Anti-CD95-induced Lethality Requires Radioresistant FcγRII⁺ Cells

A NOVEL MECHANISM FOR FULMINANT HEPATIC FAILURE*

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The Jo2 anti-mouse CD95 monoclonal antibody induces lethality in mice characterized by hepatocyte death and liver hemorrhage. Mice bearing a defect in Fas expression or in the Fas-mediated apoptotic pathway are resistant to Jo2. Here we show that FcγRII knockout mice or mice with monoclonal antibody-blocked FcγRII are also resistant to Jo2. The critical FcγRII⁺ cells are radioresistant and could not be reconstituted with splenic cells. Death of sinusoidal lining cells and destruction of sinusoids were observed, consistent with the characteristic liver hemorrhage and the selective FcγRII expression in sinusoidal lining cells but not hepatocytes. Hemorrhage developed coincident with hepatocyte death and the sharp rise of serum alanine aminotransferase and alanine aminotransferase. Invariably, moribund mice showed severe liver hemorrhage and destruction of sinusoids. The data demonstrate a novel mechanism by which the destruction of liver sinusoids, induced by the Jo2-mediated co-engagement of Fas and FcγRII, leads to severe hemorrhage and lethal fulminant hepatitis.

Cross-linking of CD95 (Fas), a type I transmembrane protein with a cytoplasmic death domain (1), induces cells to undergo apoptosis (2). Jo2, a hamster-derived mAb¹ directed against mouse Fas, is lethal when administered into mice either intravenously or intraperitoneally (3). Mice defective in either Fas expression or Fas-mediated apoptotic pathways are resistant to Jo2-induced lethality (4, 5). Jo2-induced lethality is associated with extensive hepatocyte death and marked elevation of serum AST and ALT (3). Injection of a caspase inhibitor protects mice against Jo2-induced apoptosis of hepatocytes and lethality (6). These observations suggest that hepatocytes are the primary target and that the Jo2-induced death of hepatocytes is the cause of lethality. However, freshly isolated hepatocytes are highly resistant to Jo2, displaying weak apoptosis only after prolonged incubation in the presence of cycloheximide, which facilitates Fas-mediated apoptosis (7–9). Thus, whereas lethality could be induced within 2 h after application, death of hepatocytes in in vitro experiments could only be induced after 12 h of treatment or more (3, 7–9). In addition, direct binding of Jo2 to hepatocytes in vivo has not been reported. Like hepatocyte damage, massive hemorrhage in the liver invariably accompanies Jo2-induced lethality (3). Jo2-induced hemorrhage is highly specific, restricted only in the liver but not in other tissues whose cells often express a higher level of Fas than hepatocytes. Thus, the selective toxicity of Jo2 toward liver cannot be explained either by its Fas expression level or by the in vitro sensitivity of hepatocytes. Conversely, a direct attack of hepatocytes by Jo2 cannot explain the induction of severe hemorrhage in liver.

Jo2 but not other anti-Fas mAb effectively induces lethality in mice (7). Still, the apparent liver toxicity of Jo2 has raised a serious concern regarding the use of Fas cross-linking agents as therapeutics. We noted that Jo2-mediated cytotoxicity is critically dependent on target FcγR expression. Both hepatocytes and sinusoidal lining cells express Fas, but only the latter express FcγR (10–12). Because a co-engagement of Fas and FcγR can facilitate binding and strengthen the subsequent signaling process, we asked the question of whether the Jo2-induced lethality requires co-engagement of Fas and FcγR. We found that Jo2-induced lethality is critically dependent on host FcγRII expression. This observation and additional studies described herein provide strong evidence for a novel mechanism for Jo2-induced hepatic injury in which hemorrhage and hepatocyte death are secondary to the killing of sinusoidal lining cells, which are the primary target of Jo2 and the deciding factor for Jo2-induced lethality. In addition, this study revives the possibility of using Fas cross-linking agents that lack the FcγRII binding activity as useful therapeutic agents.

MATERIALS AND METHODS

Cytotoxicity Assays—Cytotoxicity assays against the FcγR⁺ A20 and the FcγR⁻ variant IIA1.6 target cells (obtained from Dr. C. J. Janeway, Yale University, New Haven, CT) were conducted as described previously (13). Target cells (2 × 10⁶), labeled with Na₂⁵¹CrO₄ (PerkinElmer Life Sciences), were cultured with various doses of Jo2 (BD Biosciences) or FasL, vesicle preparation (FasL VP) (13, 14) in 0.2 ml in individual wells of a 96-well plate. Supernatants were removed at 5 h after culture, and radioactivity (counts/min) was determined. Background release was determined by culturing cells alone. Cytotoxicity is expressed as % specific ⁵¹Cr release, which is determined by the formula: 100% ×
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(Experimental release — background release) /total cpm released by 0.5% Nonidet P-40 — background release). Assays were carried out in duplicate, and the experiment was repeated three times. The amount of human FasL protein present in FasL VP was determined using a capture enzyme-linked immunosorbent assay kit (Oncogene, Boston, MA) (14).

Jo2-induced Lethality—B6, B6;129S-F2J, B6;129S-Fegr2tm1Rav (FcγRII KO), C57Bl/6-Fegr2tm1B6 (FcγRII KO), and B6MRL-Fas+/+ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. In accordance with IACUC guidelines, mice were treated under various conditions as described in the legends of the figures and tables. To determine the lethality, mice were injected intraperitoneally with various doses of Jo2 or were untreated. Mice were euthanized at the time that the Jo2-treated B6 mice became moribund. Tissues were fixed with 10% paraformaldehyde and photographed. Sera were collected and assayed for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a commercial kit (Sigma). In some experiments, mice were euthanized at various times after injection of Jo2. Livers were fixed and examined for apoptotic cells using the in situ apoptosis detection kit (Trengren, Inc., Gaithersburg, MD).

Radiosensitivity of the Target of Jo2—To determine the role of FcγR and target radiosensitivity in the Jo2-induced lethal fulminant hepatitis, B6 and FcγRII KO mice were irradiated with 600 rads and used 4 days later. In some experiments, mice were reconstituted 24 h before the injection of Jo2 with B6 splenic cells (10^6/mouse) by intravenous injection of single cell suspension prepared from B6 spleen cells. In other experiments, mice were injected intravenously with purified, FcγR-specific 2.4G2 or control rat Ig (Jackson ImmunoResearch, Atlanta, GA) 2 h before the intraperitoneally injection of Jo2.

Treatment with d-Gal Plus Lipopolysaccharide (LPS)—Both B6 and FcγRII KO mice, 3 mice per group, were injected with d-Gal (0.5 g/kg body weight) plus bacterial LPS (50 μg/kg body weight) intraperitoneally as described (15, 16). Neither agent alone induced lethality. Mice were observed for 24 h. Sera and tissues of moribund mice were collected. Samples of control mice were collected at the same times when moribund mice were euthanized.

RESULTS

FcγR Expression on Target Cells Regulates the Cytotoxicity of Jo2—We tested Jo2 anti-Fas mAb for FcγR-dependent cytotoxicity using ^125I-labeled, FcγR+ A20 and FcγRII+ variant IAI.6 cells (derived from A20) as targets in a 5-h cytotoxicity assay (Fig. 1). Jo2 effectively killed FcγR+ A20 cells but not IAI.6 cells (Fig. 1a). Both targets were equally sensitive to FasL VP, the apoptosis-inducing membrane vesicles that induce cell death independent of target FcγR expression (Fig. 1a).

In addition, the killing of FcγR+ A20 targets by Jo2 was inhibited by 2.4G2 mAb that blocks Fc binding to FcγRII and FcγRIII (17, 18). The control rat IgG2b did not inhibit the killing (Fig. 1c). In addition, 2.4G2 did not inhibit FasL VP-mediated killing (Fig. 1d). These results indicate that the acute cytotoxicity of Jo2 depends on FcγR binding.

FcγRII KO Mice Are Resistant to Jo2—Because 2.4G2 blocked both FcγRII and FcγRIII, we determined whether Jo2 induces lethality in FcγRII KO mice (B6;129S-Fegr2tm1B6) or FcγRII KO mice (C57Bl/6-Fegr2tm1B6). Control groups included B6129SF2J, B6, and B6MRL-Fas+/+ mice. FcγRII KO mice were completely resistant to Jo2, including those treated with a high dose of Jo2 (5 mg/kg body weight, Table I). In contrast, almost all of the control B6;129SF2J mice died even when treated with a lower dose of Jo2 (1.5 mg/kg body weight). In addition, all of the FcγRII KO mice died after injection of a lower dose of Jo2. B6MRL-Fas+/+ mice were resistant to the high dose of Jo2 (5 mg/kg body weight). Thus, Jo2-induced lethality requires the presence of FcγRII+ and Fas+ cells and correlates with FcγR-dependent cytotoxicity in vitro.

FcγRII KO Mice Are Sensitive to d-Gal/LPS-induced Lethality—To exclude the possibility that Jo2-induced lethality is due to d-Gal/LPS treatment alone, mice were treated with varying doses of Jo2 and/or d-Gal/LPS. Jo2-induced lethality was not observed in mice treated with Jo2 alone but was observed in mice treated with a high dose of Jo2 (5 mg/kg body weight) or with a low dose of Jo2 (1.5 mg/kg body weight) plus d-Gal/LPS (50 μg/kg body weight) (Fig. 2). Hemorrhage was selec-
Jo2 elevates serum transaminase of B6 but not FcγRII KO mice

Mice were either untreated, injected with Jo2 (3 mg/kg, body weight, intraperitoneally), or treated with n-Gal/LPS as described under "Materials and Methods." Mortality developed within 3–7 h after injection. All moribund mice displayed severe hemorrhage in the liver. Sera were collected from moribund mice. Sera of viable mice were collected at 7 h after treatment. The serum levels (units/ml) of AST and ALT were determined with a commercial kit.

| Strain       | Treatment   | Mortality | AST       | ALT       |
|--------------|-------------|-----------|-----------|-----------|
| B6           | Not treated | 0/3       | 46 ± 10   | 39 ± 27   |
| B6           | Jo2         | 5/5       | 5070 ± 2369 | 4520 ± 2829 |
| FcγRII KO    | Jo2         | 0/3       | 86 ± 17   | 70 ± 18   |
| B6           | n-Gal/LPS   | 3/3       | 2017 ± 1504 | 2340 ± 1502 |
| FcγRII KO    | n-Gal/LPS   | 6/7       | 3283 ± 1956 | 6332 ± 1201 |
| B6:129SF2/J | n-Gal/LPS   | 3/4       | 3163 ± 978 | 3400 ± 2208 |

![Image](Image.png)

**Fig. 2.** Jo2 induces hemorrhage and death of hepatocytes in the livers of B6 mice but not FcγRII KO mice. a, from left to right: hemorrhagic liver (top row) was observed in Jo2-treated B6 mice (2nd), but not in untreated B6 mice (1st), untreated FcγRII KO mice (3rd), or Jo2-treated FcγRII KO mice (4th). Hemorrhage was not observed in kidneys (middle row) from the corresponding mice. Hemorrhage in liver (bottom row) was observed in n-Gal/LPS-treated B6 mice (2nd) and FcγRII KO mice (4th) but not in untreated B6 mice (1st) and FcγRII KO mice (3rd). b and c, show the H&E staining of Jo2-treated B6 and Jo2-treated FcγRII KO mice, respectively. Magnification, ×200. Please note the congestion of red blood cells, the distortion of sinusoids, and nuclear condensation of hepatocytes.

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Jo2-induced toxicity of hepatocytes is prevalent around damaged sinusoids. By contrast, hepatocytes around areas where destruction of sinusoids was not induced did not become apoptotic. The data suggest that the FcγRII-dependent destruction of sinusoids is the primary mechanism for Jo2-induced lethality by causing severe hemorrhage in liver and hepatocyte apoptosis.

**DISCUSSION**

**A Novel Mechanism for Jo2-induced Lethal Fulminant Hepatitis**—Our study supports a model in which the critical targets of Jo2 are the sinusoidal lining cells that co-express FcγRII and Fas. As sinusoidal lining cells are the gateway for blood components to enter liver parenchyma, our data indicate that Jo2 travels through the blood, encounters sinusoidal lining cells, and delivers a Fas-mediated apoptotic signal that is dependent on target FcγRII expression. Under *in vitro* conditions, we showed that the co-engagement of Fas and FcγRII provided a
strong apoptotic signal, and presumably the FcγR-engaged Jo2 could enhance binding and resist endocytosis. Under in vivo conditions and within 1 h of application, Jo2 induces the detachment of sinusoidal lining cells and inhibits their microvascular perfusion function (21). Within the next hour, nuclear condensation in the sinusoidal lining cells and congestion/extravasation of erythrocytes becomes obvious (21) (Figs. 2 and 3). Death of sinusoidal lining cells could damage vascular integrity, resulting in focal hemorrhage. Our data suggest that focal hemorrhage induces secondary damage to hepatic parenchymal cells and further loss of sinusoid integrity. As the process progresses, the liver becomes severely hemorrhagic. Massive bleeding is always observed in the livers of moribund mice and is coincident with death. Together with the FcγR-dependent induction of apoptosis of targets in vitro, our in vivo study provides strong evidence that hemorrhage in the liver is responsible for the lethality induced by Jo2.

Transgenic mice in which the bcl-2 transgene is controlled by a pyruvate kinase regulatory sequence are resistant to Jo2 (23). In contrast, transgenic mice in which the bcl-2 transgene is linked with the α1-antitrypsin promoter (pAAT-bcl-2) are susceptible (9, 24). Our model can explain this conundrum. The resistance is likely due to the expression of the bcl-2 transgene in sinusoidal lining cells because the pyruvate kinase regulatory sequence used is not cell type-specific. In contrast, the α1-antitrypsin promoter is hepatocyte-specific. Therefore, the sinusoidal lining cells in the pAAT-bcl-2 transgenic mice should remain susceptible to Jo2. Also of great relevance to the present study is the observation that anti-FLAG-aggregated FasL is lethal to the pAAT-bcl-2 mice (9), whereas recombinant sFasL alone is not even toward normal mice. According to our model, the anti-FLAG-aggregated FasL and their derivatives may not induce hepatic toxicity, raising the possibility that they may be used as therapeutic agents. It should be noted, however, that lethality has been induced with an excessive amount of rsFasL (25, 26), suggesting that the relative sensitivity of target tissues versus liver to FcγRII-nonreactive, Fas-binding agents is an important factor to consider for therapeutic purposes.

Because sinusoidal lining cells form the barrier between blood and hepatocytes, their death leads to focal hemorrhage. As the welfare of hepatocytes depends on a functional barrier, hemorrhage-induced biochemical events and the loss of sinusoidal integrity may induce secondary damage to hepatocytes.
This hypothesis could reconcile the strong resistance of hepatocytes to Jo2 in vitro study and their rapid death upon Jo2 administration in vivo (3, 9, 21). This hypothesis predicts that circulating Jo2 would attack sinusoidal lining cells first. Indeed, injected fluorescein isothiocyanate-Jo2 (100 μg intravenously) strongly stained sinusoidal lining cells but not hepatocytes, whereas fluorescein isothiocyanate-conjugated hamster Ig did not stain sinusoidal lining cells (data not shown). TUNEL assays indicated focal hemorrhage and apoptotic sinusoidal lining cells around the damaged sinusoids (Fig. 3).

Jo2-induced toxicity of hepatocytes apparently emanates from damaged sinusoids, and regions where sinusoids are intact the hepatocytes remained viable. Our study suggests that sinusoidal lining cells are the primary targets, and hepatocyte death is secondary to the destruction of sinusoids. This is reasonable because the welfare of hepatocytes depends on functional and intact sinusoids. The mechanism by which hepatocyte death is induced requires further study.

Implication for Sinusoidal Lining Cells as Critical Targets for Hepatic Damage—In addition to Jo2-induced lethality, severe hemorrhage in the liver is observed in several other models of lethal hepatic failure. The acute liver toxicity of acetaminophen has been shown to be the result of a preferential toxicity toward sinusoidal endothelial cells (27). Consistent with the present study, severe hemorrhage in liver was observed in moribund mice (27). In the lethal fulminant hepatitis in which the apoptosis-inducing factor implicated is TNF-α or FasL, the lethality has been attributed to hepatocyte death (19, 26, 28). Our hypothesis suggests that hemorrhage is the underlying cause for lethality in these models. As with the FcγRII-mediated focusing of Jo2 on the sinusoidal lining cells, TNF-α produced by the LPS-activated Kupffer cells could preferentially attack the neighboring sinusoidal endothelial cells (20, 28). Although the Jo2-induced lethality is an acute model for fulminant hepatitis, sinusoidal lining cells can be critical targets in chronic hepatic injury as well. Sinusoidal endothelial cells have been implicated as early targets in veno-occlusive disease observed in the setting of hematopoietic cell transplantation (29). In this setting, following injury of the sinusoidal endothelial cells, a series of biochemical processes lead to circulatory compromise of centrilobular hepatocytes, fibrosis, and obstruction of liver blood flow. Other clinical settings include the inflammation around sinusoidal lining cells in lpr bone marrow chimera in irradiated MRL hosts (30), the reperfusion injury of sinusoidal lining cells associated with liver transplantation (31), and liver hemorrhage observed after a successful post-operative period in patients of liver transplantation (32). Finally, recent studies (6, 21) have shown that caspase inhibitors can block the lethality and hepatocyte death induced by Jo2 and TNF-α. Our study indicates that these inhibitors could potentially be used to treat certain hepatic injuries such as peritonitis or perfusion-induced liver damage by protecting the sinusoidal lining cells from apoptosis.

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