles for anti-angiogenic drugs. For optimisation of IL for use in systemic application, variation of the spacer length between the cholesterol and reactive group of the new group of cholesterol-based coupling lipids is possible.

References


