Evidence for an Asialoglycoprotein Receptor on Nonparenchymal Cells for O-Linked Glycoproteins

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ABSTRACT
B cell-activating factor receptor 3 (BR3)-Fc is an IgG1-receptor dimeric fusion protein that has multiple O-linked glycosylation sites and sialylation levels that can vary in the manufacturing process. Increased sialic acid levels resulted from increased site occupancy with the O-linked N-acetylgalactosamine (GalNAc-Gal), but because the ratio of sialic acid per mole of oligosaccharide remained approximately 1, this led to increased asialo terminal GalNAc. Previous studies have demonstrated an effect of terminal asialo Gal or GalNAc on the clearance of glycoproteins due to uptake and degradation by lectin receptors in the liver. However, the previous studies examined N-linked oligosaccharides, and there are less data regarding O-linked oligosaccharides. The objective of these studies was to determine the effects on the pharmacokinetics and distribution of the asialo terminal GalNAc and varying amounts of sialic acid residues on BR3-Fc. The results of the data presented here suggest that exposed Gal on the desialylated BR3-Fc led to rapid clearance due to uptake and degradation in the liver that was associated with nonparenchymal cells. It is interesting to note that the data indicated a decreased clearance and increased exposure of BR3-Fc as the sialic acid levels increased, even though increased sialic acid was associated with increased asialo GalNAc. Therefore, the exposed GalNAc did not seem to play a role in the clearance of BR3-Fc; although the Gal linked to the hydroxyl group at position 3 may have prevented an interaction. Because we did not see uptake of desialylated BR3-Fc in hepatocytes where the asialoglycoprotein receptor is localized, this nonparenchymal cell lectin may have preference for O-linked glycoproteins.

B cell-activating factor receptor 3 (BR3)-Fc is an IgG1-receptor dimeric fusion protein that is directed against B cell-activating factor (BAFF of the tumor necrosis factor ligand superfamily) (Gordon et al., 2003; Mackay and Ambrose, 2003; Mackay et al., 2003). BR3-BAFF interaction seems to be particularly important for the regulation of B cell survival and maturation in the spleen (Ng et al., 2004). Prevention of BAFF binding to BR3 on B cells by neutralizing BAFF via BR3-Fc results in notable peripheral and tissue B cell reduction in rodents and monkeys due to apoptosis (Vugmeyster et al., 2006; Lin et al., 2007). BR3-Fc consists of two polypeptide chains, each containing 299 amino acids, with sequences from the extracellular domain of the human BAFF receptor BR3 and the Fc domain of human IgG1. The two chains are joined by two disulfide bonds located in the IgG1 Fc domain near the junction between the BR3 extracellular domain and the IgG1 Fc region.

In addition, BR3-Fc has multiple O-linked glycosylation sites with sialylation on the glycoproteins that can vary in the manufacturing process. There are six potential sites per BR3 domain and two BR3 domains per molecule. The monosaccharide analyses of BR3-Fc lots indicate that, on average, less than half of the identified O-linked sites are occupied and that between 91 and 95% of the O-linked glycans contained the N-acetylgalactosamine (GalNAc-Gal) sequence. The fully sialylated serine O-linked Galβ1–3GalNAc-Gal sequence is presented in Fig. 1. However, sialic acid quantitation of BR3-Fc lots indicated that the O-linked oligosaccharides contained, on average, between 1.1 and 1.3 mol of sialic acid per mole of O-linked oligosaccharide. As the sialic content increased, the ratio of 1 sialic acid per O-linked oligosaccharide did not change, but rather the site occupancy increased. The analysis also indicated that the Gal was always sialylated first, and therefore the GalNAc was almost never sialylated. This increase in site occupancy resulted in an increase of asialo terminal GalNAc.

Previous studies have shown the important role of sialylation...
tion in the pharmacokinetics and distribution of glycoproteins. Morell et al. (1968) demonstrated that asialo-ceruloplasmid was cleared rapidly in rabbits, and Briggs et al. (1974) demonstrated that sialic acid capping of the glycans chains of erythropoietin reduces the clearance (CL) in vivo. Similar observations have been made with other glycoproteins including orosomucoid and human chorionic gonadotropin (Morell et al., 1971). The primary reason for the rapid clearance of the asialoglycoproteins in previous studies seems to be asialo (exposed) Gal or GalNAc leading to uptake by lectin receptors in the liver (Ashwell and Kawasaki, 1978; Tavassoli, 1985). Circulating glycoproteins usually do not contain O-linked oligosaccharides, and previous data focused on glycoproteins bearing terminal N-linked oligosaccharides; whereas our study examined a molecule with O-linked oligosaccharides. Asialoglycoproteins bearing terminal N-linked Gal or GalNAc are internalized by the asialoglycoprotein (ASGP) receptor via the clathrin-coated pit pathway and are delivered to lysosomes for degradation (Ciechanover et al., 1983; Spiess, 1990; Yik et al., 2002). The ASGP receptor has been reported to be on the plasma membrane of parenchymal cells/hepatocytes (Ashwell and Harford, 1982; Matsura et al., 1982; Steer et al., 1983). The mannose/N-acetylgalacosamine (GlcNAc) receptor has been described previously as being associated with sinusoidal/Kupffer cells, but no binding has been reported with glycoproteins bearing terminal GalNAc-Gal residues (Ashwell and Harford, 1982). However, a GalNAc-Gal-specific lectin with similar activity as the ASGP receptor has been described on Kupffer cells (Ii et al., 1990; Ozaki et al., 1992; Combs et al., 2006) and was demonstrated to play a role in the rapid uptake of asialofetuin (Kolb-Bachofen et al., 1982).

To determine whether varying levels of sialylation had an impact on the pharmacokinetic, biodistribution and biological activity of BR3-Fc, we conducted studies in mice with BR3-Fc versions that ranged from a mean of 3 to 14 mol sialic acid per mole of BR3-Fc as well as desialylated BR3-Fc. Mice were considered a relevant model to use because BR3-Fc binds BAFF in mice with similar affinity as human BAFF. The results of the data presented here suggest that exposed Gal on the desialylated BR3-Fc oligosaccharides led to rapid clearance due to uptake in the liver that was associated with nonparenchymal cells. The data also indicated an effect of sialylation levels on the clearance and exposure of BR3-Fc that was not associated with increased liver uptake. Because we did not see uptake in hepatocytes, where the ASGP receptor is localized, this nonparenchymal cell lectin may have preference for O-linked glycoproteins bearing terminal Gal or GalNAc.

**Materials and Methods**

**In Vivo Pharmacokinetic Study**

**Test Material.** Native BR3-Fc is the bulk material with an average of 7 mol of sialic acid per mole of BR3-Fc molecule. High- and low-sialylated BR3-Fc were purified from native BR3-Fc by using a Carboxymethyl Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) cation-exchange chromatography. Desialylated BR3-Fc was produced by enzymatic digestion of native BR3-Fc using Sialidase A (Prozyme, San Leandro, CA). Desialylated, low-sialylated, and high-sialylated materials have an average of 0, 3, and 14 mol of sialic acid per mole of BR3-Fc molecule, respectively.

**Monosaccharide Analysis.** Information from the monosaccharide analysis was used both to assess the structure and site occupancy of the O-linked oligosaccharides. First, the N-linked oligosaccharides were...
removed by Peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGase F; New England Biolabs, Ipswich, MA) digestion. Quantification of the released monosaccharides was accomplished by high-pH anion exchange chromatography with pulsed amperometric detection using a CarboPac PA10 column (Dionex) attached to a DX500 chromatography system (Dionex, Sunnyvale, CA). The mobile phase consisted of 18 mM sodium hydroxide at a flow rate of 1.0 ml/min. Throughout the separation, the column was maintained at a temperature of 30°C.

**Sialic Acid Quantitation.** The amount of sialic acid (N-acetyllactosaminic acid) present in BR3-Fc lots was determined by releasing sialic acid by hydrolysis, derivatizing with o-phenylenediamine, and then quantifying by reversed-phase chromatography (Anumula, 1995).

**Animal Study.** The mouse pharmacokinetic (PK)-pharmacodynamic (PD) study was approved by an institutional animal care and use committee. Two hundred twenty-four female BALB/c mice weighing between 18–24 g were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The mice were randomly divided into four dose groups (n = 56; n = 4 per group/timepoint). Each animal received a single intravenous bolus dose of 5 mg/kg of test material. Serum samples for PK analysis were collected predose as follows: at 5 and 30 min; at 1, 2, 4, and 6 h; and on 1, 2, 4, 7, 9, 11, and 14 days postdose. Blood samples for PD analysis were collected predose as follows; and on 1, 2, 4, 7, 9, 11, and 14 days postdose.

**Pharmacokinetic Assay.** Serum PK samples were analyzed using an activated enzyme-linked immunosorbent assay with plates coated with a monoclonal antibody to BR3 (BR3:3809). F(ab)/2 goat antihuman IgG Fc-hors eradish peroxidase conjugates were added for detection. The percentage coefficient of variation of intra- and interassay precision was ≤20%. The assay has a standard curve range of 0.08 to 160 ng/ml. The minimal quantifiable concentration was determined to be 2 ng/ml in neat mouse serum (accounting for the minimal sample dilution of 1/10).

**PD Assay (Fluorescent-Activated Cell Sorting).** For the analysis, 0.1 ml of whole blood was labeled with 10 μg/ml of fluorescein isothiocyanate conjugated rat anti-mouse CD45R/B220 monoclonal antibody (BD Biosciences, San Jose, CA) and 10 μg/ml of R-phycocerythrin conjugated rat anti-mouse CD19 monoclonal antibody (BD Biosciences) for 45 min at 2° to 8°C. The samples were lysed with 1× fluorescent-activated cell sorting (FACS) lysing solution (BD Biosciences). The resulting cell pellet was washed in Hanks’ balanced-salt solution and 1% bovine serum albumin, and stored at 2° to 8°C until they were analyzed on a FACSort cytometer (BD Biosciences).

**Complete Blood Count Assay.** For the analysis, 40 μl of each blood sample was diluted into 10 ml of Haemast Line Diff (10 g/l sodium sulfate, 4.2 g/l sodium chloride) (MIT Service Inc., El Cajon, CA). The samples were analyzed on a Baker 9000 Hematology Series Cell Counter (ABX Diagnostic Inc., Irvine, CA) to determine total lymphocyte counts.

**Data Analysis.** PK parameters were estimated by the noncompartmental method using WinNonlin (version 3.2) (Pharsight, Mountain View, CA). The absolute peripheral blood B cell count (CD19+ B220+) counts are presented as percentage of baseline.

**In Vivo Biodistribution Studies**

**Test Material.** All four forms of BR3-Fc were iodinated using the lactoperoxidase method (Karonen, 1990). The resulting specific activities of the dosing material for the distribution studies were of 17 to 36 μCi/μg. The 125I-labeled BR3-Fc fusion proteins were diluted to approximately 24 to 34 μCi/ml in buffer containing 10 mM Tris, pH 7.4, 140 mM NaCl, 0.01% Tween 20. The dosing solutions were tracer only doses.

**Animal Studies.** The mouse distribution studies were approved by an institutional animal care and use committee. Fifteen female BALB/c mice approximately 20 g were obtained from Charles River Laboratories (Hollister, CA). All of the mice were administered single intraperitoneal bolus doses of 5 mg of NaI at 24 and 1 h before administration of the 125I-BR3-Fc fusion proteins to block uptake of the 125I in the thyroid. In the first study, 12 female BALB/c mice from each group were administered a single intravenous bolus dose of 2.4 to 3.4 μCi of each of the four forms of 125I-BR3-Fc. In the second study, three female BALB/c mice were administered a single intravenous bolus dose of 3.7 μCi native 125I-BR3-Fc or desialylated 125I-BR3-Fc. Liver, lungs, kidneys, spleen, and stomach and blood were harvested from 3 mice out of each group at 5 min, 30 min, 4 h, and 24 h postdose. The harvested tissues, blood, and plasma were analyzed by a gamma counter (Wallac 1417; PerkinElmer Life Sciences, Waltham, MA) to determine the amount of radioactivity associated with each. Three mice from each group were also housed together in metabolic cages, and urine was collected over 24 h and analyzed by trichloroacetic acid (TCA) precipitation and a gamma counter to determine the amount of radioactivity associated with the urine.

**Microautoradiography Assay.** In the second study, liver sections were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut at 4 μm. Slides containing liver sections were dipped in Kodak NTB3 radioemulsion (Carestream Health, Rochester, NY) and allowed to expose in the dark at 4°C. After 3 days of exposure, slides were developed for 2 min in Kodak developer (Carestream Health), rinsed, then fixed in Kodak fixative (Carestream Health) for 4 min, and coverslipped. Liver sections for brightfield microscopy were stained routinely with hematoxylin and eosin and coverslipped.

**SDS-Polyacrylamide Gel Electrophoresis Analysis.** Two microliters from two plasma samples of the three from each 5-min, 30-min, and 4-h timepoints were resolved on a 4 to 12% polyacrylamide Bis-Tris gel using MOPS running buffer (Invitrogen, Carlsbad, CA) under nonreducing conditions. Liver lysates were produced from two of the 30-min liver samples of each group by homogenizing in ice-cold buffer: phosphate-buffered saline, 1% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO), 0.5% sodium deoxycholate, 0.1% SDS, and Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Liver lysate samples were resolved on a 4 to 12% polyacrylamide Bis-Tris gel as described above. Gels were then exposed to autoradiography film (Hyperfilm; GE Healthcare) for 72 h and developed using the Kodak X-OMAT 2000A processor (California Radiographics, Soquel, CA). Images were analyzed using Adobe Photoshop version 7.0.1 (San Jose, CA).

**Results**

**In Vivo PK and PD Study.** After single dose intravenous bolus administration of 5 mg/kg BR3-Fc in mice, biphasic disposition was observed in animals from all dose groups (Fig. 2A). Clearance was extremely rapid when sialic acid residues were completely absent from desialylated BR3-Fc (Fig. 2A; Table 1). Overall, there was a direct relationship between CL and the degree of sialylation of BR3-Fc (Fig. 2B), and exposure was lower as the number of sialic acid residues on BR3-Fc decreased as shown by decreasing area-under-the-concentration-time-curve (AUC) (Table 1). AUClast was presented in Table 1; 10% or less of the AUClast was extrapolated for all versions of BR3-Fc.

The PD marker measured in this study was absolute peripheral blood B cell counts because by neutralizing BAFF, we expect B cell counts to be reduced (Vugmeyster et al., 2006; Lin et al., 2007). CD19 is expressed on mouse B-lineage cells throughout their development from the early pro-B cell stages onward and was used to gate B cells from the lymphocyte population. Despite the faster clearance and lower exposure of the low and desialylated BR3-Fc, peripheral blood CD19+ B cell count versus time profiles were similar among
The differences in the amount of low-sialylated and desialylated $^{125}$I-BR3-Fc in the blood seems to be primarily due to its association with the liver. Less than 10% injected dose/g of the native and high-sialylated $^{125}$I-BR3-Fc dose was associated with the liver at 30 min (Fig. 4A). In contrast, 40% injected dose/g of the desialylated $^{125}$I-BR3-Fc and approximately 15% injected dose/g of the low-sialylated $^{125}$I-BR3-Fc dose was associated with the liver at 30 min postdose (Fig. 4A). Similar results were observed as early as 5 min postdose (data not shown). It is interesting to note that the uptake in the liver seemed to account for the rapid CL of desialylated BR3-Fc, but only a small portion of the low-sialylated BR3-Fc seemed to be taken up by the liver. This is evident by the data at 24 h postdose, which show no further uptake in the liver by the low-sialylated BR3-Fc; whereas, the majority of the desialylated $^{125}$I-BR3-Fc remained associated with the liver at 24 h (Fig. 4B).

The tissue/blood ratio of radioactivity was determined for all tissues analyzed and defined specific tissue uptake and localization as a ratio >1. The livers from mice administered desialylated $^{125}$I-BR3-Fc were the only tissue to average a tissue-to-blood ratio >1 as early as 30 min postdose (Fig. 5A). The livers from mice administered low-sialylated $^{125}$I-BR3-Fc approached a tissue-to-blood ratio of 1 (Fig. 5A). We did not observe uptake of $^{125}$I-BR3-Fc in any of the tissues examined from animals treated with native or high-sialylated forms of the molecule, even though there was a difference in the PK of these forms. In addition to livers, lung tissue from the mice administered desialylated $^{125}$I-BR3-Fc also approached a tissue-to-blood ratio of 1 (Fig. 5A). By 24 h postdose, only the liver from mice administered desialylated $^{125}$I-BR3-Fc had a tissue-to-blood ratio >1 (Fig. 5B).

SDS-PAGE analysis of the livers under nonreducing conditions indicated the presence of and the degradation of the desialylated and low-sialylated $^{125}$I-BR3-Fc evident 30 min postdose (Fig. 6A). In addition to the band visualized at approximately 64 kDa that represents intact $^{125}$I-BR3-Fc, there was a prominent band at approximately 39 kDa. Consistent with the absence of $^{125}$I-BR3-Fc localization in the livers of mice treated with native $^{125}$I-BR3-Fc and high-sialylated $^{125}$I-BR3-Fc, no bands were detected in the liver samples from these mice (Fig. 6A). However, under reducing conditions, all of the bands were visualized at approximately 39 kDa (data not shown), the same as the lower molecular mass band under nonreducing conditions. SDS-PAGE analysis of plasma samples indicated that all forms of $^{125}$I-BR3-Fc remained mostly intact in plasma; the bands were resolved at approximately 64 kDa, representing intact $^{125}$I-BR3-Fc (Fig. 6B). This suggests that the degradation of desialylated and low-sialylated $^{125}$I-BR3-Fc occurred in the liver and not in the blood. Analysis of urine collected over 24 h suggested that there was twice the amount of radioactivity in the urine of the mice administered desialylated $^{125}$I-BR3-Fc compared with the other sialylated forms (data not shown). TCA precipitation indicated that >85% of the radioactivity was not TCA precipitable (data not shown), which suggest that it is most likely free $^{125}$I generated from the degradation of desialylated $^{125}$I-BR3-Fc in the liver.

To evaluate the cell type that the $^{125}$I-BR3-Fc was associated with in the liver, we dosed mice with native and desialylated $^{125}$I-BR3-Fc and fixed the liver samples for histopathology analysis. Biodistribution results were consistent with...
the previous study and suggest a rapid clearance of desialylated 125I-BR3-Fc from the blood as a result of the uptake in the liver (Fig. 7). Analysis of the liver samples by histopathology confirmed that the desialylated 125I-BR3-Fc was distributed in a lace-like reticular pattern rather than a diffuse pattern and was localized with nonparenchymal cells rather than hepatocytes (Fig. 8).

Discussion

BR3-Fc has multiple O-linked glycosylation sites with sialylation on the glycoproteins that can vary in the manufacturing process. The average ratio of sialic acid per mole of O-linked oligosaccharide was between 1.1 and 1.3, and this ratio did not change with increasing sialic acid content. Instead, increased sialic acid was due to increased site occupancy with O-linked oligosaccharides. Because the Gal is always sialylated before the GalNAc, the ratio of sialic acid close to 1 indicates that the majority of GalNAc was not sialylated regardless of the amount of sialic acid present, but Gal was almost always sialylated. Because the ratio of sialic acid per O-linked oligosaccharide did not change, the higher sialylation resulted in an increase of asialo terminal GalNAc.

The objective of these studies was to determine the effects on the pharmacokinetics and distribution of the asialo terminal GalNAc and varying amounts of sialic acid residues on BR3-Fc.

Results suggest that BR3-Fc does have a sialic acid-dependent clearance consistent with the Webster et al. (1999) study. The effects on clearance and exposure were greatest for the desialylated BR3-Fc; however, there was still a rela-
The relationship between the sialic acid levels and clearance/exposure for the other sialylated forms of BR3-Fc. Although an increase in CL was observed with low and desialylated BR3-Fc, there was no apparent affect on the biological activity of these forms at the dose level tested of 5 mg/kg. These data suggest that all forms of BR3-Fc achieved the threshold concentration needed to neutralize existing low BAFF levels in mice, leading to similar B cell reduction. This study did not address the duration of B cell reduction and return to baseline, only the extent of reduction. It is possible that the duration of B cell reduction would be shorter for the low and desialylated forms of BR3-Fc because they are cleared faster than the native and high-sialylated forms of BR3-Fc.

The higher CL of desialylated BR3-Fc in our studies was primarily caused by the increased liver uptake of desialylated BR3-Fc. As early as 30 min postdose, the majority of the desialylated 125I-BR3-Fc was associated with the liver, and this liver association remained at 24 h postdose. In comparison, liver uptake of the low-sialylated 125I-BR3-Fc was only observed at the early timepoints, and by 24 h there was no more liver uptake. In addition to livers, lung tissue from the mice administered desialylated 125I-BR3-Fc also approached
difficult to distinguish from Kupffer cells in the histopathology. Endothelial cells cannot be ruled out because they would be associated with Kupffer cells and not hepatocytes; although sinusoidal endothelial cells that internalize and degrade glycoproteins or cells bearing terminal sugar moieties. An ASGP receptor has been reported on liver hepatocytes (Ashwell and Harford, 1982; Matsuura et al., 1982; Steer et al., 1983; Webster et al., 1999), and there has also been evidence that a similar ASGP receptor is associated with Kupffer cells (Kolb-Bachofen et al., 1982; Li et al., 1990; Ozaki et al., 1992). The mannose/N-GlcNAc receptor has been described previously as being associated with sinusoidal/Kupffer cells, but no binding has been reported with glycoproteins bearing terminal GalNac-Gal residues such as those in BR3-Fc (Ashwell and Harford, 1982). Desialylated erythrocytes have also been shown to bind to a lectin receptor on Kupffer cells with preference for GalNac (Aminoff et al., 1977; Schlepper-Schäfer et al. 1980). The scavenger receptor C-type lectin is associated with endothelial cells, but it has requirement for adjacent terminal Gal and fucose residues and does not bind Gal/GalNAc-terminating glycans unless they form part of a Lewis^a^-type epitope (Coombs et al., 2005). The results of these previous studies would support the presence of an asialoglycoprotein receptor on Kupffer cells in our studies. Based on the data demonstrating liver uptake and degradation of BR3-Fc and the previous published studies, it is reasonable to assume BR3-Fc bearing terminal Gal or GalNac binds a receptor on Kupffer cells, is internalized, and degraded.

There was no apparent affect of the terminal asialo GalNAc on the liver uptake of ^125^I-BR3-Fc because the native and high-sialylated BR3-Fc did not demonstrate liver uptake. Only the desialylated BR3-Fc would be expected to express asialo or exposed terminal Gal, and the low-sialylated BR3-Fc that averaged 3 mol sialic acid did have approximately 7% of material bearing terminal asialo GalNac with no Gal. Therefore, these data suggest that either asialo terminal Gal alone was enough to bind to the nonparenchymal cell lectin or that both terminal adjacent asialo Gal and GalNac are needed. It is also possible that the Gal associated with the GalNac at the hydroxyl group at position 3 hindered the ability of the asialo GalNac to interact with the lectin, and only when the Gal is not present can the asialo GalNac bind to the lectin. The small percentage of material with terminal asialo GalNac and no Gal may be why a portion, but not all, of the low-sialylated ^125^I-BR3-Fc seemed to be cleared through the lectin receptor. It is unclear from this data whether there is another role of sialic acid on the PK of BR3-Fc, because the desialylated and low-sialylated BR3-Fc were the only ones to bind the lectin receptor, yet there still seemed to be a sialic acid relationship to clearance. It is possible that the change in isoelectric point due to change in sialic acid content may play a role in the effect on clearance because higher sialic acid would result in a higher net negative charge.

It is interesting that we did not observe uptake by the ASGP receptor on hepatocytes because the desialylated BR3-Fc would have exposed terminal Gal. The difference in preference for the lectin receptor on hepatocytes versus nonparenchymal cells may be due to N- versus O-linked glycosylation. Another study demonstrated a difference in the pharmacokinetics of recombinant IgA1 and IgA2 in mice.

**Fig. 8.** Histologic images of liver from mice treated with desialylated ^125^I-BR3-Fc. A, a darkfield image demonstrating that the radioactivity is distributed in a lace-like reticular pattern (arrows) rather than in a diffuse pattern. B, a brightfield image stained with hematoxylin and eosin; the high magnification inset demonstrates silver grains localized to nonparenchymal cells (arrows). Scale bars in A and B = 50 µm; scale bar in B high magnification inset = 10 µm.

a tissue-to-blood ratio of 1, indicating that some of the desialylated ^125^I-BR3-Fc may also be cleared from the lungs. Our data suggest asialo BR3-Fc can be internalized and degraded as indicated by lower molecular mass bands detected by SDS-PAGE analysis of the liver lysates from mice administered low and desialylated ^125^I-BR3-Fc. By 30 min postdose, the low-sialylated ^125^I-BR3-Fc was detected only in the lower molecular mass band, indicating that the amount of low-sialylated ^125^I-BR3-Fc internalized had already been degraded, whereas the desialylated ^125^I-BR3-Fc was detected at both bands. All versions of ^125^I-BR3-Fc seemed stable in plasma, and there were very little of the lower molecular mass bands observed in the plasma, consistent with the degradation of the low and desialylated ^125^I-BR3-Fc occurring in the liver and not the blood. Taken together, these data provide evidence that in mice, BR3-Fc bearing terminal Gal or GalNac may be internalized and degraded rapidly by an asialoglycoprotein receptor.

Histopathology on liver sections was done to get an idea of the cells that were playing a role in the liver uptake. The data suggest that the desialylated ^125^I-BR3-Fc was associated with Kupffer cells and not hepatocytes; although sinusoidal endothelial cells cannot be ruled out because they would be difficult to distinguish from Kupffer cells in the histopathology analysis. Although we do not have direct data demonstrating the presence or binding to a receptor in Kupffer cells, there are many previous studies demonstrating the presence of various lectin receptors on hepatocytes, Kupffer cells, and endothelial cells that internalize and degrade glycoproteins or cells bearing terminal sugar moieties. A study reported on liver hepatocytes (Ashwell and Harford, 1982; Matsuura et al., 1982; Steer et al., 1983; Webster et al., 1999), and there has also been evidence that a similar ASGP receptor is associated with Kupffer cells (Kolb-Bachofen et al., 1982; Li et al., 1990; Ozaki et al., 1992). The mannose/N-GlcNAc receptor has been described previously as being associated with sinusoidal/Kupffer cells, but no binding has been reported with glycoproteins bearing terminal GalNac-Gal residues such as those in BR3-Fc (Ashwell and Harford, 1982). Desialylated erythrocytes have also been shown to bind to a lectin receptor on Kupffer cells with preference for GalNac (Aminoff et al., 1977; Schlepper-Schäfer et al. 1980). The scavenger receptor C-type lectin is associated with endothelial cells, but it has requirement for adjacent terminal Gal and fucose residues and does not bind Gal/GalNAc-terminating glycans unless they form part of a Lewis^a^-type epitope (Coombs et al., 2005). The results of these previous studies would support the presence of an asialoglycoprotein receptor on Kupffer cells in our studies. Based on the data demonstrating liver uptake and degradation of BR3-Fc and the previous published studies, it is reasonable to assume BR3-Fc bearing terminal Gal or GalNac binds a receptor on Kupffer cells, is internalized, and degraded.

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It is interesting that we did not observe uptake by the ASGP receptor on hepatocytes because the desialylated BR3-Fc would have exposed terminal Gal. The difference in preference for the lectin receptor on hepatocytes versus nonparenchymal cells may be due to N- versus O-linked glycosylation. Another study demonstrated a difference in the pharmacokinetics of recombinant IgA1 and IgA2 in mice.
(Rifai et al., 2000). The major difference between the IgA1 and IgA2 subclasses is a 13-amino acid sequence in the IgA1 hinge region containing 3 to 5 O-linked carbohydrate moieties lacking in IgA2. The Rifai et al. (2000) study showed the faster CL of the IgA2 was caused by greater liver uptake with approximately 30 to 40% of IgA2 associated per gram of liver at 10 min postdose compared with approximately 10% of IgA1. The liver uptake and fast CL of IgA2 was not observed in the presence of the specific ASGP receptor ligand, galactose-Ficoll, or in knockout mice lacking the ASGP receptor (Rifai et al., 2000). These results suggest that the ASGP receptor present in hepatocytes rapidly clears all three allo-
types of IgA2. The ASG receptor does not seem to recognize the O-linked galactosyl residues in the IgA1 hinge region because hinge-deleted IgA1, lacking the O-linked carbohydrates, was cleared more rapidly than the native molecule (Rifai et al., 2000). It is possible that the liver uptake observed with IgA1 containing O-linked carbohydrates, which was not blocked by an ASGP receptor antagonist or in knockout mice lacking the ASGP receptor, was due to nonparenchymal cells and the lectin receptor described in our studies. The results of the studies presented are evidence of a asialoglycoprotein receptor on mouse nonparenchymal cells that internalizes glycoproteins bearing terminal Gal and may have a preference for O-linked oligosaccharides. The absence of sialic acid on the hydroxyl group at position 6 in terminal asialo GaINAc does not seem to be a factor in the binding of desialylated BR3-Fc to this nonparenchymal cell lectin. However, it is possible that there was interference from the Gal linked to the hydroxyl group at position 3 in the binding of GaINAc to this lectin, and if the Gal was removed the GaINAc would probably play a role.

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