Clearance and Tissue Distribution of Recombinant Human Interleukin 1ß in Rats

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ABSTRACT

Clearance and tissue distribution of recombinant human interleukin 1ß (IL-1ß) were investigated by determining the growth-inhibitory activity on tumor cells in rats after i.v. or s.c. administration.

A single 100 µg/kg i.v. bolus was bi-phasically eliminated with a terminal half-life of 19.0 min in normal rats. Serum IL-1ß activity reached a maximum level 1 h after s.c. administration and then declined with a half-life of 1.59 h. The absolute bioavailability was 40.5%. IL-1ß activity was mainly located in the kidney and was particularly accumulated in the tissues. A 14-fold increase in the elimination half-life of IL-1ß activity was found in nephrectomized rats, in comparison with sham-treated control rats. Pretreatment with E-64 and leupeptin, both of which are thiol protease inhibitors, had no effect on the plasma levels of IL-1ß activity, but a 2-fold increase in plasma was found in rats pretreated with pepstatin A, a carboxyl protease inhibitor. Since excreted IL-1ß activity was not detected in urine, these results suggest that the kidney is the main site of IL-1ß degradation and that carboxyl protease is involved in its metabolic inactivation.

INTRODUCTION

IL-1ß is a cytokine produced by monocyte/macrophages (1-7) and several other cells (8), and it has multiple biological functions (9). It may be used as a therapeutic agent in cancer treatment, since these functions include induction of other cytokines such as IL-2, interleukin 6, interferon, tumor necrosis factor, and colony-stimulating factor, stimulation of bone marrow recovery after myelosuppression (10), and cytotoxic activity on tumor cells (11-14).

There have been several reports on the clearance and tissue distribution of IL-1 after systemic administration to animals (15-19). Kampschmidt and co-workers have studied the plasma clearance of leukocytic endogenous mediator (15) and IL-1 (16) by measuring plasma iron or fibrinogen concentrations and counting neutrophils, and they have reported that IL-1 was rapidly removed from the circulation. Townsend and Cranston (19) have also determined the clearance of leukocyte pyrogen by measuring body temperature. Recently, the elucidation of the fate of IL-1ß has been documented by other researchers who have used a tracer technique by radioiodinated IL-1ß. Newton et al. (17) have reported that the injection of radioiodinated IL-1ß by the i.v. route demonstrated a rapid initial loss of IL-1 from the circulation in mice, and intact radioabeled IL-1ß was excreted in the urine. Klaproth et al. (18) have also reported that the plasma clearance of radioiodinated IL-1ß showed a biphasic behavior and that the radioactivity was concentrated in the kidneys, liver, and intestines. However, they could not identify intact radiolabeled IL-1ß in the urine. Accordingly, the exact in vivo metabolism of IL-1ß has yet to be determined.

Recently, Nakai et al. (20) have developed a sensitive and specific bioassay for the determination of IL-1ß, using a human melanoma A375 subclone, which is highly sensitive to the cell growth-inhibitory activity of IL-1. Therefore, in the present study we describe the clearance, tissue distribution, and metabolism of IL-1ß in rats following systemic administration using the bioassay. Furthermore, we describe the subcellular distribution of IL-1ß in the kidneys of rats and discuss the involvement of intracellular protease in the metabolic inactivation of IL-1ß.

MATERIALS AND METHODS

Protein and Reagents. IL-1ß-Ser71, which is a recombinant variant of natural human IL-1ß substituting serine for cysteine at the 71st position, was expressed in Escherichia coli (21) and purified as previously described (22).

Leupeptin and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO) and E-64 was purchased from the Peptide Institute, Protein Research Foundation (Osaka, Japan). All other reagents were of the best commercial grade available.

Assay of IL-1ß Activity. IL-1ß activity was determined by its ability to inhibit the growth of human melanoma A375.S1 cells, which were isolated from human melanoma A375 (American Type Culture Collection, CRL-1619) by the limiting dilution procedure (20). Briefly, the IL-1ß standard or test samples were serially diluted in 0.1 ml of the basal medium (Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 60 µg/ml kanamycin) in 96-well microculture plates, and then 0.1 ml of 2 x 104 A375.S1 cells was added to each well. After 96 h of incubation in a 5% CO2 incubator at 37°C, 0.05 ml of 0.5% neutral red dye was added to each well and the microplate was incubated for 2 h at 37°C. After washing the plate with phosphate-buffered saline, 0.1 ml of 50% ethanol in 50 mM NaOH was added to each well for extraction of the dye incorporated in viable cells. The quantity of dye incorporated in viable cells was determined by measuring the absorbance at 540 nm. One unit of IL-1ß activity was defined as the reciprocal of the sample dilution needed to bring a 50% cell growth inhibition in a 4-day culture. All assays were performed at least twice. A preparation of IL-1ß-Ser71 with a specific biological activity of 4.6 x 107 units/mg protein was used in this experiment. This variant had a slightly higher biological activity than that of IL-1ß (21).

Serum or Plasma Clearance Experiments. Serum or plasma clearance experiments were performed in 6- to 8-week-old male SD strain rats (CLEA Japan Inc.). The test solution of IL-1ß (10 mg protein/ml) was diluted with physiological saline containing 1% SD strain rat serum and prepared as a solution of 100 µg/µl IL-1ß. The solution was injected s.c. into the upper region of the dorsal lumbar of the animals or i.v. into the penile vein under light ether anesthesia at a dose of 100 µg/kg. Blood was collected from the inferior vena cava or tail vein of the animals, and serum or plasma samples were prepared and frozen at −20°C until testing for IL-1ß activity.

Some plasma clearance experiments were performed in animals immediately after functional nephrectomy. The lower part of the dosal skin of each animal under ether anesthesia was incised along the media line and the right and left kidneys were identified. The renal artery, vein, and ureter on both sides were ligated, and the kidneys were removed. In these studies, sham-operated animals were used as a control group. These animals were anesthetized with ether and subjected to the same procedure except for the ligation and removal of the kidney.

To investigate the involvement of protease in the metabolic inactivation of IL-1ß, plasma clearance experiments were also performed using low molecular weight-type protease inhibitor-treated rats. Leu-
peptin was dissolved in saline and both pepstatin A and E-64 were dissolved in N,N-dimethyl formamide to make each preparation for injection at a concentration of 10 mg/ml. IL-1β was administered to each rat 1 h after a single i.p. administration of protease inhibitors at 10 mg/kg.

Preliminary pharmacokinetic analysis was performed, in which serum or plasma IL-1β activity after i.v. doses of IL-1β were fit to a biexponential equation (Eq. 1), using a computer program for a two-compartment open model:

\[ C_p = A e^{-\alpha t} + B e^{-\beta t} \]  

(1)

where \( C_p \) is the IL-1β activity in serum or plasma at time \( t \), \( A \) and \( B \) are coefficients, and \( \alpha \) and \( \beta \) are elimination rate constants for the fast (\( \alpha \)) and (\( \beta \)) phases.

The absolute bioavailability (\( F \)) of IL-1β activity after s.c. administration was calculated from Eq. 2 (23):

\[ F = \frac{AUC_{ss}}{AUC_{iv}} \times 100 \]  

(2)

The AUC from \( t = 0 \) to the last measurable data point was calculated by the trapezoidal rule.

Tissue Distribution Experiments. IL-1β was s.c. administered to rats at 100 μg/kg. Following the dosing, blood was taken from the inferior vena cava under ether anesthesia. Ice-cold saline was injected into the hepatic portal vein to remove any excess blood, and then tissues were removed for the assay. A sample solution for the assay of IL-1β activity in tissues was prepared as described below. Namely, the tissues were homogenized with 3 volumes of 0.25 M ice-cold sucrose to obtain 25% homogenates using a Polytron homogenizer (Polytron; Kinematica, Switzerland). To aliquots of the homogenates, an equal volume of 50 mM glycine NaCl-HCl buffer, pH 3.0, containing 0.25 M sucrose was added, and the contents were placed in ice for 30 min and centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a Millex-GV filter (0.22 μm; Japan Millipore Ltd.), and then the filtrate was neutralized with an equal volume of 0.25 M phosphate buffer, pH 7.4. The extracted solution was used as a sample for the assay of IL-1β activity.

Subcellular Fractionation. The animals receiving IL-1β were exsanguinated by decapitation 30 min after administration. The body was perfused by an injection of 10 ml ice-cold saline, and the kidneys were removed. Tissue homogenates were prepared and fractionated by differential centrifugation, according to the method of Maunsbach (24) with minor modifications. Briefly, each tissue was homogenized with 9 volumes of 0.3 M ice-cold sucrose solution containing 1 mM Na2EDTA, using a Potter-Elvehjem type homogenizer. The homogenates were filtered through four layers of gauze, and the resultant filtrate was centrifuged for 10 min at 140 × g. The supernatant was centrifuged again for 30 min at 9,000 × g. The resulting 9,000 × g supernatant was further centrifuged by ultracentrifugation for 60 min at 100,000 × g. Each pellet fraction was washed with the sucrose solution and used for subsequent experiments. Isolation of separate lysosomal and mitochondrial fractions from the 9,000 × g pellet fraction was also carried out by the method of sucrose density gradient ultracentrifugation. A linear sucrose gradient (40 ml/tube) ranging from a 1.1 M sucrose solution containing 1 mM Na2EDTA (top layer) to a 2.2 M sucrose solution containing 1 mM Na2EDTA (bottom layer) was formed with a density gradient fractionator (Hitachi Koki Co., Ltd., Japan). The washed 9,000 × g pellet of the kidney was suspended in a 0.3 M sucrose solution containing 1 mM Na2EDTA, and 4 ml of the suspension was layered on the top of the gradient. After centrifugation at 86,000 × g for 2 h, excluding acceleration and deceleration time, 1.5-ml fractions were obtained from the top layer of the gradient with an automatic fraction collector. After disruption of organelles contained in each fraction by sonication with an ultrasonic disruptor (TomY Seiko Co., Ltd.), IL-1β was extracted according to the method described above to determine its biological activity.

Measurement of Enzyme Activity and Protein. Cytochrome c oxidase activity was measured according to the method of Wharton and Tzagoloff (25). Acid phosphatase activity was determined by the method of Nigam et al. (26). p-Nitrophenyl phosphate was used as the substrate, and free p-nitrophenol was determined by colorimetry. Protein was determined according to the method of Lowry et al. (27).

RESULTS

Clearance of IL-1β in Normal Rats. The time-courses of serum IL-1β activity after dosing are given in Fig. 1. Serum IL-1β activity after s.c. administration at 100 μg/kg reached a maximum level of 13.36 ng-equiv/ml at 1 h and then declined with an elimination half-life of 1.59 h. When IL-1β was administered i.v., IL-1β activity was 560.97 ng-equiv/ml at 5 min and rapidly declined to 54.63 ng-equiv/ml at 20 min. The results, based on the two-compartment model, showed that the elimination half-life was 3.36 min in the α-phase and 19.0 min in the β-phase. Accordingly, this indicates that the half-life after s.c. administration was 5 times longer than that after an i.v. administration.

Tissue Distribution of IL-1β. The time-courses of IL-1β activity in tissues after a single s.c. administration of IL-1β to rats at a dose of 100 μg/kg are given in Table 1. IL-1β activity reached maximum levels 1 h after dosing in the lung, kidney, stomach, and large intestine, 2 h after dosing in the testis, and 4 h after dosing in the spleen. IL-1β activity was not detected in the brain. The highest level, 47.9 times higher than the serum level, was achieved in the kidney. The levels in the lung, spleen, stomach, large intestine, and testis were 7.1–20.7 times higher than the serum level. IL-1β activity was still detected in the spleen, testis, stomach, and large intestine 24 h after dosing.

Subcellular Localization of IL-1β in the Kidney. Subcellular localization of IL-1β activity in the kidneys of rats 30 min after single i.v. administration of IL-1β at 100 μg/kg was determined according to a fractionation method, by centrifugation. The results showed a high activity in the 9,000 × g pellet fraction, whereas the activity after the addition of IL-1β to the kidney
CLEARANCE AND DISTRIBUTION OF INTERLEUKIN 1β

Table 1 Time-courses of IL-1β activity in tissues after s.c. administration into male rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>15 min†</th>
<th>30 min†</th>
<th>1 h†</th>
<th>2 h†</th>
<th>4 h†</th>
<th>8 h†</th>
<th>24 h†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>3.69 ± 0.77†</td>
<td>4.40 ± 3.40</td>
<td>2.60 ± 2.60</td>
<td>5.86 ± 3.71</td>
<td>2.09 ± 1.22</td>
<td>ND†</td>
<td>ND†</td>
</tr>
<tr>
<td>Brain</td>
<td>10.77 ± 0.59</td>
<td>36.94 ± 6.73</td>
<td>71.72 ± 4.88</td>
<td>44.42 ± 3.80</td>
<td>55.62 ± 11.56</td>
<td>7.50 ± 3.77</td>
<td>ND†</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.72 ± 1.72</td>
<td>19.64 ± 2.13</td>
<td>36.69 ± 6.62</td>
<td>25.20 ± 5.15</td>
<td>38.20 ± 9.88</td>
<td>34.75 ± 0.51</td>
<td>0.56 ± 0.56</td>
</tr>
<tr>
<td>Spleen</td>
<td>54.94 ± 25.98</td>
<td>134.91 ± 1.47</td>
<td>229.89 ± 47.39</td>
<td>169.80 ± 38.60</td>
<td>70.28 ± 3.59</td>
<td>17.80 ± 3.70</td>
<td>ND†</td>
</tr>
<tr>
<td>Kidneys</td>
<td>3.99 ± 3.99</td>
<td>13.03 ± 1.80</td>
<td>14.52 ± 1.87</td>
<td>21.01 ± 4.24</td>
<td>17.45 ± 1.80</td>
<td>4.42 ± 2.82</td>
<td>1.22 ± 1.22</td>
</tr>
<tr>
<td>Testes</td>
<td>6.85 ± 6.85</td>
<td>26.82 ± 2.57</td>
<td>35.78 ± 8.78</td>
<td>22.66 ± 0.61</td>
<td>22.15 ± 3.42</td>
<td>22.00 ± 2.33</td>
<td>15.32 ± 2.46</td>
</tr>
<tr>
<td>Stomach</td>
<td>8.96 ± 0.96</td>
<td>21.07 ± 2.08</td>
<td>28.03 ± 6.47</td>
<td>23.37 ± 2.32</td>
<td>23.01 ± 2.34</td>
<td>7.37 ± 2.11</td>
<td>4.62 ± 2.71</td>
</tr>
<tr>
<td>Large intestine</td>
<td>3.69 ± 0.77†</td>
<td>4.40 ± 3.40</td>
<td>2.60 ± 2.60</td>
<td>5.86 ± 3.71</td>
<td>2.09 ± 1.22</td>
<td>ND†</td>
<td>ND†</td>
</tr>
</tbody>
</table>

† Time after administration.
* Mean ± SE of three animals.
*: ND, not detectable.
*: not measured.

homogenate was observed in the 100,000 × g supernatant fraction (Table 2).

Both cytochrome c oxidase and acid phosphatase activities were highest in the 9,000 × g pellet fraction. Since cytochrome c oxidase and acid phosphatase are known to be marker enzymes of the mitochondria and lysosomes, respectively, it is assumed that mitochondria and lysosomes are preferentially distributed to the 9,000 × g pellet fraction. Accordingly, the 9,000 × g pellet fraction was fractionated again by sucrose density gradient ultracentrifugation to separate mitochondria and lysosomes (Fig. 2). The results showed that the distribution pattern of IL-1β activity was similar to that of acid phosphatase activity. This suggested that IL-1β was accumulated in lysosomes.

Table 2 Subcellular distribution of IL-1β activity in the kidneys of rats

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IL-1β treatment</th>
<th>Homogenate with IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unit/mg protein</td>
<td>Unit/g tissue</td>
<td>Recovery rate</td>
</tr>
<tr>
<td>Homogenate</td>
<td>223.7</td>
<td>31,900</td>
</tr>
<tr>
<td>140 × g pellet</td>
<td>143.0</td>
<td>1,916</td>
</tr>
<tr>
<td>9,000 × g pellet</td>
<td>222.3</td>
<td>8,386</td>
</tr>
<tr>
<td>100,000 × g pellet</td>
<td>167.6</td>
<td>4,554</td>
</tr>
<tr>
<td>supernatant</td>
<td>89.6</td>
<td>4,372</td>
</tr>
<tr>
<td>Recovery rate</td>
<td>58.6%</td>
<td>128.2%</td>
</tr>
</tbody>
</table>

Clearance of IL-1β in Nephrectomized Rats. The next experiment was designed to investigate the involvement of the kidney in the prolongation of the plasma half-life of IL-1β. The results are shown in Fig. 3. When kinetic parameters of plasma IL-1β activity in nephrectomized rats after i.v. administration were compared to those in sham-operated control rats, prolongation of the elimination half-life (4.7 times in the α-phase and 14.0 times in the β-phase) and an increase of the AUC (17.1 times) were observed. From the above, it is indicated that the kidneys are a major metabolic site of IL-1β in rats.

Clearance of IL-1β in Protease Inhibitor-treated Rats. To investigate the involvement of protease in the metabolic inactivation of IL-1β, the time-courses of plasma IL-1β activity were studied using low molecular weight-type protease inhibitor-treated rats after i.v. administration of IL-1β (Fig. 4). The results showed that there was little difference in pharmacokinetic parameters between the E-64- or leupeptin-pretreated rats and the control rats. In contrast, pepstatin A-pretreated rats showed a 1.7-fold increase in the β-phase elimination half-life and a 2.2-fold increase in the AUC of IL-1β activity.

DISCUSSION

The pharmacokinetics and the fate of IL-1β were investigated in rats after systemic administration, using the A375.S1 bioassay for the determination of IL-1. This bioassay allows detection...
expression as means of four animals ± SE.

Plasma levels are expressed as means of five animals ± SE.

Interleukin-1β can distribute freely from the circulation to all organs except the brain. The failure of the penetration of administered IL-1β into the brain might be caused by the exiguous penetrability of IL-1β in the blood-brain barrier. The highest level of IL-1β was achieved in the kidney rather than in other tissues, including the liver. IL-1β activity in the liver after s.c. administration was below the detection limit at all measurable points. Accordingly, it was clear that the kidney was the primary organ, indicating the extremely high distributability of administered IL-1β, and that IL-1β particularly accumulated in the lysosomal fraction in the kidney. These results indicate that the kidney is involved in the metabolic clearance of IL-1β. Namely, the striking pattern of localization of IL-1β in the kidney strongly suggests that IL-1β permeates glomerular basement membrane, is taken up into the lysosomes of the proximal tubular cells via endocytosis, and is then degraded in the organelle. These fates of proteins in the kidney are well documented in other papers (34-35).

We also investigated the urinary excretion of IL-1β in rats after s.c. administration at a dose of 100 µg/kg. The result was that administered IL-1β was not detected in the urine, the urinary excretion rate being less than 1% of dose. This result was similar to that reported in another paper (18). Accordingly, it strongly suggests that IL-1β permeates glomerular basement membrane and is almost completely absorbed into the tubular cells in rat kidney.

Prolongation of the elimination half-life and an increase of the AUC of plasma IL-1β were observed in nephrectomized animals, in comparison with sham-operated control animals. This result also indicates that the kidney is the major metabolic site of IL-1β. Incidentally, the pharmacokinetic parameters of IL-1β in sham-operated animals were similar to those of unin-
jured animals; no effect on the bioassay based on the injury was observed.

Moreover, we made another attempt to investigate the involvement of intracellular proteases in the metabolic inactivation of IL-1β, using low molecular weight-type protease inhibitors. E-64, leupeptin, and pepstatin A were used in this study, since it is well known that E-64 and leupeptin are both specific inhibitors of thiol protease and pepstatin A is a potent inhibitor of carboxyl protease (36–38). The results showed that the plasma IL-1β activity in rats pretreated with E-64 and leupeptin was comparable to that in control rats, while an increase in the plasma levels of IL-1β activity was found in pepstatin A-treated rats.

Another set of experiments using plasma samples treated with each protease inhibitor revealed that the treatment had little effect on the plasma IL-1β activity, which was determined by the assay system used in this study. With the above results, we suggest the involvement of carboxyl protease in the metabolic inactivation of IL-1β. Cathepsin D, a well-characterized lysosomal carboxyl protease, appears to be plentiful in rat kidney (39–41). Accordingly, IL-1β taken up into renal lysosomes may be degraded by cathepsin D located in the organelle.

We investigated the clearance, distribution, and metabolism of IL-1β following systemic administration in rats, clearly identified the kidney as the main site for its metabolic degradation, and determined that carboxyl protease is involved in its metabolic inactivation. Recently, Ohnishi et al. (42) have reported that renal cathepsin D is responsible for the degradation of IL-2 in vivo experiments. Taken together, cathepsin D located in the lysosomes of the kidney might be a key enzyme in the metabolic degradation of lymphokines. Investigations concerning the enzymatic degradation of IL-1β due to lysosomal protease in the kidney are now in progress.

REFERENCES
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