Cytotoxic Activity of a Tumor Protease-Activated Pore-Forming Toxin

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Equinatoxin II is a pore forming toxin produced by the sea anemone Actinia equina. It is able to kill very unspecifically most cell types by the membrane-perturbing action of an amphiphilic α-helix located at its N-terminal. A normally active N-terminal mutant, containing one single cysteine in the amphiphilic α-helix, becomes totally inactive when it is bound to avidin via a biotinylated linker. By choosing, as a linker, a peptide containing a tumor protease cleavage site, we were able to construct an enzymatically activatable conjugate which should be selective for tumor cells. The introduced cleavage site was designed in order to be digested by both cathepsin B and matrix metalloproteases (MMPs). We confirmed that this conjugate could be activated in vitro by cathepsin B and MMPs. After having measured the enzymatic activity of fibrosarcoma and breast carcinoma cells, we analyzed the cytotoxic effect of the conjugate on the same lines and on human red blood cells (HRBC) as controls. We found that the conjugate was activated, at least in part, by the tumor cell lines used, whereas it was inactive on HRBC. That the activation process was dependent on the enzymatic action of cathepsin B and MMPs, was indicated by three lines of evidence: (1) binding occurred normally on all type of cells including HRBC which however were insensitive being devoid of enzymes; (2) the cytotoxic effect correlated with the amount of cathepsin B activity expressed by the cells; (3) conjugate activation was reduced by specific inhibitors of cathepsin B and MMPs. These results demonstrate the possibility of tumor cell killing by a pore-forming toxin conjugate specifically activated by tumor proteases.

INTRODUCTION

Immunotoxins and mitotoxins are chimeric molecules in which specific ligands are coupled to toxins. If the ligand moiety is specific for a tumoral cell line, the chimeric toxin can kill those cells selectively. Several reviews describe the construction and the activity of chimeric toxin produced by genic fusion or chemical linking to either antibodies (immunotoxins) or growth factors (mitotoxins) (1, 2).

The toxins usually used for conjugation are derived either from bacteria (e.g., Pseudomonas exotoxin A or diphtheria toxin (3, 4)), or from plants (e.g., ricin or abrin (5)). Both types of toxins kill cells by inhibiting protein synthesis (6). To exert their effect these complexes must enter the cell, where only a portion of the internalized immunotoxin escapes degradation and reaches the cytoplasmic target. In some cases internalization can be a rate-limiting step, which reduces the immunotoxin efficacy. One way to overcome this problem is to use membrane-active toxins (7), such as hemolysins and cytolsins (8, 9). These pore forming toxins attack the lipid phase of cell membranes disintegrating vital membrane functions. They could provide very powerful antitumoral drugs, when conjugated with an appropriate targeting molecule. Some examples of the construction of such molecules have been reported, using membrane damaging toxins of different origin e.g. from animals (cytolysins from sea anemones (10–13), plants (Pyricularia thionin (14)) or from bacteria (Bacillus thuringiensis δ-endotoxin (15), Staphylococcus aureus α-toxin (16), Bacillus anthracis anthrax toxin (17)). However, the main problem of most of the immunotoxins constructed with membrane active proteins is the poor cellular specificity associated to the capacity of the toxin to bind to almost any cell membrane. The purpose of this study was to overcome much unspecificity. To this aim we present the construction of a modular inactive conjugate between a sea anemone cytolysin (the EqTII-I18C mutant) and avidin and its activation mediated by tumor cell proteases. To construct this tumor protease-activated pore-forming toxin we chose two classes of proteases, cathepsins and metalloproteinases (MMPs), which are known to be associated to several tumors. Numerous reports in the literature have demonstrated the possibility of the activation of such conjugates by tumor proteases.

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[1] Abbreviations: BMH: bismaleimidohexane; biotin-HPDP: N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; DTT: dithiothreitol; E64: trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA: ethylenediaminetetraacetic acid; EqTII: Actinia equina equinatoxin II.; FBS: fetal bovine serum; GM 6001: N-(2′-[4–(diethylamino)benzyl)-4-methylpen- tanoyl]-1-tryptophan methylamide; HC50: concentration which causes 50% of hemolysis; HIC: hydrophobic interaction chromatography; HRBC: human red blood cells; HT 1080: human fibrosarcoma; IC50: concentration which reduces by 50% viability in a citotoxicity assay; MCP 7: human breast adenocarcinoma; MMPs: matrix metalloproteinases; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS: sodium dodecyl sulfate; ZR 751: human breast carcinoma.

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the literature describe circumstantial evidence that cathepsin B and L contribute to tumor progression and metastasis (18, 19). Cathepsin B is found at higher levels in a number of human cancers (i.e. colorectal, lung and bladder), suggesting that it may be useful as a diagnostic and prognostic marker. The MMPs are involved in a wide range of proteolytic events, in normal and pathological circumstances (20). Pathological processes involving MMPs include tumor growth and migration (21).

This approach is intended as a conceptual proof to demonstrate that a relatively simple chemistry could be used to conjugate a cytolytin to a ligand moiety via a peptide containing cleavage sites of tumor-associated proteolytic enzymes. We further demonstrated that the conjugate could be specifically cleaved by these enzymes, leading to its possible application as an antitumoral drug. The advantage of the activable pore-forming system, for the construction of immunotoxins, is the reduction of the unspecific activity. In this way two mechanisms of target would be present in a single hybrid molecule, the recognizing of tumor cell through the ligand moiety and the specific activation of the toxin by the tumor-associated proteases.

EXPERIMENTAL PROCEDURES

Materials. *Actinia equina* protein toxin I-II18C mutant was produced in an *E. coli* expression system as described previously (22). N-{6-(Biotinamido)hexyl}-3′-(2′-pyridyl-dithio)propionamide (biotin-HPPD) and bismaleimidohexane (BMH) were purchased from Pierce, the peptide NH2-C-N-K-S-R-L-G-L-G-K-biotin-COOH was from Primm (Milan), cathepsin B, matrix metalloproteinase 7 (MMP-7), avidin, N-{(2′)-(hydroxamidocarbonylmethyl)-4-methylpentanoyl}{-l-tryptophan methylmamide (GM 6001) and cathepsin B substrate III (Z-R-R-AMC) from Calbiochem; 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman’s reagent) from Acros; trans-epoxyssuccinyl{l-lyeucylamido}{-4-guanidinobutane (E64), dithiothreitol (DTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. The metalloproteinase specific substrate Mca-P-L-G-L-Dap(Dnp)-A-R-NH2 was purchased from Bachem (catalog number M-1895), while Pronex (porcine hydrolyzed collagen of average mass 20 kDa) was purchased from Pentapharm Ltd.

Cell Lines. Cells used in these assays were ZR 751 (human breast carcinoma), MCF 7 (human breast adenocarcinoma) and HT 1080 (human fibrosarcoma) obtained from the Istituto Zooprofilattico Sperimentale (Brescia, Italy). After every week new cells were thawed and cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin. The MCF 7 cell line was also cultured in RPMI 1640 (ZR 751) or Eagle’s minimum essential medium (MCF 7 and HT 1080) with 10% fetal bovine serum (Gibco), 2 mM glutamine and 0.15 mg/mL gentamicin.

Conjugation via HPPD. EqTII-I18C was incubated with biotin-HPPD in a 1:8 molar ratio in 20 mM Na2HPO4, 150 mM NaCl, 1 mM EDTA pH 7.4 for 90 min at room temperature. EqTII-I18C, that was S–S linked to biotin via a long spacer arm, was separated from unreacted HPPD using a HIC column (Metachem) equilibrated with 25 mM Na2HPO4, 2M (NH4)2SO4 pH 7; the bilinear gradient of (NH4)2SO4 was decreasing from 100% to 15% in 12 min and from 15% to 0% in 8 min. The concentration of the conjugate among the collected aliquots was estimated by measuring the absorbance at 280 nm and by titrating the hemolytic activity of the fractions before and after reduction with 2.5 mM DTT. The concentration of the conjugate was approximately calculated using a molar extinction coefficient of 132 840 M⁻¹ cm⁻¹ at 280 nm on the basis of a molar composition of one toxin per avidin molecule (confirmed by electrophoretic experiments, see Figure 3E). The conjugate produced with BMH was EqTII-I18C-S-maleimide{-CH2}_{10}-maleimide-S-peptide-biotin_avidin, while by using Ellman’s reagent, or no reagents at all, we obtained the following molecule: EqTII-I18C-S–S-peptide-biotin_avidin.

Enzymatic Digestion. Cathepsin B was first activated with 10 mM DTT at 37 °C for 15 min. For peptide digestion, a 1.2 mM peptide solution was incubated with activated cathepsin B (at final concentration of 3.5 μM) in 30 mM sodium acetate pH 5, or 0.35 mM peptide was incubated with 3.8 μM MMP-7 in 0.2 M NaCl, 50 mM Tris/HCl, 5 mM CaCl2, 20 μM ZnCl2 pH 7.6, both at 37 °C for 2 h. The specificity of the cleavage was checked by adding 50 μM E64 for cathepsin B or 8 μM GM6001 for MMP-7.

For conjugate digestion a 6.3 μM conjugate solution was incubated with activated cathepsin B (at final concentration of 0.9 μM) in 80 mM Na2HPO4 pH 5 or 2.8 μM conjugate was digested by 2.3 μM MMP-7 in 0.2 M NaCl, 50 mM Tris/HCl, 5 mM CaCl2, 20 μM ZnCl2 pH 7.6, at 37°C for 2 h. To check the specificity of the cleavage, parallel experiments were performed by adding 11.4 μM E64 or 4.7 μM GM6001, respectively.

In both cases the products were separated by hydrophobic interaction chromatography under the same conditions used for conjugate separation. The products of the conjugate digestion were also tested by hemolytic assays.

Mass Spectrometry Analysis. Digested peptides (same conditions as described above) were separated on a Jupiter C4 column by Phenomenex with a 15 min linear gradient from 5 to 95% acetonitrile in water. LC-MS analysis was performed on a Hewlett-Packard Series 1100 liquid chromatograph coupled to a Bruker Esquire LC ion trap mass spectrometer equipped with an atmospheric pressure ESI interface. A Rheodyne 7725 (Cotati, CA) injection valve equipped with a 200 μL internal loop was used for the injections. The PDA (photodiode-array) detector (HP Series 1100) was set at a wavelength of 215 nm, and a 7:3 split of the column effluent was used to achieve a flow rate of 300 μL/min into the ESI source.
Hemolytic Assays. Hemolytic activity of EqTII-I18C and different conjugates was determined turbidimetrically at 650 nm with a microplate reader (UVmax from Molecular Devices, Sunnyvale, CA) supported by the computer program SOFTmax. Briefly, human RBC were prepared from fresh heparinized blood by washing thrice (700 g for 10 min) and resuspending in the saline buffer: 140 mM NaCl, 10 mM Tris/HCl, pH 7.4. Finally, the concentration of HRBC was adjusted to an apparent absorbance of 0.1 at 650 nm with the buffer. Toxin and conjugates were 2-fold serially diluted in saline buffer using flat-bottom 96-well microplates, previously treated for 30 min with 0.1 mg/mL Prionex to saturate the unspecific protein binding sites of plastic which can reduce toxin concentration. One volume of HRBC was added to each well, and the time course of hemolysis was followed for 30 min at room temperature. Percentage of hemolysis was determined as follows:

\[
\text{hemolysis} \% = \frac{A_{\text{max}} - A_{10}}{A_{\text{max}} - A_{\text{min}}} \times 100
\]

where \(A_{\text{max}}\) and \(A_{\text{min}}\) represent the absorbance values for intact and completely hemolysed RBC respectively and \(A_{10}\) is the absorbance value in each well after 10 min from the beginning of the kinetic.

Electrophoretic Analysis. SDS–PAGE was performed according to Laemmli using precast minigels with density gradients ranging 8–25% (Amersham Pharmacia, Uppsala, Sweden). A semiautomatic horizontal unit (PhastSystem by Pharmacia) was used. Gels were stained with Coomassie brilliant blue followed by silver stain.

Fluorescence Measurements. Cathepsin B and MMP activities associated to the tumor cells were evaluated using specific fluorogenic peptides and choosing conditions as similar as possible to the citotoxicity test. In fact cells were prepared as in the cytotoxicity assay using the appropriate media without serum. In the citotoxicity test 1 x 10^5 cells were incubated with cathepsin B substrate III (final substrate concentration 40 μM; \(\lambda_{\text{exc}} = 320\,\text{nm}, \lambda_{\text{em}} = 460\,\text{nm}\) at room temperature and fluorescence was measured for 15 h every 5 min with a fluorescence microplate reader (Fluostar, SLT, Austria). In the MMP test, the quantity of cells used was between 0.6 and 0.9 x 10^5 and the specific MMP substrate was at 10 μM. The fluorescence was monitored at \(\lambda_{\text{exc}} = 320\,\text{nm}, \lambda_{\text{em}} = 395\,\text{nm}\) for 30 min at 25 ± 3°C with a photon-counting fluorimeter (SPEX Fluoromax, JY Horiba, Edison, NJ). Ultramicro quartz cuvettes of 100 μL volume and 1 cm path length were used. In both tests the pH was about 7.

Tetrazolium-Based Assay of Cytotoxicity. In vitro toxicity against three tumor cell lines (MCF 7, ZR 751 and HT 1080) was measured by the MTT reduction assay. Cells were grown as monolayers in a humidified air atmosphere with 5% CO2, at 37°C, and when reaching 80% confluence, they were trypsinized (2.5 g/L porcine trypsin in Hanks's balanced salt solution), washed three times, resuspended in the appropriate media (without FBS) and added to 96-well microplates at similar cellular densities. The conjugate, treated with or without 1 mM DTT, was serially diluted (2-fold at each step) with appropriate medium without FBS on 96-well microtiter plates treated with Prionex. In some cases 0.1 mM MMP inhibitor GM6001 was added. All solutions used were filtered for sterility. A range 1.6 to 38 h. MTT was finally added to each well (0.2 mg/mL) and the cells were cultured for two more hours before adding 100 μL of 10% SDS in 0.01 N HCl to dissolve the dark blue crystals. After overnight incubation under the same conditions, the optical density at 575 nm of each well was measured with a microplate reader (UVmax from Molecular Devices).

Percentage of cell viability was determined as follows:

\[
\text{viability} \% = \frac{A_i - A_c}{A_i - A_0} \times 100
\]

where \(A_i\) represents the absorbance value of the sample, \(A_c\) is the absorbance of nontreated cells (control) and \(A_0\) is the blank value.

Binding of the Conjugate to HRBC and Cells. To determine the binding of the conjugate to human red blood cells, the hemolytic activity of 0.19 μg/mL conjugate with or without 10 mM DTT was measured. Erythrocytes incubated with nonactivated conjugate (in the absence of DTT) were sedimented by centrifugation (3 min at 700 g), resuspended in 10 mM Tris/HCl 140 mM NaCl pH 7.4 and treated with or without 10 mM DTT. Binding to ZR 751 and HT 1080 cells was studied performing a citotoxicity assay as described previously. The conjugate was serially diluted (2-fold at each step) beginning from the nontoxic concentration of 1.5 μg/mL, and cells were added. To activate the conjugate 10 mM DTT was added at different times (after 1, 2 and 3 h). Cell viability was then determined by MTT test.

RESULTS AND DISCUSSION

Inhibition of EqTII Activity by Conjugation with Avidin. As a preliminary step biotin was directly linked to EqTII-I18C by using biotin-HPDP. In this case, the addition of an excess of avidin to the biotinylated mixture removed the hemolytic activity (Figure 1, solid circles). Only at the highest concentration (about 40 nM), the fraction of biotinylated mutant treated with avidin showed a modest activity (6% of the DTT treated sample) due probably to the presence of a small amount of nonbiotinylated EqTII-I18C. The inactivation due to avidin was reversible. In fact, it was possible to restore the hemolytic activity by removing it with DTT (Figure 1, open circles). DTT reduced the disulfide bond between the EqTII cysteine and the biotin, thus releasing the active free toxin. The biotinylated EqTII-I18C appeared...
to be slightly less hemolytic (HC_{50} = 5.7 nM) than EqTII-I18C released from the conjugate by DTT (HC_{50} = 2.9 nM). This could be due to a steric effect introduced by the presence of biotin which is removed by DTT. These experiments demonstrated that avidin, bound to the N-terminal of EqTIII, was able to block the hemolysis by introducing a sterical hindrance into the mutant (the molecular weight of avidin, 66 kDa, is roughly thrice that of the toxin).

**Construction of an Enzymically Activable Conjugate. Choice of the Peptide.** To conjugate EqTII-I18C to avidin we next decided to use a biotinylated peptide containing a general cleavage sites for matrix metalloproteinases (MMPs) and cathepsin B, two classes of enzymes that are associated to tumor cells. The peptide sequence is given in Figure 2. The presence of one cysteine at the N-terminus allowed its coupling to the EqTII mutant; it was followed by an asparagine, that increased solubility and served to link the cysteine to the cathepsin B cleavage site: KSRL [16, 23, 24]. The general MMP cleavage site, LGL [21], was inserted next. We have chosen this site because it could be cleaved by a large number of soluble MMPs (for example MMP-2, MMP-7, MMP-9) and by membrane MMPs such as MMP-14 [25, 26]. Moreover, the LGL sequence is present in the fluorescent metalloproteinase specific substrate we used to evaluate the MMPs activity of our cell lines. The remaining amino acids, GK, made the linking to biotin more flexible and introduced one additional charge to further increase solubility. Biotin was used for avidin binding.

That the enzymes could cut this peptide was confirmed by exposing it to human cathepsin B or MMP-7 and separating the digested products by hydrophobic interaction chromatography (Figure 2A and 2B, respectively). We chose MMP-7 as a representative member of the MMP family. The peak corresponding to the peptide disappeared almost completely from the chromatogram when incubated with cathepsin B or with MMP-7. Meanwhile, two main peaks appeared, marked by arrows in Figure 2, indicating digestion. On the contrary, when the peptide was incubated with the enzyme and the appropriated inhibitor, no cleavage was observed (comparison between the dashed and the dashed—dotted line, Figure 2A). We further demonstrated that the enzymatic cleavage was specific through LC-ESI-MS measurements. From the digestion with MMP-7 we found two major peaks which UV absorption well correlated with the MS signal. In fact, the fragments for LGK-biotin and LGLGK-biotin were detected as M + Na\(^+\) ions at the nominal mass of 678 Da and at 848 Da respectively, as expected from the complete digestion. Similarly, when we treated the peptide with cathepsin B, three major peaks have been detected both by UV detector and by ESI ionization. In particular, by the latter technique [M + Na\(^+\)] ions corresponding to the fragments (i) K-biotin at m/z 508, (ii) GK-biotin at m/z 565 and (iii) LGK-biotin at m/z 565 have been found in order of increasing elution time. These fragments derive from the carboxypeptidase action of the cathepsin B, which is preferred in the presence of a free C-term. It was possible to detect also LGLGK-biotin (m/z 848 as [M + Na\(^+\)]\(^+\) and CNKSR (m/z 607 as M\(^+\)), the fragments expected from the classical endopeptidase activity of cathepsin B (16).

**Preparation and Characterization of the Conjugate.** EqTII-I18C was S–S linked to the biotinylated peptide. The disulfide bond between the cysteine of the mutant and the cysteine of the peptide, which formed spontaneously at low rate, was favored by activating the mutant with Ellman’s reagent. As an alternative to the disulfide bond, we used BMH to form a thioether bond. This reagent increased the toxin–avidin distance, allowing the construction of a conjugate potentially more sensitive to the protease action. Furthermore, BMH was chosen to have a control conjugate free of the reducible disulfide bond. Unfortunately, this goal was only partially obtained since, from hemolytic assays, we noticed the permanence of some DTT-induced activity, which could be attributed to the presence of spontaneously formed conjugate (not shown).

The toxin was first separated from the excess of reagent and then from the excess of peptide by filtration on Sephadex G25. The resulting fraction was incubated with an excess of avidin to give a conjugate which was separated from unreacted components by HIC (Figure 3A). As indicated by comparison with the chromatograms of free proteins, the conjugation mixture contained free toxin (nonbiotinylated and, possibly, some biotinylated, but not avidin bound), avidin and conjugate (marked by an arrow). In some cases avidin eluted as a double peak, the minor one corresponding to avidin bound to the peptide without toxin. All peaks, collected after HPLC, were tested for the hemolytic activity before and after
reduction with DTT. This analysis confirmed the presence of the conjugate in the peak indicated by the arrow. In fact the protein in that fraction was able to permeabilize the erythrocyte membrane only after DTT reduction of the S–S bridge (Figure 3D). An HC$_{50}$ of 1.1 nM of toxin was determined, for the reduced conjugate, after 30 min. As a control we observed that, under the same conditions, avidin was not hemolytic at all whereas the hemolytic activity of the mutant itself was always expressed independently of DTT. SDS–PAGE analysis of the purified conjugate under nonreducing conditions (Figure 3E, lane 2) confirmed the presence of high molecular weight compounds (around 90 kDa), as expected from the binding of one molecule of toxin (lane 3) to one molecule of avidin (lane 4, monomeric form). A minor band corresponding to monomeric avidin was also present. From the HPLC profiles (Figure 3A) we estimated that about 25% of the EqTII-I18C used was present in the conjugate. The yield varied from one preparation to another and sometimes was higher. The HPLC-purified conjugate was rechromatographed and it appeared to be free of other components (not shown).

**Activity of the Conjugate by Human Cathepsin B and Matrix Metalloproteinase 7.** That either human cathepsin B or MMP-7 were able to cleave the conjugate had been shown by HIC of the digestion products (Figure 3B and 3C). The HPLC profile demonstrated that cathepsin B and MMP-7 could cleave the conjugate releasing free avidin and toxin. In both cases, the first peak obtained (centered at about 13 min) corresponded to avidin (compare Figure 3A) while the peak corresponding to the free toxin was centered at about 22 min. The positions of the enzymes are marked by an arrowhead in Figure 3B and 3C, indicating that the cathepsin B peak (Figure 3B) was observed at the same position as the conjugate peak. The latest peak was still present when we treated it with the inhibitors (dashed line, Figure 3B and 3C), showing that the cleavage was specific.

In agreement, the hemolytic assay (Figure 3D, line marked by cathB) clearly demonstrated that cathepsin B can cut the linker peptide and restore the hemolytic activity of EqTII-I18C. The HC$_{50}$ after 30 min, due to the mutant released by cathepsin B, was 1.0 nM (concentration of toxin), almost the same as that obtained by treating the conjugate with DTT (Figure 3D, line DTT). In a similar way we found that also trypsin could cleave the conjugate producing a hemolytic fragment (HC$_{50}$ = 0.8 nM; data not shown), thus confirming that the peptide inside the conjugate was accessible by enzymes.

**Enzymatic Activity of ZR 751, HT 1080 and MCF 7 Tumor Cell Lines.** All cell lines used were characterized for the presence of MMPs and cathepsin B activity in our experimental conditions using specific fluorogenic peptides (Figure 4). Cells were trypsinized, washed and resuspended in the appropriate culture media (without FBS) to reproduce similar conditions as the cytotoxicity assay. After addition of cells, the fluorescence of the substrate was monitored (Figure 4). The MMP activities, calculated as the relationship between the initial slopes and the total number of cells in the assay, of the three cell lines were quantitatively similar. The ratio between the enzymatic activities of ZR 751 and MCF 7, with respect of that of HT 1080 cells, was approximately one in both cases, with values ranging from 0.8 ± 0.2 for ZR 751 to 1.1 ± 0.6 for MCF 7 (mean of three experiments). Cathepsin B activities, instead, were different in the three lines. In particular, the higher activity was found for MCF 7 (10.0 ± 3.6 higher than HT 1080) and an intermediate value for ZR 751 (3.4 ± 0.8 higher than HT 1080). In our experimental conditions, MMP activity was always better than cathepsin activity (Figure 4).

To check if the resulting activity was due to the specific enzymes or if there was any unspecific cleavage, we...
added appropriate inhibitors. The enzymatic activities were significantly reduced by the MMP inhibitor. As an example, MCF 7 MMP enzymatic activity was inhibited from 47 to 98\% when the GM6001 inhibitor ranged from 3 to 25 \textmu M. Some inhibition was also verified when the cathepsin inhibitor E64 was added. In this case MCF 7 enzymatic activity was inhibited by 16 or 30\% using the inhibitor at a concentration of 50 and 100 \textmu M, respectively. This suggested that the measured enzymatic activity was due, at least in part, to the specific enzymes studied.

**Cytotoxic Effects of the Conjugate on the Tumor Cells.** Contrary to the case of erythrocytes (Figure 5A, insert), even in the absence of DTT the conjugate displayed cytotoxic activity on the tumor cell lines studied (Figure 5). The MCF 7 cell line was the most sensitive, with an IC\textsubscript{50} of 0.3 \textmu g/mL of the conjugate in the absence and 0.2 \textmu g/mL in the presence of DTT (Figure 5A). Both the other cell lines were less affected by the conjugate; in particular, the IC\textsubscript{50} was 5.8 \textmu g/mL for ZR 751 and 14.2 \textmu g/mL for HT 1080 cells (Figure 5B and C).

This correlates nicely with the cathepsin B activity (see Figure 4), but not with MMP activity, that was similar in all three cell lines. However, by studying the effect of the specific MMP inhibitor GM6001 on the conjugate activity toward MCF 7 tumor cells, we observed that some MMP enzymatic activity was also involved in cytotoxicity. In fact, in the presence of 100 \mu M inhibitor and 0.2 \mu g/mL of conjugate (Figure 6) we observed that the cell fraction killed by the conjugate treated with the inhibitor was one-fifth in comparison with the one without inhibitor.

**Binding of the Conjugate to the Cell Membrane.** Finally, we wanted to evaluate if the conjugate was able to bind to the cells even when not permeabilizing them (Figure 7). The EqTII-I18C\_S\_S-peptide-biotin avidin conjugate was added to HRBC at the concentration of 0.19 \mu g/mL (Figure 7A). The cells were then washed, resuspended in physiological buffer and treated or not with DTT. Only under reducing conditions we observed hemolytic activity, indicating that the conjugate was indeed bound to the erythrocyte membrane but could not permeabilize them until activated by DTT. In this way, we could demonstrate that membrane-bound conjugate is stable and active for a long time; in fact even when DTT was added after 80 min, the permeabilization was complete. Similarly we found that, at sub-cytotoxic concentrations, the same conjugate could bind to tumor cells and that it could be activated by DTT even after 1 h from the washout (Figure 7B). We tested the time-course of this availability, finding that after 2 h the
conjugate was no longer available for cleavage. This was true for ZR 751 (Figure 7) as well as for HT 1080 cells (data not shown).

CONCLUSIONS

The biotin–avidin conjugation method, pioneered for anticancer immunotoxins (27) and already exploited also for the construction of an antiparasite (11) and an anticancer immunotoxin (12), is presented here in a version with a linker-peptide. This has the advantage of being very versatile. Simply by changing the sequence of the biotinylated peptide it is possible, at least in principle, to activate a pore-forming toxin like EqTII onto different cell types. In fact, by introducing in the peptide the cleavage sequence for an enzyme expressed by a certain tumor cell, or even by tumor surrounding stromal cells, only in the proximity of that cell will the peptide be cleaved. The killer toxic moiety will be released, directly killing the tumor cell itself or the cells which support the tumor.

The use of peptides to construct immunotoxins was already described (28, 29) but only to help the transport of the toxic moiety, or to improve the extent of covalent conjugation between an antibody and a toxin. Here we propose a new system, which is useful to specifically target the conjugate. In fact we could show that a peptide, containing the cleavage sites for cathepsin and MMP, renders the conjugate active against several tumoral cell lines expressing those enzymes, but not against erythrocytes, which are devoid of them. For the conjugation we selected EqTII-I18C mutant, because this links the avidin molecule to the N-terminal α-helix of EqTII (30), which is a crucial element for the toxin–membrane interaction and should be free to move for activity (31–33).

The most sensitive tumor cells were found to be MCF 7 which also express the highest enzymic activity. The extent of cytotoxicity on the different cell lines correlated with the amount of cathepsin B activity expressed; however, at least on MCF 7 cells, the conjugate activity was also significantly inhibited by the specific MMP inhibitor GM6001, suggesting a contribution of both cathepsin B and MMP to the conjugate activation. This conclusion is supported by the fact that erythrocytes, which do not possess such enzymes, are not damaged by the conjugate. At subtoxic concentrations, the conjugate is still capable of binding to the cells in a form that can be activated, e.g. by DTT, for at least 1 h. Such binding is not cell specific and may be mediated by the toxin moiety. To avoid such unspecific binding we are planning to select for the conjugation a different EqTII mutant, which could not bind to the cell surface. Attaching a biotinylated peptide to the avidin of such conjugate, we could produce a promising immunotoxin with two levels of specific activation: antibody targeting and enzymic activation.

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LITERATURE CITED


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