TISSUE DISTRIBUTION, STABILITY, AND PHARMACOKINETICS OF APO2 LIGAND/TUMOR NECROSIS FACTOR-RELATED APOPTOSIS-INDUCING LIGAND IN HUMAN COLON CARCINOMA COLO205 TUMOR-BEARING NUDE MICE

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ABSTRACT:

Apo2L/TRAIL [Apo2 ligand/tumor necrosis factor (TNF)-related apoptosis-inducing ligand], a member of the TNF cytokine superfamily, induces cell death by apoptosis in a number of human cancer cells and is a potential agent for cancer therapy. We have characterized the in vitro stability of Apo2L/TRAIL in human serum and the tissue distribution and metabolism of Apo2L/TRAIL in a xenograft model of human colon carcinoma (COLO205). Apo2L/TRAIL was stable after incubation in human serum, with no significant high molecular weight complexes or degradation products observed. After i.v. administration of 125I-Apo2L/TRAIL to mice, a small percentage of the radiolabeled drug was seen as high molecular weight complex or as low molecular weight degradation products in plasma. However, the most abundant radioactive species corresponded to the intact Apo2L/TRAIL monomer, indicative of the relative stability of this recombinant protein in blood. Distribution of 125I-Apo2L/TRAIL to organs and solid xenograft tumors was limited. Intact 125I-Apo2L/TRAIL was detectable in the solid tumor at all time points and was the only tissue in which radioactivity transiently increased over time. Kidney contained the highest levels of radioactivity. Radioactive signal reached a tissue-to-blood ratio of 18 in the kidney cortex region when 125I-Apo2L/TRAIL was given in the presence of excess unlabeled ligand. In contrast to blood, extensive 125I-Apo2L/TRAIL degradation was observed in the kidney and, to a lesser degree, in the solid tumor and other organs, including liver, spleen, and lung. Our studies demonstrated that Apo2L/TRAIL is stable in the circulation, localizes to human solid xenograft tumors, and is primarily eliminated through the kidney.

This work is subject to a collaboration and development agreement between Genentech, Inc. and Immunex Corporation.

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Apo2 ligand/tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) is a member of the TNF gene superfAMILY that induces apoptosis in a number of human cancer cell lines (Pitti et al., 1996). A recombinant, soluble form of the endogenous molecule, referred to as Apo2L/TRAIL, is under investigation as a potential therapeutic agent against malignant solid tumors.

Apo2L/TRAIL is a type II transmembrane homotrimer (60 kDa) (Wiley et al., 1995; Pitti et al., 1996) belonging to the TNF cytokine family, which includes TNF-α and Fas ligand. Apo2L/TRAIL binding to functional receptors results in selective, p53-independent apoptosis of tumor cells (Wiley et al., 1995; Pitti et al., 1996; Rieger et al., 1998). Contrary to other members of the TNF family with antitumor activity, induction of apoptosis by Apo2L/TRAIL is independent of NF-κB activation. Apo2L/TRAIL interacts specifically with five different receptors (Griffith and Lynch, 1998; Ashkenazi and Dixit, 1999): DR4, DR5, DcR1, DcR2, and osteoprotegerin. DR4 and DR5 are type I transmembrane proteins that contain conserved cytoplasmic death domains responsible for recruiting adaptor proteins to the receptor complex. Upon binding of Apo2L/TRAIL, DR4 and DR5 can recruit and activate apoptosis-initiating proteases (caspase 8 and 10), through the death domain-containing adaptor molecule Fas-associated death domain. These initiating caspases in turn activate “effector” caspases such as caspase-3, -6, and -7, which result in tumor cell apoptosis. DcR1 is a glycosylphosphatidylinositol-linked protein, and DcR2 is a type I membrane protein with an incomplete cytoplasmic death domain. Consequently, binding of Apo2L/TRAIL to these decoy receptors does not activate the apoptotic machinery. Apo2L/TRAIL binds osteoprotegerin with low affinity, and this interaction appears to be of minimal physiological significance.

Apo2L/TRAIL has shown antitumor activity in tumor xenograft models (Ashkenazi et al., 1999; Walczak et al., 1999; Kelley et al., 2001) through induced tumor cell apoptosis, suppressed tumor progression, and improved survival. To date, there are no published data concerning the in vivo distribution and stability of Apo2L/TRAIL. To gain a better understanding of the pharmacological potential of this therapeutic candidate, we have assessed the stability and interaction of Apo2L/TRAIL in human serum in vitro and its tissue distribution and metabolism in mice bearing human tumor xenografts. These studies demonstrate that Apo2L/TRAIL administered intravenously is stable in vivo. After i.v. administration of 125I-Apo2L/TRAIL to mice, a small percentage of the radiolabeled drug was seen as high molecular weight complex or as low molecular weight degradation products in plasma. However, the most abundant radioactive species corresponded to the intact Apo2L/TRAIL monomer, indicative of the relative stability of this recombinant protein in blood. Distribution of 125I-Apo2L/TRAIL to organs and solid xenograft tumors was limited. Intact 125I-Apo2L/TRAIL was detectable in the solid tumor at all time points and was the only tissue in which radioactivity transiently increased over time. Kidney contained the highest levels of radioactivity. Radioactive signal reached a tissue-to-blood ratio of 18 in the kidney cortex region when 125I-Apo2L/TRAIL was given in the presence of excess unlabeled ligand. In contrast to blood, extensive 125I-Apo2L/TRAIL degradation was observed in the kidney and, to a lesser degree, in the solid tumor and other organs, including liver, spleen, and lung. Our studies demonstrated that Apo2L/TRAIL is stable in the circulation, localizes to human solid xenograft tumors, and is primarily eliminated through the kidney.

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Apo2L/TRAIL, Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; TCA, trichloroacetic acid; ELISA, enzyme-linked immunosorbent assay; SEC, size exclusion chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PK, pharmacokinetic; AUC, area under the concentration curve; CL, clearance; ADME, absorption, distribution, metabolism, and excretion.
in the circulation, that it localizes to human solid xenograft tumors expressing Apo2L/TRAIL receptors, and that the kidney is the major organ of Apo2L/TRAIL elimination in tumor-bearing nude mice.

Materials and Methods

Labeling Procedures. Recombinant Apo2L/TRAIL was produced in Escherichia coli cells at Genentech, Inc. Twenty micrograms of Apo2L/TRAIL was labeled with 1 mCi of 125I (PerkinElmer Life and Analytical Sciences, Boston, MA) using the lactoperoxidase method. Four reactions were performed to prepare the dose material required for this study. The pooled 125I-Apo2L/TRAIL was 97% trichloroacetic acid (TCA)-precipitable.

In Vitro Stability Studies. Apo2L/TRAIL was incubated at different concentrations in pooled human serum and in vehicle (10 mM Tris, 8% trehalose, 0.05% Tween 20, pH 7.5) at 37°C for up to 2 days. Apo2L/TRAIL recovery was measured by enzyme-linked immunosorbent assay (ELISA) and alamarBlue (BioSource International, Camarillo, CA) assays. In addition, 125I-Apo2L/TRAIL (1.6 μg/ml) was analyzed by SDS-size exclusion chromatography (SEC) HPLC.

<table>
<thead>
<tr>
<th>Group No./Sex</th>
<th>Nominal Dose 125I-Apo2L/TRAIL</th>
<th>Specific Activity</th>
<th>Postdose Sampling Time</th>
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<tbody>
<tr>
<td>1</td>
<td>3/F</td>
<td>2.0</td>
<td>0.054</td>
</tr>
<tr>
<td>2</td>
<td>4/F</td>
<td>2.0</td>
<td>0.054</td>
</tr>
<tr>
<td>3</td>
<td>3/F</td>
<td>2.0</td>
<td>0.054</td>
</tr>
<tr>
<td>4</td>
<td>4/F</td>
<td>2.0</td>
<td>0.054</td>
</tr>
<tr>
<td>5</td>
<td>3/F</td>
<td>2.0</td>
<td>0.054</td>
</tr>
<tr>
<td>6</td>
<td>3/F</td>
<td>2.0</td>
<td>0.054</td>
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<tr>
<td>7</td>
<td>3/F</td>
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<tr>
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<tr>
<td>12</td>
<td>3/F</td>
<td>2.0</td>
<td>15</td>
</tr>
</tbody>
</table>

Each animal received ~45 μCi of 125I-Apo2L/TRAIL.

In the circulation, that it localizes to human solid xenograft tumors expressing Apo2L/TRAIL receptors, and that the kidney is the major organ of Apo2L/TRAIL elimination in tumor-bearing nude mice.
In Vivo Study Design. Forty athymic nude nu/nu female mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Mouse body weights ranged from 22 to 26 g.

 Xenograft model and tumor measurements. COLO205 colon tumor cells in log phase (5 × 10^6 cells in 0.2 ml of Hanks' buffered saline solution) were implanted subcutaneously in the right dorsal flank of each mouse. Mice were kept in micro-isolator cages until tumors grew to approximately 450 to 480 mm³. The average tumor volume was ~475 mm³. Tumor growth was measured using the following equation (Corbett et al., 1997): Tumor volume (mm³) = length (mm) × width, (mm) × width, (mm) × 0.5.

 Group assignment and dosing. Mice with established tumors were randomized by tumor volume and assigned to one of 12 treatment groups (n = 3–4/group). Within each group, mice were identified as A, B, C, and, if applicable, D. Mice in groups 1 to 6 were given 0.16 ml of ¹²⁵I-Apo2L/TRAIL (2.0 mCi/kg, 54 μg/kg) as an i.v. bolus dose in the tail vein. Mice in groups 7 to 12 were given the same dose of ¹²⁵I-Apo2L/TRAIL, with the addition of a 311-fold excess of unlabeled Apo2L/TRAIL (15 mg/kg). Table 1 summarizes the study design.

 Blood collection and termination. Mice were anesthetized with an intraperitoneal administration of ketamine (60–80 mg/kg) and xylazine (10–15 mg/kg), prior to euthanasia. Blood (0.5 ml) was collected via cardiac puncture in 3.6% citrate and kept at 4°C. Plasma was separated from each blood sample by centrifugation and stored at −70°C. Blood (0.3 ml) was also collected for serum harvest.

 Tissue collection. For microautoradiography analysis, a 5- to 10-mm³ sample from the kidney, liver, and tumor was removed from animals A, B, C, and, if applicable, D. Mice in groups 1 to 6 were given 0.16 ml of ¹²⁵I-Apo2L/TRAIL to determine tissue-to-blood ratios. The value of the blood pool was quantitated by ImageQuant (Version 5.0) software (Amersham Biosciences, Inc.) to determine tissue-to-blood ratios. The value of the blood pool (heart chamber) was set to equal one.

 Emulsion Microautoradiography. Cryosections (3–5 μm) of collected kidney and tumor tissues were dipped in Kodak NTB3 emulsion (165-4441; Eastman Kodak) and allowed to develop in the dark at 4°C. At the end of the exposure period, the sections were developed using Kodak D19 developer (146-4593; Eastman Kodak) and then fixed using Kodak fixer (190-2485; Eastman Kodak). After routine hematoxylin and eosin counterstaining, the sections were evaluated using bright- and dark-field microscopy.

 Quantitation of Total and TCA-Precipitable Radioactivity in Plasma, Urine, and Tissues. Total radioactivity per gram of tissue was quantitated (MiniAx Auto Gamma 5000 Series; PerkinElmer Life and Analytical Sciences). Partially frozen tissue samples were minced and homogenized; 1 g of wet tissue sample per 10 ml of phosphate-buffered saline-lysine buffer containing 1% Triton X-100 and protease inhibitor cocktail (1836153; Roche Diagnostics, Indianapolis, IN) using a probe-type tissue homogenizer (Tekmar Tissumizer; Tekmar-Dohrmann, Cincinnati, OH). The tissue slurry was centrifuged (2000g for 20 min, 8°C) and the tissue supernatant was stored at −70°C until TCA precipitation and SDS-PAGE.

 Total radioactivity. The total radioactivity present in 20 μl of plasma, 10 μl of urine, or 100 μl of tissue homogenate supernatants was quantitated in a gamma counter.

 TCA-precipitable radioactivity. Two hundred microliters of ice-cold bovine serum albumin (1%) in phosphate-buffered saline (pH 7.2) was added to each sample (20 μl of plasma, 10 μl of urine, or 100 μl of tissue homogenate supernatants) and vortexed. An additional 50 μl of ice-cold 50% TCA was added and vortexed again. After 30 min of incubation on ice, 700 μl of ice-cold 10% TCA was added, vortexed, and incubated for 5 min over ice. Samples were centrifuged at 14,000 rpm for 3 min, and the supernatant was aspirated. The radioactivity in the pellet was quantitated and the percentage of TCA-precipitable radioactivity was calculated.

 Radioactivity Analysis. Plasma and tissue data was tabulated and presented as mean ± S.D. (n = 3), unless otherwise noted. The nanogram-equivalent of precipitable ¹²⁵I-Apo2L/TRAIL was calculated by dividing the TCA-precipitable cpm/g of tissue, or cpm/ml of plasma or urine, by the specific activity of the dosed material (cpm/μg). Measurements of radioactivity were normalized for iodine decay and specific activity of dosed material.

 SDS-PAGE of Tissue Homogenates. Fifty microliters of tissue lysate supernatant was suspended in 50 μl of plasma, 10 μl of urine, or 100 μl of tissue homogenate supernatants and vortexed. An additional 50 μl of ice-cold 50% TCA was added and vortexed again. After 30 min of incubation on ice, 700 μl of ice-cold 10% TCA was added, vortexed, and incubated for 5 min over ice. Samples were centrifuged at 14,000 rpm for 3 min, and the supernatant was aspirated. The radioactivity in the pellet was quantitated and the percentage of TCA-precipitable radioactivity was calculated.

 Pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Apo2L/TRAIL (ELISA)</th>
<th>¹²⁵I-Apo2L/TRAIL (TCA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (h × ng/ml)</td>
<td>22,400</td>
<td>19,100</td>
</tr>
<tr>
<td>K10 Half-life (min)</td>
<td>4.8</td>
<td>8.4</td>
</tr>
<tr>
<td>α-Half-life (min)</td>
<td>4.2</td>
<td>4.8</td>
</tr>
<tr>
<td>β-Half-life (min)</td>
<td>20</td>
<td>83</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>199,000</td>
<td>92,000</td>
</tr>
<tr>
<td>CL (ml/h)</td>
<td>16.8</td>
<td>19.7</td>
</tr>
<tr>
<td>V₁ (ml)</td>
<td>1.88</td>
<td>4.09</td>
</tr>
<tr>
<td>V₂ (ml)</td>
<td>2.48</td>
<td>19.0</td>
</tr>
</tbody>
</table>

K10, elimination rate constant; Cmax, estimated volume of distribution of the central compartment (serum); V₁, estimated volume of distribution at steady state.
with MaxiSorp surface; Nalge Nunc International, Rochester, NY) overnight at 4°C. After blocking, sample or recombinant Apo2L/TRAIL standard was added. Captured Apo2L/TRAIL was detected with a biotinylated secondary monoclonal antibody (clone SC2.8.16; Genentech, Inc.) followed by streptavidin-horseradish peroxidase.

**alomarBlue Bioassay.** Two-fold serial dilutions of standard Apo2L/TRAIL and Apo2L/TRAIL-containing samples were performed in 96-well tissue culture plates seeded with SK-MES-1 (20,000 cells/well) human lung carcinoma cells. The plates were incubated at 37°C for 24 h. alamarBlue was added to the wells for the last 2 h of the 24-h incubation time. Fluorescence was measured using a 96-well fluorometer at an excitation of 530 nm and an emission of 590 nm. Measurements were expressed in relative fluorescence units. For data analysis, a four-parameter curve-fitting program (KaleidaGraph, Version 3.09; Abelbeck/Synergy Software, Reading, PA) was used.

**SDS-SEC and Standard HPLC.** Samples were analyzed on a 1090 Series II HPLC apparatus (Hewlett Packard, Palo Alto, CA). The effluent was monitored optically at 280 nm. Radiolabeled Apo2L/TRAIL was detected with an in-line gamma detector (RAMONA 90; Raytest USA Inc., Wilmington, NC). Gel filtration protein standards were used to calibrate the column (data not shown; 151-1901, Bio-Rad). Plasma samples containing 125I-Apo2L/TRAIL were diluted 1:1 with an SDS buffer containing 25 mM sodium phosphate/200 mM NaCl/0.2% SDS. The mixture was heated in a 50°C water bath for 5 min. Mobile phase contained 25 mM sodium phosphate/200 mM NaCl/0.1% SDS, pH 7.0, at a flow rate of 0.5 ml/min and a 30-min assay time. Under these conditions, all trimeric Apo2L/TRAIL (but not covalently linked dimers) will dissociate into monomers. A TSK G3000SWXL column (Tosoh Bioscience, Montgomeryville, PA) with a precolumn filter (Upchurch Scientific, Oak Harbor, WA) was used to identify the relative amounts of dissociated monomers and dimers of Apo2L/TRAIL.

A different protocol was used to characterize the presence of 125I-Apo2L/TRAIL aggregates or high mol. wt. complexes. Samples were resolved by a Superose 12 HR 10/30 column (Tosoh Bioscience) in 13 mM sodium phosphate, 400 mM ammonium sulfate, pH 6.5, at a flow rate of 0.6 ml/min with a 40-min assay time.

**Pharmacokinetic Analysis.** Concentration versus time profiles were made using KaleidaGraph (Version 3.09). Nominal doses and sample collection times were used for PK analyses. Apo2L/TRAIL concentrations were determined by ELISA, which detects Apo2L/TRAIL specifically, and by analysis of TCA-precipitable radioactivity, which could contain nonbiologically active Apo2L/TRAIL aggregates or high mol. wt. complexes. Only data from animals receiving 125I-Apo2L/TRAIL plus cold Apo2L/TRAIL were used for pharmacokinetic analysis. Both ELISA and TCA-precipitable data were modeled using a two-compartment model with first-order elimination and uniform weighting (WinNonlin, Version 3.1, Model 7; Pharsight, Mountain View, CA). Pharmacokinetic parameters were computed as described elsewhere (Gibaldi and Perrier, 1982).

**Results**

**Characterization of 125I-Apo2L/TRAIL.** The purity and integrity of the dosing material was characterized by several methods. Figure

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**FIG. 4.** SDS-PAGE analysis of plasma samples. Plasma samples (1.5 µl) collected at 5 min, 10 min, 15 min, 1 h, 2 h, and 4 h after dosing with 125I-Apo2L/TRAIL (A) or 125I-Apo2L/TRAIL plus unlabeled Apo2L/TRAIL (B) were analyzed by 16% SDS-polyacrylamide gels. The control lane was the predosing material. Molecular weight markers are shown in the right lane. The arrow indicates Apo2L/TRAIL monomer (20 kDa). C, TCA precipitation of 125I-Apo2L/TRAIL in plasma and urine. Plasma and urine samples collected at 5 min, 15 min, 30 min, 1 h, 2 h, and 4 h after dosing with 125I-Apo2L/TRAIL or 125I-Apo2L/TRAIL plus unlabeled Apo2L/TRAIL were precipitated with 10% TCA. Precipitated pellets were quantitated in a gamma counter. Nanogram-equivalents of precipitable 125I-Apo2L/TRAIL were calculated and are shown on the y-axis.
1A shows radiolabeled lots of Apo2L/TRAIL analyzed by SDS-PAGE autoradiography. 125I-Apo2L/TRAIL resolved as a single band at 20 kDa that corresponds to the monomeric form of Apo2L/TRAIL. The purity and relative amounts of 125I-Apo2L/TRAIL dimer versus trimer in the dosing material were also determined by SEC-HPLC. A SEC-HPLC profile of the dosing material on a Superose column indicated a single peak (Fig. 1B). The SDS-SEC-HPLC chromatograph showed that >91% of the dosing material was monomeric (SDS mobile phase dissociates Apo2L/TRAIL trimers but not dimers to a monomeric species; Fig. 1C). The bioactivity of the labeled dosing material is comparable with the standard Apo2L/TRAIL control, suggesting that radiolabeling did not impact the biologic activity of the ligand (data not shown).

Analysis of 125I-Apo2L/TRAIL Stability and Interactions in Biological Matrices in Vitro. In vitro stability of Apo2L/TRAIL in human serum was characterized by ELISA and alamarBlue bioassay (Fig. 2, A and B, respectively), as well as SDS-SEC-HPLC (data not shown). Apo2L/TRAIL was incubated for up to 2 days at 37°C (1 μg/ml, 10 μg/ml, and 25 μg/ml) in pooled human serum. Apo2L/TRAIL bioactivity remained above 90% for as long as 12 h of incubation. After a 2-day incubation period, 79% of the test material was recovered by ELISA (Fig. 2A) and more than 56% remained bioactive (Fig. 2B). No high molecular weight complexes, degradation products, or increased presence in disulfide-linked dimers were detected in human serum (data not shown).

Pharmacokinetics of Apo2L/TRAIL after a Single i.v. Administration. The Apo2L/TRAIL serum concentration versus time profiles are presented in Fig. 3. Because of limitations on blood sampling in mice, these data were pooled with each mouse contributing a portion of the total serum versus time profile (n = 3/time point). A two-compartment model provided a good fit to the observed data and generated the PK parameters as summarized in Table 2. Differences in serum concentration curves are likely due to the analytical techniques used. Whereas the ELISA detects Apo2L/TRAIL specifically, TCA precipitation will also detect nonbiologically active Apo2L/TRAIL fragments. However, estimates of exposure (AUC) are similar using both techniques. Additionally, when curves begin to separate (~1 h after dosing), 98% and 79% of the total drug exposure has been captured for the ELISA- and TCA-detected Apo2L/TRAIL, respectively. After i.v. bolus dosing, Apo2L/TRAIL was eliminated rapidly from the serum (elimination half-life ~5–8 min). Estimates of Cmax were approximately 2-fold higher using ELISA-derived data. PK analysis of ELISA and TCA profiles resulted in similar α-elimination phases (4.2 versus 4.8 min, respectively). Results from earlier pharmacokinetic studies in mice (Kelley et al., 2001) showed similarly rapid in vivo clearance (9.8 ml/h/kg) and elimination half-life (3.6 min) compared with those reported from our ELISA-derived data.

FIG. 5. Nanogram-equivalents of total 125I-Apo2L/TRAIL measured in tissues and tumor. Total radioactivity of 125I-Apo2L/TRAIL in kidney, liver, lung, spleen, tumor, and plasma was measured at 5 min, 15 min, 30 min, 1 h, 2 h, and 4 h after dosing with 125I-Apo2L/TRAIL (A) or 125I-Apo2L/TRAIL plus unlabeled Apo2L/TRAIL (B). Nanogram-equivalents of 125I-Apo2L/TRAIL were calculated as described under Materials and Methods and are shown on the y-axis.

FIG. 6. Total 125I-Apo2L/TRAIL in kidney and tumor. Figures represent 125I-Apo2L/TRAIL radioactivity (cpm/g) in kidney (A) and tumor (B) at 5 min, 15 min, 30 min, 1 h, 2 h, and 4 h after dosing 125I-Apo2L/TRAIL or 125I-Apo2L/TRAIL plus unlabeled Apo2L/TRAIL.
shown on the autoradiographs of the plasma samples following dosing with 125I-lyzed by SDS-PAGE and TCA precipitation. Figure 4 shows film collected from each animal. Citrated plasma was harvested and analyzed by SDS-PAGE film autoradiography (Fig. 8). Noticeable differences in band intensity between 125I-Apo2L/TRAIL and 125I-Apo2L/TRAIL plus excess unlabeled Apo2L/TRAIL (Fig. 8). Nanogram-equivalents of 125I-Apo2L/TRAIL were calculated and are shown on the y-axis.

supporting similar disposition of labeled and unlabeled Apo2L/TRAIL.

Analysis of 125I-Apo2L/TRAIL Stability and Interactions in Plasma and Urine in Vivo. Prior to euthanasia, blood and urine were collected from each animal. Citrated plasma was harvested and analyzed by SDS-PAGE and TCA precipitation. Figure 4 shows film autoradiographs of the plasma samples following dosing with 125I-Apo2L/TRAIL (Fig. 4A) and 125I-Apo2L/TRAIL with excess unlabeled Apo2L/TRAIL (Fig. 4B). The dominant band (~20 kDa) corresponds to intact monomeric Apo2L/TRAIL. Bands representing dimeric Apo2L/TRAIL (40 kDa) and high molecular weight species (>100 kDa) were visible at the earlier time points. There were only minor degradative products (~15 kDa), indicating stability of the labeled Apo2L/TRAIL in blood.

Nanogram-equivalents (ng-Eq) of radioactivity were determined by TCA precipitation. Levels of Apo2L/TRAIL in plasma decreased over 4 h to ~10 ng/ml and 1 μg/ml for 125I-Apo2L/TRAIL alone, and with excess unlabeled material, respectively (Fig. 4C). Levels in the urine peaked at 30 min postdose (Fig. 4C). The TCA-precipitable ng-Eq of radioactivity in urine were less than 10% of total radioactivity, suggesting that radioactivity in urine was primarily free iodine or lower molecular weight degradation products. This was corroborated by SDS-PAGE analysis of urine that showed no protein-associated radioactivity, confirming the lack of intact 125I-Apo2L/TRAIL in the urine (data not shown).

Analysis of 125I-Apo2L/TRAIL Distribution in Tissues and Solid Tumor. Total and TCA-precipitable radioactivity profiles of 125I-Apo2L/TRAIL and 125I-Apo2L/TRAIL with unlabeled Apo2L/TRAIL after i.v. administration were measured over time in various organs and tumors. The highest level of total radioactivity after 125I-Apo2L/TRAIL administration alone (Fig. 5A) was detected in the kidney (1 μg-Eq/g tissue at 5 min postdose). In all tissues analyzed except the tumor, radioactivity decreased continually with time. Total radioactivity was initially lowest in the tumor (10 ng-Eq/g tissue) but doubled and peaked at approximately 30 min postdose. The increase of total radioactivity in the tumor was also observed after administration of 125I-Apo2L/TRAIL + 311-fold excess unlabeled Apo2L/TRAIL (Fig. 5B). Coadministration of excess unlabeled Apo2L/TRAIL resulted in a noticeable increase of radioactive signal in the kidney (Fig. 6A). This observation is consistent with saturation of a nonspecific clearance/degradation mechanism resulting in more labeled Apo2L/TRAIL being present in the kidney over time. Contrary to the increase in radioactive signal disposition to the kidneys, the presence of excess unlabeled Apo2L/TRAIL had a small but measurable competitive effect on the amount of 125I-Apo2L/TRAIL present in the tumor (Fig. 6B).

To determine the relative degree of 125I-Apo2L/TRAIL processing and protein-binding interactions in vivo, tissue and solid tumor lysates were analyzed by SDS-PAGE film autoradiography (Fig. 8). Noticeable differences in band intensity between 125I-Apo2L/TRAIL and 125I-Apo2L/TRAIL plus excess unlabeled material (Fig. 8, A and B, respectively) were detected in the kidney, where coadministration of unlabeled material resulted in more degradative products of similar molecular weight. The kidney also had the highest level of TCA-precipitable radioactivity, in decreasing order, were kidney, plasma, liver, lung, and spleen. Percentage TCA precipitability was initially high (approximately 80%) 15 min postdose but decreased markedly with time (approximately 20–30% at 1, 2, and 4 h postdose).

Characterization of 125I-Apo2L/TRAIL Organ Disposition by Whole-Body Autoradiography Analysis. Phosphorimaging of whole-body sections was conducted to complement the characterization of 125I-Apo2L/TRAIL organ disposition. Figure 9 shows the localization of radioactivity at 15 min and 1 h after administration of 125I-Apo2L/TRAIL and 125I-Apo2L/TRAIL + excess unlabeled Apo2L/TRAIL, respectively. In agreement with the TCA precipitate
radioactivity and SDS-PAGE autoradiography data, whole-body autoradiography showed a rapid uptake of radioactivity in the kidney. Consistent with the TCA and SDS-PAGE data, the autoradiographic signal was stronger in the 125I-Apo2L/TRAIL + excess Apo2L/TRAIL groups (Fig. 9). At 15 min postdose, the entire kidney contained high levels of radioactivity. At 1 h, the distribution of radioactivity was mostly concentrated in the renal medulla. To a much lesser degree, radioactivity was also detected in the liver. Traces of radioactivity were observed in the urinary bladder at 1 h postdose, which may reflect the excretion of 125I-Apo2L/TRAIL degradation products and processing of the 125I moiety. No radioactive signal was found in brain tissues at any time point. Measurements of tissue-to-blood ratios of radioactive signal in the 125I-Apo2L/TRAIL alone, or in the presence of excess unlabeled ligand, are shown in Fig. 10, A and B, respectively. Strong radioactivity uptake from the blood was observed mainly in the kidney with tissue-to-blood ratios as high as 9 (125I-Apo2L/TRAIL) and 18 (125I-Apo2L/TRAIL plus unlabeled Apo2L/TRAIL) in the kidney cortex. The small tissue-to-blood ratio in other tissues is indicative of the low distribution of Apo2L/TRAIL to these organs.

Characterization of 125I-Apo2L/TRAIL Organ Disposition by Emulsion Microautoradiography. Figure 11A is an emulsion microautoradiograph of kidney tissue 15 min after administration of 125I-Apo2L/TRAIL. The autoradiograph shows a marked epithelial signal in a subset of proximal convoluted renal tubules and along a portion of parietal epithelium in Bowman’s capsule. A weaker epithelial signal was also found in a subset of proximal straight tubules at the medullary-cortical junction. A background signal was observed in collecting tubules and weak radioactivity was present in blood vessels (predominately glomerular and interstitial capillaries). At 1 h postdose, the labeled Apo2L/TRAIL signal had a similar tubular distribution but decreased intensity (data not shown). The proximal renal tubular localization is consistent with glomerular filtration, physiologic protein degradation, and resorption of metabolites by the proximal convoluted tubular epithelium. Increased signal and more extensive tubular distribution was found in animals dosed with excess unlabeled Apo2L/TRAIL (data not shown). These results are in agreement with the strong staining for Apo2L/TRAIL in tubular epithelial cells and glomerular vascular loops following i.v. dosing of Apo2L/TRAIL in monkeys (Hartmut Koeppen, Genentech, personal communication).

Figure 11B is a microautoradiographic analysis from a tumor sample 15 min after administration of 125I-Apo2L/TRAIL. The hematoxylin and eosin-stained section is of a poorly differentiated carcinoma with a high mitotic rate, small areas of central necrosis, and occasional perivascular apoptosis. A radioactive signal of moderate to strong intensity is present in blood vessels and increased signal was observed in tumor cells directly over vascular channels, in their immediate vicinity, and in areas of extravasation of red blood cells. Analysis of tumor sections from an animal 15 min after
administration of $^{125}$I-Apo2L/TRAIL + excess unlabeled Apo2L/TRAIL indicated a similar distribution pattern, but of lower intensity (data not shown). Analysis of tumor sections from another animal 1 h after administration of $^{125}$I-Apo2L/TRAIL + excess unlabeled Apo2L/TRAIL showed a markedly increased apoptotic index in the section of tumor (data not shown), consistent with drug activity, which was not seen at the 15-min time point.

**Discussion**

Apo2L/TRAIL is a potential candidate for therapeutic intervention in oncology and is currently evaluated in the clinic. To gain a better understanding of the therapeutic potential of Apo2L/TRAIL, we assessed the in vitro stability and interaction of Apo2L/TRAIL in human serum and its in vivo stability, tissue distribution, and metabolism in mice bearing human tumor xenografts.

Apo2L/TRAIL was stable in human serum in vitro and mouse serum after i.v. administration. Incubation in human serum for 2 days resulted in modest loss of Apo2L/TRAIL as determined by ELISA (79% recovered) or bioassay (56% recovered). Although a 2-day incubation in human serum resulted in a 21% loss of Apo2L/TRAIL, this decrease may not be significant considering the rapid in vivo clearance of Apo2L/TRAIL: $t_{1/2}$ observed by us was 4.2 min and by Kelley et al. (2001) was 3.6 min in nude mice and ~30 min in nonhuman primates. Furthermore, no high molecular weight complexes, degradation products, or increased presence of disulfide-linked dimers were detected in human serum. In vivo data also supported the stability of the labeled Apo2L/TRAIL in murine blood.

Using ELISA results, we showed that $t_{1/2}$ of Apo2L/TRAIL in this study were similar to results previously reported (Kelley et al., 2001). However, differences in Apo2L/TRAIL serum concentrations were obtained using TCA and ELISA. These differences likely reflect a degree of biotransformation of Apo2L/TRAIL in vivo, resulting in positive radioactivity values by TCA precipitation of Apo2L/TRAIL species no longer recognized as Apo2L/TRAIL by ELISA. Similar changes in $\beta$-half-life using radioimmunoassay versus TCA for TNF-α were reported by Pang et al. (1991).

Based on our findings, the kidney appears to be the major organ of clearance for Apo2L/TRAIL. Our data suggest that the kidney may also play an important catabolic role. This was also the case for another member of the tumor necrosis factor family, TNF (Pessina et al., 1987). Kelley et al. (2001) showed that Apo2L/TRAIL clearance across various species (mouse, rat, cynomolgus monkey, and chimpanzee) correlate with glomerular filtration rate, corroborating the importance of this organ in the elimination of Apo2L/TRAIL. However, our data indicate that no intact Apo2L/TRAIL is excreted in the urine and that the large amount of radioactivity in the urine most likely reflects degradation products of Apo2L/TRAIL. Indeed, SDS-
Page analysis of urine showed no protein-associated radioactivity, suggesting that the Apo2L/TRAIL was extensively metabolized. Interestingly, the coadministration of excess unlabeled Apo2L/TRAIL resulted in a noticeable increase of radioactive signal in the kidney, suggesting the potential saturation of an Apo2L/TRAIL degradation mechanism in this organ. This seems consistent with the good plasma stability of Apo2L/TRAIL during the same examination period. The relevance of the kidney in Apo2L/TRAIL clearance could not be predicted from the molecular weight of the trimeric molecule (60 kDa), and it is likely that other physicochemical properties, including Apo2L/TRAIL’s high, 9.1, isoelectric point (Roger Pai, Genentech, personal communication), potential dissociation into monomers, or proteolysis within the kidney, may play an active role in the renal elimination of this molecule. It is also possible that other organs may play a relevant catabolic role and the resulting degradation products are selectively accumulated in the kidney. Nevertheless, the potential effect of kidney impairment to clearance and exposure of Apo2L/TRAIL should be monitored in the clinic.

In contrast to all other tissues analyzed, disposition of $^{125}$I-Apo2L/TRAIL to tumor increased slightly over time and peaked at approximately 30 min postdose. Coadministration of excess unlabeled Apo2L/TRAIL resulted in a small but measurable decrease in the radioactivity within the COLO205 tumor, perhaps indicative of competition at the receptor level. However, these studies have shown that extravasal distribution of Apo2L/TRAIL is low, despite significant antitumor efficacy observed in vivo in these models. This apparent paradox is in part explained by the high affinity of Apo2L/TRAIL for the death receptors $[K_d\approx1\text{nM (156 ng/ml)}]$ and potency in cell killing (effective dose is approximately 50-100 ng/ml), suggesting that only 30 to 60% receptor occupancy is needed for maximum killing of this human colon cancer cell line.

In summary, our results demonstrated that Apo2L/TRAIL is stable in blood and reaches the intended target tumor tissue in vivo, and that kidney is the major organ of clearance and elimination for this recombinant molecule in mice.

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**References**


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