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Charge-dependent Targeting: Results in Six Tumor Cell Lines

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Abstract. *Background: Many previous studies show that cell surface sialylation of malignant cells is enhanced compared to normal tissue. The carboxyl group of the sialic acid yields a negative surface charge of the tumor cells. This study investigates how tumor cell growth is affected when a cationic polymer is incubated with six different tumor cell lines. Materials and Methods: Cationic dextran (CatDex) was prepared by periodate oxidation and subsequent coupling of cationic sidegroups by reductive amination. A fluorimetric cytotoxicity assay (FMCA) was used for the cell survival assay. Six different tumor cell lines (lung, breast, ovarian, prostate, colon, urinary bladder) were seeded into 96-well microtiter plates. CatDex was added at different μM concentrations and incubated for 72 h. Additionally, CatDex was fluorescence-labeled (FITC) and the interaction with the tumor cells was studied using fluorescence microscopy. The presence of sialic acid in the different cell lines was confirmed by using a FITC-labeled sialic acid binding lectin. Results: CatDex showed a concentration-dependent growth inhibitory effect (i.e. the number of cationic side groups/ dextran molecule and the molarity used). If the substitution was <20%, the growth inhibitory effect was small and difficult to reproduce. With 20-22% substitution, the growth inhibition varied between 20-95% depending on the molarity and the tumor type. Higher substitution resulted in complete cell death in all the cell lines. The fluorescent images showed intensive cell membrane interaction. Conclusion: Incubation with cationic dextran caused cell death in all six tumor cell lines. Our hypothesis is that CatDex binds to the anionic sialic acid residues and causes fatal disturbances in the cell membrane. However the exact mechanism remains to be elucidated. The results may indicate a new method of general interest for intra/local/regiolocal treatment of cancer. Clinical studies to explore this concept are pending.*

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In an earlier study it was demonstrated that intravesical instillation of a polymer with cationic charge resulted in selective accumulation in the tumor tissue in patients with superficial bladder cancer. The effect was pronounced and dependent on the cationic charge of the polymer. High ratios between normal and tumor tissue were observed (1).

As a consequence of these findings, and to use the mechanism for "drug-delivery", attempts were made to develop cytotoxic cationic polymer derivatives suitable for instillation therapy on patients with superficial bladder cancer. Anthracyclines were coupled to a neutral and a cationic dextran and their effects on tumor cell growth were tested in different bladder tumor cell lines (2). A secondary finding in this study was that cationic dextran alone could affect the cell growth in one of the cell lines.

There are numerous studies showing that cell surface sialylation of malignant cells is considerably enhanced compared to normal tissue. Sialic acid (neuraminic acid, lactamic acid, NANA) expression seems to be a requirement for the malignant phenotype, affecting the metastatic potential (3-8). Sialic acid has a strong electronegative charge at physiological pH, a property that enables the electrostatic interaction with cationic polymers.

This study investigates how the tumor cell growth of six different tumor cell lines is affected when incubated with a cationic polymer. The interaction between the cationic polymer and the tumor cells was demonstrated by fluorescent labeling. Photographic images were made in a fluorescent microscope.

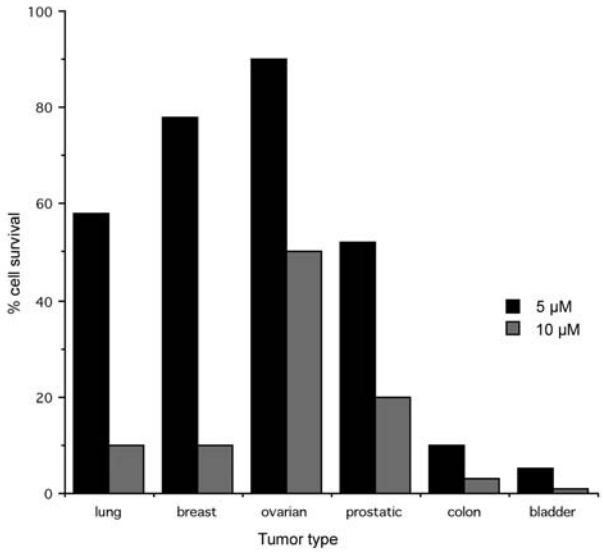

Figure 1. *One representative example of the FMCA assay showing the growth inhibition in six tumor cell lines using 5 and 10 µM concentration of CatDex (72h incubation). The Catdex conjugate has 22 % amine substitution (n=3, rel. s.d. < 5 %).*

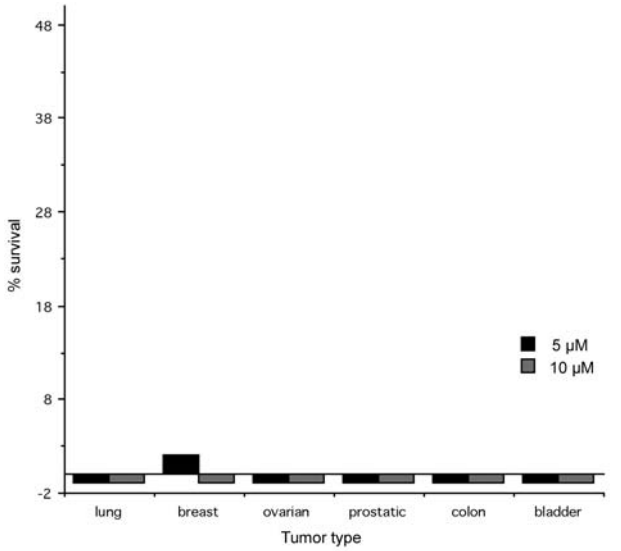

Figure 2. *The same FMCA assay as in Figure 1 however using a CatDex conjugate with 25 % amine substitution (n=3, rel. s.d. < 5 %, negative value=100 % cell death).*

Materials and Methods

Dextran PM70 (Pharmacia Amersham Biotech AB, Uppsala, Sweden) was used as conjugate backbone. Sodium meta-periodate (Merck AG, Darmstadt Germany) was used for dextran activation and 1,6-diaminohexane (Sigma-Aldrich, Sweden) was used for the amine coupling. Sodium cyanoborohydride (Chemicon, Stockholm, Sweden) was used for reductive amination. NAP-5 and PD-10 disposable Sephadex G-25 columns were used for separation and purification (Pharmacia Amersham Biotech AB).

Activation. Activation and coupling was performed as described previously (9, 10). Briefly, 20 mg of dextran PM70 dissolved in 0.5 mL of a 0.25 M sodium acetate buffer (pH 5.5) +15 mg of sodium periodate was incubated gently shaking for 24 h (dark, room temperature). After incubation the solution was purified on a NAP-5 column equilibrated with 0.25 M sodium acetate buffer at pH 6.5.

Coupling. One hundred and fifty mg diaminohexane + 5 mg sodium cyanoborohydride were mixed with 20 mg of activated dextran, all in 1 mL of 0.25 M sodium acetate at pH 6.5. The solution was incubated gently shaking for 4 h (dark, room temperature). After the incubation, the solution was purified on two PD-10 columns; one using phosphate-buffered saline (PBS) as eluent and the other with borate 0.02 M (pH 9.5) as eluent.

Total nitrogen determination. The substitution degree of the dextran-amine conjugate was determined by analysis of the total nitrogen content (by Mikrokemi AB, 75228 Uppsala, Sweden, elemental analysis, method MK2062).

Fluorescein isothiocyanate labeling (FITC). Forty µL FITC solution (50 mg , Sigma-Aldrich) was mixed with 1 mL of the dextran conjugate (5 mg), all in 0.02 M borate buffer at pH 9.5. The solution was incubated on a shaker, overnight in the dark and at room temperature. After incubation the solution was purified on a PD-10 column equilibrated with PBS (11).

Lectin and CatDex binding studies. A fluorescein-elderberry bark lectin *Sambucus nigra* (SNA) from Immun Kemi F & D AB Järfälla, Sweden, was used as sialic acid binding control ligand. The cell lines were cultured on chamber slide glass 4-well (Nunc, WVR) for 24 h. The cells were rinsed in 0.15 M NaCl +10 mM HEPES two times. Twenty mg/mL of SNA lectin in NaCl-HEPES buffer, was added to the cells and incubated for 2 h in the dark at room temperature. After the incubation the cells were rinsed with NaCl-HEPES buffer three times. The cells were fixed for 15 min in 70% ethanol and mounted in mounting medium with DAPI (Vectashield, Immunkemi F & D AB, Järfälla, Sweden).

CatDex binding. The cell lines were cultured on chamber slide glass 4-well for 24 h. Twenty-five ml of the FITC-dextran conjugate (~ 62 µg) was added to the cells in 500 µl PBS buffer. The incubation time was 2 h in the dark and at room temperature. After the incubation the cells were rinsed with PBS three times, and then fixed for 15 min in 70% ethanol. The cells were then mounted in mounting medium with DAPI.

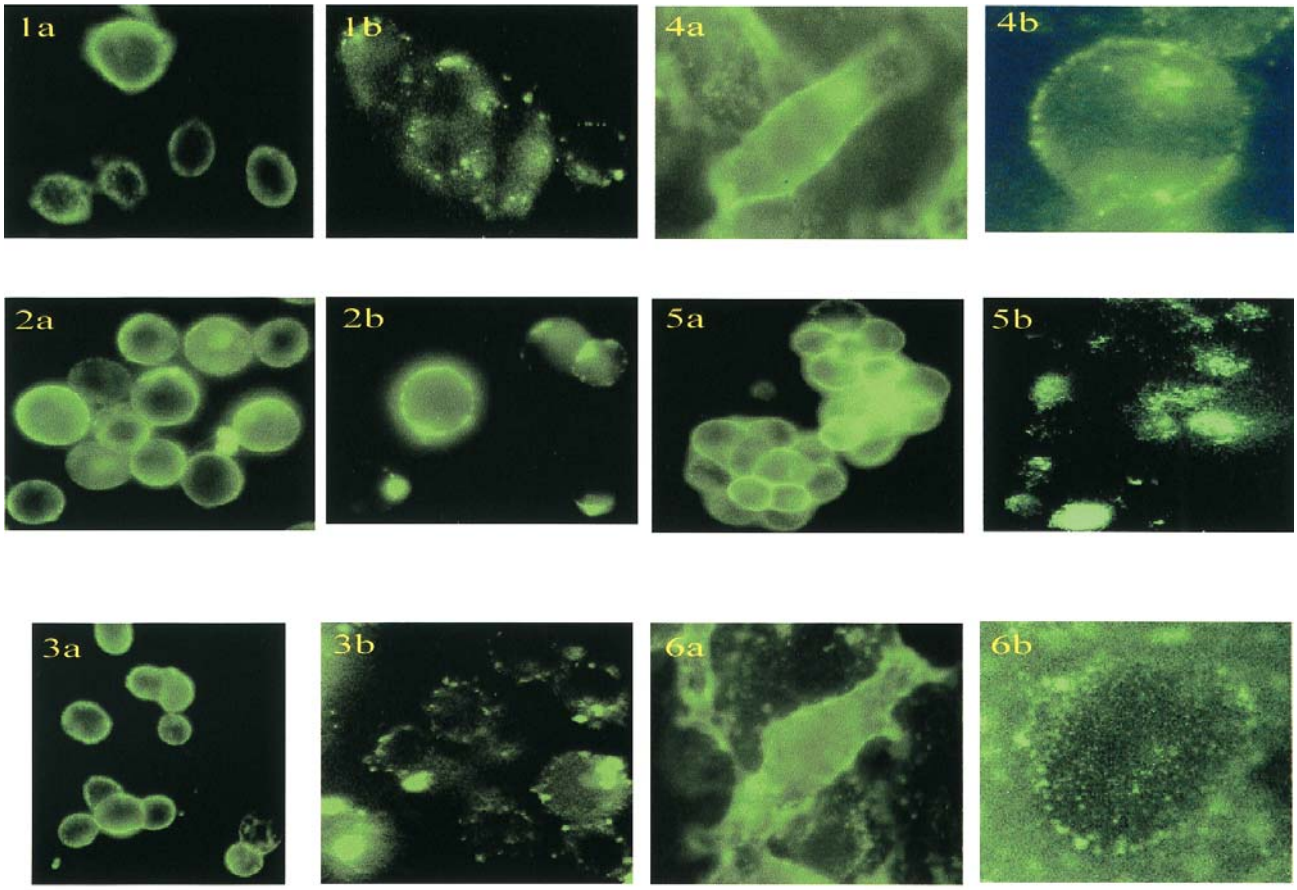

Figure 3. *Examples of typical fluorescence images using FITC-labeled sialic acid binding lectin (a) or FITC-labeled CatDex (b). 1=Urinary bladder cancer (5637), 2=Colorectal adenocarcinoma (Colo205), 3=Breast carcinoma (MDA-MB-453), 4=Prostatic cancer (PC-3), 5-6=Ovarian cancer (OVCAR-3 and SK-OV-3).*

Confocal microscopy (CLSM) was used for studying the interactions with the cells.

Fluorimetric cytotoxicity assay (FMCA). The assay was performed as described before by Larsson and Nygren (12). The following cell lines were used: ovarian cancer, OVCAR-3 and SK-OV-3 (chemotherapy resistant), urinary bladder carcinoma, 5637, colorectal adenocarcinoma, colo 205, breast carcinoma, MDA-MB-453, prostate adenocarcinoma, PC-3, all from ATTC, Manassas, USA. Lung squamous cell carcinoma cell line (1752) was a gift from Prof. Jonas Berg, CCK, Karolinska Institute, Sweden.

Briefly, approximately 10,000 cells/well were seeded (96-well microtiter plates, Falcon, Becton Dickinson, Meylan, France). Dextran and Catdex were added at equimolar concentrations (5 μM and 10 μM). The control wells were given the same amount of PBS. After 72 h incubation the microtiter plates were centrifuged (200 x g for 3 min) and the medium was removed by flicking the plates. The cells were washed in PBS. Fluorescein diacetate (FDA, Sigma, Stockholm, Sweden) was dissolved in DMSO and kept frozen at -20˚C as a stock solution (10 mg/mL). The FDA was diluted in PBS at a concentration of 10 μg/mL and 200 μL was added to each well. The plates were then incubated for 30 min at 37˚C. A 96-well scanning fluorometer (Fluoroscan 2, Labsystems, Helsinki, Finland) was used to count the emitted fluorescence. The data were transferred to a Macintosh SE computer and the results were calculated.

Results

CatDex showed a concentration-dependent growth inhibitory effect (*i.e.* the number of cationic side groups per dextran molecule and the molarity of the conjugate). If the substitution was < 20%, the growth inhibitory effect was small and difficult to reproduce (substitution=% of the glucose units with a coupled amine side group). With 20-22% substitution, the growth inhibition varied between 20-95% depending on the

molarity and the tumor type (Figure 1). Higher substitution resulted in complete cell death in all the cell lines (Figure 2).

Dextran alone did not have any effect on the cell growth (data not shown). The fluorescent images (Figure 3) showed intensive cell membrane interaction. The sialic acid binding FITC-labeled lectin indicated the presence of sialic acid in the different cell lines. The binding was uniform and homogeneous over the cell membrane. FITC-labeled CatDex showed different interaction images. CatDex was binding in a punctillate manner with a tendency to be internalised. If the free amine groups of the CatDex molecule were minimised by labeling with a large excess of FITC, no interaction with the cells could be observed (data not shown).

Discussion

Aberrant glycosylation seems to be present in almost all types of human cancers. Sialic acid is a significant part of this expression. It is usually found at the end position of oligosaccharide chains attached to glycoproteins and glycolipids. Many forms of sialic acid exist, however all of them have a carboxylate at the 1-carbon position that is typically ionised at physiological pH. Because of their terminal position and electronegative charge, they can affect many intermolecular and intercellular interactions (13-15). Increased sialylation in human malignancy ("selectivity") and the electronegative charge, enabling electrostatic interaction with a cationic polymer ("the targeting"), are the foundations for the hypothesis presented in this study. The FITC-labeled lectin could demonstrate the presence of the sialic acids in the different cell lines. Different images were obtained with the FITC-labeled CatDex. The interaction was quite fast and could be observed even after ~30 min. There is an intensive interaction with the cell membranes of the cells. Probably this interaction eventually causes a collapse of the cell, seemingly through membrane rupture. This could be observed even after less than 24 h. The cell killing effect of the CatDex seemed to have a steep "threshold". The cationic dextran needed to have a sufficient amount of cationic groups (>20 % substitution) to be lethal to the tumor cells. This could mean that a minimum interaction between cell and polymer is necessary to obtain a lethal effect. Thus, CatDex may be tumor selective since normal tissue has a lower and "normal" expression of sialic acid . Earlier observations indicate selectivity, where radio-labeled CatDex showed minimal uptake in normal tissue compared to tumor tissue (1).

One important limitation of the tumor targeting hypothesis presented here is that its potential therapeutic usefulness is limited to local, regiolocal or intratumoral applications. It is not suitable for systemic treatment mostly because of the abundant sialic acid expression of the red blood cells and the strong tendency of the unspecific immune system (RES) to remove cationic molecules from the circulation (16-18).

In conclusion, our hypothesis that cell surface sialic acid can be targeted with a cationic polymer, causing membrane collapse and cell death, may indicate a new method of general interest for intra/local/regiolocal treatment of cancer. Clinical studies to explore this concept are pending.

Acknowledgements

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