Absence of Lysosomal Cleavage in the Cytotoxicity Mechanism of an Immunoconjugate Composed of Anti- α -fetoprotein Monoclonal Antibody and Vindesine Analog

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The effect of lysosomal enzyme inhibition on the cytotoxic activity of an immunoconjugate composed of anti- α -fetoprotein monoclonal antibody and vindesine analog (VDS) was studied *in vitro* using human tumor clonogenic assay (HTCA). Addition of the lysosome enzyme inhibitors, leupeptin and ammonium chloride, to the HTCA system had little influence on the cytotoxicity of this immunoconjugate. In separate experiments, no released VDS was detected by HPLC after incubation with the supernatant of rat liver homogenate without inhibitor. These results show that the immunoconjugate may bypass the lysosomal process and exert its activity as an intact or similar form.

Key words α -fetoprotein; human tumor clonogenic assay; lysosome; immunoconjugate; monoclonal antibody; vindesine

Monoclonal antibodies (MoAbs) are superior carriers for delivering anticancer drugs selectively to neoplastic cells. The cytotoxic activity of an immunoconjugate composed of anti-tumor MoAb and anticancer drug is considered to occur *via* one of the following three mechanisms: a) the immunoconjugate incorporated into the target cells is transferred to lysosomes and thereafter liberates an active drug; b) the immunoconjugate directed to the target cells is not incorporated into the cells but releases an active drug outside the tumor cells; or c) the immunoconjugate incorporated into the target cells is not transferred to lysosomes and bypasses the lysosomal process, then damages the cells in an almost intact form.

The first mechanism is considered to be the main one for many types of immunoconjugates.¹⁻³⁾ Several features (low pH, rich digestive enzymes, *etc.*) specific to lysosomes are utilized to develop immunoconjugates. For example, *cis*-aconitic acid, which is easily cleaved in the acidic condition, was adopted as a linker.⁴⁾ Tripeptide (Leu-Ala-Leu) and tetrapeptide (Ala-Leu-Ala-Leu), which are susceptible to the lysosomal enzymes, have also been used for the same purpose.⁵⁾ As for the second mechanism, Starling *et al.* reported that the immunoconjugate (anti-TAG-72 MoAb B72.3 and vinca alkaloid) released a drug at the cell surface after binding to a non-internalizing TAG-72 antigen.⁶⁾ And Lavie *et al.* reported that non-internalized immunoconjugates (anticarcinoma MoAb L6 and daunomycin) could release free drug at the tumor tissue because the pH of the tumor tissue is 0.5–1.0 unit more acidic than that of healthy tissues.⁷⁾ The third mechanism was proposed to explain the cytotoxic activity of immunotoxins. If an immunotoxin is incorporated into lysosomes, it will be easily digested by lysosomal enzymes and lose its activity. Thus, the immunotoxin must bypass the lysosomal process to act against the tumor cells.^{8,9)} However, few immunoconjugates have been reported whose cytotoxicity can be explained by this mechanism.

Recently, we reported that an immunoconjugate composed of anti- α -fetoprotein MoAb (anti-AFP MoAb),

35G, and a vindesine (VDS) analog was one hundred times more active against human hepatoma cells (HuH-7) than VDS alone in a human tumor clonogenic assay (HTCA).¹⁰⁾ In the present study, we investigated the effect of lysosomal enzyme inhibitors on the cytotoxic activity of this immunoconjugate, VDS-35G. The purpose of this study was to clarify whether lysosomal enzymes were involved in the selective and excellent action of VDS-35G conjugate against HuH-7 cells.

MATERIALS AND METHODS

Materials and Synthesis of Immunoconjugate The production of anti-AFP MoAb, 35G, was reported in the previous paper.¹¹⁾ Cells from a human hepatoma cell line (HuH-7)¹²⁾ was used as the target cells, and HuH-7 cells were cultured in serum-free medium. Leupeptin and ammonium chloride were purchased from Sigma (St. Louis, U.S.A.) and Wako (Osaka, Japan), respectively. Other chemicals were of reagent grade.

The synthesis of the immunoconjugate, VDS-35G, was based on the procedure described for VDS-bovine serum albumin.¹³⁾ The detailed process is shown in the following Chart 1.

***In Vitro* Cytotoxic Assay** The *in vitro* cytotoxicity of the immunoconjugate was evaluated using a modified HTCA. HTCA, a colony assay developed by Hamburger and Salmon,¹⁴⁾ was modified to evaluate the cytotoxicity of immunoconjugates. After exposure of HuH-7 cells to the immunoconjugate for a given period (contact time), the cells were centrifuged and rinsed with cell-free conditioned medium (CFCM) to remove the unbound immunoconjugate. Then the cells were dispersed in CFCM and cultivated in double layer soft agar gel for two weeks. The details of this procedure were described in our previous paper.¹⁰⁾ Leupeptin or ammonium chloride was added to the medium during both the drug exposure and the cell culture.

Stability of Immunoconjugate in Liver Homogenate A male Wistar rat (Shionogi, Osaka, Japan) was anesthe-

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Chart 1. Synthetic Procedure of VDS-35G

Reaction of vinblastine (1) with hydrazine gave 4-desacetylvinblastine monohydrazide (2). 4-Desacetylvinblastine acid azide (3) was obtained from 2 by nitrosation. Reaction of anti-AFP MoAb (35G) dissolved in 0.2 M borate buffer (pH 9.0) with 3 gave VDS-35G. VDS-35G was dialyzed with 0.1 M PBS and purified using Sephadex G-100.

tized with ether. The liver was excised and washed to remove blood with saline. Six grams of the liver was homogenized with 15 ml of saline in an ice bath by Polytron (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged (3000 rpm, 10 min) and the supernatant was diluted with an equivalent volume of saline. Three ml of the dilute supernatant was added to 2 ml of 0.5 M phosphate-buffered saline (pH 5.5) and this was used as the test incubation medium. Thirty μ g (VDS equivalent) of VDS-35G was added to 0.2 ml of this medium, which was then incubated at 37°C for a maximum of 25 h. Aliquots of the medium were sampled at 2, 8, and 25 h, and were mixed with an equal volume of methanol and centrifuged at 4°C. VDS concentration in the supernatant was measured by HPLC.

The destruction of lysosomes was certified by the activity of acid phosphatase in the homogenate compared with it in serum. The activity of acid phosphatase was measured by an assay kit (Wako, Osaka, Japan).

HPLC VDS and its analogs in the incubation medium were measured under the following HPLC conditions. Apparatus: Waters M-6000A (Millipore, Milford, U.S.A.), column: Nucleosil 100-CN (Chemco, Osaka, Japan), 10 μ m, 4 mm i.d. $\times$ 25 cm, Eluent: 0.01 M phosphate buffer (pH 3.0)/acetonitrile = 25/75, flow rate: 1.5 ml/min, injection volume: 30 μ l, detector: UV 220 nm (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Targeting Efficacy of VDS-35G VDS exhibits its cytotoxic activity by binding to the protein tubulin and thereby inhibiting tubulin polymerization to form microtubules.¹⁵⁾ Thus, VDS must be incorporated within the cells to exert its effect.

After HuH-7 cells were exposed to VDS-35G for 60 min at 37°C, the excess unbound VDS-35G was removed from the medium and the cells were cultured in soft agar gel

without VDS-35G. In this assay, the effective concentration of VDS-35G was more than 0.5 ng/ml (VDS equivalent), one-hundredth that of VDS (50 ng/ml). However, neither MoAb (35G) itself nor a nonspecific conjugate (VDS-n-IgG) in which VDS was linked to normal mouse IgG was effective against HuH-7 cells. VDS-35G was ineffective against HuH-13 cells producing no AFP. These data suggested that the anti-AFP MoAb, 35G, was a suitable carrier for targeting AFP-producing hepatoma cells.¹⁰⁾

To investigate the contact time required for VDS-35G to exhibit cytotoxicity against HuH-7 cells, the exposure periods were varied from 5 to 120 min. The maximum cytotoxic activity of VDS was exhibited after it had been in contact with HuH-7 cells for 1 h. However, that of VDS-35G was seen after only 10 min of exposure.¹⁰⁾ These results indicated that VDS-35G could bind to AFP on the cell surface and be incorporated into the cells. Generally, specific ligands bound by receptor proteins on the cell surface are incorporated into the cells by endocytosis and sent into the digestive pathway by lysosomal enzymes.¹⁶⁾ If VDS-35G incorporated within the cells was kept in the same pathway as the proteins taken up by receptor-mediated endocytosis, it would be degraded by the lysosomal enzymes. We evaluated whether lysosome enzyme inhibitors affected the cytotoxicity of VDS-35G against HuH-7 cells in HTCA to determine the fate of VDS-35G within the cells.

Effect of Leupeptin Leupeptin strongly inhibits cathepsin B, which is one of the major lysosomal enzymes.¹⁷⁾ In the present study, leupeptin was added to the HTCA medium. Kyriakos *et al.* reported that the ID₅₀ of leupeptin against cathepsin B was 0.44 μ g/ml (1.03 nM), and that 0.1 mM of leupeptin strongly inhibited the *in vitro* degradation of MoAb (MJ37), which directed the cell surface antigen.¹⁸⁾ The degradation of MoAb (anti-CD3) was evaluated with 0.05 mM of leupeptin by Press *et al.*¹⁹⁾ In our investigation, the effect of leupeptin on the

Fig. 1. Effect of Leupeptin on Cytotoxic Activity of VDS-35G against HuH-7 Cells

The cytotoxic activity of VDS-35G was evaluated using modified HTCA. Leupeptin (0–0.4 mM) was added to the medium during the contact time between VDS-35G (5 ng/ml of VDS equivalent) and HuH-7 cells (1×10^6). HuH-7 cells were incubated with leupeptin in soft agar gel. An index greater than 50% was considered to be effective against the target cells. The sensitivity index was calculated by the equation described below. Values are the means $\pm$ S.D.s for 3–5 experiments.

$$\text{sensitivity index} = \left(1 - \frac{\text{number of colonies for a test sample}}{\text{number of colonies for control}}\right) \times 100$$

cytotoxicity of VDS-35G was examined by varying the leupeptin concentration from 0.1 to 0.4 mM. The concentration (VDS equivalent) for VDS-35G was 5 ng/ml. When the sensitivity index in HTCA is greater than 50%, the drug is regarded as being effective against the target cells. The sensitivity indices of VDS-35G were not altered for all the concentrations of leupeptin (Fig. 1). These data mean that the cytotoxic activity of VDS-35G is not affected by cathepsin B.

Effect of Ammonium Chloride Leupeptin inhibits a specific group of lysosomal enzymes. In contrast, ammonium chloride is known to be a non-specific inhibitor which inactivates lysosomal enzymes by raising the pH level inside the lysosomes.²⁰⁾ According to previous studies, the concentrations of ammonium chloride sufficient to inhibit the lysosomal enzymes were 10 mM for anti-carcinoembryonic antigen (CEA) MoAb²⁰⁾ and diphtheria toxin,²¹⁾ and 20 mM for anti-CD3 MoAb.¹⁹⁾ We studied whether ammonium chloride would affect the cytotoxic activity of VDS-35G when its concentration was varied from 10 to 40 mM.

The effect of ammonium chloride on the cytotoxic activity of VDS-35G is shown in Fig. 2. The concentration (VDS equivalent) for the immunoconjugate was 5 ng/ml. Although the sensitivity indices of VDS-35G were slightly reduced upon increasing the concentration of ammonium chloride, the indices were more than 50% below 20 mM of ammonium chloride. The cytotoxic activity of VDS-35G was inhibited with 40 mM of ammonium chloride. However, the cytotoxic activity of VDS (50 ng/ml) was also diminished with the same concentration of ammonium chloride in HTCA (data not shown). At a high concentration of ammonium chloride, VDS may be prevented from binding to the tubulin.

From these results, ammonium chloride was considered to scarcely affect the activity of VDS-35G by inhibiting

Fig. 2. Effect of Ammonium Chloride on Cytotoxic Activity of VDS-35G against HuH-7 Cells

The cytotoxic activity of VDS-35G was evaluated using modified HTCA. Ammonium chloride (0–40 mM) was added to the medium during the contact time between VDS-35G (5 ng/ml of VDS equivalent) and HuH-7 cells (1×10^6). HuH-7 cells were incubated with ammonium chloride in soft agar gel. An index greater than 50% was considered to be effective against the target cells. The sensitivity index is explained in Fig. 1. Values are the means $\pm$ S.D.s for 3–5 experiments.

Fig. 3. Fate of VDS-35G after Endocytosis

The fate of VDS-35G is illustrated after it is bound to AFP on the cell surface and incorporated into HuH-7 cells by endocytosis. All the immune complex taken up may not be exposed to the lysosomal enzymes. Some escape from the endosomes and may bind to the tubulin.

the lysosomal enzymes.

Fate of VDS-35G in HuH-7 Cells Shih *et al.*, using fluorescence microscopy, showed that an intact immunoconjugate composed of the anti-CEA MoAb (NP-4) and doxorubicin was internalized by the target cells (LoVo cells).²⁰⁾ They concluded that the immunoconjugate was transferred to lysosomes because its degradation was markedly inhibited in the presence of ammonium chloride. The immune complex between VDS-35G and AFP might be internalized by HuH-7 cells similarly to the anti-CEA MoAb. However, the *in vitro* cytotoxic activity of VDS-35G was not affected by the lysosomal enzyme inhibitors, differing from the case of anti-CEA MoAb.

In separate experiments, VDS or VDS analogs were not detected in the supernatant by HPLC when VDS-35G was incubated with homogenized rat liver suspension (pH 5.5) for a maximum of 25 h at 37 °C. The lower detection limit of VDS concentration under these conditions was about

1 ng per 1 mg of the liver. If VDS had been released from the immunoconjugate, it would have been detected by HPLC. Also, VDS was not degraded in the liver-homogenate at least for 8 h. Such indirect evidence supports the idea that the chemical bond between VDS and 35G monoclonal antibody may also be stable in HuH-7 cells, and that the fate of VDS-35G in the cells may be different from that of the NP-4 immunoconjugate.

This hypothesis, that VDS-35G may pass through endosomes and escape from the lysosomal enzymes, was supported by the results using immunotoxins composed of gelonin and anti-AFP MoAb, as reported in the previous paper.²²⁾ Gelonin is a type I ribosome-inactivating protein. Therefore, these immunotoxins would be inactivated by lysosomal enzymes if they were incorporated into lysosomes. However, they exerted cytotoxic activity against HuH-7 cells both *in vitro* and *in vivo*. These findings strongly suggest that some VDS-35G may avoid the lysosomal cleavage and bind to tubulin in the cytoplasm. Figure 3 summarizes the proposed behavior of VDS-35G in HuH-7 cells.

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