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A new animal model for evaluation of long-term growth rate over one month by rhGH/PLGA microcapsule formulations

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Abstract

A new animal model to evaluate the long-term growth rate produced by a sustained-release formulation of recombinant human growth hormone (rhGH) over one month was developed and the usefulness of our microcapsule formulations was demonstrated in this model. Long-term pharmacological effects by subcutaneous injection of microcapsules for sustained release of rhGH were evaluated in hypophysectomized (Hpx) rats treated with immunosuppressive agent along with hormone supplement. Copoly($_{DL}$-lactic/glycolic)acid (PLGA) microcapsules for sustained release of rhGH, a two-week sustained-release formulation (rhGH-SR-2W) and a one-month sustained-release formulation (rhGH-SR-1M), were prepared by a solid-in-oil-in-water emulsion solvent evaporation technique. Body-weight gain, body-length gain and serum levels of rat insulin-like growth factor-I (rIGF-I) induced by subcutaneous injection of rhGH-SR were compared with those by daily injections of rhGH solution in Hpx rats for 35 days. Serum IGF-I levels in Hpx rats after the injection of rhGH-SR-2W microcapsules were higher than those after daily injections of rhGH solution. Body-length gain, a new parameter, after single injection of rhGH-SR-1M microcapsules demonstrated the higher growth rate than that after daily injections of rhGH solution for 35 days. Thus, single injection of rhGH-SR microcapsules demonstrated long-term pharmacological effects greater than those by daily injections of rhGH solution in a newly developed model, immunosuppressed Hpx rats.

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Introduction

Recombinant human growth hormone (rhGH) is used to treat short stature in children caused by growth hormone deficiency (GHD), and promising clinical results have been obtained in the treatment of adult GHD, human immunodeficiency virus-associated wasting, Turner's syndrome and growth failure due to chronic renal insufficiency. In addition, because of its anabolic effects, there is clinical evidence that hGH may be useful in treating trauma, clinical malnutrition, osteoporosis and to facilitate wound healing. Currently, patients receive daily subcutaneous injections of rhGH solution over a period of several years. A reduction in frequency of administration would greatly improve patient compliance and increase patient convenience in terms of quality of life. In several papers, clinical studies demonstrated that continuous infusion of rhGH from external pumps provides both IGF-I levels and growth rate in GHD children comparable with those achieved by daily injections (Jorgensen et al 1990; Tauber et al 1993; Cavallo et al 1994; Laursen et al 1994, 1995). However, the pumps or implant devices are not ideal because some surgical procedures are required. In this context, we considered that an injectable microcapsule formulation is one of the most desirable dosage forms of rhGH to maintain the pharmacological efficacy over at least two weeks following a single subcutaneous injection with a standard-gauge needle (generally 23-gauge). The injectable microcapsules for sustained release of rhGH (rhGH-SR) can be prepared by a solvent evaporation technique, which is most commonly used for microencapsulation, mainly because it has been demonstrated that a sterile microcapsule product can be produced — Lupron Depot

has been commercially available since 1989 in the United States (Okada et al 1988; Ogawa et al 1988).

In the last decade, protein delivery using biodegradable microcapsules has been intensively investigated and several articles regarding copoly(DL-lactic/glycolic)acid (PLGA) microspheres incorporating rhGH by solvent evaporation techniques have already been published (Cleland et al 1995; Jones & Cleland 1996; Kim & Park 1999). However, most of them reported difficulty in retaining the integrity and bioactivity of labile proteins through the microencapsulation process, because the preparation process consists of various conditions imposing chemical and physical stresses on labile proteins (e.g. lyophilization, shearing forces, exposure to hydrophobic interfaces and polymer adsorption) (Park et al 1995; Jones & Cleland 1996; Kim & Park 1999). The most commonly used water-in-oil-in-water (W/O/W) emulsion solvent evaporation technique has been associated with severely diminished bioactivity of many proteins (Lu & Park 1995; Yang et al 1999). This method may not be suitable for unstable proteins because the proteins might be denatured at the W/O interface (Sah 1999), and thus a solid-in-oil-in-water (S/O/W) emulsion method will be suitable for protein drugs, because solid-state proteins retain their activity in organic conditions (Castellanos et al 2001). Much of the research on the structural stability of proteins during the microencapsulation process reported the usefulness of additives such as sugars (e.g. trehalose), surfactants (e.g. Tween 20) and poly(ethylene glycol) in S/O/W emulsion methods (Cleland & Jones 1996; Bam et al 1998; Constantino et al 1998; Morita et al 2000; Castellanos et al 2001). However, it is generally difficult to produce injectable microcapsules with high entrapment and small initial release by utilizing the hydrophilic additives, because they would decrease the entrapment and increase the initial burst of proteins (Carrasquillo et al 2001). Therefore, we considered that volatile salts might be desirable additives to obtain fine microparticles of proteins for PLGA microcapsules prepared using an S/O/W emulsion method because most of them would disappear after lyophilization. In our previous studies, we have demonstrated that the microparticles of rhGH were obtained by lyophilizing with ammonium acetate at a molar ratio of 20 times against rhGH (Takada et al 2003). Also, addition of zinc oxide into microcapsules resulted in higher serum levels than those prepared without zinc oxide, suggesting the stabilizing effect of zinc oxide after subcutaneous injection to rats (Yamagata et al 2003). The advantage of zinc oxide as a stabilizer is the ease of assuring the sterility of the oil phase, since it is easy to filter the dichloromethane solution, dissolving PLGA and zinc oxide through a membrane filter with a pore size of 0.2 μm.

The first sustained-release microsphere product of rhGH, Nutropin Depot, has been launched in the United States in 1999. The cryogenic process for producing the microspheres is novel and unique, but it seems rather difficult to scale up for reproducible production and assure the sterility of the product because the handling of liquid nitrogen in an aseptic environment is not easy (Johnson et al 1996; Herbert et al 1998; Tracy 1998). They also reported a month-long effect of rhGH from microspheres, elevated serum levels of insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3) in rhesus monkeys (Johnson et al 1996; Lee et al 1997), although there is no report regarding long-term growth rate of any animals over one month, probably because there is no animal model to evaluate it.

Body-weight gain and tibial growth in hypophysectomized (Hpx) rats have been reported as indices in animal models for pharmacological activity of rhGH for some decades (Van Dyke & Wallen-Lawrence 1930; Evans et al 1943). However, it was impossible to evaluate the long-term pharmacological effects over 10 days due to the antibody formation in rats against rhGH (Cleland et al 1995). We have also observed the saturation of body-weight gain on day 10 after subcutaneous injection of our rhGH-SR-2W microcapsules in Hpx rats without immunosuppression (unpublished data). In normal rats, we studied the effect of tacrolimus on antibody formation to establish the in-vivo release test. Without immunosuppression, the anti-hGH IgG titre in rat serum was approximately 100 000 on day 9 after implantation of two-weeks type mini-osmotic pump (Alzet model 2002, ALZA, Corporation, Palo Alto, CA) providing rhGH solution. However, the titer of antibodies was within the range of detection limit (approximately 1000) on day 9 after implantation, when rats were immunosuppressed with subcutaneous injections of tacrolimus at a dose of 0.4 mg/rat (approximately 2 mg kg^{-1}) three days before the implantation of the mini-osmotic pump and also at a dose of 0.2 mg/rat (approximately 1 mg kg^{-1}) twice a week after the implantation. At the same time, serum levels of rhGH in immunosuppressed rats were maintained almost at a constant level for 14 days, while those in normal rats were remarkably decreased after day 7 (unpublished data). These results indicated that this dose of tacrolimus controlled the immune response in rats and that this dosing schedule could be used for in-vivo release test to determine the serum levels of rhGH in rats after subcutaneous injection of rhGH-SR microcapsules. Similar doses of tacrolimus might be used in Hpx rats for immunosuppression. The use of immunosuppressed animals by intraperitoneal treatment with cyclosporin and hydrocortisone has been reported for the pharmacokinetic study of rhGH in rats, although the precise dosing schedule was not disclosed (Herbert et al 1998).

The objectives of this study were to develop a new animal model to evaluate the long-term growth rate produced by rhGH-SR microcapsules over one month, and to demonstrate the usefulness of our rhGH-SR microcapsules. This paper describes the characteristics of the rhGH-SR microcapsules, including bioactivity of rhGH extracted from microcapsules and long-term pharmacological effects, body-weight gain, serum level of IGF-I and a new parameter, body-length gain, in immunosuppressed Hpx rats after subcutaneous injection of rhGH-SR in comparison with those obtained by daily injections of rhGH solution.

Materials and Methods

Materials

Recombinant human growth hormone (rhGH) was produced by genetic engineering technology at the Pharmaceutical

Table 1 Preparation conditions and characteristics of microcapsules prepared using PLGA with different L/G ratio and weight-average molecular weight.

PLGA		rhGH (%)	Zinc oxide (%)	Size of microcapsules (μm)	Entrapment (%)	Initial release (%)
L/G	MW					
50/50	12 800	7	0.5	36.8	83.3	29
65/35	13 500	15	0.5	35.1	83.1	22
65/35	15 500	15	0.5	34.2	84.0	15

Discovery Center of our company (Nishimura et al 1998; Suenaga et al 1999). Copoly(DL-lactic/glycolic)acid (PLGA) with different L/G ratio and weight-average molecular weight, zinc oxide and D-mannitol were supplied by Wako Pure Chemical Industries, Ltd (Osaka, Japan). Tacrolimus (Prograf) was purchased from Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan). All other chemicals were of reagent grade.

Animals

All procedures involving animals used in this study were consistent with the guidelines set by the National Institutes of Health (NIH publication no. 85-23, revised 1985) and approved by the animal use ethical committee of Takeda Chemical Industries Ltd. Male Sprague-Dawley rats (6 weeks of age) were purchased from Clea Japan, Inc. (Tokyo, Japan). Female Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) after hypophysectomization at 4 weeks of age.

Preparation of microcapsules

Biodegradable microcapsules for sustained release of rhGH were prepared by an S/O/W emulsion solvent evaporation technique using lyophilized rhGH microparticles. rhGH microparticles were obtained by lyophilization with ammonium acetate at a molar ratio of 20 times against rhGH (Takada et al 2003). A weighed amount of rhGH microparticles (140 or 300 mg) was dispersed in 2.7 mL of dichloromethane (DCM) dissolved PLGA (1850 or 1690 mg) with zinc oxide (10 mg). The mixture was homogenized with a Polytron (Kinematica GmbH, Luzern, Switzerland) on ice to make an S/O dispersion at 23 000 rev min^{-1} for 30 s. This S/O dispersion was poured into an 0.1% polyvinyl alcohol aqueous solution (800 mL) cooled to 18 °C under stirring with an Autohomomixer (Tokushu Kika Kogyo Co., Osaka, Japan) at 6000 rev min^{-1}. The resulting mixture was stirred to make an S/O/W emulsion. To evaporate DCM, the S/O/W emulsion was further stirred gently for 3 h. After removing coarse particles larger than 74 μm by sieving, the resulting microcapsules were collected by centrifuging at 2000 rev min^{-1}, rinsed with water and then lyophilized with D-mannitol (200 mg) to yield a powder (rhGH-SR microcapsules). Two-week sustained-release microcapsules (rhGH-SR-2W) were prepared using PLGA with an L/G ratio of 50:50 and weight-average molecular weight of 12 800, where the loading amounts of rhGH and zinc oxide were 7% and 0.5%, respectively. One-month sustained release microcapsules (rhGH-SR-1M) were prepared in the same manner using PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 13 500–15 500, where the loading amounts of rhGH and zinc oxide were 15% and 0.5%, respectively (Table 1).

Characterization of microcapsules

Microcapsules (20 mg) were dissolved in 1.75 mL of acetonitrile and then 3.25 mL of 5 mM phosphate buffer containing 0.15 M sodium chloride (pH 8) was added to extract rhGH. The concentration of rhGH was determined by HPLC using a size-exclusion column (TSK gel G2000SW$_{XL}$; Tosoh) with 50 mM ammonium bicarbonate as mobile phase. The flow rate was 0.6 mL min^{-1} and the eluate was monitored at 214 nm by ultraviolet detection. Particle size distributions of microcapsules were determined using a Coulter Multisizer (Coulter Electronics, UK). The shape and surface structure of the microcapsules were observed using a scanning electron microscope (Model ABT-60; Topcon Co. Ltd, Tokyo, Japan).

Bioactivity of rhGH extracted from microcapsules

Microcapsules (20 mg) were dissolved in 7 mL of acetonitrile and then 13 mL of 5 mM phosphate buffer containing 0.15 M sodium chloride (pH 8) was added to extract rhGH. The concentration of rhGH was determined by HPLC using a size-exclusion column (TSK gel G2000SW$_{XL}$; Tosoh) with 50 mM ammonium bicarbonate as mobile phase. The flow rate was 0.6 mL min^{-1} and the eluate was monitored at 214 nm by ultraviolet detection. Biological activity of the extracted rhGH was determined using an Nb_2 cell proliferation assay (Tanaka et al 1980).

Release profile of rhGH from microcapsules in immunosuppressed rats

The rhGH-SR microcapsules were suspended in a vehicle consisted of 5% mannitol, 0.5% sodium carboxymethyl cellulose and 0.1% Tween 80 in an aqueous solution and injected subcutaneously into the nape of male Sprague-Dawley rats (6 weeks of age) at a dose of 6 mg or 12 mg (as rhGH)/ rat. To eliminate the influence of anti-hGH antibody formation, rats were immunosuppressed with a single subcutaneous injection of tacrolimus at a dose of

0.4 mg/rat three days before the administration of microcapsules and also at a dose of 0.2 mg/rat twice a week after the administration of microcapsules. Serum was periodically collected from the tail vein of the immunosuppressed rats and its hGH levels were determined in duplicate by an immunoradiometric assay (IRMA) kit (Ab Bead HGH 'Eiken'). The detection limit of serum hGH level was 0.3 ng mL^{-1}.

Body-weight gain and body-length gain in hypophysectomized (Hpx) rats treated with immunosuppressive agent

Five-week-old female Sprague-Dawley rats, one week after hypophysectomy, were obtained from Japan SLC Inc. (Shizuoka, Japan). Hpx rats were kept housed under controlled conditions (light from 0700–1900 h, constant temperature at 25 °C) and were allowed free access to standard food and water. The experiment was begun after a 6-day acclimation period. On day 0, rats were randomized into four or five groups. During the experiment, Hpx rats were immunosuppressed by subcutaneous injections of tacrolimus solutions (50 μg/rat on days −3, 0, 4, 7 and 11, and 75 μg/rat on days 14, 18, 21, 25, 28 and 32). L-Thyroxine sodium salt (1 μg/rat) and hydrocortisone succinate (50 μg/rat) were also given by subcutaneous injection three times a week for hormone supplementation. Body weights were measured in the morning between 0800 and 0930 h. Body lengths, from nose to tip of the tail, were measured under anaesthesia with diethyl ether.

Body-weight gain in Hpx rats was determined after subcutaneous injection of rhGH-SR-2W microcapsules or daily subcutaneous injections of an equivalent overall dose of rhGH solution (6 mg or 12 mg/rat/2 weeks). The control group was given 5% mannitol solution. The number of rats in each group was twelve. Subsequently, to assure the reproducibility of pharmacological effect, rhGH-SR-2W microcapsules were injected on day 14 at a dose of 6 mg/rat/2 weeks and the body-weight gain was compared with that after daily subcutaneous injections of rhGH solution for 28 days. The body lengths of eight Hpx rats, except the rats for serum sampling (four rats in each group), were measured on day 35 and the body-length gain was calculated by subtracting the mean value of the control group in the experiment using rhGH-SR-2W microcapsules. As for rhGH-SR-1M microcapsules comprising PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 13 500 or 15 500, body weights and body lengths were measured periodically for 35 days after subcutaneous injection of the microcapsules and were compared with those after daily subcutaneous injections of an equivalent overall dose of rhGH solution (12 mg/rat/4 weeks). The control group was given 5% mannitol solution. The number of rats in each group was eight to ten.

Serum levels of hGH and rat IGF-I in immunosuppressed Hpx rats

Serum was periodically collected from the tail vein of the immunosuppressed Hpx rats and the hGH levels were determined in duplicate by an immunoradiometric assay (IRMA) kit (Ab Bead HGH 'Eiken'). The detection limit of serum hGH level was 0.3 ng mL^{-1}. Serum rat IGF-I (rIGF-I) levels were determined in duplicate by the rIGF-I radioimmunoassay kit (DSL-2900, Diagnostic Systems Laboratories, Inc.).

Statistical analysis

All results were expressed as the mean ± standard deviations (s.d.) of 4–12 experiments. Statistical analysis was performed using Statistical Analysis System (SAS) program. The significance of differences in body-weight gain on day 14 and AUC values of serum rIGF-I levels during the first 14 days between the rhGH-SR-2W microcapsule group and the daily injection group, at each dose, was statistically analysed using Student's t-test with Holm's correction for multiple comparison. The numbers of rats for determination of body-weight gain and serum rIGF-I levels in each group were twelve and four, respectively. Statistical analysis of significant differences in body-weight gain on day 35 between the rhGH-SR-2W (12 mg × 1) group and daily injection (0.86 mg × 14) group, and also between rhGH-SR-2W (6 mg × 2) group and daily injection (0.43 mg × 28) group was performed using Student's t-test with Holm's correction for multiple comparison. The number of rats in each group was twelve. In body-length gain on day 35, the significance of differences between the rhGH-SR-2W (12 mg × 1) group and the daily injection (0.86 mg × 14) group, and also between the rhGH-SR-2W (6 mg × 2) group and the daily injection (0.43 mg × 28) group were statistically analysed using Student's t-test with Holm's correction for multiple comparison. The number of rats in each group was eight. In the pharmacological study of rhGH-SR-1M microcapsules, the significance of differences in body-weight gain and body-length gain on day 28 and day 35 of the microcapsules groups versus the daily injection group were statistically analysed using Dunnett's test with Bonferroni's correction for multiple comparison. The number of rats in each group was eight to ten. $P < 0.05$ was regarded as statistically significant.

Results

Characteristics of rhGH-SR microcapsules

PLGA microcapsules incorporating rhGH, both rhGH-SR-2W and rhGH-SR-1M, had a spherical shape and smooth surface (Figure 1). The entrapment efficiency of rhGH into microcapsules was 83–84% in either rhGH-SR-2W or rhGH-SR-1M (Table 1). The mean particle size of the microcapsules was approximately 35 μm, and the suspension after dispersion in a vehicle was easily injectable with a 23-gauge or 25-gauge needle at a concentration of microcapsule 200 mg/mL vehicle. A smaller gauge needle would reduce the pain at the injection site and greatly benefit pediatric GHD patients in terms of quality of life and compliance.

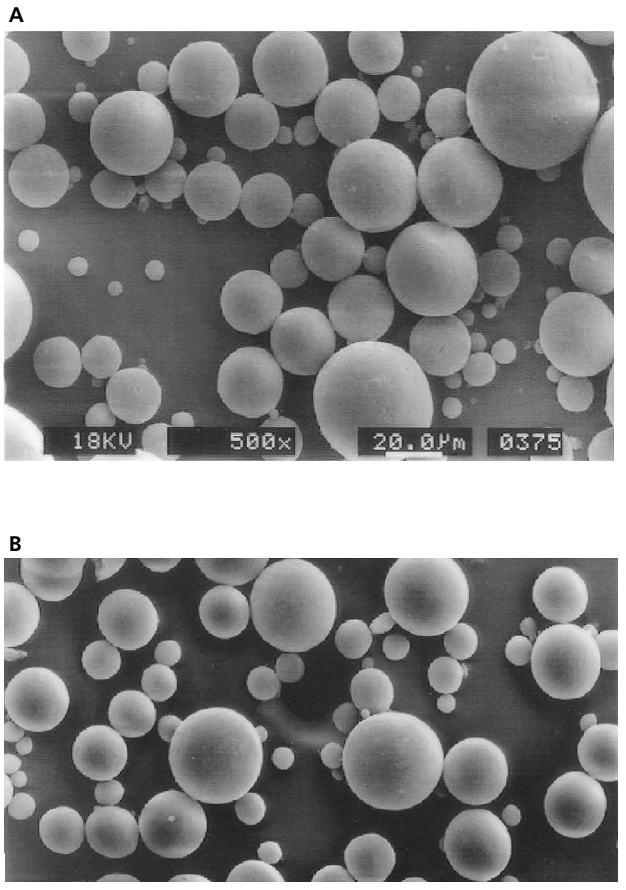

Figure 1 Scanning electron micrographs of rhGH-SR-2W microcapsules (A) and rhGH-SR-1M microcapsules (B).

Bioactivity of rhGH extracted from microcapsules

The specific bioactivity of intact rhGH was 3.43 IU mg^{-1} in the Nb_2 cell proliferation assay ($n = 2$). rhGH extracted from rhGH-SR-2W microcapsules demonstrated a similar bioactivity (3.56 ± 0.294 IU/mg (mean $\pm$ s.d., $n = 6$)). As for rhGH-SR-1M microcapsules comprising PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 14 200, the specific bioactivity of rhGH extracted from microcapsules was 3.20 ± 0.261 IU mg^{-1} ($n = 6$) and was as potent as that of lyophilized rhGH (3.34 ± 0.364 IU mg^{-1} ($n = 6$)). All of these values are considered within the range of experimental variance in this assay. The results of specific bioactivity of rhGH extracted from both rhGH-SR-2W and rhGH-SR-1M microcapsules demonstrated that rhGH was not inactivated during the microencapsulation process.

Release profile of rhGH from microcapsules in immunosuppressed rats

Serum levels of rhGH were determined after a single subcutaneous injection of microcapsules in rats immunosuppressed with tacrolimus. The microcapsules comprising PLGA with an L/G ratio of 50/50 and weight-average molecular weight of 12 800 (rhGH-SR-2W) showed serum rhGH levels of approximately 10 ng mL^{-1} for 9 days and then maintained the levels higher than the pre-dose level for 2 weeks in rats (Figure 2). The rhGH-SR-1M microcapsules comprising PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 13 500 or 15 500 demonstrated one-month sustained-release profiles in rats. The amount of rhGH released during the first 24 h after the injection of microcapsules was calculated from the dose–AUC calibration curve because there was linearity between the dose and AUC of serum rhGH levels after subcutaneous bolus injection of rhGH solution (Takada et al 2003). The values of initial release of rhGH from rhGH-SR-2W, rhGH-SR-1M (65/35-13500) and rhGH-SR-1M (65/35-15500) microcapsules against the administered dose were 29% , 22% and 15% , respectively (Table 1).

Serum levels of hGH and rIGF-I in immunosuppressed Hpx rats

Serum levels of hGH in female immunosuppressed Hpx rats, after subcutaneous injection of rhGH-SR-2W microcapsules at the higher dose of 12 mg, were maintained at higher levels than those obtained at the lower dose of 6 mg for two weeks dose dependently (Figure 3A). Repeated injections of rhGH-SR-2W microcapsules at the dose of 6 mg/2 weeks demonstrated good reproducibility in serum hGH profiles. The elevated serum hGH levels induced serum rIGF-I levels higher than basal levels in immunosuppressed Hpx rats for

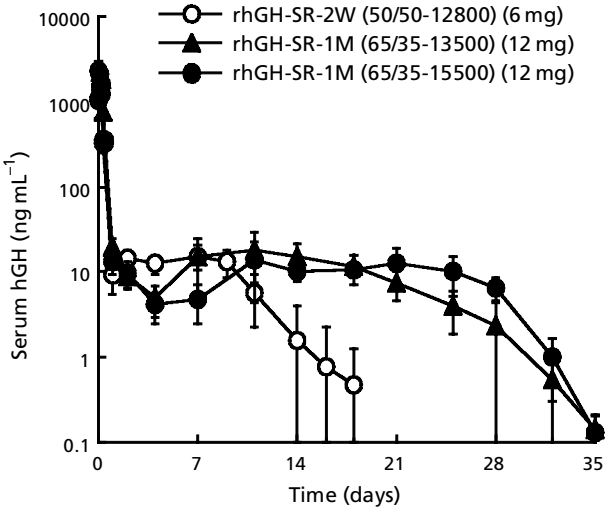

Figure 2 Serum levels of rhGH in male rats immunosuppressed with tacrolimus after single subcutaneous injection of rhGH-SR-2W microcapsules comprising PLGA with an L/G ratio of 50/50 and weight-average molecular weight of 12 800 (6 mg as rhGH) or rhGH-SR-1M microcapsules comprising PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 13 500 or 15 500 (12 mg as rhGH). Each point represents the mean $\pm$ s.d. of data from 4–6 rats.

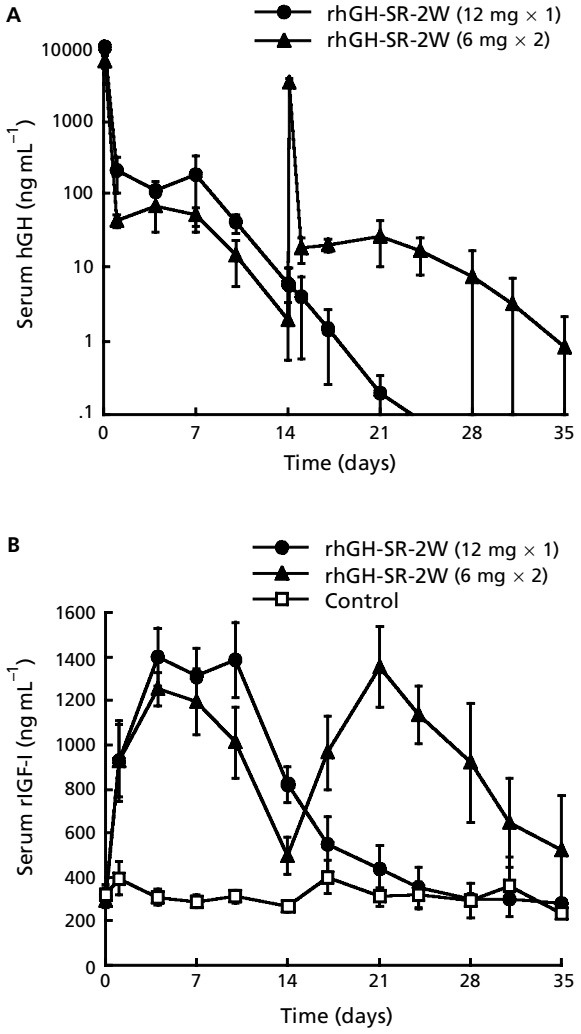

Figure 3 Serum levels of rhGH (A) and rIGF-I (B) in female immunosuppressed Hpx rats after single subcutaneous injection (12 mg as rhGH) or repeated injections (6 mg/2 weeks as rhGH) of rhGH-SR-2W microcapsules. PLGA: L/G = 50/50, MW = 12 800. Each point represents the mean ± s.d. of data from 4 rats.

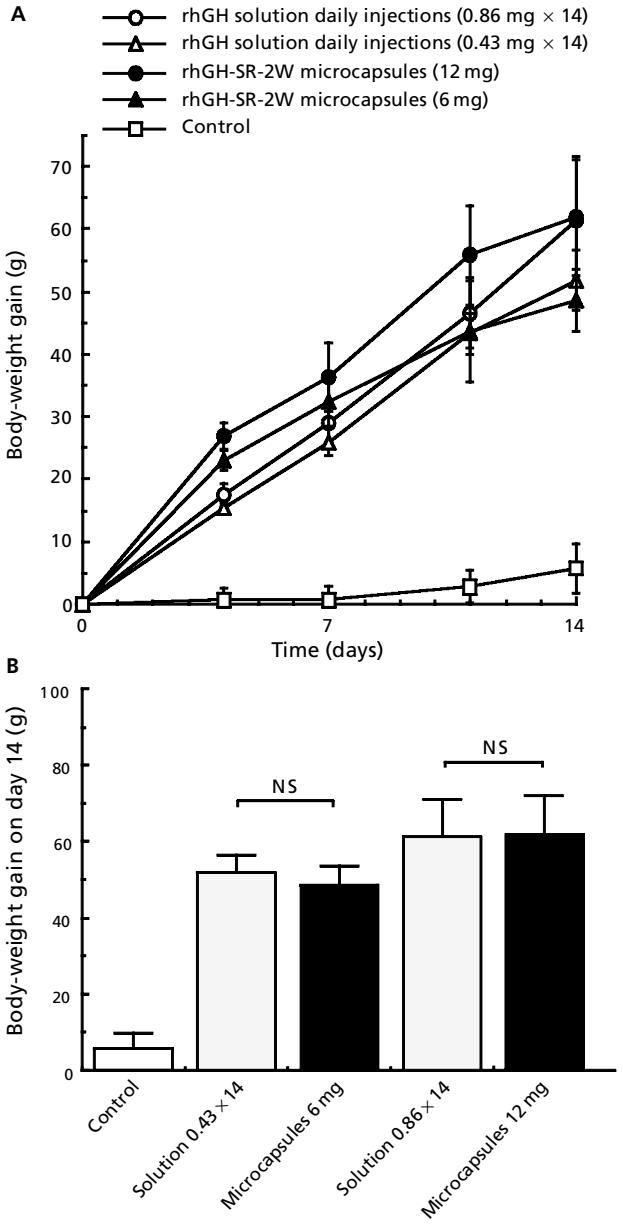

Figure 4 Body-weight-gain profiles (A) and the body-weight gain on day 14 (B) in female immunosuppressed Hpx rats after single sc injection of rhGH-SR-2W microcapsules (6 mg or 12 mg as rhGH) or daily sc injections of rhGH solution (0.86 mg × 14 or 0.43 mg × 14). PLGA: L/G = 50/50, MW = 12 800. Each point represents the mean ± s.d. of data from 12 rats. NS, not significant vs respective daily injection group (Student's t-test with Holm's correction for multiple comparison).

two weeks (Figure 3B). Also, the serum rIGF-I profiles showed dose dependency and good reproducibility.

Body-weight gain and serum rIGF-I levels in immunosuppressed Hpx rats after a single injection of rhGH-SR-2W microcapsules or daily injections of rhGH solution

The body-weight gain in the rhGH-SR-2W microcapsule group showed a higher growth rate than that in the rhGH solution daily injection group for about 10 days and both became equivalent on day 14 (Figure 4A). At the higher dose of 12 mg/2 weeks, the body-weight gain by both the daily injection and microcapsule group were larger than that obtained at the lower dose of 6 mg/2 weeks (Figure 4B).

Single subcutaneous injection of rhGH-SR-2W microcapsules induced higher serum rIGF-I levels than daily injections of an equivalent overall dose of rhGH solution for two weeks at both doses (Figure 5A). The AUC values of serum rIGF-I levels during the 14 days in the rhGH-SR-2W microcapsule group were significantly larger than those in the hGH solution daily injection group at both doses (Figure 5B).

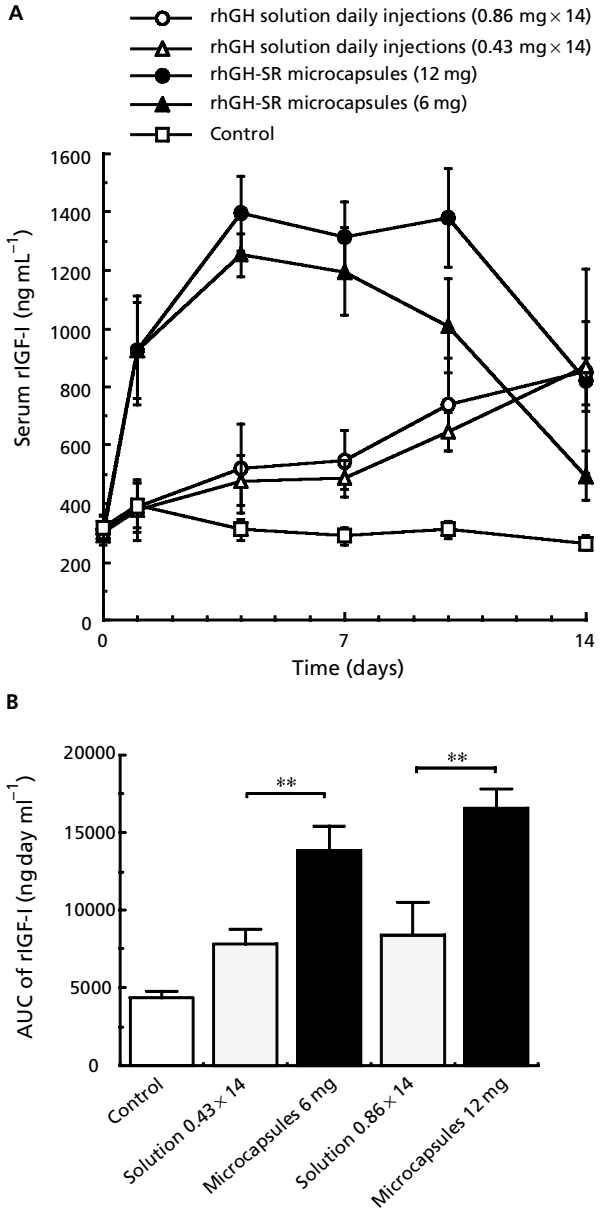

Figure 5 Serum rIGF-I levels (A) and the AUC values of serum rIGF-I levels during the first 14 days (B) in female immunosuppressed Hpx rats after a single subcutaneous injection of rhGH-SR-2W microcapsules (6 mg or 12 mg as rhGH) or daily subcutaneous injections of rhGH solution (0.86 mg × 14 or 0.43 mg × 14). PLGA: L/G = 50/50, MW = 12 800. Each point represents the mean ± s.d. of data from 4 rats. **P< 0.01 vs respective daily injection group (Student's t-test with Holm's correction for multiple comparison).

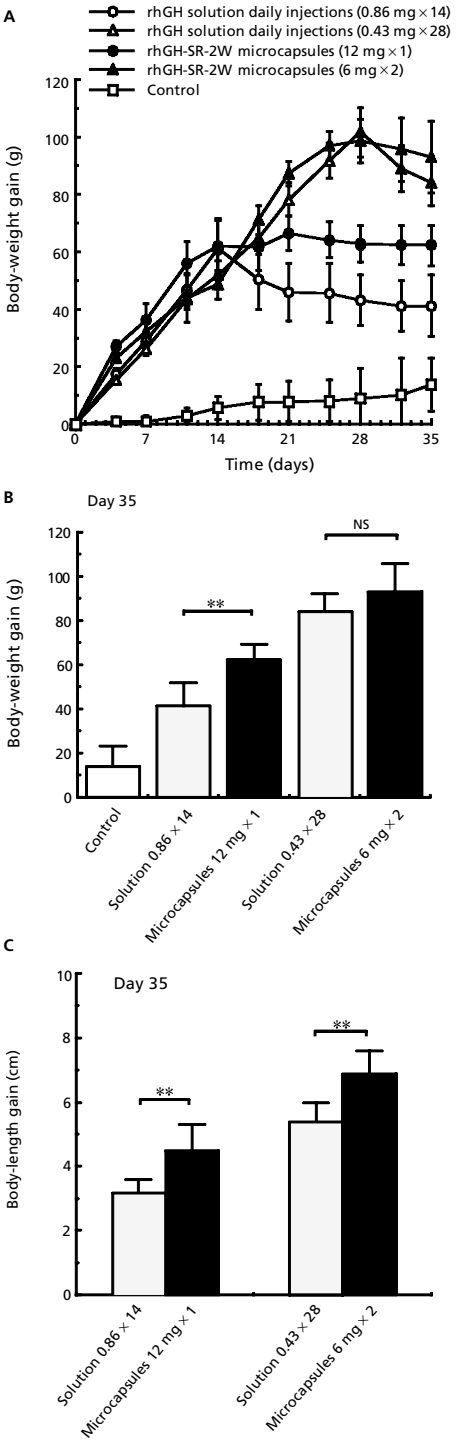

Figure 6 Body-weight-gain profiles (A), the body-weight gain on day 35 (B) and body-length gain on day 35 (C) in female immunosuppressed Hpx rats after a single subcutaneous injection (12 mg as rhGH) or repeated injections (6 mg/2 weeks as rhGH) of rhGH-SR-2W microcapsules (PLGA: L/G = 50/50, MW = 12 800). An equivalent overall dose of rhGH solution was administered as daily subcutaneous injections (0.86 mg × 14 or 0.43 mg × 28). Each point represents the mean ± s.d. of data from 12 (A, B) or 8 (C) rats. **P < 0.01 vs respective daily injection group (Student's t-test with Holm's correction for multiple comparison). NS, not significant.

Reproducibility of body-weight gain by repeated injections of rhGH-SR-2W microcapsules

The body-weight gain in Hpx rats by repeated injections of rhGH-SR-2W microcapsules showed good reproducibility (Figure 6A). During the first 14 days, the body-weight gain was 48.6 ± 5.0 g after subcutaneous injection

of microcapsules at the dose of 6 mg/2 weeks, and the gain during the next 14 days was 50.3 ± 7.1 g after the second injection of the microcapsules on day 14. In the group receiving daily injections of rhGH solution, the body-weight gain remarkably decreased after daily injections had stopped on day 14 and day 28, respectively. On day 35, the body-weight gain of the rhGH-SR-2W ($12\,mg \times 1$) group was significantly larger than that of the daily injection ($0.86\,mg \times 14$) group by statistical analysis using Student's t-test with Holm's correction for multiple comparison (Figure 6B, $P < 0.01$). At the end of the study on day 35, we measured the body lengths of Hpx rats to compare the body-length gain. The body-length gain on day 35 in Hpx rats of the rhGH-SR-2W microcapsule groups was significantly larger than that of the daily injection groups at both doses (Figure 6C, $P < 0.01$).

Body-weight gain and body-length gain in immunosuppressed Hpx rats after single injection of rhGH-SR-1M microcapsules

The body-weight gain in Hpx rats produced by a single injection of rhGH-SR-1M microcapsules demonstrated a growth rate higher than or equal to that produced by daily injection of rhGH solution for 28 days (Figure 7A). On day 28, the body-weight gain of the rhGH-SR-1M microcapsule (65/35-15500) group was significantly larger than that of the daily injection ($0.43\,mg \times 28$) group by statistical analysis using Dunnett's test for comparison (Figure 7B, $P < 0.05$). Just as shown in Figure 6A, the body-weight gain remarkably decreased after daily injections had stopped on day 28. On day 35, the body-weight gain in Hpx rats produced by single injection of both kinds of rhGH-SR-1M microcapsules was significantly larger than that produced by daily injections of rhGH solution (Figure 7B, $P < 0.01$). Also, body-length-gain profiles revealed that a single injection of rhGH-SR-1M microcapsules produced higher growth rate than that by daily injections of rhGH solution (Figure 7C). On day 28 and day 35, the body-length gains in Hpx rats produced by both kinds of rhGH-SR-1M microcapsules were significantly larger than that produced by daily injections of rhGH solution (Figure 7D, $P < 0.01$).

Discussion

This must be the first report to demonstrate the linear long-term growth in body weight and body length of Hpx rats produced by rhGH over one month. Previously, we observed the saturation of body-weight gain on day 10 after subcutaneous injection of rhGH-SR-2W microcapsules in Hpx rats without immunosuppression (unpublished data). This saturation phenomenon of body-weight gain in Hpx was supposed to be due to anti-hGH antibody formation in rats. By subcutaneously injecting tacrolimus twice a week at a dose of 0.1 mg/rat (approximately $1.2\,mg\,kg^{-1}$), the body-weight gain in Hpx rats increased over 14 days in the rhGH-SR-2W microcapsule group, although the body weight of the control group decreased gradually and some of them died, probably as a result of a serious condition due to the combination of hypophysectomization and immunosuppression. As a supplement of hormones, thrice-weekly injections of both L-thyroxine and hydrocortisone were effective to keep female Hpx rats in good condition (Maiter et al 1988). Thus, we have established a new animal model for evaluating the long-term pharmacological effects of rhGH-SR over 35 days. Although the tibial epiphyseal widths in these rats were measured at the same time, the growth rate during the second week was much slower than that during the first week after rhGH treatment. As reported previously, the width of the cartilage increases during the first 6–8 days of treatment with growth hormone until the normal equilibrium between chondrogenesis and osteogenesis is re-established (Evans et al 1943). Therefore, the tibial epiphyseal width was not suitable for an index of long-term pharmacological effects of rhGH-SR over 14 days.

In our previous report, we have described the preparation conditions and characteristics of an injectable sustained-release formulation of rhGH prepared by incorporating lyophilized rhGH microparticles into PLGA microcapsules by a solvent evaporation technique through an S/O/W emulsion (Takada et al 2003). The mean particle size of rhGH in S/O dispersions showed minimum value when ammonium acetate was added at a molar ratio of 20 times against rhGH and was a critical factor for reducing the initial release. The release profile was adjustable by selecting the proper PLGA and the microcapsules prepared utilizing PLGA with an L/G ratio of 50/50 and weight-average molecular weight of approximately 13 000 demonstrated a two-week sustained-release profile. In this study, one-month sustained-release profiles were obtained by a single subcutaneous injection of rhGH-SR microcapsules prepared using PLGA with an L/G ratio of 65/35 and weight-average molecular weight of 13 500 or 15 500 (Figure 2).

In immunosuppressed Hpx rats, rhGH-SR-2W microcapsules provided sustained serum levels of rhGH for two weeks dose dependently (Figure 3A). Also, the polymer matrix had almost completely disappeared from the injection site in rats on day 21. Repeated injections of rhGH-SR-2W demonstrated good reproducibility in serum hGH profiles. Serum rIGF-I levels in immunosuppressed Hpx rats were upregulated by the elevated serum hGH levels (Figure 3B). Serum IGF-I levels in Hpx rats, after the injection of microcapsules, were higher than those after daily injections of rhGH solution (Figure 5A). A similar phenomenon has been reported in humans treated with continuous infusion and daily injections (Tauber et al 1993; Laursen et al 1994, 1995). IGF-I is generally recognized as the mediator of the growth-promoting effect of growth hormone and serum IGF-I levels are low in GHD patients and high in acromegaly. Nevertheless, the precise correlation between serum IGF-I level profiles and growth rate is still unknown (Tauber et al 1993; Cavallo et al 1994). When the indications, such as the treatments of

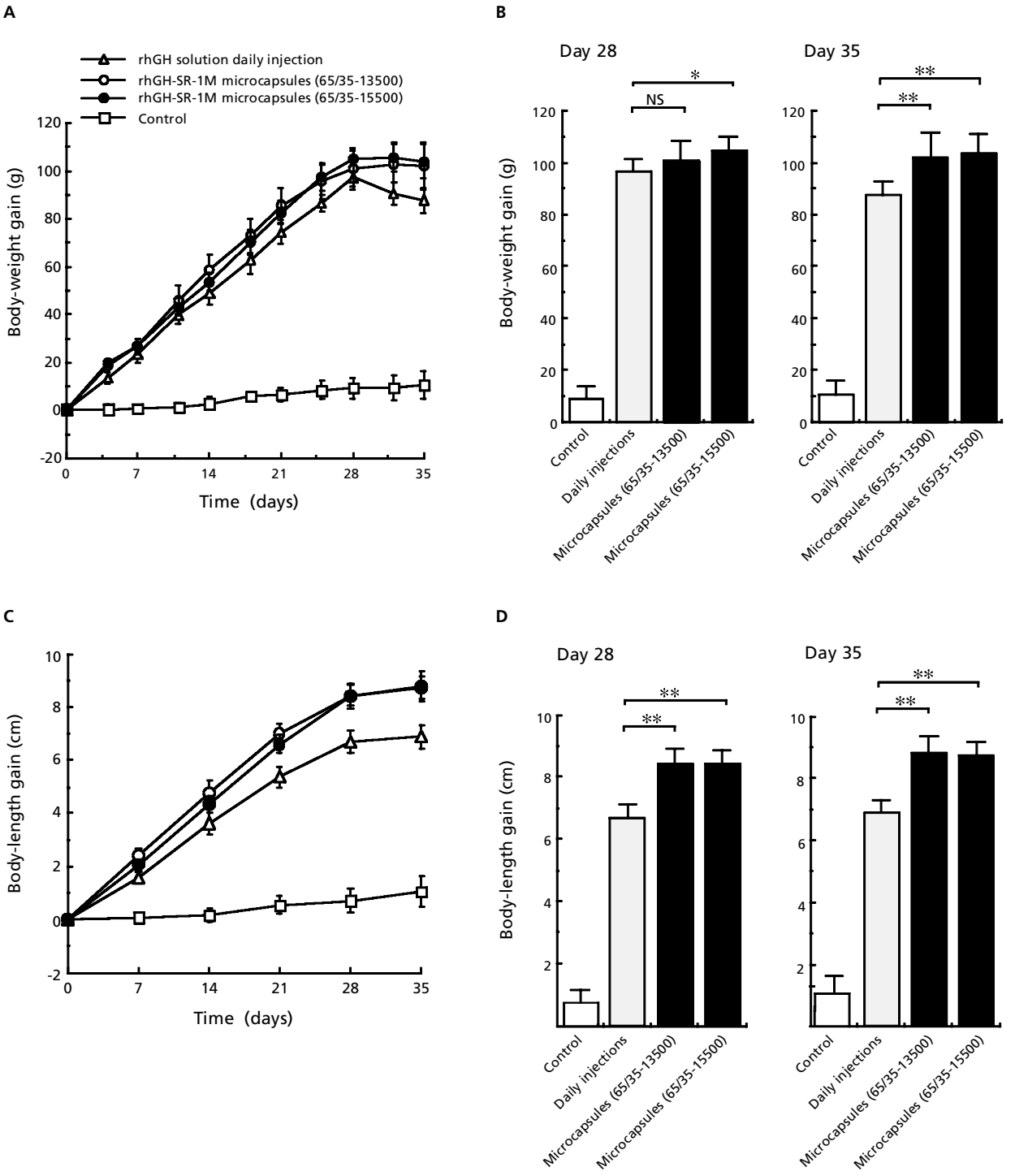

Figure 7 Body-weight-gain profiles (A, B) and body-length-gain profiles (C, D) in female immunosuppressed Hpx rats after a single subcutaneous injection (12 mg as rhGH) of rhGH-SR-1M microcapsules (PLGA: L/G = 65/35, MW = 13 500 or 15 500). An equivalent overall dose of rhGH solution was administered as daily subcutaneous injections (0.43 mg × 28). Each point represents the mean ± s.d. of data from 8–10 rats. *$P < 0.05$, **$P < 0.01$ vs respective daily injection group (Dunnett's test with Bonferroni's correction for multiple comparison). NS, not significant.

adult GHD, human immunodeficiency virus-associated wasting, trauma, clinical malnutrition, osteoporosis and wound healing, were considered, the higher serum IGF-I levels induced by the microcapsules might be more effective than those by conventional daily injections of rhGH solution.

On day 35, the body-weight gain and body-length gain of the rhGH-SR-2W (12 mg × 1) group were lower than those of the daily injection (0.43 mg × 28) group (Figure 6B, C). Also, the serum levels of rhGH and IGF-I in Hpx rats after a single injection of rhGH-SR-2W (12 mg × 1) did not show the one-month release profile (Figure 3A, B). These results indicate that it is impossible to achieve the pharmacological action equivalent to daily injection over one month by a single injection of rhGH-SR-2W at a double dose (12 mg × 1).

The body-weight gain in immunosuppressed Hpx rats increased dose dependently by both modes (continuous infusion and pulsatile injection) of rhGH administration over two weeks (Figure 4B). However, the latter remarkably decreased after daily injections had stopped on day 14 and day 28, respectively (Figure 6A). The reason for this decrease in weight gain is considered to be a return from a temporal excessive circulatory plasma volume increased condition due to growth-hormone-induced renal sodium retention, for which clinically reported common side effects are fluid retention, swelling or oedema (Meling & Nylen 1996; Loon 1998). On the contrary, body-weight gain in Hpx rats after the injection of rhGH-SR microcapsules at both doses did not decrease, suggesting that the gain was due to the substantial growth of bone and muscles. The difference between body-length gain on day 35 after the injections of microcapsules and the gain after daily injections (Figure 6C) was consistent with the difference in body-weight gain on day 35 (Figure 6B). The body-weight gain and body-length gain in immunosuppressed Hpx rats increased linearly for one month by single injection of rhGH-SR-1M microcapsules or by daily injections of rhGH solution (Figure 7A and 7C). The advantage of body-length gain in immunosuppressed Hpx rats over body-weight gain, as a new index for evaluation of pharmacological efficacy of rhGH, is the possibility of avoiding the overestimation observed in the body-weight gain that might result from the phenomena such as fluid retention, swelling or oedema (Figure 6A and 7A), and the capability of evaluating the substantial growth of bone and muscles over one month without any reduction (Figure 7C). Body-length gain in Hpx rats might be useful as a new index for evaluation of pharmacological efficacy of rhGH in relation to the growth rate in height of GHD children.

Conclusions

We have successfully developed a new animal model for evaluating the long-term growth rate produced by an rhGH-SR formulation over one month and demonstrated the usefulness of our rhGH-SR microcapsules prepared by an S/O/W emulsion solvent evaporation technique in this model. This must be the first report to demonstrate the linear long-term growth in body weight and body length of Hpx rats induced by rhGH over one month. The body-weight gain induced in immunosuppressed Hpx rats by a single injection of rhGH-SR-2W was equivalent to that produced by daily injections of rhGH solution on day 14. Serum IGF-I levels in Hpx rats after the injection of rhGH-SR-2W microcapsules were higher than those after daily injections of rhGH solution. The body-weight gain and serum IGF-I levels in Hpx rats produced by repeated injections of rhGH-SR-2W microcapsules showed good reproducibility. Although it was impossible to achieve the pharmacological action equivalent to daily injection over one month by a single injection of rhGH-SR-2W at a double dose, one-month sustained release profiles were obtained by a single injection of rhGH-SR microcapsules prepared using PLGA with an L/G ratio of 65/35 and a weight-average molecular weight of 13 500 or 15 500. Body-length gain and body-weight gain on day 35 after single injections of rhGH-SR-1M microcapsules were significantly larger than those after daily injections of an equivalent overall dose of rhGH solution. Body-length gain in Hpx rats might be useful as a new index for evaluation of pharmacological efficacy of rhGH in relation to the growth rate in height of GHD children. This new animal model may contribute to the development of a more reliable evaluation method in preclinical studies that include such formulation screening.

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