Effect of Gelonin Immunoconjugate with Monoclonal Antibody MSN-1 to Endometrial Adenocarcinoma on Antigen-producing Tumor Cells in vivo

Yoshibumi Kaneta,1 Katsumi Tsukazaki,1 Kaneyuki Kubushiro,1 Rui Aoki,1 Motoko Sakayori,1 Masakazu Ueda2 and Shiro Nozawa1

1Department of Obstetrics & Gynecology and 2Department of Surgery, Keio University School of Medicine, Shinnomachi 35, Shinjuku-ku, Tokyo 160-0016

Missile therapy, which destroys cancer cells specifically, has been advocated as an effective modality for the treatment of carcinoma. We have developed an immunoconjugate consisting of the monoclonal antibody MSN-1 (IgM), which reacts strongly with endometrial adenocarcinomas, combined with a plant hemitoxin named gelonin via a disulfide bond using N-succinimidyl-3-(2-pyridylthio)propionate and 2-iminothiolane, and examined its selective cytotoxicity in athymic mice. The reductions in resected weights of target tumor cells, at the local site of MSN-1-gelonin immunoconjugate treatment, were 96% with local administration and 75% with caudal vein administration, as compared with the untreated group. There was no weight loss in treated mice. Our results suggest that this MSN-1-gelonin immunoconjugate has potential clinical applications in the treatment of endometrial adenocarcinomas.

Key words: Missile therapy — Endometrial adenocarcinoma — Gelonin — MSN-1 — in vivo

Recently, the incidence of endometrial adenocarcinoma has been increasing in Japan,1) and chemoresistant and/or radiation-resistant cases are found frequently among those with advanced clinical stages and recurrence. Given these circumstances, new therapies for endometrial adenocarcinoma are needed.

Missile therapy, employing an immunoconjugate which binds carcinostatics and toxins to tumor-associated antibodies against epitopes specific to the carcinomas, thereby destroying only cancer cells, has been considered to be an effective modality for the treatment of carcinoma. However, most epitopes utilized in previous missile therapies2–4) leak into the bloodstream, causing decreased anti-tumor activity secondary to unintended immunoreaction, and thus reducing the effectiveness of the agent.

We have produced a monoclonal antibody named MSN-1.5) MSN-1 is advantageous because its epitope is located on the surface of the endometrial adenocarcinoma cell membrane,6) and does not leak into the bloodstream.5)

Using this antibody and a plant hemitoxin named gelonin,7) we investigated selective in vitro cytotoxicity against the endometrial adenocarcinoma cell line SNG-II.8) Ricin (true toxin), which was utilized in previous studies, consists of a B chain, which binds nonspecifically to sugar chain antigens on the cell membrane surface, and an A chain, and thus has little selective cytotoxicity. On the other hand, gelonin (hemitoxin) consists of an A chain alone, does not require purification, and has low inherent toxicity. The cytotoxicity of the MSN-1-gelonin immunoconjugate was 188 times more than that of gelonin alone against SNG-II.8)

In this study, we have investigated the selective cytotoxicity of the MSN-1-gelonin immunoconjugate in athymic mice in vivo, to examine whether this agent might have potential for the clinical treatment of endometrial adenocarcinoma.

MATERIALS AND METHODS

Two cell lines, both of which were established in our laboratory, were used for all experiments. One, named “SNG-II,” was derived from a well-differentiated endometrial adenocarcinoma.5) The other, named “SKG-IIa,” was derived from a uterine cervical squamous cell carcinoma.9) SKG-IIa was used as a control cell line for SNG-II. It has been confirmed that SKG-IIa has no epitope for MSN-1 by means of immunohistochemical staining and flow-cytometric analysis.10) Gelonin7) was purchased from Pierce (Rockford, IL). The monoclonal antibody MSN-1 is a murine immunoglobulin class IgM monoclonal antibody raised against SNG-II.5) Its characteristics were described in detail previously.5,6,11) Female athymic BALB/c nu/nu mice (Clea Japan, Inc., Tokyo), 8 to 9 weeks old, were used for the experiments.

Preparation of the MSN-1-gelonin immunoconjugate

Gelonin was conjugated to MSN-1 by using a procedure described previously.9) Briefly, after modification of MSN-1 with N-succinimidyl-3-(2-pyridylthio)propionate, and modification of gelonin with 2-iminothiolane, excess reagents were removed by gel filtration on a column of Sephadex G-25 (Pharmacia, Sweden). The modified MSN-1 was mixed with modified gelonin, and allowed to react. Free gelonin was removed from the mixture of the MSN-1-gelonin immunoconjugate, MSN-1 and gelonin by passage of the solution through a column of Sephacryl S-300 (Pharmacia), and the solution was...
immunoconjugate was 1:13.8. The MSN-1-gelonin immunoconjugate used contained 50 µg of gelonin and 100 µg of MSN-1. Thus, the selective cytotoxicity of the MSN-1-gelonin immunoconjugate was 188 times greater than that of gelonin alone against SNG-II in vitro.

Cytotoxicity of the MSN-1-gelonin immunoconjugate to SNG-II (or SKG-IIIa) in vivo According to the method of Hirota et al.,12) mice were given subcutaneous injections of SNG-II cells in the left flank. Four days later, the same mice were given an injection of SKG-IIIa cells. The estimated tumor weight was determined by measuring the major and minor tumor axes, which are perpendicular, according to Geran et al.’s method.13) Tumor weight was calculated by means of the formula $W = 0.5 \times L \times S^2$, where $L$ is the major axis (mm), and $S$ is the minor axis (mm). After tumor cell inoculation, when the estimated tumor weight had reached 100–150 mg (7 to 10 days after inoculation), the mice were randomly divided into 5 groups ($n=5$) consisting of 5 mice each, and treated. The five groups were 1) MSN-1-gelonin immunoconjugate-treated via the caudal vein, 2) MSN-1-gelonin immunoconjugate-treated by local administration, 3) gelonin-treated via the caudal vein, 4) gelonin-treated by local administration, and 5) 0.1 M phosphate-buffered saline (PBS)-treated via the caudal vein (control group).

The dose of gelonin for each treatment group was 50 µg per day for 5 consecutive days. Various intervals of immunoconjugate therapy have been reported. We used the 5 consecutive days method as described by Hirota et al.12) for selective cytotoxicity with an immunoconjugate of gelonin and IgG.

The relative mean tumor weight (the tumor weight at that time/the estimated tumor weight at initial treatment) in the treated groups and the control group was calculated every week. Mice were killed on day 35, their weights were determined, the tumor and vital organs (heart, lungs, liver, spleen, kidneys, uterus and ovaries) were harvested, and each organ was weighed. Tumors and vital organs were stained with hematoxylin-eosin (HE), and histological effects were judged using Shimosato et al.’s histological criteria for evaluation of therapeutic effects.14)

Statistical analysis The statistical significance of differences in tumor volume was examined by using Student’s $t$ test.

RESULTS

Cytotoxicity of the MSN-1-gelonin immunoconjugate to SNG-II (or SKG-IIIa) in vitro By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of each ion-exchange chromatography (CM-52) fraction as described previously,8) we confirmed the purity of the MSN-1-gelonin immunoconjugate.

Based on the SDS-PAGE analysis and densitometry, the average ratio of gelonin to MSN-1 in the MSN-1-gelonin immunoconjugate was 1:13.8. The MSN-1-gelonin immunoconjugate used contained 50 µg of gelonin and 100 µg of MSN-1. Thus, the selective cytotoxicity of the MSN-1-gelonin immunoconjugate was 188 times greater than that of gelonin alone against SNG-II in vitro.

Cytotoxicity of the MSN-1-gelonin immunoconjugate to SNG-II (or SKG-IIIa) in vivo In a preliminary study, doses of 50 and 100 µg of the MSN-1-gelonin immunoconjugate per day were ineffective, but doses of 150, 200, and 250 µg were effective. The dose of 150 µg of the MSN-1-gelonin immunoconjugate per day was chosen because it was the minimum dose showing cytotoxicity against the SNG-II tumor.

In athymic mice bearing SNG-II cell tumors (Fig. 1), there was a significant difference in relative tumor growth between the group given MSN-1-gelonin immunoconjugate by local administration and that given PBS (control) via the caudal vein ($t$ test $P<0.01$), and between the group given MSN-1-gelonin immunoconjugate via the caudal vein and the control ($t$ test $P<0.01$). There was no significant difference among the group given gelonin by local administration, that given gelonin via the caudal vein and the control. On the other hand, in the athymic mice bearing SKG-IIIa cell tumors, there was no significant suppression of tumor growth in any of the treatment groups (Fig. 2).
There was no weight loss in mice bearing tumors consisting of SNG-II cells or SKG-IIIa cells after treatment (Table I). None of the mice was found to have ascites at the time of resection. There was also no statistically significant difference in the resected tissue weights of vital organs before and after treatment among treatment groups (Table II).

There was significant suppression of the resected SNG-II tumor weight between the group given MSN-1-geloin immunoconjugate via the caudal vein (final resected weight: mean±SE=0.49±0.28 g) and the control (1.99±1.10 g) (t test P<0.01), and between the group given MSN-1-geloin immunoconjugate by local administration (0.08±0.04 g) and the control (t test P<0.05) after treatment. There was no significant suppression of the resected SNG-II tumor weight in the group given geloin via the caudal vein (2.78±0.65 g), the group given geloin by local administration (2.67±0.64 g) and the control. On the other hand, there was no significant suppression of resected SKG-IIIa tumor weight in any of the treated groups (Table II).

The HE histological evaluations (using Shimosato et al.'s classification) of the SNG-II tumors in the group given MSN-1-geloin immunoconjugate by local administration, that given MSN-1-geloin immunoconjugate via the caudal vein, that given geloin by local administration, that given geloin via the caudal vein, and the control were grade IIa, grade I, grade 0, grade 0 and grade 0 (n=5), respectively, i.e., none had histological damage of grade IIb or worse. On the other hand, the HE histological

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**Table I. Weights (g) of Mice before and after Treatment**

| Treatment                        | Before treatment | After treatment |
|----------------------------------|------------------|-----------------|
| MSN-1-geloin immunoconjugate     |                  |                 |
| via caudal vein                  | 20.4±0.4         | 25.7±1.8        |
| MSN-1-geloin immunoconjugate     |                  |                 |
| by local administration          | 20.4±0.6         | 25.3±2.0        |
| Geloin via caudal vein           | 20.1±0.6         | 30.4±1.8        |
| Geloin by local administration   | 20.5±0.5         | 28.6±2.4        |
| PBS (control) via caudal vein    | 20.4±0.6         | 27.1±2.0        |

Values are mean±SE (n=5).

**Table II. Tissue Weights (g) of Resected Vital Organs and SNG-II and SKG-IIIa Cell Tumors of Mice after Treatment**

| Treatment                        | Heart | Lungs | Liver | Spleen | Kidneys | Uterus and ovaries | SNG-II cell tumor | SKG-IIIa cell tumor |
|----------------------------------|-------|-------|-------|--------|---------|-------------------|-------------------|-------------------|
| MSN-1-geloin immunoconjugate     | 0.10±0.01 | 0.18±0.01 | 1.73±0.02 | 0.12±0.01 | 0.31±0.03 | 0.15±0.02 | 0.49±0.28         | 4.18±1.26          |
| via caudal vein                  |       |       |       |        |         |                   |                   |                   |
| MSN-1-geloin immunoconjugate     | 0.11±0.01 | 0.19±0.01 | 1.72±0.19 | 0.12±0.01 | 0.29±0.03 | 0.15±0.03 | 0.08±0.04†         | 2.89±0.61          |
| by local administration          |       |       |       |        |         |                   |                   |                   |
| Geloin via caudal vein           | 0.11±0.00 | 0.20±0.01 | 1.75±0.16 | 0.12±0.01 | 0.29±0.02 | 0.12±0.03 | 2.78±0.65         | 7.72±3.18          |
| Geloin by local administration   | 0.10±0.01 | 0.20±0.02 | 1.84±0.07 | 0.12±0.01 | 0.30±0.03 | 0.14±0.03 | 2.67±0.64 *         | 5.16±0.33          |
| PBS (control) via caudal vein    | 0.10±0.01 | 0.19±0.02 | 1.63±0.24 | 0.12±0.01 | 0.30±0.02 | 0.15±0.04 | 1.99±1.10         | 3.53±1.29          |

Values are mean±SE (n=5). * P<0.01, † P<0.05.
evaluations of SKG-IIIa tumors were grade 0 in all treatment groups (n=5) (Table III).

There was no marked histological damage to vital organs in any of the groups.

DISCUSSION

Since Gilliland et al.\(^{13}\) reported in 1980 the first missile therapy using monoclonal antibody against colon adenocarcinoma with diphtheria toxin or ricin A chain, basic studies, both in vitro and in vivo, and clinical applications have been reported.\(^{2-4, 8, 12, 16-30}\)

The monoclonal antibody MSN-1, used in this study, has been shown to have the following characteristics: it reacts with endometrial adenocarcinomas at a high frequency, but very seldom with normal endometrial cells\(^{13}\); its epitope is present on the surfaces of endometrial carcinoma cells\(^{12}\); its epitope shows minimal leakage into the bloodstream.\(^{59}\) Gelonin (hemitoxin),\(^{71}\) as used in this study, consists of an A chain alone, without a B chain that binds nonspecifically to sugar chain antigens on the surface of cell membranes. It does not readily bind to sugar chains, so it has low inherent toxicity. Ozawa et al. reported missile therapy with gelonin and antibody B4G7 (IgG) recognizing epidermal growth factor receptor in vitro.\(^{19}\) and Hirota et al. reported a similar study in vivo.\(^{12}\) However, the present report is the first to describe missile therapy using gelonin and IgM that recognizes epitopes on the surface of cell membranes. The MSN-1-gelonin immunoconjugate appears to be an ideal agent for missile therapy against endometrial adenocarcinomas. We found that the cytotoxicity of the MSN-1-gelonin immunoconjugate is 188 times greater than that of gelonin alone in vitro.\(^{89}\) In order to avoid problems arising from differences among athymic mice, the same mouse was inoculated with SNG-II cells at the left back, and SKG-IIIa cells at the right back for the in vivo study. The relative mean tumor weight was used to evaluate whether the character of SNG-II cells, or SKG-IIIa cells, influenced, for example, the feeding artery volume.

Weights of resected SNG-II cell tumors were reduced 96% in the group given MSN-1-gelonin immunoconjugate by local administration, and 75% in the group given MSN-1-gelonin immunoconjugate via the caudal vein, as compared with the PBS-treated control. On the other hand, the SKG-IIIa cell tumor weights in the groups given MSN-1-gelonin immunoconjugate by local administration and via the caudal vein showed no cytotoxic effect as compared to the control. Thus, the MSN-1-gelonin immunoconjugate showed highly selective cytotoxicity in vivo as well as in vitro.

In this study, the group given MSN-1-gelonin immunoconjugate via the caudal vein showed less cytotoxicity than the local administration group. This suggests that in mice given the MSN-1-gelonin immunoconjugate via the caudal vein, the immunoconjugate was diluted by systemic blood, and a part of it was trapped in the reticuloendothelial system (liver, spleen), before it could reach the target cells, so that the activity against the target cells was decreased. This study did not examine the passage of the MSN-1-gelonin immunoconjugate (M.W. 1,300,000) through the target cell membrane. However, the cell can take up huge molecules by pinocytosis, and isotope-labeled IgM entered its target cells after intravenous injection into mice.\(^{31}\) After \(^{125}\)I-labeled MSN-1-gelonin immunoconjugate was injected into the caudal vein, it showed little accumulation in SNG-II or SKG-IIIa tumors. Nevertheless, the MSN-1-gelonin immunoconjugate showed selective cytotoxicity. The mechanism involved may be similar to that in the case of ricin immunoconjugate. First, the IgM component in the MSN-1-gelonin immunoconjugate binds specifically to the surface antigen on the cell membrane of SNG-II cells. Next the bound immunoconjugate was considered to have reached target cells via the cardiovascular system, and to have shown cytotoxicity secondary to pinocytosis at the cell membrane.

Prior to clinical application of immunoconjugates, we must examine the therapeutic efficacy of the MSN-1-gelonin immunoconjugate against orthotopic tumors. Byers et al. reported that XomaZyme-791, an immunoconjugate of antibody against human colon carcinoma with ricin, showed unexpected neural toxicity in 17 patients.\(^{161}\) Thus,
Toxins have unknown pharmacological dynamics, are heterogeneous proteins, may have severe side effects, and are associated with capillary leak syndrome. In our in vivo study in athymic mice, it was not clear whether neural toxicity, for example, capillary leak syndrome, occurred. However, vital organs were unaffected by the MSN-1-gelonin immunoconjugate treatment, suggesting that this immunoconjugate might be suitable for clinical application.

An immunohistochemical study of MSN-1 in human systemic organs revealed slight uriniferous tubule positivity, indicating that the MSN-1-gelonin immunoconjugate may have few side effects. However, it was reported that after several weeks of missile therapy using monoclonal antibody derived from mice, monoclonal antibody activity decreased and allergic reactions, most seriously anaphylaxis due to human antimouse antibody (HAMA), appeared. With regard to HAMA production, the use of 1) antibody fragments, 2) mouse-human chimera antibody and 3) CDR graft has been tried, but did not appear to decrease the side effects. Recently, a new missile therapy was designed, using growth factor to target the growth factor receptor, rather than monoclonal antibody. However, for endometrial adenocarcinomas, no growth factor receptor or hormone receptor has yet been identified, making it necessary to use antibodies for missile therapy. If the cytotoxicity of the MSN-1-gelonin immunoconjugate is time-dependent, intraarterial injection may be superior to intravenous injection. Recently, an ultra-selective intra-arterial infusion therapy, balloon occluded arterial infusion (BOAI) therapy, has been developed for tumors in the small pelvis, and reportedly achieves higher concentrations of carcinostatics than local administration of these drugs. BOAI therapy using MSN-1-gelonin immunoconjugate may be an effective form of neoadjuvant chemotherapy for advanced endometrial adenocarcinomas and for inducing remission of recurrent endometrial adenocarcinomas.

In this study, the MSN-1-gelonin immunoconjugate was found to be safe and effective against MSN-1 epitope-producing tumors, not only in vitro, but also in vivo. We are continuing to develop human MSN-1 in the hope of expediting clinical application.

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