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Role of Fas ligand in uveal melanoma-induced liver damage

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Abstract *Background*: Uveal melanoma, the most common adult intraocular malignancy, metastasizes preferentially to the liver. Areas of cell death surrounding uveal melanoma metastases were observed in the livers of mice. We hypothesized that uveal melanoma cells might express Fas ligand (FasL), facilitating FasL-mediated apoptosis of Fas-expressing hepatocytes. *Purpose*: To determine whether Fas ligand (FasL)-expressing human uveal melanoma cells induce apoptosis of human hepatocytes in vitro and in vivo. *Methods*: Human uveal melanoma cell lines were assayed for FasL expression by flow cytometry and immunohistology. A human hepatocyte cell line was assayed for Fas expression by flow cytometry. Apoptosis of hepatocytes was detected by annexin V staining in vitro, and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) in vivo. *Results*: Human uveal melanoma cell lines expressed FasL, as determined by flow cytometry and immunohistology. Human hepatocytes were Fas-positive by flow cytometry. In vitro, annexin V staining revealed that human uveal melanoma cells induced apoptosis of human hepatocytes. TUNEL staining of liver metastases revealed apoptosis of murine hepatocytes in contact with metastatic human uveal melanoma cells. *Conclusion*: FasL-induced apoptosis of hepatocytes in contact with FasL-positive human uveal melanoma cells may contribute to hepatic failure during metastatic disease.

Introduction

Uveal melanoma is the most common intraocular malignancy in adults and can arise in all three components of the uveal tract, the iris, ciliary body and choroid. Uveal melanoma preferentially metastasizes to the liver, resulting in liver failure whose pathophysiology is not understood, and which affects up to 95% of uveal melanoma patients at the time of death [1]. This propensity to colonize the liver differentiates uveal melanoma from skin melanoma, which can metastasize to virtually any organ and, in fact, is one of the few tumors that regularly metastasizes to the heart [6]. Although uveal melanoma can disseminate to multiple organs, several studies have reported that the liver is frequently affected by metastases [6, 7, 14, 19, 26]. In three separate studies, 87–94% of uveal melanoma patients harbored liver metastases [6, 7, 14]. Since the liver is one of the organs frequently affected in uveal melanoma patients, it is important to understand the pathophysiology of uveal melanoma liver metastases.

In the course of examining the livers of mice bearing metastases from human uveal melanomas, we observed areas of cell death, which were confined to the metastatic foci, and we wondered whether the metastatic tumor cells had induced apoptosis of hepatocytes. Fas ligand (FasL), a cell surface protein that induces apoptosis of cells bearing its receptor (Fas), is expressed on cells of the uveal tract [9, 31]. We suspected that uveal melanoma cells might also express FasL and induce apoptosis

of hepatocytes, which are known to express large amounts of the Fas receptor. Moreover, previous studies have shown that the liver is highly susceptible to Fas-induced apoptosis since infusion of anti-Fas antibody produces acute liver failure and death in mice [23, 34]. The present study examined the hypothesis that FasL expression by human uveal melanoma might contribute to liver disease by inducing apoptosis of hepatocytes by a FasL-dependent mechanism. This was tested in vitro and in a nude mouse model of intraocular melanoma.

Materials and methods

Animals

Athymic nude mice on a Balb/c (H-2^d) background were purchased from Jackson Laboratories (Bar Harbor, Me., USA). All animals were treated in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell lines

The human liver cell line CCL-13 (Chang cells) and human embryonic kidney HEK293 cells were purchased from American Type Culture Collection (ATCC, Rockville, Md., USA). We thank Dr. June Kan-Mitchell, University of California (San Diego, Calif., USA) for OCM1 uveal melanoma cells, and Dr. Gregorius P. Luyten, University Hospital Rotterdam (Rotterdam, Netherlands) for OMM1 cells which were isolated from a subcutaneous metastasis originating from a uveal melanoma [13, 15]. Dr. Martine Jager, Leiden University Hospital (Leiden, Netherlands), kindly provided 92-1 cells [5]. The liver cells were maintained in complete BME media (JRH Biosciences, Lenexa, Kan., USA) containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah, USA), 2 mM L-glutamine (JRH Biosciences), 10 mM HEPES buffer solution (JRH Biosciences), 1% non-essential amino acids solution (BioWhittaker, Walkersville, Md., USA), 1% penicillin-streptomycin-fungizone solution (BioWhittaker), 1 mM sodium pyruvate (JRH Biosciences) and 2 mM Minimum Essential Medium (MEM) vitamins (JRH Biosciences). HEK293 cells were grown in complete Dulbecco's modified Eagle's medium (DMEM; JRH Biosciences) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin-fungizone solution and 1 mM sodium pyruvate. OCM1 cells were maintained in Ham's F-12 containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES buffer solution, 1% non-essential amino acids solution, 1% penicillin-streptomycin-fungizone solution and 1 mM sodium pyruvate. OMM1 and 92-1 cells were maintained in complete RPMI-1640 medium (JRH Biosciences) containing the same additives as Ham's F-12 medium with the addition of 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo., USA).

Flow cytometry

Flow cytometry was used to determine the relative expression of FasL among the various uveal melanoma cells lines. Uveal melanoma cells were trypsinized, centrifuged, fixed in 2% formalin for 10 min, and then treated for 30 min in 0.025% saponin at room temperature. The monoclonal antibody used for flow cytometry was the same commercial antibody (NOK-1, Pharmingen, SanDiego, Calif., USA) that has been used for staining FasL in formalin-fixed tissue specimens [8]. Anti-FasL antibodies have been used for staining FasL in formalin-fixed tissue specimens [9, 24]. Cells were washed once in complete RPMI-1640 and stained for 3 h at 37°C with anti-FasL antibody at a concentration of 50 µg/ml or with an isotype control antibody. Cells were washed three times with 0.2% bovine serum albumin in phosphate-buffered saline (PBS) and then stained with a goat anti-mouse IgG-FITC antibody (Accurate Chemical Co., Westbury, N.Y., USA) for 1 h at 37°C. After washing cells as before, 10% formalin was added and staining intensity was analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif., USA) using CellQuest software (Becton Dickinson).

Fas expression on human liver cells was assayed in a similar way, using anti-Fas antibody AM01 (Calbiochem, Cambridge, Mass., USA) at 2.5 µg/ml and a FITC-conjugated anti-mouse IgG 55496 (Cappel, Aurora, Ohio, USA) at a dilution of 1:50 as a secondary to stain cells.

Immunoperoxidase staining

OMM1 cells were grown to form a monolayer in chamber slides and fixed with 2% formalin for 10 min, then treated with 0.025% saponin for 30 min and washed for 1 h with three changes of 0.5% Tween 20 in PBS. Cells were stained with 50 µg/ml anti-FasL antibody (Pharmingen NOK-1) or a nonspecific antibody. Antibody binding was detected with a Zymed histostain kit (Zymed Laboratories, San Francisco, Calif., USA), with developing done with 0.05% diaminobenzidine and 0.02% H_2O_2 in PBS.

Annexin V staining

Human uveal melanoma cell lines OMM1 and OCM1 were labeled with Syto 59 Red fluorescent nucleic acid stain (Molecular Probes, Eugene, Ore., USA) at a concentration of 5 µM for 1.5 h, washed three times in RPMI and incubated with hepatocytes in 6-well plates for 24 h at 37°C at an effector-to-target ratio of 5:1. Cells were then trypsinized and stained with annexin V and propidium iodide according to the manufacturer's protocol (R&D Systems, Minneapolis, Minn., USA). Annexin V staining detects surface exposure of phosphatidylserine, which appears on the outside of the plasma membrane in apoptotic, necrotic and dead cells, while propidium iodide stains necrotic and dead cells [18, 33]. Double staining allows one to identify apoptotic cells as annexin V-positive and propidium iodide-negative. Although a 24-h assay provides more time than is generally necessary for a cell to apoptose, the apoptotic process may initiate at various time points during the assay and this method of gating-out propidium iodide-stained dead and necrotic cells ensures that we are quantifying apoptotic cells. Flow cytometry was used to gate-out Syto 59-stained melanoma cells in the co-culture and quantify the apoptosis of remaining hepatocytes. Percentage apoptosis was calculated as the percentage of cells that were Syto 59 Red-negative (hepatocytes), propidium iodide-negative (neither dead nor in late apoptosis), and annexin V-positive (apoptotic). Anti-FasL antibody (Pharmingen NOK-1) was used at a concentration of 0.1 µg/ml to inhibit hepatocyte cytolysis.

Mouse model of metastatic intraocular melanoma

This animal model closely parallels the course of disease in human patients, because human uveal melanoma cells injected into the anterior segment of an athymic nude mouse eye rapidly invade and metastasize to the liver as they do in humans [16, 17, 21]. Nude mice were injected intracamerally with either 10^5 OMM1 or 10^5 OCM1 FasL-expressing human uveal melanoma cells. Briefly,

an 80-µm diameter glass micropipette was mounted on a sterile 5-French infant-feeding tube (Professional Medical Products, Ocala, Fla., USA), which was mounted onto a Hamilton automatic dispensing apparatus (Hamilton, Whittier, Calif., USA) and 5 µl tumor cell suspensions were dispensed into the anterior segments of the eyes of anesthetized mice. At day 50, animals were killed and livers were harvested.

TUNEL staining

As a means of looking for hepatocyte apoptosis in vivo, livers from tumor-bearing nude mice described above were sectioned and stained using a TdT-dependent dUTP-biotin nick end labeling (TUNEL) staining kit as per the manufacturer's protocol (Oncor, Gaithersburg, Md., USA).

Statistics

Differences between groups were analyzed by Student's *t*-test; *P*-values <0.05 were considered significant.

Results

FasL expression by human uveal melanoma cell lines

In order to investigate the hypothesis that uveal melanoma cells might be inducing apoptosis of hepatocytes via a Fas-FasL pathway, we evaluated expression of FasL by melanoma cells. Immunohistological staining for FasL on OMM1 and OCM1 cells revealed strong staining with some punctate patterning suggestive of either clustered surface expression or storage within cytoplasmic vesicles (Fig. 1A, 1C). Shown in Fig. 1B and 1D are negative staining with an isotype control antibody for OMM1 and OCM1, respectively. In this experiment, HEK293 cells were used as a negative control (Fig. 1E). Human uveal melanoma cells were assayed by flow cytometry for expression of FasL. Cell lines OMM1 and OCM1 strongly expressed FasL, while 92-1 cells were negative (Fig. 1F). OMM1 cells were 34% positive over background, and OCM1 cells were 15% positive over background. Human corneal endothelial cells were included as a positive control, and were 10% positive.

Fas expression by human hepatocytes

The central hypothesis for this study proposes that human hepatocytes express Fas receptor, which transmits an apoptotic signal following engagement with FasL on uveal melanoma cells. In order to test this mechanism in vitro, it was necessary to confirm that the human hepato-

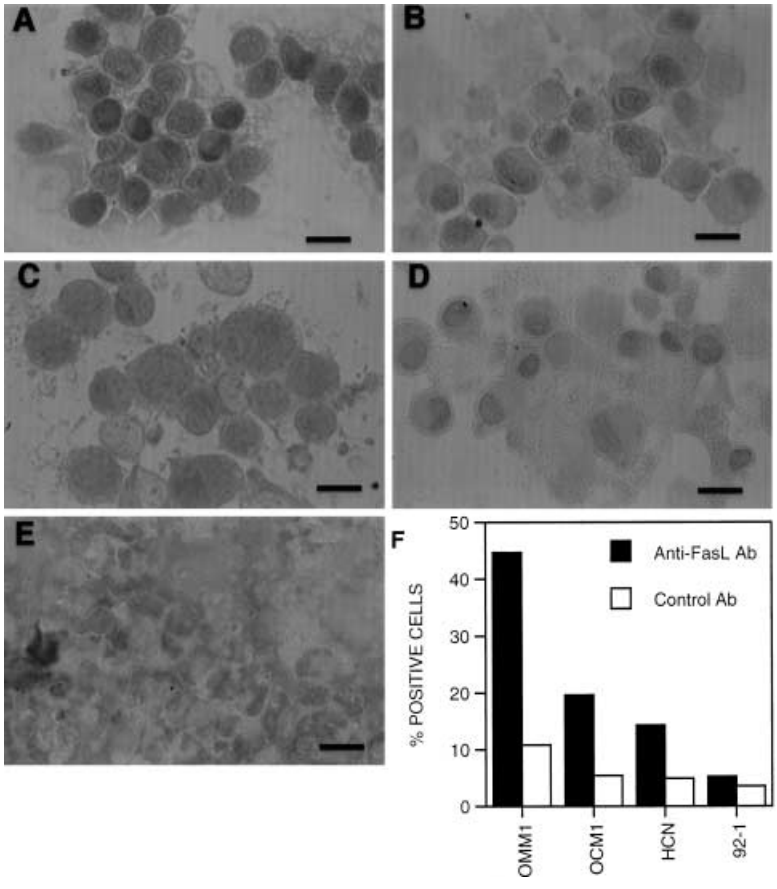

Fig. 1A–F Expression of FasL by human uveal melanoma cell lines. FasL expression was assayed by immunohistology (**A–E**) and flow cytometry (**F**). OMM1 cells were stained with anti-FasL antibody (**A**) or control antibody (**B**), as were OCM1 cells (**C, D**, respectively). As a negative control, HEK293 cells were stained with anti-FasL antibody (**E**). In (**F**), cells were stained with anti-FasL antibody or a control antibody, and human corneal endothelial cells (*HCN*) and 92–1 uveal melanoma cells were included as positive and negative staining controls. *Scale bars* 4 µm

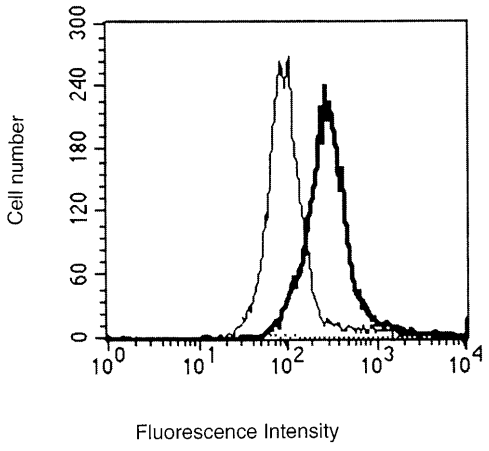

Fig. 2 Expression of Fas by human hepatocytes. The hepatocyte cell line used as targets for subsequent in vitro apoptosis assays was assayed for cell surface Fas expression by flow cytometry. Cells were stained with anti-Fas antibody and FITC-conjugated secondary antibody (*bold line*) or with secondary antibody only (*thin line*)

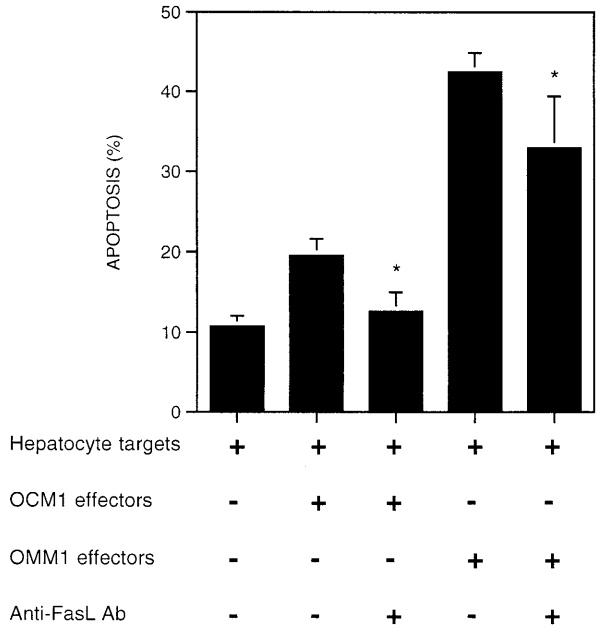

Fig. 3 Induction of hepatocyte apoptosis in a FasL-dependent manner by human uveal melanoma cells. In vitro, apoptosis was detected by annexin V staining in hepatocytes cultured with (+) or without (–) OCM1 or OMM1 cells. Treatment with (+) anti-FasL antibody (*Anti-FasL-Ab*) significantly inhibited apoptosis of hepatocyte target cells. Bars represent mean +SEM values. **P*<0.05, for anti-FasL antibody versus untreated control

cyte cell line expressed Fas. Accordingly, surface expression of Fas was examined by flow cytometry. The results in Fig. 2 confirm the abundant expression of Fas.

Induction of hepatocyte apoptosis by FasL-positive uveal melanoma cells

The capacity of FasL-expressing melanoma cells to induce apoptosis of hepatocytes was assessed in vitro using annexin V flow cytometry, and in vivo by TUNEL staining of liver lesions in nude mice bearing metastatic intraocular melanomas. Annexin V flow cytometry results provided evidence for apoptosis of hepatocytes in the presence of FasL-positive uveal melanoma cells (Fig. 3). OMM1 and OCM1 cells induced significant hepatocyte apoptosis, which was significantly inhibited by anti-FasL antibody.

The annexin V results indicated that FasL-bearing melanoma cells were capable of inducing apoptosis of hepatocytes in vitro. In order to determine the in vivo relevance of this observation, $FasL^+$ uveal melanoma cells were transplanted into the eyes of nude mice. We have previously shown that human uveal melanoma cells transplanted orthotopically into the eyes of nude mice metastasize to the liver and produce hepatic metastases [16, 17, 21]. Therefore, this model replicates the human counterpart. Mice were necropsied at day 50 when liver metastases are consistently observed. Livers were examined by TUNEL staining for the presence of apoptosis. The results showed apoptosis of hepatocytes, identifiable by their distinctive morphology, in livers that harbor metastases from intraocular OCM1 and OMM1 melanomas (Fig. 4A and 4B). Figure 4B contains a high magnification inset showing apoptotic hepatocytes adjacent to an OMM1 metastasis. Morphology of a typical OMM1 liver metastasis is shown by H&E staining in Fig. 4C, and as a negative control, TUNEL-stained normal liver tissue is shown (Fig. 4D).

Discussion

The rationale for the current study was based on a previous report that murine skin melanomas constitutively expressed FasL and were protected from immune elimination [10]. Subsequent studies challenged these findings and suggested that FasL expression on tumors and allografts might provoke, rather than prevent immune attack [for review, see 27]. Although the role, if any, for FasL in protecting tumors from immune surveillance remains contentious and unresolved, it may be irrelevant in terms of primary uveal melanomas. In contrast to skin melanomas, which are exposed to the full array of immune effector elements, uveal melanomas can arise within an immunologically privileged organ (i.e. the eye) and would not necessarily benefit from FasL expression. Since uveal melanomas reside beneath the retina – a site that provides immune privilege to foreign tissue grafts – the expression of FasL on primary uveal melanomas

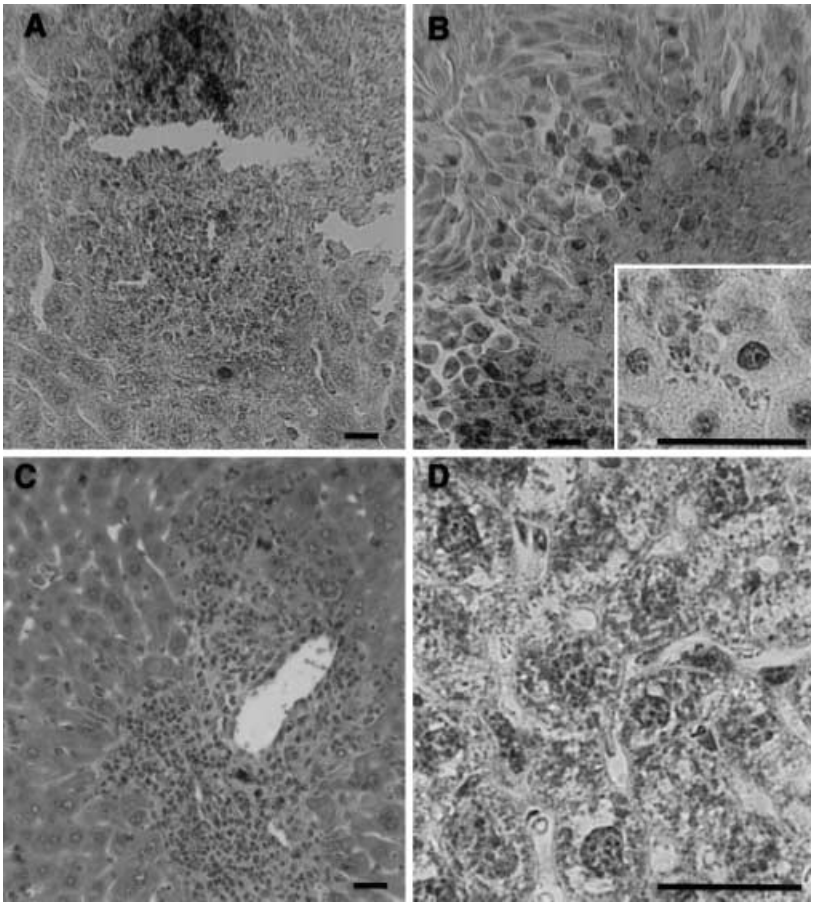

Fig. 4A–D Apoptosis of hepatocytes in contact with FasL-bearing human uveal melanoma cells. In vivo, nude mice were injected intracamerally with 10^5 human uveal melanoma cells. At day 50, livers were harvested and TUNEL stained to detect apoptotic cells in mice bearing OCM1 cells (**A**) or OMM1 cells (**B**). Apoptosis of hepatocytes adjacent to an OMM1 metastasis are shown at increased magnification (**B inset**). OMM1 tumor morphology is shown by H&E staining (**C**), and TUNEL-stained normal liver tissue is included as a negative control (**D**). *Scale bars* 20 μm

would have significantly less impact on their survival and escape from immune surveillance than on tumors arising in non-privileged sites [11, 12].

When we discovered that the uveal melanoma cell lines expressed FasL, we theorized that the destruction, which we consistently see, of hepatic tissue surrounding human uveal melanoma metastases in livers of nude mice could be due to FasL-mediated apoptosis of Fas expressing hepatocytes. Fas-mediated apoptosis has been shown to play a role in liver failure in other systems. Intravenous injection of anti-Fas antibody causes massive liver destruction and death in mice [23]. FasL has also been implicated in murine hepatitis [28]. Human colon cancer may use the same mechanism of hepatocyte destruction as uveal melanoma does, since colon cancer has been shown to express FasL and induce apoptosis of hepatocytes [29, 35].

Although uveal melanoma arises in an immune-privileged site, its pattern of metastasis to the liver suggests that the tumor is capable of evading immune surveillance after leaving the immune-privileged sanctuary of the eye. We propose that the melanoma-induced hepatocyte apoptosis reported here might be a byproduct of selection for tumors that are able to induce apoptosis of Fas-expressing lymphocytes. Induction of lymphocyte apoptosis has been observed in several types of tumors, including melanoma, breast cancer, and colon cancer [10, 20, 22]. It is possible that FasL-expressing uveal melanoma cells are initially successful outside of the immune-privileged ocular environment because they inactivate lymphocytes that they encounter in the circulating blood during the metastatic process. Once in the liver, the same tumor cells induce apoptosis of the hepatocytes, even though the liver cells pose no threat to the survival of the metastatic tumor cells. While we have not used anti-FasL antibodies in vivo because of the enormous expense incurred for a treatment protocol that would need to span a 50-day period, the fact that anti-FasL antibody inhibits hepatocyte apoptosis in vitro suggests that hepatocyte lysis in mice could also be a FasL-mediated event, and not simply the result of a mechanical crowding of hepatocytes [4]. However, this hypothesis remains to be proven.

Expression of FasL by various tumor types has been reported recently, but some controversy exists in the literature as to the specificity of certain anti-FasL antibodies. One group concluded that the Santa Cruz Biotechnology polyclonal rabbit anti-FasL IgG antibody C-20

does not specifically detect FasL, and another group questioned the A11 FasL antibody produced by Alexis Corp. (San Diego, Calif., USA) because they were unable to detect a FasL reverse transcriptase-polymerase chain reaction (RT-PCR) product in skin melanomas when the same type of tumor had been shown to express FasL using the A11 antibody [3, 30]. The FasL antibody from Transduction Laboratories (Lexington, Ky., USA) was demonstrated to produce a band of approximately the correct size in a Western blot when FasL-negative cells were tested [8]. In the present study, we have used the Pharmingen NOK-1 antibody, which has been shown to be specific for FasL [8]. In support of our data showing the presence of FasL protein on uveal melanoma cells, others have found expression of FasL on these cell lines by RT-PCR [2, 32]. Finally, our FasL expression data are supported by experiments demonstrating functionality of FasL on the human uveal melanoma cell lines used in the present experiments.

FasL expression by primary uveal melanomas may or may not play a role in the tumor's capacity to escape immune attack within the eye, but the present results suggest that it may be important for the survival of uveal melanoma cells once they leave the eye and form metastases. A similar proposition has been suggested in the case of liver metastases in colon cancer patients. In a previous study on colon cancer patients, only two of seven primary colon cancers expressed FasL, yet all of the hepatic metastases were FasL-positive [29]. Although one can only speculate as to whether FasL-induced apoptosis contributes to liver failure in uveal melanoma patients, it is noteworthy that prospective studies in a hamster model have shown that inducing injury to the liver results in a two- to three-fold increase in melanoma liver metastases [25]. Also, it has been suggested that FasL expression facilitates liver colonization by colon cancer metastases in human patients [29]. Thus, expression of FasL on uveal melanoma metastases may exacerbate liver disease by inducing apoptosis of hepatocytes, by facilitating liver colonization, by enhancing immunologic escape, or by a combination of all three.

We have shown that FasL expressed on human uveal melanoma cells can be used to induce apoptosis of Fas-positive hepatocytes in vitro and in vivo, where hepatocytes in close proximity to FasL-positive tumor cells are seen to undergo apoptosis. Patients with metastatic uveal melanoma experience liver failure, possibly in part due to apoptosis of hepatocytes in contact with FasL-bearing uveal melanoma cells. Treatment for metastatic uveal melanoma is at this point largely ineffective. However, understanding the pathological mechanisms of liver metastases is crucial for improving the status of treatment options, and this study provides evidence for one contributing mechanism.

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