A Novel Microbial Infection-Responsive Drug Release System

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Abstract □ The aim of this study was to construct a novel drug delivery system suitable for controlled release of antibiotics. There is a need for devices that release antibiotics only during microbial infection, because prophylactic or prolonged use of antibiotics leads to serious problems, such as renal and liver toxicity and the emergence of drug-resistant bacteria (e.g., meticillin-resistant *Staphylococcus aureus*). We found previously that *Staphylococcus aureus*-infected wound fluid showed high thrombin-like activity; therefore, in this study we designed an antibiotic release system triggered by thrombin activity. We synthesized an insoluble polymer–drug conjugate in which gentamicin was bound to poly(vinyl alcohol) hydrogel through a newly developed thrombin-sensitive peptide linker. The conjugate released gentamicin when it was incubated with *Staphylococcus aureus*-infected wound fluid, with thrombin and leucine aminopeptidase, or with human plasma and Ca^{2+}, whereas no biologically active gentamicin was released when the conjugate was incubated with noninfected wound fluid, with leucine aminopeptidase alone, with thrombin alone, or with plasma. Furthermore, the conjugate reduced the bacterial number in an animal model of *Staphylococcus aureus* infection. These results demonstrated that the conjugate has sufficient specificity and excellent potential as a stimulus-responsive, controlled drug release system.

Introduction

Improper usage of antibiotics is very serious because of the appearance of renal and liver toxicity and the emergence of drug-resistant bacteria (e.g., meticillin-resistant *Staphylococcus aureus*).[1−4] In the ideal case, the proper amount of antibiotics is used at the site and during the period of the infection. For this purpose, many kinds of drug delivery systems have been investigated, including several antibiotic-impregnated polymer systems;[5−11] however, these systems are not appropriate for controlled release of antibiotics. Therefore, a system that detects the occurrence of bacterial infection and releases the proper amount of antibiotics relative to the degree of infection is needed. In this study, we constructed a system in which antibiotics are bound to an insoluble polymer matrix through a linker that acts as an infection sensor.

First, we investigated biological signals, such as enzyme activities, which appeared in infected wound fluid and found a remarkable increase of thrombin-like activity in *Staphylococcus aureus* (SA)-infected wounds.[12] This enzyme activity was thought to be mainly attributed to staphylocoagulase produced by SA, which forms an enzymatically active complex with prothrombin named staphylothrombin.[13] Second, we developed a novel peptide linker, Gly-(D)-Phe-Pro-Arg-Gly-Phe-Pro-Ala-Gly-Gly, which could connect insoluble polymer matrix and drug, and was specifically cleaved by thrombin.[14] The ideal insoluble polymer matrix should be biocompatible, noninflammative, and nontoxic, and should have good mechanical properties. A new hydrogel made of a poly(vinyl alcohol) derivative was synthesized in this study. Introduction of carboxyl groups to partially saponified polyvinyl pivalate gave a transparent, soft, and high-water-content hydrogel that is cross-linked by hydrophobic bonds between tertiary butyl groups in pivalate.[15]

Our preliminary study revealed that gentamicin was selectively released from the device when it was incubated with infected wound fluid and that the device showed bacteriocidal effect in vitro.[12] In this report, we describe the synthesis of the device combining these components to attain a suitable polymer−drug conjugate system, and the specificity of drug release properties in vitro and bacteriocidal effect in vivo. The results showed that the device is sufficiently potent and specific for treating SA-infected wounds.

Methods

Materials—Gentamicin sulfate, thrombin (human plasma, EC 3.4.21.5), and leucine aminopeptidase (cytosol, Type V: Porcine kidney, EC 3.4.11.1) were purchased from Sigma Chemical Company, St. Louis, MO. Fluorogenic peptide substrates, WSCD (1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride), and HOSu (*N*-hydroxysuccinimide) were from Peptide Institute Inc., Minoh, Osaka, Japan. Other reagents were from Wako Pure Chemicals, Doshomachi, Osaka, Japan. Peptide linker (Gly-(D)-Phe-Pro-Arg-Gly-Phe-Pro-Ala-Gly-Gly) was synthesized by Peptide Institute Inc.; purity: 95.0%, as determined by reversed-phase HPLC [column: YMC PACK ODS-A (4.6 mm i. d. × 150 mm) + (4.0 mm i. d. × 10 mm); eluent: 10− 60% acetonitrile gradient (25 min) in 0.1% trifluoroacetic acid−water, flow rate: 1 mL/min, detection: 220 nm OD). Amino acids analyzed (calcd): Arg 1.00 (1), Pro 1.99 (2), Gly 4.01 (4), Ala 1.01 (1), Phe 1.99 (2). Mass analysis: 962.8 $[M + H]^+$ (calcd for $[M + H]$: 962.5).

Synthesis of PVA−Linker−Gentamicin (Scheme 1)—*Polyvinyl Pivalate* (***1***)—Freshly distilled vinyl pivalate (200 g, 1.56 mol) was dissolved in 70 g of methanol in a reaction vessel, and purged with nitrogen gas with stirring. The mixture was heated to 60 °C, and 2, 2′-azobis(isobutylonitrile) (0.04 g, 0.24 mmol) in 5 g of methanol was added to start the polymerization. When the polymerization ratio reached 40%, about 5 h after starting of the polymerization, the reaction mixture was cooled to 20 °C. Several volumes of *tert*-butyl alcohol were added to the reaction mixture, and residual vinyl pivalate was distilled off under reduced pressure. Then several volumes of tetrahydrofuran were added and *tert*-butyl alcohol was distilled off under reduced pressure. Finally the solution of polyvinyl vivalate in tetrahydrofuran (concentration: 45.7 wt %) was obtained. Yield: 78 g (39%).

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*Partially Saponified Poly(vinyl alcohol) (**2**)*—Fifty grams of the solution of polyvinyl vivalate in tetrahydrofuran was heated to 60 °C with stirring in a reaction vessel equipped with a reflux condenser. The vessel was purged with nitrogen gas, and 20 g of a 25% solution of KOH was added. After the reaction mixture was kept at 60 °C for 6 h, it was neutralized by adding 5.5 g of acetic acid and 5.5 g of methanol. The solidified product, partially saponified poly(vinyl alcohol), was washed with methanol using a Soxhlet extractor and dried under reduced pressure. Yield: 10.7 g (46.8%), P = 1650, determined by [η] of the polyvinyl acetate at 30 °C, which was acetylated after full saponification of the partially saponified poly(vinyl alcohol), according to the following equation:

$$P = \{[\eta] \times (1000/7.94)\}^{(1/0.62)} \quad (1)$$

The saponified ratio was 0.811, determined from 500 MHz ^{1}H NMR (d_6-dimethyl sulfoxide): δ (ppm) 1.11 (s, 9H, $C(CH_3)_3$, integration: 89 au), 1.4−1.77 (complex, 2H, CH_2, integration: 106 au).

*Carboxylated Poly(vinyl alcohol) Hydrogel (PVA−COOH) (**3**)*—Ten grams of partially saponified poly(vinyl alcohol) was dissolved in 300 g of dimethyl sulfoxide at 70 °C, followed by the addition of succinic anhydride (1.64 g, 16.4 mmol) and pyridine (0.64 g, 8.1 mmol). After the reaction mixture was kept at 70 °C for 4 h, it was cast in a glass tray, followed by hydration in a humidified chamber. After 1 or 2 days of incubation in a humidified chamber, a sheet of transparent hydrogel (PVA−COOH) was obtained. This hydrogel was thoroughly washed with water to remove the residual reagents. The water content of the hydrogel was 91 wt % and the carboxyl group content was 12 μmol/g-hydrogel (= 1.3×10^{-4} mol/g-dry weight).

*Poly(vinyl alcohol) Hydrogel−Peptide Linker Conjugate (PVA−Linker) (**4**)*—PVA−COOH (10 g, 0.12 mmol carboxyl group) was washed several times with dimethylformamide, and to the resultant dimethylformamide-swollen gel, HOSu (46 mg, 0.4 mmol) and WSCD (81 mg, 0.42 mmol) in 10 mL of dimethylformamide were added, followed by shaking overnight. The gel was washed several times each with dimethylformamide and water, followed by the addition of the peptide linker, Gly-(D)-Phe-Pro-Arg-Gly-Phe-Pro-Ala-Gly-Gly (116 mg, 0.12 mmol), in 10 mL of 20 mM phosphate buffer (pH 7.0). After shaking the reaction mixture overnight, the resultant PVA−linker was washed several times with phosphate buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, pH 7.4) and Gly-HCl buffer (0.1 M, pH 2.5), and then washed several times with 20 mM phosphate buffer (pH 7.0). The yield of this coupling reaction was ~80%, as determined by HPLC analysis of the residual peptide linker in the reaction mixture.

*Poly(vinyl alcohol) Hydrogel−Peptide Linker−Gentamicin Conjugate (PVA−Linker−Gentamicin) (**5**)*—PVA−linker (10 g) was immersed in 10 mL of 20 mM phosphate buffer (pH 7.0), and gentamicin (1.28 g, 1.68 mmol) and WSCD (46 mg, 0.24 mmol) were added, followed by overnight shaking. The resultant product, PVA−linker−gentamicin, was thoroughly washed with phosphate buffered saline (PBS) and water until the concentration of total organic carbon of the supernatant decreased below 10 ppm.

Bacterial Culture—*Staphylococcus aureus* (FDA209P, Institute of Fermentation Osaka, Osaka, Japan) was used. This strain was stored in trypticase soy broth with glycerol (20%) at −70 °C. Before the experiments, the strain was grown in Brain-Heart Infusion (BHI) broth overnight at 37 °C on a rotary shaker. The concentration of cells was measured by turbidity at 600 nm, and adjusted to the desired concentration by diluting with PBS.

SA-Infected Wounds—Male Wistar rats weighing 150 to 200 g were acclimatized to laboratory conditions for 7 days prior to use. The animals were fed a standard commercial rat chow ad libitum and housed individually in facilities that complied with the requirements of the Kyoto University Animal Experiment Committee, with controlled temperature (22−23 °C) and light (12 h light/12 h darkness). Each rat was anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). The dorsal paravertebral fur was then removed with electric clippers and a depilatory agent. The skin was cleansed with an iodophor solution followed by a 70% alcohol rinse. Air was sucked into a syringe through a sterile cotton filter, and 10 mL of air was injected into the loose subcutaneous tissue between the shoulders of the rat. One day after creation of the air pouch, 3 g of PVA−linker−gentamicin (n = 8) or PVA−COOH (n = 8) was inserted into the pouch with 5 mL of a suspension of 10^5 SA per milliliter in PBS. After 4 h, the bacterial number in the fluid from each pouch was estimated by limiting dilution methods using BHI agar plates.

Sampling of wound fluids was done by almost the same method just described except for the following points. One day after creation of the air pouch, animals were randomly assigned to the infected wound group or the noninfected wound group, and 10 mg of Montmorillonite clay soil fraction, a well-known infection-potentiating factor,[16] was inserted into each air pouch instead of PVA−COOH or PVA−linker−gentamicin as already described. Five days after creation of the infected air pouch, the animals were anesthetized as already described, and the wounds were opened. The pus was removed and the inner surface of the pouch was gently wiped with wet gauze to expose the fresh wound bed. Freeze-dried agarose sponges were inserted into the pouch to absorb the wound fluid, and the incision was closed. After 20 min, the pouch was reopened. The wound fluid contained in the agarose sponges was weighed and the amount of fluid obtained from the pouch during 20 min was calculated. The fluid contained in the agarose sponges was stored at −150 °C until assay. Simultaneously, the wound tissues were removed, weighed, and used to count the bacterial number by limiting dilution methods using BHI agar plates. Then, all the animals were sacrificed with an overdose of pentobarbital.

Assay for Enzymatic Activity of Infected Wound Fluid and Plasma—The wound fluid contained in the agarose sponges was extracted by addition of 9 volumes of PBS and filtered by centrifugation. Plasma was also diluted by addition of 9 volumes of PBS with or without 10 mM Ca^{2+}. Thrombin-like activity and aminopeptidase activity were measured using the fluorogenic peptide substrates (0.2 mM) Boc-Val-Pro-Arg-MCA[17,18] (MCA = 4-methylcoumaryl-7-amide) and Ala-MCA,[19] respectively. Enzymatic activity was calculated from the fraction of each substrate hydrolyzed after incubation for 30 min at 25 °C.

Assay of Gentamicin Release—The wound fluid in the agarose sponges was extracted by addition of two volumes of PBS and filtered by centrifugation. After incubation of 0.05 g of PVA−linker−gentamicin with 250 μL of the wound fluid, Ca^{2+}-supplemented plasma, plasma, or enzyme solution for 24 h at 37 °C, the supernatant was used to measure the concentration of gentamicin by the following two methods.

Width of Zone of Inhibition of SA Growth on BHI Agar Plates—Seventy-five microliters of the supernatant was adsorbed onto a paper disk, the disk was placed on a BHI agar plate inoculated with SA, and the plate was incubated overnight at 37 °C. The concentration of gentamicin was calculated from the width of the zone of inhibition around the disk compared with the widths of zones around standard gentamicin solutions with concentrations from 0 to 30 μg/mL of solution.

2. Fluorescence Polarization Immunoassay (FPIA)—A fully automatic FPIA apparatus (TDX analyzer, DAINABOT, Tokyo, Japan) and the corresponding reagents kit for gentamicin were used.

Statistical Analysis—All statistical evaluations were performed by unpaired t test and analysis of variance (ANOVA). All values were expressed as mean ± standard deviation.

Results

Enzymatic Activity in SA-Infected Wound Fluid—We found significantly elevated thrombin-like activity in SA-infected wound fluid compared with the level in noninfected wound fluid (Table 1). On the other hand, SA-infected wound fluid did not differ from noninfected wound fluid in aminopeptidase activity. Therefore, the thrombin-like activity in wound fluid seemed to be a specific signal of SA-infection. The amount of wound fluid also increased significantly in SA-infected wounds compared with noninfected wounds. The elevated thrombin-like activity in SA-infected wound fluid may be attributable to both α-thrombin and staphylothrombin.[12] Furthermore, high levels of aminopeptidase activity existed in both noninfected and SA-infected wound fluid.

Table 1—Enzymatic Activities and Weight of Exudate From Infected Wounds[a]

variable	digested substrate (nmol/30 min) non-infected ($n = 16$)	SA-infected ($n = 12$)
thrombin-like activity	0.376 ± 0.104	0.518 ± 0.084**
alanine amino peptidase activity	0.938 ± 0.372	1.402 ± 0.206*
amount of exudate (g/wound)	0.190 ± 0.051	0.449 ± 0.116***
bacterial number (CFU/mL)	0	5900***

[a] Key to statistical significance: (*) $p > 0.05$, not significant compared with non-infected control; (**) $p < 0.01$, significant compared with non-infected control; (***) $p < 0.001$, significant compared with non-infected control.

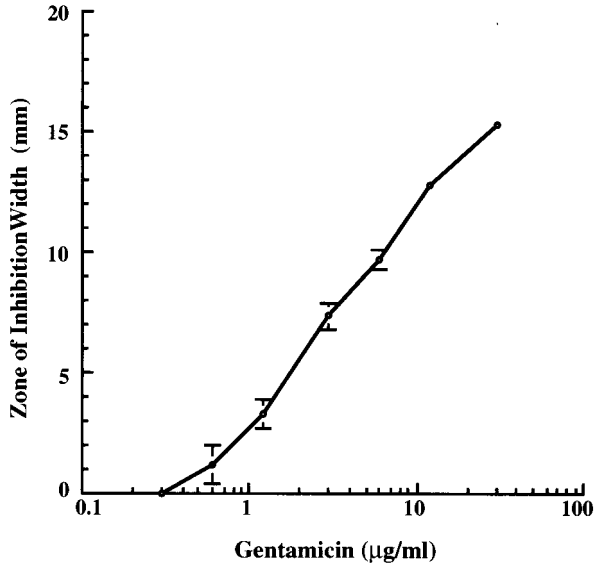
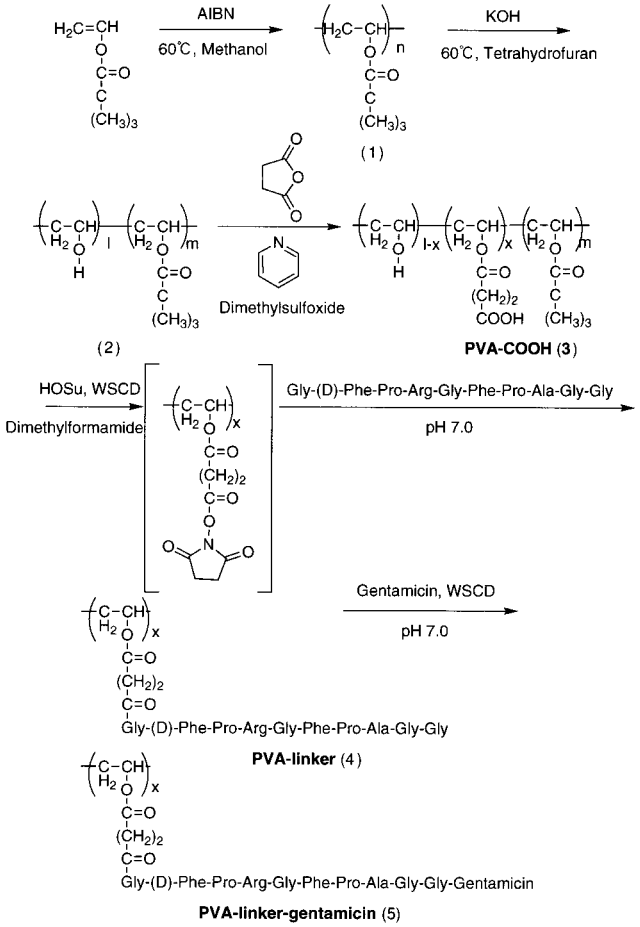


Figure 1—A representative standard curve showing the relationship between the width of the zone of inhibition and concentration of gentamicin. Each value represents mean ± SD of two or three experiments.

Gentamicin Release from PVA–Linker–Gentamicin—one representative standard curve showing the relationship between the width of the zone of inhibition and gentamicin concentration is shown in Figure 1. Because such curves showed a linear relation in the concentration range from 1 to 30 μg/mL, the concentration of released gentamicin was calculated from the width of the zone of inhibition using the standard curve obtained in each experiment.

As shown in Scheme 2, at least two enzyme activities were needed for gentamicin release from PVA–linker–gentamicin. That is, the Arg–Gly bond in the peptide linker was cleaved, and the resultant Gly-Phe-Pro-Ala-Gly-Gly gentamicin was released and digested by aminopeptidase to produce free gentamicin. Figure 2 shows the dose dependence of gentamicin release on thrombin concentration in the presence of leucine aminopeptidase (LAP). Almost no gentamicin was released without thrombin, as measured by both the zone of inhibition and FPIA methods. The amount of gentamicin released increased with increasing thrombin concentration, and seemed to reach a maximum at a thrombin concentration of 2 U/mL. At concentrations of thrombin >4 U/mL, gentamicin release measured by zone of inhibition decreased. LAP may be deactivated by excess thrombin.

The effects of LAP on gentamicin release in the presence of thrombin at 8 U/mL are shown in Figure 3. Gentamicin release was nearly undetectable in the absence of LAP when measured by the zone of inhibition method; however, a considerable amount of gentamicin released was detected by FPIA. This discrepancy seemed to arise from the difference of the specificity of the two methods. Because Gly-Phe-Pro-Ala-Gly-Gly-gentamicin is not biologically active, it was not detected by the zone inhibition method. In contrast, the anti-gentamicin antibody used in FPIA could bind with Gly-Phe-Pro-Ala-Gly-Gly-gentamicin; therefore, it was detected as gentamicin by FPIA.



Scheme 1—Synthesis of PVA-linker-gentamicin.

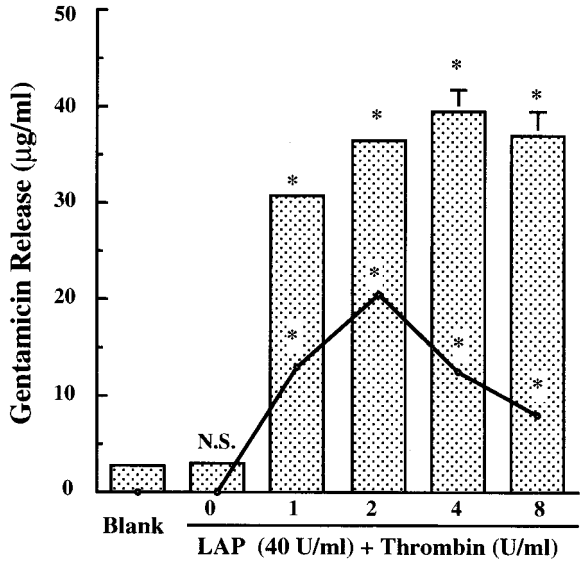


Figure 2—Dose dependence of gentamicin release on thrombin concentration in the presence of LAP: FPIA (bar); zone of inhibition (–○–). Each value represents mean ± SD of triplicate experiments. The data marked with * are significantly high values compared with blank ($p < 0.001$), calculated by ANOVA. N. S. denotes no significant difference compared with blank ($p > 0.05$), calculated by ANOVA.

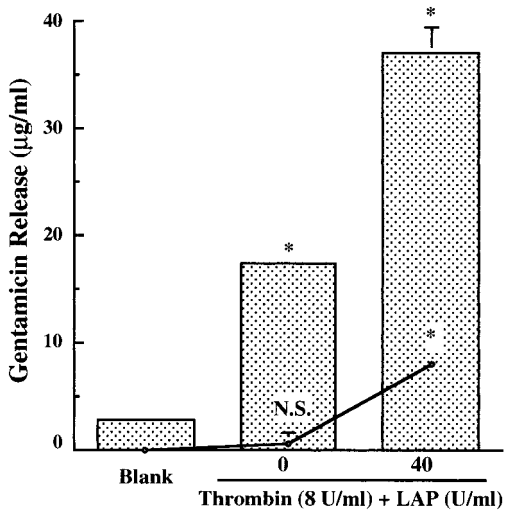


Figure 3—Effects of LAP on gentamicin release in the presence of thrombin: FPIA (bar); zone of inhibition (–○–). Each value represents mean ± SD of triplicate experiments. The data marked with * are significantly high values compared with blank ($p < 0.001$), calculated by ANOVA. N. S. denotes no significant difference compared with blank ($p > 0.05$) calculated by ANOVA.

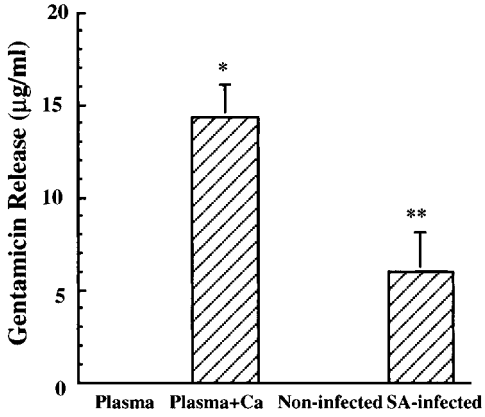
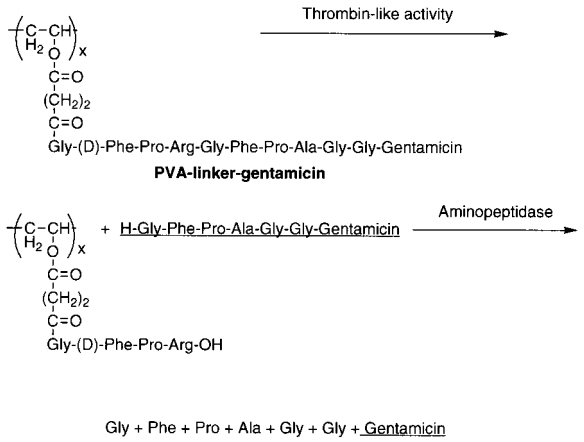


Figure 4—Gentamicin release detected by the zone of inhibition assay when PVA–linker–gentamicin was incubated with Ca^{2+}-supplemented human plasma ($n = 3$) or plasma alone ($n = 3$) in glass tubes, and SA-infected ($n = 13$) or noninfected wound fluid ($n = 3$). Key: (*) plasma + Ca shows a significantly higher value than plasma ($p < 0.001$), by the unpaired t test. (**) SA-infected wound fluid shows a significantly higher value than noninfected wound fluid ($p < 0.001$), calculated by the unpaired t test.

Figure 4 shows the release of biologically active gentamicin when PVA−linker−gentamicin was incubated with Ca^{2+}-supplemented human plasma and plasma alone in glass tubes. Because Ca^{2+} addition to human plasma in glass tubes causes thrombin activation, biologically active gentamicin was released. In plasma alone there was no thrombin activity; therefore, gentamicin release was not detected. Figure 4 also shows that PVA−linker−gentamicin could release biologically active gentamicin when it was incubated with SA-infected wound fluid, however, it could not release a detectable amount of gentamicin when incubated with noninfected wound fluid.

In Vivo Bacteriocidal Effect—The logarithm of the bacterial number (1.96 ± 0.79 CFU/mL, $n = 8$, $p < 0.001$) in an animal model of SA-infection in rats in which PVA−linker−gentamicin was applied was significantly lower than that in rats in which PVA−COOH was applied (5.74 ± 0.29 CFU/mL, $n = 8$) or that in rats in which no treatment was administered (3.76 ± 0.37 CFU/mL, $n = 9$). This result showed that PVA−linker−gentamicin was effective in vivo.



Scheme 2—Gentamicin release from PVA-linker-gentamicin by enzymes.

Discussion

LAP is a typical aminopeptidase present in human serum at a concentration of 8−12 mU/mL.[20] It is thought that LAP is also contained at about the same concentration in wound fluid. Actually, aminopeptidase activity was present in wound fluid and its activity was not changed by SA infection. Therefore, Gly-Phe-Pro-Ala-Gly-Gly-gentamicin produced from PVA−linker−gentamicin by the thrombin-like activity in SA-infected wound fluid was rapidly digested by aminopeptidase, and biologically active free gentamicin was generated. It was also shown that active gentamicin was released from PVA−linker−gentamicin in Ca^{2+}-supplemented human plasma; however, no active gentamicin was released by incubation in human plasma alone (Figure 4). When PVA−linker−gentamicin was incubated with only PBS, human plasma, or LAP, gentamicin release was not detected by either the zone of inhibition or FPIA methods (Figures 2 and 4). These results clearly show that significant contaminations of free gentamicin and peptide linker−gentamicin were not present in the PVA−linker−gentamicin.

Because the amount of released gentamicin was dependent on the thrombin concentration (Figure 2) and thrombin-like activity increases with SA-infection,[12] PVA−linker−gentamicin was expected to release an amount of active gentamicin proportional to the degree of SA-infection. In fact, PVA−linker−gentamicin did release biologically active gentamicin in SA-infected wound fluid (Figure 4), and showed a bacteriocidal effect in the animal model of SA infection. A considerable amount of thrombin-like activity was detected in noninfected wound fluid (Table 1); however, no gentamicin release was detected (Figure 4). This result might be because of failure to reach the threshold of the concentration of thrombin-like activity that could digest the macromolecular substrate, PVA−linker−gentamicin, and/or the threshold of gentamicin concentration that could be detected by the zone of inhibition assay (Figure 1).

The zone of inhibition method depends on the biological activity of intact gentamicin. In contrast, the FPIA method uses antibody that binds immunologically active gentamicin and therefore detects molecules that retain the required antigenic properties of gentamicin, such as, peptide-attached gentamicin, amino acid-attached gentamicin, and damaged gentamicin, as well as intact gentamicin. This point is very important in the study of drug delivery systems because there is a considerable chance that released drug may be damaged by the modification. In our synthesis of PVA−linker−gentamicin, shown in Scheme 1, the possibility of damaging the drug is minimized. According to the reaction shown in Scheme 2, PVA−linker−gentamicin can release intact gentamicin (Figures 2−4) in

the presence of thrombin and LAP. These results demonstrated that PVA−linker−gentamicin is an ideal system for the controlled release of this antimicrobial drug.

Systemic and local administration of antibiotics for wound infection is well established. The efficacy of antibiotics, however, depends on factors such as the pharmacokinetics, the timing of infusion, tissue concentrations, and the antibacterial spectrum of the drug. Because systemic administration of antibiotics is associated with certain drawbacks, such as systemic toxicity and poor penetration into severely infected or necrotic tissues, many physicians favor the use of local antibiotics and other topical measures. Controlled local delivery of antibiotics has been shown to reduce the number of microorganisms with minimal side effects compared with systemic administration. Thus, we have developed a new local, controlled-release system of an antibiotic stimulated by bacterial infection. PVA−linker−gentamicin showed specific release of gentamicin in SA-infected wound fluid and caused a significant reduction in SA number in vivo. These results show that this system allows gentamicin to be released at specific times and locations, namely, when and where SA infection occurs. Because PVA−COOH is a potential occlusive dressing material, it is expected that PVA−linker−gentamicin would inhibit bacteria from colonizing the dressing surface when applied to wounds infected with SA. In addition, if proteinases specific to each bacterium could be used for the triggering signal, different spectra of antibiotics could be released from the same material, depending on the strain of bacterium. That is, for bacterium X, use of bacterium-specific proteinase Y and proteinase Y-sensitive linker Z, would result in release of bacterium X-specific antibiotic. Furthermore, it may also be possible to make tumor-specific devices by utilizing peptide linkers sensitive to tumor-producing enzymes, such as some collagenases.[21,22]

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