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Inactivation of harmful tumour-associated proteolysis by nanoparticulate system

Janko Kos[a,b,*], Nataša Obermajer[a], Bojan Doljak[a], Petra Kocbek[a], Julijana Kristl[a]

[a] University of Ljubljana, Faculty of Pharmacy, Askerceva 7, SI-1000 Ljubljana, Slovenia
[b] Jožef Stefan Institute, Department of Biotechnology, Jamova 39, SI-1000 Ljubljana, Slovenia

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ABSTRACT

The primary aim in cancer therapy is to deliver anti-cancer drugs to their specific molecular targets in the tumour. Here we present a system composed of poly(D,L-lactide-co-glycolide) nanoparticles, cytokeratin specific monoclonal antibody and cystatin, a potent protease inhibitor, that can neutralize the excessive proteolytic activity associated with the invasive and metastatic potential of breast tumour cells. The antibody provides specific targeting of the delivery system to invasive breast epithelial cells and, additionally, prevents the generation of plasmin, a central extracellular protease involved in malignant progression. Polymeric nanoparticles rapidly enter the targeted cells and release the inhibitor cargo within the endosomes/lysosomes. The inhibitor is capable to inactivate lysosomal cysteine proteases, in particular cathepsin B, which is involved in the degradation of extracellular matrix inside the tumour cells. Our approach, which combines nanoparticulate delivery system with the inhibitory potential against extracellular and intracellular proteases, may improve the efficacy of therapy in patients with breast tumours compared to the application of individual protease inhibitors.

1. Introduction

The remodelling of the extracellular matrix (ECM) and the basement membrane by proteolytic enzymes is required in the processes of the progression of malignant disease, such as tumour cell migration, angiogenesis, invasion and metastasis. Four different types of proteases are involved in these processes, i.e. serine, cysteine, aspartate and metalloproteases, acting in well-coordinated proteolytic cascade (Schmitt et al., 1992). According to the proposed mechanism, the metallo proteases are ultimately involved in the degradation of the protein components of the ECM, whereas the others, in particular plasminogen activators and cathepsins act upstream in the cascade activating the protease precursors. In a number of studies it has been demonstrated that the inhibitors of metallo, serine and cysteine proteases decrease the degradation of ECM and consequently, the progression of malignant disease (Turk et al., 2004).

Plasminogen activator, either tissue (tPA) or urokinase (uPA) type has been determined as a protease, the most strongly associated with an aggressive malignant phenotype (Dano et al., 2005). Cell surface bound PA activates plasminogen into plasmin, a central player in cancer progression and metastasis. Plasmin activates precursors of metallo proteases such as procollagenases, which are capable of extensive degradation of ECM, however, it can affect malignant phenotype also by activating or inactivating growth factors, or by modifying growth factor and adhesion receptors (Lyons and Ashman, 1988; Gonias et al., 1989; Pasche et al., 1994). PA binds concomitantly with plasminogen on the cell surface via the PA receptor (uPAR). Activation of plasminogen on the cell surface is much faster compared to its activation in solution due to the interaction of plasminogen with proteins that promote a plasminogen conformation more open for a proteolytic attack (Ellis et al., 1991). Besides uPAR several other cell surface plasminogen-binding proteins have been identified, including ectoplasmatic tail of the cytokeratin 8 (Doljak et al., 2008; Kos et al., 2009).

Cathepsins are another group of proteases (Turk, 2006), implicated in a various processes of tumour progression (Kos and Lah, 1998). In particular, high expression and activity of cathepsins B and L have been identified as important tumour promoting factors. For cathepsin B it has been demonstrated that, besides the extracellular, its intracellular fraction is involved in degrading of the ECM, which is internalized by tumour cells and exposed to lysosomes (Sameni et al., 2000). We showed that inhibitors that are able to enter cells and thus, to inactivate lysosomal cathepsin B, effectively reduce ECM degradation and consequently cell invasiveness (Premzl et al., 2003). However, the cellular uptake of cathepsin inhibitors, either small molecules, protein inhibitors or neutralizing monoclonal antibodies is a slow process with very limited final effect. Also, cathepsins possess several functions in normal cells, such as intracellular protein catabolism, pro-hormone processing and regulation of cytotoxic immunity (Turk and Turk, 2001; Turk et al., 2002; Honey and Rudensky, 2003), which should not be affected

* Corresponding author at: University of Ljubljana, Faculty of Pharmacy, Askerceva 7, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 4769 604; fax: +386 1 4258 031.
E-mail address: janko.kos@ffa.uni-lj.si (J. Kos).

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during anti-tumour therapy. Therefore, for effective inactivation of tumour-associated intracellular cathepsins the delivery system, which is capable to recognize specifically the tumour cells, to enter them and to accumulate in the lysosomes is the most desired.

The aim of this study was to prepare a delivery system, recognizing specifically breast tumour cells and inactivating simultaneously the generation of serine protease plasmin on their surface and the activity of cysteine cathepsins within the lysosomes. For this purpose we prepared PLGA nanoparticles, loaded with cysteine protease inhibitor and coated with anti-cytokeratin monoclonal antibody (anti-CK MAb) recognizing tumour specific epitope on cell surface which is in a close vicinity to the binding site for plasminogen and PA. After binding the nanoparticle to the cell surface, the plasmin generation is impaired whereas the internalization of the nanoparticle results in the release of cathepsin inhibitor in the lysosomes and inactivation of lysosomal proteolytic activity.

2. Materials and methods

2.1. Materials

Poly(lactic-co-glycolic acid) (PLGA), lactic acid:glycolic acid 50:50, Resomer RG^{TM} 503H) was obtained from Boehringer (Ingelheim, Germany), polyvinyl alcohol (PVA, Mowiol^{TM} 4–98, KSE) from Hoechst (Frankfurt, Germany), ethyl acetate from Merck (Darmstadt, Germany), Alexa Fluor®-546 labelled goat anti-mouse immunoglobulin and Blue Cell Tracker from Molecular Probes (Carlsbad, CA, USA), bovine serum albumin (BSA) and fluorescein from Sigma (St. Louis, MO, USA) and Coomassie Plus reagent from Pierce (Rockford, IL, USA). Rabbit anti-uPA polyclonal antibody (PAb) or goat anti-plasmin(ogen) PAb were from Santa Cruz Biotechnology, CA. Anti-CK8 rabbit PAb was from Abcam, UK. Anti-rabbit antibody labelled with FITC or Alexa Fluor®-488 were from Invitrogen, CA, USA. Prolong Antifade kit was from Molecular Probes, Carlsbad, CA, USA. Chicken cystatin was isolated in our laboratory as reported (Kos et al., 1992). Z-Arg-Arg cresyl violet substrate was a gift from Prof. van Noorden, Amsterdam, NL. All other reagents were of standard quality for laboratory use.

2.2. Cell culture

MCF-10A neoT cells were provided by Prof. Bonnie F. Sloane (Wayne State University, Detroit, MI). Their origin was a human breast epithelial cell line (MCF-10) transformed with a neomycin resistance gene and c-Ha-ras oncogene. MCF-7 cells were obtained from ATCC (HTB 22) (Rockville, MD). Cells were cultured in a monolayer to 80% confluence in DMEM/F12 medium supplemented with 12.5 mM HEPES, 2 mM glutamine, 5% fetal bovine serum (FCS), insulin, hydrocortisone, epidermal growth factor, and antibiotics, at 37 °C in a humidified atmosphere containing 5% CO_2. Prior to use in an assay, cells were detached from culture flasks with 0.05% trypsin and 0.02% EDTA in PBS, pH 7.4. The viability of cells in the experiments was at least 90%, as determined by staining with nigrosin. Caco-2 cells were cultured in MEM supplemented with 2 mM glutamine, 1% non-essential amino acids and 10% FCS.

2.3. Antibody preparation

The mouse monoclonal antibody, anti-CK MAb used in this study, was prepared against soluble membrane proteins of MCF-7 human invasive ductal breast carcinoma as reported (Doljak et al., 2008). Using immunocytochemical analysis its positive staining was detected predominantly in primary breast carcinomas and in metastatic lymph nodes. The antibody recognizes a specific cytokeratin profile (cytokeratines 1, 2, 8, 10, 18) as determined by 2D electrophoresis, immunoblot and mass spectroscopy in a number of breast cancer cell lines (MCF-7 and MCF-10A neoT). The antibody was isolated by affinity chromatography on Protein G Sepharose using standard procedure and labelled with Alexa Fluor®-546 fluorescent dye according to the manufacturer's instructions. The labelled antibody was stored at −20 °C.

2.4. Immunofluorescence labelling

For immunofluorescence detection, MCF-10A neoT cells were cultured on glass coverslips to 70% confluence. Cells were fixed with 4% paraformaldehide at room temperature for 40 min and permeabilized with Triton-X 100, 0.01% for 10 min. Non-specific staining was blocked with 3% BSA in phosphate buffer saline (PBS), pH 7.4, for 1 h. After 1.5 h of incubation with primary antibody (Alexa 546® labelled or unlabelled anti-CK MAb, anti-CK8 rabbit PAb—used to detect CK8 in MCF-10A neoT cells, rabbit anti-uPA polyclonal antibody or goat anti-plasmin(ogen) polyclonal antibody), secondary antibody was incubated for 1 h (anti-rabbit antibody labelled with FITC or Alexa Fluor®-488, Alexa Fluor®-488 labelled anti-goat or Alexa Fluor®-555 labelled anti-mouse). Prolong Antifade kit was used for mounting coverslips on glass slides. Fluorescence microscopy was performed using Carl Zeiss LSM 510 confocal microscope. Alexa 546 was excited with an He/Ne (543 nm) laser and emission was filtered using narrow band LP 560 nm filter. Images were analyzed using Carl Zeiss LSM image software 3.0.

2.5. Preparation of antibody-coated nanoparticles loaded with cystatin

Nanoparticles were prepared by the double emulsion solvent diffusion method under mild experimental conditions as described (Cegnar et al., 2004a). Chicken cystatin, either labelled with Alexa Fluor®-488 dye or unlabelled was dissolved in deionised water and dispersed in ethyl acetate solution of PLGA with free carboxylic end groups. The primary w/o emulsion was stirred at 7000 rpm (Omni Labteh, Omni Int. Inc., Warrington, VA, USA) with simultaneous sonication (ultrasonic bath: 500 W, 30 kHz, UZ 4P Iskra Sentjernej, Slovenia) for 2 min. Then 5% PVA in aqueous solution was added to the water-in-oil emulsion to form a double emulsion (w/o/w) and stirred further for 5 min. The nanoparticles were formed after dilution of w/o/w double emulsion with 200 mL of with 0.1% PVA in aqueous solution by stirring at 5000 rpm for 5 min. The resulting nanoparticles were recovered by centrifugation at 15,000 rpm for 15 min (ultracentrifuge Sorvall RC5C Plus, MD, USA) and washed three times with purified water and if not used for antibody coating the same day, the samples were placed in liquid nitrogen and freeze-dried (−57 °C, 0.090 mbar, 24 h, Christ Beta 1-8K, Germany) as reported (Cegnar et al., 2004b).

Nanoparticles were coated with anti-CK MAb using adsorbtion method (Kocbek et al., 2007). Either Alexa Fluor®-546 labelled or non-labelled anti-CK MAb was used for the coating. Dispersed nanoparticles were incubated overnight with the antibody (0.85:1, w/w), pH 5.0, and 4 °C. In a control experiment the nanoparticles were incubated in the absence of antibody in the same volume of the buffer. The resulting immuno-nanoparticles were then washed twice with PBS, pH 5.0 and recovered by centrifugation at 10,000 rpm for 15 min (Ultracentrifuge Sorvall RC 5C plus, MD, USA). To study the coating efficiency, the fluorescence intensity of both fluorescent dyes (Alexa Fluor®-488 and Alexa Fluor®-546) was observed with fluorescence microscopy using a Carl Zeiss LSM 510 confocal microscope. Alexa Fluor®-488 and Alexa Fluor®-546 were excited with an argon (488 nm) or He/Ne (543 nm) laser and the emission was filtered using a narrow band LP 505–530 nm (green fluorescence) and 560 nm (red fluorescence) filter. Images were analyzed using Carl Zeiss LSM image software 3.0.

2.6. Internalization assay

Cellular uptake of non-coated and immuno-nanoparticles was investigated with a mono-culture of MCF-10A neoT cells. Internalization of non-coated and immuno-nanoparticles into MCF-10A neoT cells was evaluated using flow cytometry by a shift in a fluorescence intensity. Cells were placed into twelve well plates (3×10^5/well) and left to attach. 200 μL of Alexa Fluor®-488 cystatin-loaded immuno- or non-coated nanoparticles (0.3 mg/mL) were added to each well and incubated for 8 h. As a control, cells were grown separately in the absence of nanoparticles. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Inc., USA).

2.7. Cellular targeting

To determine specific delivery of immuno-nanoparticles MCF-10A neoT cells were placed in LabTek chambered coverglass system (Nalge Nunc International, Germany) in a co-culture with Caco-2 cells. Cells were grown to 80% confluence in six well plates (Corning Costar, MD, USA). Prior to labelling, the medium was removed and the cells washed with PBS. The medium with 10 μM Blue (Caco-2 cells) or Orange (MCF-10A neoT cells) Cell Tracker (Molecular Probes, Carsbad, CA) was added and cells were incubated for 40 min. The medium was changed and the cells incubated for another 30 min. The cells were then detached and placed in the LabTek chambered coverglass system (Nalge Nunc International, Germany) at concentration of 1×10^5 cells/mL in a co-culture and allowed to attach. Nanoparticles labelled with Alexa Fluor®-488 cystatin were added and the cells were observed for particle internalization. Nanoparticles incubated in the absence of anti-CK MAb were used as a control. Fluorescence microscopy was then performed using an Olympus IX/81 inverted fluorescence microscope equipped with a Dapi/FITC/TxRed filter set (E0435016) capable of distinguishing between the green (Alexa Fluor®-488 in nanoparticles), blue (Blue Cell Tracker staining of Caco-2 cells) and red (Orange Cell Tracker staining of MCF-10A neoT cells) fluorescence. Images were analyzed using Cell R Imaging software.

2.8. Proteolysis assay

A specific fluorogenic substrate, Z-Arg-Arg cresyl violet, was used to detect intracellular proteolytic activity of cathepsin B and the inhibitory effect of cystatin. Cleavage by cathepsin B of one or both arginine residues converts the molecule into a red fluorescent product (van Noorden et al., 1998). Cells were grown in a chambered coverglass system (LabTek, Nalge Nunc International) as described above. Before the assay, culture medium was removed and the cells were washed twice with PBS. The cells were then pre-incubated for 2 h with immuno-nanoparticles loaded with cystatin in serum free culture medium. Control cells were incubated in the absence of the nanoparticles. After incubation, the inhibitor solution was removed from the cells and substituted by the substrate (10 mM in serum free medium) and monitored for fluorescent product. Fluorescence was measured using Carl Zeiss LSM510 confocal microscope under the conditions described above, combined with a differential interference contrast (DIC) imaging module.

To determine the impact of anti-CK MAb to the generation of plasmin, MCF-10A neoT cells were suspended in complete medium to a final concentration of 3–10^5 cells/mL and 100 μL aliquots added to wells of a 96-well microtiter polystyrene plate (Costar, Schiphol-Rijk, The Netherlands). Cells were allowed to attach at 37 °C with 5% CO_2 overnight. Cells were then washed once with PBS and serum-free medium was added. Anti-CK MAb was added to final concentrations of 2 and 0.5 μM and the cells were incubated at 37 °C for another 24 h. The control cells were cultured in the absence of anti-CK MAb. Then, urokinase type plasminogen activator (uPA) (Sigma) (50 pM), plasminogen (Sigma) (500 nM) and plasmin specific fluorescent substrate D-Ala-Leu-Lys-AMC (Sigma) (0.5 mM) were added to each well of the microtiter plate and plasmin formation was monitored over 1.5 h at 37 °C at 370 nm and 470 nm using a fluorescence plate reader (Safire 2, Tecan) (Lopez-Alemany et al., 2003). An irrelevant MAb of the same subtype (anti-cathepsin L N135) was used with MCF-10A neoT cells in the experiment as a control.

2.9. Cell adhesion assay

A 96-well culture plate was pre-coated with 50 μL of fibronectin (1 μg/mL) in carbonate buffer, pH 9.6, overnight at 4 °C. Wells were then washed once with PBS and incubated with 1% BSA in PBS for 30 min at room temperature. MCF-10A neoT cells were harvested, washed with PBS and resuspended in the serum-free medium. Anti-CK MAb was added to the medium at the final concentration 1 μM. 50 μL of MCF-10A neoT cell suspension was added to each well of a 96-well culture plate pre-coated with fibronectin. Cells were allowed to attach for 15 min, 45 min, 1.5 h, 3 h and 5 h, after which wells were washed twice with PBS and 100 μL of complete growth medium was added. The assay was performed in quadruplicate. Control wells were washed with 50 μL of medium. Finally, CellTiter 96® One Solution was added, formazan absorbance measured, and cell adherence was calculated from the equation:

$$\text{cell adherence}\,(\%) = \left(\frac{A_{\text{tx}}}{A_{\text{control}}}\right) \times 100$$

where the absorbance of formazan was determined for cells washed at different time points (A_{tx}) and for cells not being washed (A_{control}).

Differential interference contrast images were taken with an Olympus IX 81 motorized inverted microscope and Cell® software.

3. Results

3.1. Localisation of cytokeratin, plasminogen and uPA in breast tumour cells by immunofluorescence

The staining pattern of plasminogen on the plasma membrane of MCF-10A neoT cells is shown to be similar to that obtained with anti-CK MAb (Fig. 1). Further, uPA and plasminogen were co-localized with cytokeratins, respectively, in MCF-10A neoT cells cultured on fibronectin (Fig. 1).

3.2. Characteristics of immuno-nanoparticles

Cystatin-loaded PLGA nanoparticles exhibited a mean diameter of 320–360 nm and polydispersity index 0.3. Non-covalent binding of anti-CK MAb to the nanoparticle surface was evaluated by fluorescence microscopy, using Alexa Fluor®-546 labelled anti-CK mAb to visualize the adsorption of anti-CK MAb on the surface of the Alexa Fluor®-488 cystatin loaded PLGA nanoparticles. Unlike the non-coated nanoparticles (data not shown) the immuno-nanoparticles show red fluorescence, indicating a certain proportion of nanoparticles with adsorbed MAb (Fig. 2).

3.3. Cellular uptake of immuno-nanoparticles by MCF-10A neoT cells

The uptake of nanoparticles, loaded with Alexa Fluor®-488 labelled cystatin and coated with anti-CK MAb, into MCF-10A neoT cells, in comparison to non-coated nanoparticles, was monitored by fluorescence microscopy. The uptake was similar for immuno-nanoparticles, as for the non-coated nanoparticles (Fig. 3). After the internalization of immuno-nanoparticles, green Alexa Fluor®-488

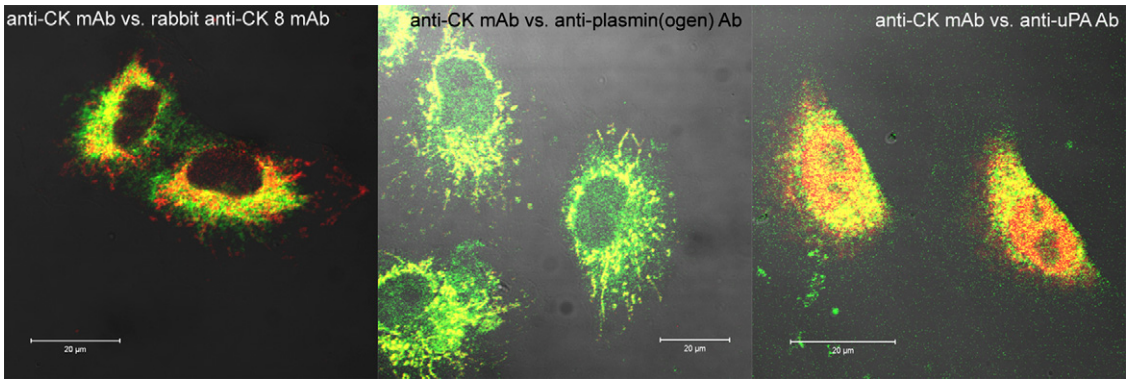

Fig. 1. Localization of cytokeratins on MCF-10A neoT cells. MCF-10A neoT cells were stained with anti-CK MAb (red fluorescence) and anti-CK8 antibody (left, green fluorescence) or anti-plasminogen antibody (middle, green fluorescence) or anti-uPA MAb (left, green fluorescence) and imaged sequentially in a line-interlace mode to eliminate cross talk between the channels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

labelled cystatin fluorescence could be observed in the perinuclear region, corresponding to lysosomal vesicles, similarly to internalized non-coated nanoparticles. Flow cytometry analysis for both types of nanoparticles showed a shift in green fluorescence intensity due to internalization of Alexa Fluor®-488 labelled cystatin-loaded nanoparticles into MCF-10A neoT cells (Fig. 4). After 8 h 78.2% and 79.4% of cells had internalized immuno-nanoparticles and non-coated nanoparticles, respectively (Fig. 4).

3.4. Cell targeting and internalization of immuno-nanoparticles in co-culture

The ability of immuno-nanoparticles in targeting MCF-10A neoT cells was evaluated in a co-culture with Caco-2 cells which lack the antigen for the anti-CK MAb. Prior to internalization, immuno-nanoparticles localized in the vicinity of the MCF-10A neoT cells and not the Caco-2 cells, indicating their ability to target antigen-specific cells (Fig. 3). By contrast, non-coated nanoparticles did not show such a specific localization towards MCF-10A neoT cells, as they were randomly distributed in the co-culture. Internalization of nanoparticles was checked after 24 h of incubation, and the specific

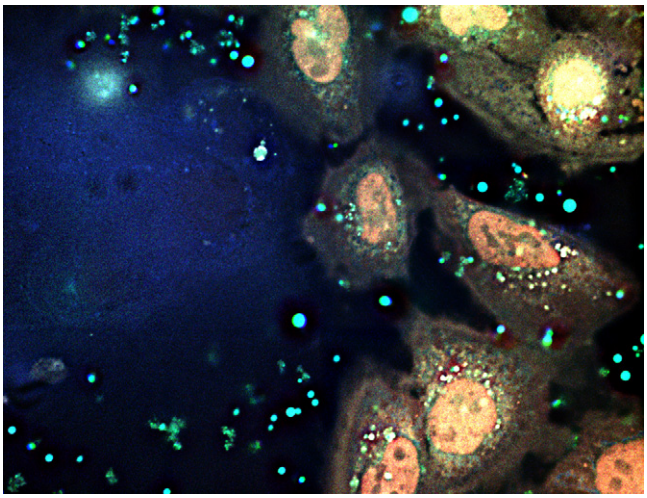

Fig. 3. Fluorescence microscope image of a co-culture of MCF-10A neoT (red fluorescence) and Caco-2 (blue fluorescence) cells incubated with fluorescein-loaded PLGA nanoparticles coated with anti-CK MAb after 24 h of incubation. Immuno-nanoparticles (green) entered solely MCF-10A neoT cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

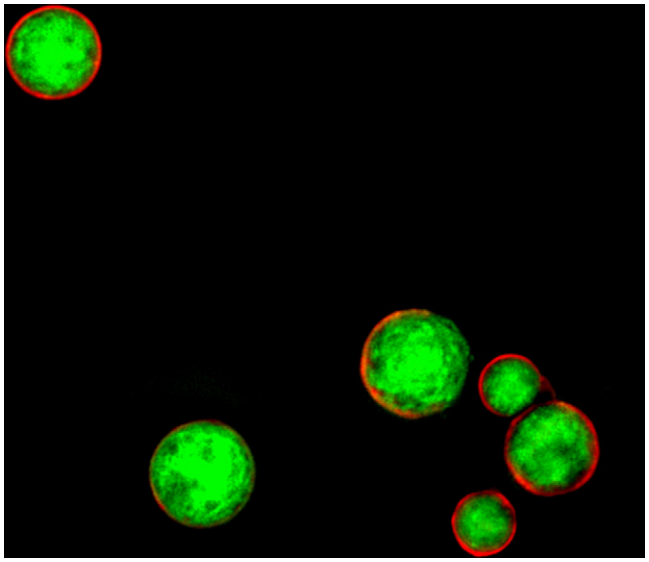

Fig. 2. PLGA micro- and nanoparticles with incorporated FITC (green fluorescence) and coated with Alexa 546® labelled anti-CK MAb (red fluorescence). Due to visualization, larger particles in diameter up to 1–2 μm were selected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

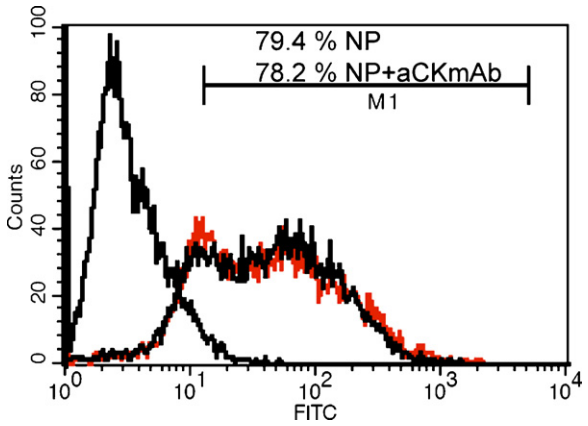

Fig. 4. Flow cytometry of anti-CK MAb-coated nanoparticle internalization into MCF-10A neoT. The cells were incubated with anti-CK MAb-coated (thick red line) or non-coated (thick black line) nanoparticles loaded with cystatin for 8 h, prior the analysis. As a control, MCF-10A neoT cells were grown in the absence of nanoparticles (thin black line). The percentages indicate the proportion of MCF-10A neoT cells, that have internalized noncoated and anti-CK MAb-coated nanoparticles, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

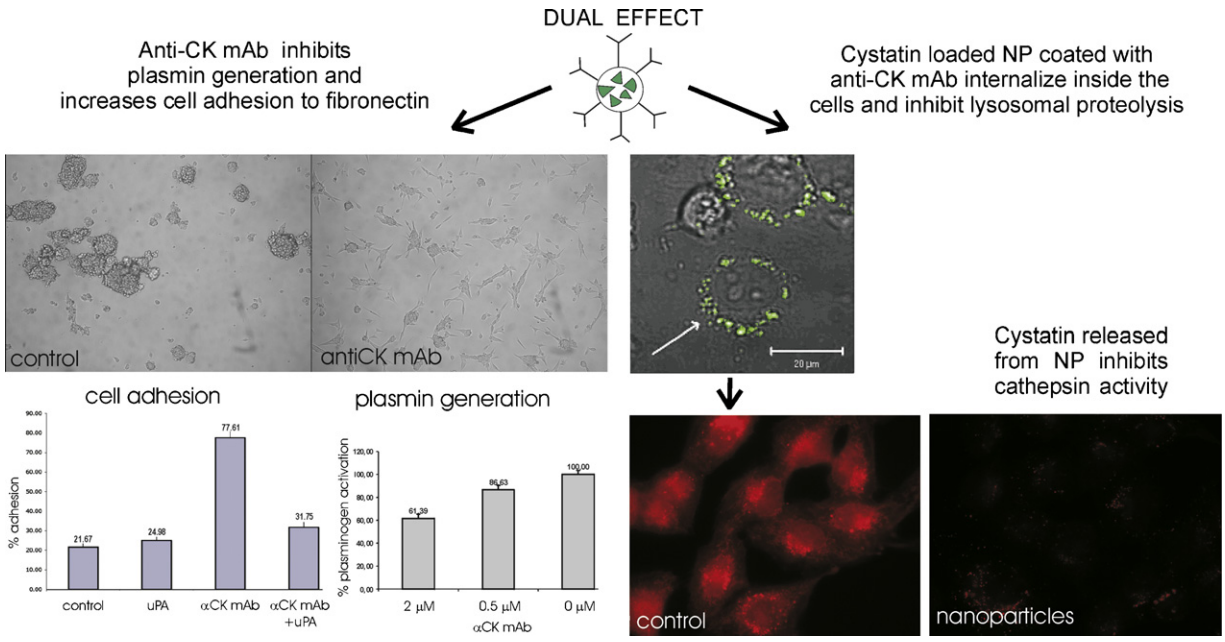

Fig. 5. A scheme representing double inhibitory effect of immuno-nanoparticles loaded with cystatin. Anti-CK MAb inhibits pericellular serine protease activity, whereas cystatin reduces intracellular cysteine protease activity.

uptake in MCF-10A neoT cell was observed only for nanoparticles having adsorbed anti-CK MAb.

3.5. Proteolysis assay

Immediately after treatment of living MCF-10A neoT cells with specific cathepsin B fluorogenic substrate Z-Arg-Arg cresyl violet a strong red fluorescence appeared in the vesicles in the perinuclear region. The fluorescence matched well the intracellular localization of cathepsin B, confirming that a large part of the lysosomal cathepsin B was present in its active form (Cegnar et al., 2004a).

Pre-incubation of cells with cystatin loaded immuno-nanoparticles almost completely abolished the substrate fluorescence, showing that cathepsin B activity was strongly inhibited (Fig. 5). The amount of cystatin released from the nanoparticles during the pre-incubation period was negligible, as determined from the loading capacity and a release profile. Also, as shown in our previous report, the free cystatin added to the MCF-10A neoT cells was not effective against intracellular cathepsin B (Cegnar et al., 2004a). The instrument settings, such as intensity of excitation light, magnification, and exposure time on digital camera, were kept constant following the inhibition of cathepsin B activity.

Plasmin generation in MCF-10A neoT cells was reduced by anti-CK MAb, as shown at 2 μM and 0.5 μM concentration of the antibody (Fig. 5).

3.6. Increased adhesion of breast tumour cells to fibronectin by anti-CK MAb

When MCF-10A neoT cells were seeded on fibronectin, they adhered more rapidly and to a greater extent in the presence of anti-CK MAb than in its absence (Fig. 5). At 30 min post seeding, 77.6% of MCF-10A neoT cells were already attached in the presence of anti-CK MAb as compared to 21.7% for untreated cells. The fact that the effect of anti-CK MAb was evident so quickly can be explained by its binding to the cell surface epitope and not to its intracellular activity. However, when uPA was added to the culture medium, the anti-CK MAb-stimulated adhesion of MCF-10A neoT cells was diminished (31.8%), demonstrating that uPA mediated increased plasmin activity reduces the adhesion of breast cancer cells to fibronectin (Fig. 5).

4. Discussion

Proteolytic enzymes have emerged as important players in a multistep process of tumour progression, facilitating invasion and metastasing of tumour cells. The application of protease inhibitors, inactivating harmful tumour-associated proteolytic activity, but not affecting normal cell functions, represents a new challenge in cancer therapy. We developed a nanoparticulate delivery system, which is able to reduce the activity of two types of tumour-associated proteases, i.e. serine protease plasmin and lysosomal cysteine proteases cathepsins.

In numerous preclinical studies the application of protease inhibitors provided promising effects with regard to reduction of tumour growth, invasion and metastatic spread. However, there are several factors that greatly limit the general application of protease inhibitors in cancer therapy, which presumably caused failures of protease inhibitors in clinical studies (Coussens et al., 2002). Proteases, causing harmful tumour-associated proteolytic activity, are involved also in several normal physiological processes and their total inhibition could result in severe complications. Moreover, many inhibitors, used in clinical studies, exhibited broad specificity and could inactivate the proteases with tumour suppressive functions, such as regulation of anti-tumour immune response or apoptosis (Kos and Lah, 2006). Some tumour-associated proteolytic processes can even take place at different locations, as is the case with the degradation of ECM, which may occur extracellularly and intracellularly (Sameni et al., 2000; Premzl et al., 2003). To improve the outcome in clinical studies, future approaches in targeting proteases in anti-tumour therapy should consider two important criteria, first, the inhibitors have to be selective against the proteases, involved in tumour progressive functions and second, specific delivery of anti-tumour agents to the spots of harmful proteolytic action has to be included in the treatment protocols.

Polymeric nanoparticles are promising systems for delivering anti-tumour agents, since they accumulate in tumours due to the

enhanced permeation and retention (EPR) effect and enter tumour cells by means of endocytosis (Cegnar et al., 2005). Moreover, they can protect the drug from premature degradation and control its release at the site of action, enhancing therapeutic efficacy and reducing undesirable side effects. Polymeric nanoparticles can also bind specific recognition molecules, enabling specific targeting of tumour cells. The ligands on polymeric nanoparticles are usually monoclonal antibodies (MAbs) that recognize tumour-associated antigens, such as receptors, enzymes, glycoproteins, etc., which are expressed uniquely on the surface of targeted tumour cells.

The MAb we used for specific targeting of nanoparticles was prepared against membrane proteins of MCF-7 human invasive ductal breast carcinoma as reported (Obermajer et al., 2007; Doljak et al., 2008). Mab recognizes an epitope on the ectoplasmic tail of cytokeratin 8, which is overexpressed by MCF-7 and other invasive breast tumour cells. Cytokeratin 8 is an intermediate filament protein and associates with cytokeratin 18 to form an insoluble matrix within the cell. However, the C-terminal end of cytokeratin 8 also penetrates the cellular membrane, as shown for hepatocellular and breast carcinoma cells (Ditzel et al., 1997; Kralovich et al., 1998). Differential expression of the cytokeratin membrane fraction may serve for diagnostic applications to assess the aggressiveness of tumour cells (Kasper and Singh, 1995). Moreover, they represent also useful targets for specific drug delivery in cancer patients.

The selectivity of the antibody used in the new delivery system was tested in a co-culture of invasive breast cells (MCF-10A neoT) and enterocytes (Caco-2). As determined by fluorescence microscopy and flow cytometry the non-coated nanoparticles entered both cell types, although the internalisation rate in Caco-2 cells was significantly lower probably due to the lower metabolic activity of these cells. Nanoparticles coated with the antibody only entered the MCF-10A neoT cells and not the Caco-2 cells demonstrating selectivity of the antibody against cytokeratin present on the cell surface of the breast MCF-10A neoT cells but not on enterocytes.

Besides the selective targeting of tumour cells the advantage of anti-CK Mab is also its ability to reduce the generation of plasmin, a key extracellular enzyme involved in cell adhesion, invasion and signalling of breast tumour cells. We showed that the binding of anti-CK Mab to the epitope localized within the VKIALEVEIATY sequence in the α-helical B2 domain of cytokeratin 8 prevents the binding of plasminogen and uPA to the cell surface and consequently, the plasmin generation (Doljak et al., 2008, Kos et al., 2009). Additionally, the co-localisation of plasminogen and cytokeratin 8, we determined in this study by fluorescence microscopy, confirms the interaction between these molecules on the surface of breast MCF-10A neoT cells. Treatment of breast tumour cells with anti-CK MAb greatly enhanced their adhesion to fibronectin, due to the reduced pericellular proteolysis of fibronectin by plasmin (Tan et al., 2006). Stronger adhesion of MCF-7 and MCF-10A cells to fibrinogen and presumably other components of the extracellular matrix could be a reason for lower Matrigel invasion (results not shown).

Rapid internalization of our new delivery system into tumour cells by means of endocytosis and its trafficking to endosomic/lysosomic vesicles enables inactivation of excessive activity of intracellular cysteine cathepsins. Polymeric nanoparticles are degraded within the vesicles releasing the incorporated protease inhibitor. The accumulation of the inhibitor released within the endosomes/lysosomes can be visualized by the bound fluorescent dye and is very evident in Fig. 3. The question arises whether the inhibitor-loaded nanoparticles reach the same vesicles as the fragments of the internalized ECM, since the mechanism of endocytosis could be different. Whereas the PLGA nanoparticles are internalized by clathrin-mediated endocytosis (Nori and Kopecek, 2004), a collagen, the main component of the ECM is internalized by the endocytic receptor uPARAP (urokinase plasminogen activator receptor-associated protein) (Mohamed and Sloane, 2006). In MCF-10A neoT cells we co-localized the degraded products of the internalized collagen and cathepsin B activity in the same vesicles (Premzl et al., 2006). Therefore, the inactivation of cathepsin B activity by the inhibitor, delivered by PLGA nanoparticles could indicate the same vesicle destination as for collagen fragments. Indeed, the activity of cathepsin B was almost completely abolished by cathepsin B inhibitor cystatin, incorporated to our nanoparticle delivery system, as determined by Z-Arg-Arg-cresyl violet, a specific fluorescent substrate. Its inactivation may therefore, prevent the degradation of internalized ECM and contribute to lower invasiveness of tumour cells.

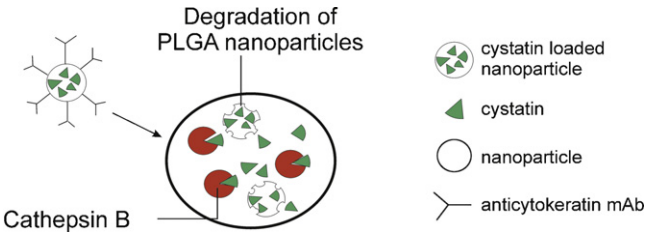

Fig. 6. A scheme of the immuno-nanoparticle and its action in the lysosomes of breast tumour cell.

In conclusion, a new delivery system, comprising biodegradable PLGA polymeric nanoparticles (Fig. 6), which can be internalized into cells, specific anti-CK MAb, which contributes specific targeting to invasive breast epithelial cells and simultaneously reduces the generation of plasmin, and the inhibitor, which inactivates lysosomal cysteine proteases and consequently ECM degradation, may reduce invasive and metastatic potential of tumour cells without affecting proteolytic functions in normal cells. The method has the potential to improve the efficacy of anti-cancer therapies directed against tumour-associated proteolytic system.

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References

Cegnar, M., Premzl, A., Zavašnik-Bergant, V., Kristl, J., Kos, J., 2004a. Poly(lactide-co-glycolide) nanoparticles as a carrier system for delivering cysteine protease inhibitor cystatin into tumour cells. Exp. Cell. Res. 301, 223–231.

Cegnar, M., Kos, J., Kristl, J., 2004b. Cystatin incorporated in poly(lactide-co-glycolide) nanoparticles: development and fundamental studies on preservation of its activity. Eur. J. Pharm. Sci. 22, 357–364.

Cegnar, M., Kristl, J., Kos, J., 2005. Nanoscale polymer carriers to deliver chemotherapeutics to tumours. Exp. Opin. Biol. Ther. 5, 1557–1569.

Coussens, L.M., Fingleton, B., Matrisian, L.M., 2002. Matrix metalloprotease inhibitors and cancer: trials and tribulations. Science 295, 2387–2392.

Dano, K., Behrendt, N., Hoyer-Hansen, G., Johnsen, M., Lund, L.R., Ploug, M., Rømer, J., 2005. Plasminogen activation and cancer. Thromb. Haemost. 93, 676–681.

Ditzel, H., Garrigues, U., Andersen, C., Larsen, M., Garrigues, H., Svejgaard, A., Hellström, I., Hellström, K., Jensenius, J., 1997. Modified cytokeratins expressed on the surface of carcinoma cells undergo endocytosis upon binding of human monoclonal antibody and its recombinant Fab fragment. Proc. Natl. Acad. Sci. U.S.A. 94, 8110–8115.

Doljak, B., Obermajer, N., Jamnik, P., Kos, J., 2008. Monoclonal antibody to cytokeratin VKIALEVEIATY sequence motif reduces plasminogen activation in breast tumour cells. Cancer Lett. 267, 75–84.

Ellis, V., Behrendt, N., Danø, K., 1991. Plasminogen activation by receptor-bound urokinase. A kinetic study with both cell-associated and isolated receptor. J. Biol. Chem. 266, 12752–12758.

Gonias, S., Young, W.J., Fox, J., 1989. Cleavage of recombinant murine interferon-gamma by plasmin and miniplasmin. J. Interferon Res. 9, 517–529.

Honey, K., Rudensky, A.Y., 2003. Lysosomal cysteine proteases regulate antigen presentation. Nat. Rev. Immunol. 3, 472–482.

Kasper, M., Singh, G., 1995. Epithelial lung cell marker: current tools for cell typing. Histol. Histopathol. 10, 155–169.

Kocbek, P., Obermajer, N., Cegnar, M., Kos, J., Kristl, J., 2007. Targeting cancer cells using PLGA nanoparticles surface modified with monoclonal antibody. J. Control Release 120, 18–26.
Kos, J., Dolinar, M., Turk, V., 1992. Isolation and characterisation of chicken L- and H-kininogens and their interaction with chicken cysteine proteineses and papain. Agents Actions 38, 331–339.
Kos, J., Lah, T., 1998. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer. Oncol. Rep. 5, 1349–1361.
Kos, J., Lah, T., 2006. Cystatins in cancer. In: Žerovnik, E., Kopitar Jerala, N., Uversky, V. (Eds.), Human Stefins and Cystatins. Nova Science Publisher, New York, pp. 153–165.
Kos, J., Jevnikar, Z., Obermajer, N., 2009. The role of cathepsin X in cell signalling. Cell Adhes. Migrat., 3, 164/166.
Kralovich, K., Li, L., Hembrough, T., Webb, D., Karns, L., Gonias, S., 1998. Characterization of the binding sites for plasminogen and tissue-type plasminogen activator in cytokeratin 8 and cytokeratin 18. J. Protein Chem. 17, 845–854.
Lopez-Alemany, R., Longstaff, C., Hawley, S., Mirshahi, M., Fabregas, P., Jardı, M., Merton, E., Miles, L., Felez, J., 2003. Inhibition of cell surface mediated plasminogen activation by a monoclonal antibody against alpha-Enolase. Am. J. Hematol. 72, 234–242.
Lyons, A., Ashman, L., 1988. The effect of recombinant cytokines on the proliferative potential and phenotype of cells of the human myelomonocytic leukaemia line, RC-2A. Leuk. Res. 12, 659–666.
Mohamed, M.M., Sloane, B.F., 2006. Cysteine cathepsins: multifunctional enzymes in cancer. Nat. Rev. Cancer 6, 764–775.
Sameni, M., Moin, K., Sloane, B.F., 2000. Imaging proteolysis by living human breast cancer cells. Neoplasia 2, 496–504.
Nori, A., Kopecek, J., 2004. Intracellular targeting of polymer-bound drugs for cancer chemotherapy. Adv. Drug Deliv. Rev. 57, 609–636.
Obermajer, N., Kocbek, P., Repnik, U., Kužnik, A., Kristl, J., Cegnar, M., Kos, J., 2007. Immuno-nanoparticles: an effective tool to impair harmful proteolysis in invasive breast tumour cells. FEBS J. 274, 4416–4427.
Pasche, B., Ouimet, H., Francis, S., Loscalzo, J., 1994. Structural changes in platelet glycoprotein IIb/IIIa by plasmin: determinants and functional consequences. Blood 83, 404–414.
Premzl, A., Zavašnik Bergant, V., Turk, V., Kos, J., 2003. Intracellular and extracellular cathepsin B facilitate invasion of MCF-10A neoT cells through reconstituted extracellular matrix. Exp. Cell Res. 283, 206–214.
Premzl, A., Turk, V., Kos, J., 2006. Intracellular proteolytic activity of cathepsin B is associated with capillary-like tube formation of endothelial cells in vitro. J. Cell. Biochem. 97, 1230–1240.
Schmitt, M., Jänicke, F., Graeff, H., 1992. Tumour associated proteases. Fibrinolysis 6, 3–26.
Tan, X., Egami, H., Nozawa, F., Abe, M., Baba, H., 2006. Analysis of the invasion-metastasis mechanism in pancreatic cancer: involvement of plasmin(ogen) cascade proteins in the invasion of pancreatic cancer cells. Int. J. Oncol. 28, 369–374.
Turk, V., Turk, B., 2001. Lyzosomal cystein proteases: facts and opportunities. EMBO J. 20, 4629–4633.
Turk, B., Turk, D., Salvasen, G.S., 2002. Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. Curr. Pharm. Dis. 8, 1623–1637.
Turk, V., Kos, J., Turk, B., 2004. Cysteine cathepsins (proteases)—on the main stage of cancer? Cancer Cell 5, 409–410.
Turk, B., 2006. Targeting proteases: successes, failures and future prospects. Nat. Rev. Drug Discov. 5, 785–799.
Van Noorden, C.J.F., Jonges, T.G.N., Van Marle, J., Bissell, E.R., Griffini, P., Jans, M., Snel, J., Smith, R.E. 1998. Heterogenous suppression of experimentally induced colon cancer metastasis in rat liver lobes by inhibition of extracellular cathepsin, B. Clin Exp Metastasis 16,159/167.