Advances in Brief

Antitumor Activity of the Type 1 Plasminogen Activator Inhibitor and Cytotoxic Conjugate in Vitro

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Abstract

Many cancer cell lines and cancers overexpress receptor bound urokinase plasminogen activator on the cell surface. The urokinase plasminogen activator/receptor complex binds plasminogen activator inhibitor (PAI-1), the inhibitor triggers a series of events leading to internalization of the entire complex. This mechanism makes a very attractive target for localization and internalization of PAI-1-based cytotoxic compounds in cancer treatment. We investigated the antitumor activity of PAI-1/A-chain cholera toxin in vitro. Fibrosarcoma-derived HT1080 cells treated with PAI-1 conjugate showed at least 4 times higher cell killing than the control KD normal fibroblast cell line.

Introduction

Around the turn of the century, Paul Ehrlich developed the idea of targeted drug delivery. He referred to the target drug as a "magic bullet" that would seek out and attack its target without damaging any other tissue (1, 2). If the cytostatic compound is made more tumor specific by this method, the therapeutic index is automatically increased and thus also is the efficacy of treatment. It is therefore not surprising that antibody-drug conjugates are considered a way to increase significantly the selectivity of treatment. In most cases drug or toxin conjugates must be internalized to act. Internalization of cell surface-bound antibodies, however, do not necessarily happen in every case (1). In addition, most monoclonal antibodies applied in immunocytotoxic research and clinical applications are of murine origin (3). This leads to negative side effects from allergic reactions to anaphylactic shock (1, 2).

The most important consideration in the selection of a cancer-specific antigen is the number of cancer cell lines that express or overexpress it. The most notable property of cancer is its ability to invade and to metastasize. The invasive properties of a particular tumor depend on the production of degenerative enzymes such as metalloproteinases, cysteine and serine proteinases able to degrade the extracellular matrix. Biochemical markers of malignant tumors should be found among the proteolytic enzymes. Indeed, highly metastatic cells synthesize various classes of degenerative enzymes and release them at higher concentrations or activities than their normal counterparts (4, 5). In a number of different tumor models, as well as in tissues derived from human malignancies, a direct correlation has been found between levels of urokinase plasminogen activator activity and/or concentration and metastatic potential (4–7). The uPA can be secreted to intracellular spaces or it can be bound to the uPA receptor on the cell surface. The bound enzyme is not internalized or rapidly degraded. Receptor-bound uPA can still convert plasminogen to plasmin, creating areas of high proteolytic activity in the vicinity of cancer cells. The receptor-bound uPA can bind plasminogen activator inhibitor. The binding is with high affinity (10^{-13} \text{M}) and only then is the PAI-1/uPA/uPAR complex internalized and most likely degraded in the lysosomes. The receptor is probably recycled, and products of degradation are released to the cytosol (8, 9). Since uPA is so commonly overexpressed on the cell surface of cancer cells, we hypothesize that it can serve as a tumor-associated antigen to distinguish between malignant and normal cells on the molecular level. PAI-1 will be used to localize the cancer cells. We hypothesize that cancer cells will be properly recognized and killed by the following mechanisms: (a) PAI-1 and a cytotoxic component coupled to the carrier will bind in high numbers to the cancer cells; (b) upon binding to the receptor-bound uPA, the PAI-1 will trigger a series of events leading to internalization of PAI-1/uPA/uPAR/carrier/cytotoxic complex. The complex will be dissociated in the lysosomes, the receptor will be recycled and the cytotoxic component will be released to the cytosol.

Using the PAI-1 colloidal gold as a carrier and A-chain of cholera toxin conjugate we observed at least 4 times higher cell killing of fibrosarcoma HT1080 [expressing 22.7 ± 2.0 (SD) IU/10^{6} cells of receptor bound uPA] than normal fibroblastic KD cells [expressing 0.2 ± 0.1 IU/10^{6} cells of receptor bound uPA]. These results demonstrate the therapeutic value of localization and destruction of the cancer cells by the proposed method. Truncated cholera toxin alone as well as the complete conjugate but cointroduced with very high concentration of uncomplexed PAI-1 (estimated as 50 times higher) do not show any cell killing at all.

Materials and Methods

Colloidal Gold Preparation. The colloidal gold was prepared as described previously (10). In brief, the 80 ml of 0.01% tetrachloroauric acid were reduced by 4 ml of 1% trisodium citrate with 0.03 ml of 1% tannic acid at 60°C. Next, the solution was boiled for 2 min and colloidal gold was formed. Under these conditions the solution yielded gold particles with a 12-nm diameter. Binding of Proteins to Colloidal Gold Nanoparticles. The 300 µl of colloidal gold were mixed with 50 µl of PAI-1 solution, 1 mg/ml (1090 from American Diagnostica, Inc., Greenwich, CT). After 3 min, colloidal gold was centrifuged at 60,000 × g for 1 h at 4°C. The

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2 The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; PAI-1, type 1 plasminogen activator inhibitor; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TRITC, tetramethylrhodamine B isothiocyanate; MoAb, monoclonal antibody.
pelleted colloidal gold was resuspended to original volume with phosphate-buffered saline and cholera toxin was added. Binding of the A-chain of cholera toxin (C 2650 from Sigma Chemical Company, St. Louis, MO) was achieved basically in the same manner as binding of PAI-1. The 350 µl of colloidal gold solution bearing PAI-1 were mixed with 50 µl of the A-chain cholera toxin, 0.1 mg/ml. After centrifugation, colloidal gold was resuspended with 2 mg/ml BSA in phosphate-buffered saline and stored at 4°C for future use.

Enzyme-linked Immunosorbent Assay. Testing biochemical activity of the PAI-1/colloidal gold conjugate was done by enzyme-linked immunosorbent assay method (11) (Fig. 1). Briefly, the flat-bottomed polystyrene 96-well microtiter plates were incubated with anti-PAI-1 monoclonal antibody (# 3780 from American Diagnostica, Inc.; concentration, 0.01 mg/ml) in carbonate buffer (0.1 MNa2CO3, pH 9.3, containing 0.4 MNaCl. TRITC was blocked with blocking solution (0.2% BSA, 0.01% Tween 80, and 0.9% NaCl in water), and stored until used.

Conjugation of Fluorochrome to PAI-1. The conjugation was performed as described previously (11). In brief, PAI-1 was dissolved (2 mg/ml) in 50 mm borate, pH 9.3, containing 0.4 M NaCl. TRITC was dissolved in dimethyl sulfoxide (3 mg in 0.2 ml) and then dissolved in 100 ml of borate buffer. PAI-1 was dialyzed against dissolved fluorochrome overnight at 4°C. Next, conjugated PAI-1 was dialyzed against several changes of PBS at 4°C in the dark for 3 more days. Conjugated PAI-1 was stored frozen for future use.

Determination of PAI-1 on Colloidal Gold Particles. To 1 ml of TRITC-conjugated PAI-1, complexed with gold particles, 1 ml of 10% Tween in 0.01 M NaOH was added. The colloidal gold was centrifuged at 60,000 × g for 1 h and amount of PAI-1 in supernatant was determined by fluorescence spectroscopy (12).

Fluorochrome Localization of PAI-1. The living HT1080 cells were incubated with gold sol-bearing TRITC-labeled PAI-1 and A-chain of cholera toxin. The cells were exposed to gold sol for 60 min in serum-free medium. Then cells were washed three times with ice cold PBS, fixed with 100% ethanol, and mounted in glycerol.

Assay of Receptor-bound uPA. The assay was performed as described previously (6, 13). In brief, cells were treated for 3 min at room temperature with 3 ml of glycine-HCl buffer containing 0.1 M NaCl, pH 3.0. The acid eluate was quickly neutralized by mixing 3 ml of glycine-HCl buffer with 0.9 ml of 0.5 M Tris-HCl, pH 7.8. This neutralized buffer was frozen at −80°C until assayed. Next, 4 volumes of buffer were mixed with 1 volume of 5× loading buffer (14) and the proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gel. To retain the enzymatic activity β-mercaptoethanol was not included in the loading buffer, and the samples were not heated before loading. After electrophoresis gel was placed in 500 ml of 2.5% Triton X-100 (2 changes, 45 min each) to remove SDS. Meanwhile, an indicator gel was prepared. The indicating gel contains 50 ml of 1.56% agarose in PBS, 20 ml of casein solution (10 mg/ml), and 2 Sigma units of plasminogen. The gel was formed by mixing agarose (50°C) with casein/plasminogen solution (37°C). The solution was poured into a 16 × 18-cm mold. After the gel hardened, it was laid on the top of the SDS-polyacrylamide gel electrophoresis gel. The unit was sealed with plastic wrap and incubated in a moist chamber at 37°C for 24 h. Lysed zones were easily seen as clear areas surrounded by cloudy rings of und lysed casein. The level of activity was determined from the area of lysed zones (6).

Cell and Cell Culture Conditions. The HT1080 fibrosarcoma cells, expressing a high activity of receptor-bound uPA, and KD normal fibroblasts expressing a very low activity of receptor-bound uPA, were routinely grown in α-minimal essential medium, supplemented with 10% newborn calf serum, l-streptomycin, and penicillin at 37°C in a humidified incubator (5% CO2–95% air). Both cell lines were obtained from The American Type Culture Collection.

In Vitro Survival Studies. Cells were plated in 60-mm tissue culture plates in their medium. One day later, the medium was removed and medium containing the PAI-1/colloidal gold/A-chain cholera toxin and the colloidal gold/A-chain cholera toxin conjugates were added. Cells were treated in triplicate and maintained at 37°C and 5% CO2. The cytotoxicity study was completed in serum-free conditions. The cells were incubated for 48 h in various concentrations of colloidal gold solutions. At the end of the incubation period, cells were washed with PBS (Ca2+ and Mg2+ free), trypsinized, and counted. The results are expressed as the mean cell number from treated plates, normalized to media controls, as a function of concentration of PAI-1 cytotoxic conjugates.

Results and Discussion

Estimating the Minimal Amount of Protein Needed to Stabilize a Gold Sol. The stability of the gold sol in water is maintained by repulsion of the electrostatic charges on each gold particle. The addition of strong electrolytes compresses the ion layers around each particle and allows the particles to approach each other closely. If a critical distance is reached, the particles aggregate and flocculation of the colloidal gold occurs. Proteins adsorbed on the surface of the gold inhibit the electrolyte-induced coagulation of the gold. This characteristic has been exploited in the form of a simple titration to determine the minimum amount of a protein needed to stabilize the gold particles. Flocculation can be judged by a color change from red (stabilized gold) to blue (flocculated gold). Using a series of dilutions of BSA, the minimum stabilizing amount of the bovine serum albumin has been determined for 0.125 mg/ml of gold sol. Since the concentration of PAI-1 used in the gold conjugate was very close to this critical value, all gold conjugate preparations were supplemented with BSA (2 mg/ml) to avoid any unwanted flocculation.

Detection of PAI-1 on the Surface of Colloidal Gold. When colloidal gold particles bearing the PAI-1 were incubated in microtiter plates coated with MoAb against PAI-1, a very light pink color of colloidal gold on the first two wells was observed. After silver enhancing, the gray-metallic color of metallic silver has been detected on all plates, except the negative control ones. As expected, the intensity of the color produced by silver enhancement was proportional to the amount of antigen present in each well.

Detection of the Receptor-bound uPA on the Cell Surface. Only cells that express cell surface receptor-bound uPA will be sensitive to PAI-1-mediated therapy. We checked in triplicate the expression of this enzyme on the surface of normal KD and

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Fig. 1. Enzyme-linked immunosorbent assay for presence of PAI-1 on the surface of colloidal gold. Row 1, colloidal gold containing BSA only; Row 2, colloidal gold containing PAI-1/A-chain cholera toxin (127 µg PAI-1/mg of gold particles). Wells 1–7 incubated with series dilutions of gold; Wells 8 incubated with distilled water to show lack of unspecific silver enhancing.

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malignant HT1080 fibroblasts. The malignant HT1080 expressed 22.7 ± 0.2 IU/10^6 cells of receptor-bound uPA when normal KD fibroblast expressed 0.2 ± 0.1 IU/10^6 cells of the same enzyme.

Detection of Internalized TRITC-labeled PAI-1 by Fluorescence Microscopy. The gold particles complexed with proteins (PAI-1 and A-chain of cholera toxin) are relatively large. This size (on the scale of the living cell) does not guarantee that the particle can be bound to a cell surface and enter into the cell cytoplasm. It is important to know if this binding will be strong enough to keep the gold particle in place and internalize it. To answer this question a fluorescence study of binding and internalization was conducted. Fluorescence has been observed in cytoplasm, and a somewhat higher intensity has been observed in the perinuclear region, where lysosomes have been shown to localize (data not shown). This suggests that the process of internalization was successful and probably was executed by endocytosis as we hypothesized earlier.

Inhibition of Tumor Cell Proliferation. Experiments described before explicitly demonstrated that beside the size and noncovalent binding to the colloidal gold, the PAI-1 is able to deliver the gold sol into the cells. However, it was not clear if A-chain cholera toxin can survive the harsh condition of lysosomes (pH < 5.0 and proteolytic enzymes), leave them, and still be able to effectively poison the cells. Complete cholera toxin enters the cells in this way, but A-chain cholera toxin lacks the B-chain. This modification can have a profound effect on the ability of A-chain to persist in its undenatured form through lysosomal pathway. We hypothesize that it can. To rigorously test this hypothesis the HT1080 and KD cells were poisoned for 48 h in serum-free conditions. Serum contains many, very often undefined, proteins. Some of them can bind to gold particles. This can lead to undefined alternative pathways of internalization. To avoid this uncertainty, the experiment was performed in serum free conditions. The cells were divided into three groups. The first contained cells treated with PAI-1/A-chain cholera toxin/gold complex with 7 serial dilutions (127 μg of PAI-1/mg of gold particles; starting concentration of PAI-1, 740 ng/ml). We expected to observe differences in the cell killing between cancer and normal cells. Concentration-dependent cell killing has been observed. More fibrosarcoma-derived cells were killed than normal fibroblastic ones. The second group contained cells treated with A-chain cholera toxin/gold complex. We expected that this complex would not be internalized into the cells, since it was not equipped with molecules responsible for internalization. No cell killing was observed. The third group contained cells treated with PAI-1/A-chain cholera toxin/gold complex and treated with excess unbounded PAI-1 (estimated amount, 50 times more than the cytotoxic component). We expected that the high amount of free PAI-1 will saturate uPAR/uPA complex on the cell surface and abolish the cytotoxic effect of the PAI-1/A-chain cholera toxin/gold complex. No cell killing was observed. The results are shown in Fig. 2.

As we expected, the result stated above demonstrated the therapeutic value of localization and destruction of cancer cells by the proposed method. This concept of cancer therapy is based on the ability of PAI-1 to locate cancer cells. Therefore, the PAI-1 conjugated with toxins can function in the same way as antibody-drug conjugates do. If PAI-1 possesses only this property, it can be eventually used in cancer therapy. However, PAI-1 has several advantages over antibody-mediated therapy. The PAI-1, upon binding to the cell surface, will initiate a series of events leading to active delivery of the cytotoxic component into the cell. The second advantage of the proposed method is the human origin of plasminogen activator inhibitor. The PAI-1 part of conjugated cytotoxin will cause very little, if any, immunogenic response.

The ultimate goal of this work was to demonstrate the effectiveness of the PAI-1 in recognition of cancer cells and the proficiency of the plasminogen pathway in delivery of the cytotoxic compound into the cell. This goal has been reached. However, it must be mentioned that, as toxin, the cholera toxin has been used only for economical and safety reasons. The compounds used here as a model are not necessarily the best for therapeutic purposes. Cholera toxin was not able to kill all cancer cells. Complete cancer cell destruction is likely by using much stronger cytotoxic compounds like ricin or cobra venom factor. The protein carrier, if needed, can be replaced or modified too. The selection includes polymer and metallic nanoparticles, liposomes, and artificial cells.

In-depth toxicological studies are needed to fully prove therapeutic value of this method. Particularly critical in the delineation of PAI-1-mediated toxicities will be the binding of PAI-1 cytotoxic conjugates within the normal tissues expressing uPA and uPAR. However, in conclusion, we believe that therapies utilizing cytotoxic designed to target cells expressing cell surface antigens will provide a viable alternative approach to conventional cancer treatments. Toxins coupled to fibroblast growth factor or interleukin 2 are already in different stages of preclinical or clinical trials, and some excellent clinical responses have been reported (15, 16).

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References

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