Pharmacokinetics and Safety of Human Recombinant Hepatocyte Growth Factor Administered to Vocal Folds

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Objectives/Hypothesis: Previous animal studies demonstrated that hepatocyte growth factor (HGF) has the potential to regenerate scarred vocal folds. In addition, HGF is now produced under a good manufacturing practice (GMP) procedure. Therefore, human clinical trials of HGF are warranted in patients with vocal fold scarring. In the current study, we investigated the pharmacokinetics and the local tissue responses of HGF administered to rat vocal folds.

Study Design: Prospective animal experiment.

Methods: Five μg of recombinant human HGF was administered to the vocal folds of Sprague-Dawley rats (n = 60) using a microsyringe. The concentration of HGF in larynges and blood was investigated by enzyme-linked immunosorbent assay. To evaluate the local tissue responses caused by HGF administration, endoscopic and histological examinations were performed.

Results: HGF concentration in the larynges was 50.1 μg/g tissue 5 minutes after administration. The concentration decreased rapidly to 1.71 μg/g tissue at 12 hours after administration and to 0.29 ng/g tissue at 24 hours after administration. Seven days after administration, HGF concentration was minimal in one-half of the cases and was not detected in the other cases. Transmission of HGF to blood was detected in two of six cases at 5 minutes after administration, but was no longer detected 12 hours later. Endoscopic and histological examinations revealed no edema or erythema of the vocal folds in any of the cases.

Conclusions: The current results contribute to the safety and pharmacokinetic management of future clinical trials using HGF administered to vocal folds.

Key Words: Hepatocyte growth factor, vocal fold, pharmacokinetics, safety.

Level of Evidence: N/A.

INTRODUCTION

Vocal fold scars result from a disruption of the layer structure in the vocal fold mucosa. This is caused by injury and inflammation, including chronic vocal abuse and laser surgery of the vocal folds. Once vocal folds are scarred, the vocal mucosa stiffens, the viscoelasticity of vocal folds is decreased, and permanent dysphonia occurs. Histological studies revealed dense disorganized collagen deposition and a reduction of hyaluronic acid (HA), elastin and, decorin. To date, no consistent treatment has been developed to treat vocal fold scarring. Voice therapy cannot restore the histological alteration. Augmentation procedures, including fat injection and medialization thyroplasty, can lessen the glottic gap but do not significantly improve mucosal function. Technology to improve mucosal function has been warranted.

Hepatocyte growth factor (HGF) was originally purified from the plasma of the patients with fulminant hepatic failure. Human HGF has two natural forms: 1) full-length HGF composed of 697 amino acid residues and 2) a five amino acid-deleted type HGF (dHGF), which lacks the F-L-P-S-S amino acid residues in the first kringle domain. HGF plays a significant role in embryogenesis, angiogenesis, organ regeneration, and wound healing—and has been reported to have beneficial effects in several organs including kidney, liver, and lung. HGF also has a favorable potential for the treatment of vocal fold scarring. Animal studies demonstrated that local administration of full-length HGF to scarred vocal folds increased HA and reduced collagen deposition in the lamina propria of the vocal folds, leading to an improvement of viscoelasticity of the vocal folds. In vitro studies using fibroblasts derived from vocal folds showed that full-length HGF increased the mRNA expression of hyaluronic acid synthase (HAS)—1 and HAS-2 and increased HA production.
comparison study of dHGF with full-length HGF showed that dHGF had a similar potential for regenerative effects on vocal fold scars as full-length HGF. Recently, dHGF has begun to be produced under good manufacturing practice (GMP) procedures. Therefore, human clinical trials of dHGF in patients with vocal fold scarring are warranted.

Pharmacokinetic and safety tests using animals are essential before human clinical trials can commence. Previous pharmacokinetic studies using rats indicated that liver is the major clearance organ for HGF. Sugiura et al. investigated the pharmacokinetics of dHGF administered intravenously, which demonstrated that the kidneys have a minimal contribution to clearance of dHGF. In addition, a pharmaceutical company has performed various pharmacokinetic and safety tests of dHGF administered intravenously (unpublished data). However, there are no pharmacokinetic and safety tests on dHGF administered to vocal fold mucosa.

In the current study, we investigated the concentration of dHGF in vocal folds and transmission to blood after administration of dHGF to rat vocal folds. In addition, we specifically address concerns regarding the administration of HGF to the vocal fold center and whether administration to vocal folds causes swelling of vocal folds or narrowing of the airway. Endoscopic and histological examinations were performed to evaluate local tissue responses caused by administration of dHGF.

MATERIALS AND METHODS

Animals and Drug Treatment

Recombinant human hepatocyte growth factor, which is the five amino acid-deleted type, was supplied by Kringle Pharma Inc. (Osaka, Japan) and diluted to 1 mg/mL in 2 mM citrate buffer (pH 6.0) containing 0.002% polysorbate 80, 0.15 M NaCl, 0.16 mg/mL glycine, and 3 mg/mL lactose.

Sixty 13-week-old male Sprague-Dawley rats were used in this study. All experimental protocols were approved by the animal research committee of Kyoto University Graduate School of Medicine. Animal care was provided by the Institute of Laboratory Animals of Kyoto University.

Evaluation of HGF Concentration of the Solution Discharged Through the Microsyringe

A low concentration of dHGF may be absorbed within the microsyringe (22-gauge needle; Hamilton Co., Reno, NV) used in the following experiments. To evaluate the loss of dHGF in the microsyringe, the concentration of the HGF solution discharged through the microsyringe was measured by enzyme-linked immunosorbent assay (ELISA). One mg/mL dHGF was drawn into and discharged through the microsyringe. After the microsyringe was washed with more than five repeated procedures of suction and discharge of normal saline (NS), the same procedure was repeated (n = 8).

Injection of HGF or Saline to Vocal Folds

The rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (4.5 mg/kg) after inhalative sedation with diethyl ether. Atropine sulfate (0.005 mg/kg) was also injected intraperitoneally to reduce the secretion of saliva and sputum in the laryngeal lumen. Rats were positioned on a custom-made operation platform. Visualization of the larynx was achieved with a transoral endoscope connected to video equipment. Topical anesthesia (5% lidocaine) was applied to the vocal folds to reduce their movement. Five μL per vocal fold of 1 mg/mL dHGF or NS was injected into the vocal folds with the microsyringe. To evaluate the concentration of dHGF and transmission to blood, dHGF was injected to the bilateral vocal folds. To evaluate the nonspecific background of the ELISA kit, NS was injected to the bilateral folds (n = 6). In addition, for endoscopic and histological examinations, dHGF was injected into one side of the vocal folds and NS was injected into the other side.

Extraction of HGF in Larynges

At each time point (n = 6), blood was sampled through intracardiac injection after rats were anesthetized, as described above. After placing on ice for 2 hours, the blood was centrifuged at 3,000 rpm for 15 minutes to obtain serum. After sampling the blood, the rats were humanely euthanized with an intracardiac injection of pentobarbital sodium (200 mg/kg), and whole larynges were harvested from each animal. HGF in larynges was extracted using HGF extraction reagent (Institute of Immunology Co., LTD., Tokyo, Japan) following the manufacturer’s protocol. Phenylmethanesulfonyl fluoride (PMSF) was prepared by dissolving 17.4 mg of PMSF in 1 mL of isopropyl alcohol, and 100 mM PMSF was added to the extraction buffer to obtain a final concentration of 1 mM PMSF. After measuring the weight of the tissue, the larynges were cut into small pieces and added to 500 μL of extraction buffer prepared as above. After homogenizing the mixture of larynges and extraction buffer with a Polytron homogenizer (Central Scientific Commerce, Inc., Osaka, Japan) and a manual homogenizer (As One Inc., Osaka Japan), the mixture was centrifuged at 15,000 rpm for 30 minutes at 4℃. The intermediate layer (300 μL) was then sampled.

Measurement of HGF Concentration

ELISA was performed using the Immunos HGF EIA (Institute of Immunology Co., Ltd.), which is reported to specifically detect human HGF and not rat HGF. Because the recommended measurement range was under 3 ng/mL, the extracted samples were diluted to an adequate concentration with sample diluent in the Immunos HGF EIA kit. Standard solution was prepared by diluting 1 mg/mL dHGF with sample diluent. The following procedures were performed according to the manufacturer’s protocol. After 50 μL of sample diluent was pipetted into the wells of a microplate coated with anti-HGF monoclonal antibody, 50 μL of standard solution, or samples were dispensed into each well. The microplate was then shaken at 500 rpm for 1 hour at room temperature (primary reaction). After washing the wells with the washing solution, 100 μL of enzyme-labeled monoclonal antibody was pipetted into each well, and the microplate was shaken at 500 rpm for 1 hour at room temperature. After washing wells with the washing solution, 100 μL of the enzyme substrate solution containing the color developer was pipetted into each well, and the microplate was left at room temperature in the dark for 30 minutes. Fifty μL of stop solution was then added to all wells. The absorbance of each well at 485 nm was measured using a microplate reader (Fluostar Optima, BMG Labtech, Ortenburg, Germany). Standard curves were used to determine HGF concentration in each sample. Because the minimum concentration of standard solution was 0.5 ng/mL, the limit of quantification was designated as 0.5 ng/mL. When HGF concentration was below the limit of quantification (BLOQ), the concentration was regarded as zero. The average and standard deviation were calculated.

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Endoscopic Examination
Vocal folds were observed by endoscopy at each of the following time points (n = 6): 5 minutes, 1 hour, 3 hours, 12 hours, 24 hours, 3 days, and 7 days after administration of dHGF to the vocal folds. Before endoscopic examination, the rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (4.5 mg/kg) after inhalative sedation with diethyl ether. After the images were recorded and randomized, the presence of swelling or redness was evaluated.

Histological Examination
Histological examinations were performed 24 hours, 3 days, and 7 days after administration of dHGF (n = 6). Whole larynges were harvested from each animal after humane euthanasia with an intracardiac injection of pentobarbital sodium (200 mg/kg). The harvested whole larynges were immersed in 4% paraformaldehyde for 24 hours and then in 30% sucrose for 24 hours. Tissues were then soaked in embedding medium (Optimum Cutting Temperature Compound; Tissue-Tek Inc., Kyoto, Japan) and frozen quickly with liquid nitrogen. Ten μm-thick cryostat sections (Leica, CM 1850 Kryostat; Leica Instruments GmbH, Nussloch, Germany) of whole larynges were made vertical to the glottis (coronal sections), air-dried, and stored at −80°C until use. For histological examination, hematoxylin and eosin staining was performed. Images were captured at 10× magnification with a Biorevo BZ-9000 microscope (Keyence Corp., Osaka, Japan). After the images were randomized, the presence of swelling of the vocal folds or infiltration of inflammatory cells into the vocal folds was evaluated.

RESULTS
Loss of HGF During Suction Into and Discharge Through the Microsyringe
When 1 mg/mL dHGF was drawn into and discharged from the microsyringe used in the current study, HGF concentration of the discharged solution was an average of 0.891 mg/mL (standard deviation 0.128 mg/mL) (Fig. 1).

Non-specific Background of ELISA
HGF concentration was detected at 2.44 ng/g tissue in one of six NS-injected control rats, whereas HGF was not detected in the other cases.

Concentration of HGF in Larynges After Local Administration
HGF concentration was 50061.07 (SD 7556.66) ng/g tissue 5 minutes after the administration of dHGF to the vocal folds. HGF remaining after 12 hours and 24 hours was 1709.76 (SD 1411.19) ng/g tissue and 290.69 (SD 170.2) ng/g tissue. Three days after administration, HGF concentration was below the BLOQ in one of six cases, and the other cases showed concentrations ranging from 2.06 to 29.17 ng/g tissue (Fig. 2). Seven days after administration, HGF concentration was BLOQ in three of six cases, whereas a maximum concentration of 2.49 ng/g tissue HGF was detected in the other cases (Table I).

Transmission of HGF to Blood After Administration to the Vocal Folds
Five minutes after the administration of dHGF to the vocal folds, two of six cases showed 0.736 and 0.767 ng/g tissue HGF in serum, whereas HGF concentration was BLOQ in the other cases. Twelve hours and 24 hours after administration, HGF concentration was BLOQ in all cases (Table II).

Endoscopic and Histological Examinations
Endoscopic examinations revealed that vocal folds were swollen immediately after administration on both the dHGF-injected and the NS-injected sides (Fig. 3). However, no swelling of the vocal folds was observed in any cases at any of the subsequent time points. There were no cases where redness of the vocal folds was...
observed. Swelling of the vocal folds or infiltration of inflammatory cells into the vocal folds was not detected in any of the cases upon histological examination (Fig. 4).

DISCUSSION
The current study investigated HGF concentration in the solution discharged through a microsyringe to be used in the experiments. A low concentration of dHGF has the possibility to be adsorbed especially to glass and to be discharged at a concentration less than we assume. We found that the HGF concentration of the discharged solution was 0.891 mg/mL (89.1%) when 1 mg/mL dHGF was drawn into and discharged through a microsyringe. This demonstrated that 1 mg/mL dHGF was discharged with an acceptable loss through the microsyringe used in the current study.

Next, we investigated the nonspecific background response of the ELISA using the Immunis HGF EIA kit. One of six NS-administered control larynges had 2.44 ng/g tissue of human HGF. This revealed that the background nonspecific measurement of human HGF in rat larynges was approximately 2.5 ng/g tissue.

Because 89.1% of dHGF was discharged through the microsyringe and the average weight of the rat larynges was 141 mg, 8.91 μg (63.19 μg/g tissue) of dHGF was calculated to be administered to the rat larynges. The concentration of dHGF 5 minutes, 12 hours, and 24 hours after administration was 50.06 μg/g tissue, 1.71 μg/g tissue, and 0.29 μg/g tissue, which were calculated to be 79.22%, 2.71%, and 0.46% of the administered dHGF. Considering that the ELISA showed approximately 2.5 ng/g tissue as a nonspecific background, the larynges where the concentration of dHGF was approximately 2.5 ng/g tissue were considered to have no dHGF. This suggests that three of six larynges had no dHGF 3 days after administration, and no larynges had any HGF 7 days after administration.

Regarding transmission of dHGF administered to a larynx to the blood, the serum concentration of HGF was 0.73 to 0.76 ng/mL in two of six cases and BLOQ in the other cases 5 minutes after local administration to the vocal folds. Twelve and 24 hours after administration, there were no cases where HGF was detected in the serum. In phase I/II clinical trials of patients with fulminant hepatitis in Japan, 0.6 mg/m² of HGF was administered. Consideration of the ELISA showed approximately 2.5 ng/g tissue as a nonspecific background, the larynges where the concentration of dHGF was approximately 2.5 ng/g tissue were considered to have no dHGF. This suggests that three of six larynges had no dHGF 3 days after administration, and no larynges had any HGF 7 days after administration.

### TABLE I.
Concentration of Human Hepatocyte Growth Factor in a Rat Larynx (ng/g tissue).

<table>
<thead>
<tr>
<th></th>
<th>Rat A</th>
<th>Rat B</th>
<th>Rat C</th>
<th>Rat D</th>
<th>Rat E</th>
<th>Rat F</th>
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</thead>
<tbody>
<tr>
<td>Three Days After Administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat A</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat B</td>
<td>2.66</td>
<td>2.06</td>
<td>4.14</td>
<td>6.94</td>
<td>29.17</td>
<td></td>
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<tr>
<td>Rat C</td>
<td>2.00</td>
<td>1.98</td>
<td></td>
<td>2.49</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>Rat D</td>
<td>1.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat E</td>
<td>1.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat F</td>
<td>1.98</td>
<td></td>
<td></td>
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</tbody>
</table>

### TABLE II.
Serum Concentration of HGF After Administration of HGF into Vocal Folds (ng/mL).

<table>
<thead>
<tr>
<th></th>
<th>5 minutes</th>
<th>12 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat A</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat B</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat C</td>
<td>BLOQ</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat D</td>
<td>0.736</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat E</td>
<td>0.767</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
<tr>
<td>Rat F</td>
<td>0.767</td>
<td>BLOQ</td>
<td>BLOQ</td>
</tr>
</tbody>
</table>

BLOQ = below the limit of quantification; HGF = hepatocyte growth factor.

Fig. 3. Endoscopic examinations were performed at each of the following time points: immediately, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 3 days, and 7 days after administration of Hepatocyte growth factor (HGF) to the vocal folds. HGF was administered to the right side of the vocal folds. Normal saline (NS) was administered to the left side. Although both sides of the vocal folds were swollen immediately after administration, no swelling of the vocal folds was observed in any case at any of the other time points. There were no cases where redness of the vocal folds was observed. Ary = arytenoid cartilage.

Fig. 4. Histological examinations were performed 24 hours, 3 days, and 7 days after administration of hepatocyte growth factor (HGF) to the vocal folds. HGF was administered to the right side of the vocal folds. Normal saline (NS) was administered to the left side. There were no cases where the swelling of the vocal folds or infiltration of inflammatory cells into the vocal folds was detected. Scale bar = 200 μm.
administered intravenously and revealed a maximum drug concentration (Cmax) of 22.3 ng/mL. Nevertheless, no severe adverse reactions were observed. This suggests that the concentration of dHGF transmitting to the blood after administration to the vocal folds was too little to cause any adverse problems.

Possible unfavorable responses of administration of HGF to the larynx include swelling of the vocal folds and the surrounding tissues, which leads to narrowing or closure of the airway. In endoscopic examinations, although swelling of the vocal folds was observed immediately after injection in all cases, this occurred similarly on both the HGF-injected side and the NS-injected side. Therefore, this was considered to be caused by the injection of fluid. At all time points after 1 hour, no swelling or redness suggesting local tissue responses was observed. In addition, histological examinations showed no significant swelling of the vocal folds or infiltration of inflammatory cells into the vocal folds. These endoscopic and histological examinations suggested that administration of HGF into the vocal folds does not cause any local tissue responses.

When human clinical trials are planned in the future, the dosage to be used is determined by previous animal studies. The optimal dosage of HGF for regenerative effects in rat vocal folds was reported to be 100 ng HGF per vocal fold. In a canine model, 0.5 μg HGF per vocal fold showed regenerative effects for scarred vocal folds. Table III shows the sizes of larynges in humans, dogs, and rats. The size of human larynges was measured in cadavers. This demonstrates that the size of human vocal folds is approximately 2 and 100 times larger than that of dogs and rats, respectively. Due to the differences in size, 100 ng dosage in rats or 0.5 μg dosage in dogs correlates to 10 μg or 1 μg HGF in humans. Therefore, a dosage of HGF from 1 μg to 10 μg per vocal fold was calculated to be an effective dose for human vocal folds. In the current study, 5 μg HGF per vocal fold was administered to rat vocal folds. Five μg in rats corresponds to 500 to 1,000 μg in humans, which is 50 times greater than the maximum dosage suggested to be used in future clinical trials. The current results suggest that as much as 5 μg HGF per rat vocal fold disappeared 7 days after administration, resulted in minimal transmission to the blood 5 minutes after administration, and caused no local tissue responses. Therefore, a maximum dosage of 10 μg per human vocal fold is considered to be safe and acceptable.

### CONCLUSION

The current study demonstrated that dHGF administered to rat vocal folds disappeared after 7 days, with minimal transmission to the blood. Endoscopic and histological examinations revealed no edema or erythema of the vocal folds in any case. The current findings are expected to contribute to the safe and pharmacokinetic management of future clinical trials when dHGF is administered into human vocal folds.

### Acknowledgment

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