Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis Is an Important Endogenous Mechanism for Resistance to Liver Metastases in Murine Renal Cancer


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ABSTRACT

Recent reports have suggested that the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) may partially limit the formation of hepatic metastases of a variety of mouse tumors, and the major source of TRAIL in the liver was shown to be local natural killer cells. We isolated a clone (R331) of the murine renal cancer cell line Renca that was strikingly more sensitive to both Fas and TRAIL death receptor-mediated apoptosis in vitro. R331 grew in tissue culture in vitro at a rate similar to that of the parental Renca cell line but formed larger and more numerous colonies than parental Renca in soft agar. After s.c. implantation, R331 tumors progressed more rapidly than parental Renca tumors. However, R331 formed far fewer lung and liver metastases in wild-type (WT) BALB/c mice. Administration of antibodies that neutralized TRAIL dramatically increased the number of R331 liver metastases. Furthermore, numbers of R331 liver metastases were much greater in TRAIL−/− than in WT BALB/c mice. In contrast, no difference was seen in numbers of lung metastases when comparing TRAIL−/− and WT mice, suggesting that the antitumor effects of TRAIL in vivo were compartment specific. Transfection of cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein into R331 increased the numbers of liver metastases in BALB/c mice by up to 10-fold, indicating that local TRAIL in the liver was directly mediating tumor cell apoptosis. These organ-specific differences in the endogenous levels of death ligands may apply different selective pressures on the development of liver or lung metastases. Consequently, the efficacy of TRAIL therapy may vary depending on the location of the tumor metastases.

INTRODUCTION

Tumor metastasis is usually defined clinically and experimentally by evidence of the end point of the process, that is, the presence of metastatic tumors. Although end point assays are suitable for determining whether a therapeutic approach is effective, they provide little information on what steps are crucial in a particular organ for the establishment of tumor metastases there (1). Recent studies using in vivo video microscopy and techniques for “cell accounting” in tissues have shed new light on the metastatic process (1–3). Therefore, a major determinant in the arrest of solid tumor cells in the target tissues is their size. Only a small proportion of cancer cells that enter the circulation go on to form metastases. Metastatic inefficiency has generally been considered the result of massive destruction of cancer cells within the circulation, due to the immune system and hydrodynamic forces. However, a number of more recent studies using non-toxic markers suggest that for many different types of tumor cells, the vast majority of the cells survive arrest in the vasculature and go on to extravasate (4, 5). From such studies, it was concluded that two factors determine the development of organ-specific metastases: (a) the number of cells arrested in that organ; and (b) the ability of the environment of that organ to support the growth of these cells. The ability of cancer cells to only grow where they find a supportive microenvironment has been termed the “seed and soil” hypothesis. Therefore, development of metastases involves interactions between cancer cells (the “seeds”) and specific organs or microenvironments within them (the “soil”).

Death receptor-mediated apoptosis plays an important role in tissue homeostasis. This is particularly evident in the immune system, where mutations in the death receptor Fas or its ligand (FasL)3 result in massive lymphoproliferation in both mice and humans (6–8). Furthermore, death ligands such as TNF-α, FasL, or TRAIL may be used by immune cytotoxic effector cells to eliminate virally infected cells or tumor cells (9). Several studies have suggested that a reduction in tumor cell sensitivity to apoptosis via death receptors can promote tumor growth and development of metastases in vivo (10–13). More recently, it has been proposed that TRAIL may act as a tumor suppressor molecule in vivo (14, 15), and TRAIL restricted the ability of several tumors to form experimental liver metastases in vivo. IFN-γ was shown to be essential for the antimitastatic effects of TRAIL, and liver NK cells were shown to express TRAIL, particularly after activation with IFN-γ. Because tumors are heterogeneous, it would be predicted that the degree of sensitivity or resistance of individual tumor cells to TRAIL-mediated apoptosis could significantly influence their ability to form metastases, particularly in the liver.

Using the murine renal cancer cell line Renca, we isolated clones that had a growth rate similar to that of the parental tumor in vitro yet were much more sensitive to death receptor-mediated apoptosis. We have used one of these clones (R331) as a model to determine the relative importance of sensitivity to death receptor-mediated apoptosis in controlling tumor metastases in vivo. We therefore compared R331 with the parental Renca tumor for their ability to grow in different primary tissue locations as well as their propensity to form liver metastases after i.s. injection or lung metastases after i.v. injection. A thorough analysis of the importance of death receptor-mediated apoptosis in vivo was performed using death receptor-resistant c-FLIP transfectants of Renca and R331 tumor cells, neutralizing Abs to TRAIL and FasL, and appropriate gene-targeted or mutant mouse strains.

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3 The abbreviations used are: FasL, Fas ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WT, wild-type; c-FLIP, cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein; NK, natural killer; TNF, tumor necrosis factor; mAb, monoclonal antibody; Ah, antibody; AMC, amido-4-methylcoumarin; i.s., intrasplenic or intrasplenically; Ox, oxime.
TRAIL CONTROLS LIVER METASTASES

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c mice were obtained from the Animal Production Area, National Cancer Institute (Frederick, MD) or The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at the National Cancer Institute or The Peter MacCallum Cancer Institute (East Melbourne, Australia): BALB/c perforin-deficient (BALB/c ppf−/−) mice (16); and BALB/c TRAIL−/− mice back-crossed to BALB/c for five generations (Immunex Corp., Seattle, WA; Ref. 15). C57BL/6 Tnfsf6gld (gld) mice (where mutated, nonfunctional FasL has been previously described) (17). Briefly, Renca cells (2 × 104 cells/well) were plated in 24-well plates 24 h before transfection. Transfection was performed with 0.8 μl of Plus reagents, 1 μl. to evaluate the sensitivities of tumor cell lines to FasL or TRAIL, d11S or mTRAIL/2PK-3 cells were used as effector cells. Alternatively, recombinant human FasL was added in the culture to evaluate susceptibility to Fas-induced apoptosis. After incubation, supernatants were harvested and counted on a gamma counter. Specific killing (percentage of cytotoxicity) was calculated as follows: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100. All groups were run in triplicate, and all experiments were performed three or more times with similar findings.

Fluorometric Assay of Caspase-3. Caspase-3 activities of tumor cells, induced on TRAIL exposure, were measured by a fluorometric method using a caspase-3 assay kit (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, 2 × 104 tumor cells were plated in 6-well plates and left to settle overnight. The wells were washed with media twice, followed by a 4-h incubation in the complete media with or without TRAIL (1000 ng/ml). Extracts of 2 × 105 cell equivalents were incubated in the reaction buffer [20 mM HEPES (pH 7.4), 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5 mM DTT, and 2 mM EDTA] containing fluorogenic synthetic substrates acetyl-asp-Glu-Val-Asp-AMC (16 μM) at room temperature for 2 h. Free AMC was quantified fluorometrically at excitation and emission wavelengths of 360 and 460 nm, respectively, on a Cytofluor multilabeled reader series 4000 (Perspective Biosystems, Framington, MA). Standard curves were generated with AMC.

Tumor Proliferation Assays in Vivo. On day 0, tumor cells were plated in a 96-well tissue culture plate at the indicated dose in 100 μl of complete medium. On days 1, 2, and 5, viable cells were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Roche, Mannheim, Germany) or 3-[4,5-dimethylthiazol-2-yl]-2-[4-sulfophenyl]-2H-tetrazolium cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Spectrophotometric absorbance at 595 nm or 490 nm was measured with absorbance at 655 nm for reference in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or 3-[4,5-dimethylthiazol-2-yl]-2-[4-sulfophenyl]-2H-tetrazolium assay, respectively. All groups were run in triplicate, and all experiments were performed three or more times with similar findings. Proliferation of Renca or R331 tumor cells was also assessed by using a soft agar colony assay (13). Briefly, single-cell suspensions of tumor cells in complete medium were mixed with agarose in a final concentration of 0.3% agarose and 1× complete medium with agarose in a final concentration of 0.3% agarose and 1× complete medium and allowed to gel. The dishes were incubated at 37°C in 5% CO2 for 14 days and stained with 0.005% crystal violet solution.

Tumor Growth and Experimental Metastasis Assay in Vivo. To examine primary tumor growth, male WT BALB/c mice (6–12 weeks of age) were inoculated s.c. or in the kidney capsule with Renca or R331 tumor cells on day 0 at the doses indicated. S.c. tumors were measured after tumor inoculation over the course of 27 days with a caliper square as the product of two perpendicular diameters (mm2). The mice in which tumor cells had been injected into the kidney were sacrificed on day 24, and the tumor-bearing kidneys were weighed. BALB/c WT and gene-targeted mice were injected i.s., followed by splenectomy, or i.v. with tumor cells at the indicated doses. Mice were killed 14 days after tumor inoculation, and liver (after i.s.) or lung (after i.v.) metasteses were quantified with the aid of a dissecting microscope. Some mice also received injections of antitumor TRAIL mAb, control rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or antitumor FasL mAb (250 μg) i.p. on days 0, 1, and 7 after tumor inoculation. Data were presented as the mean ± SE of 5–15 mice in each group. All data are representative of two to three independent experiments with similar results.

Statistical Analysis. The significance of difference in the number of metastases and tumor sizes between experimental groups was determined by the Mann-Whitney t test. Two-sided Ps of <0.05 are considered significant.

Flow Cytometric Analysis. Fas or MHC class I (H-2Kb) expressions of tumor cell lines were analyzed after overnight treatment with IFN-γ (500 units/ml) and TNF-α (400 units/ml) or media alone by flow cytometry using Phycocerythrin-labeled mAbs or isotype-matched mAbs. Flow cytometry analysis was performed on a FACSscan (BD Biosciences, Mountain View, CA) using CellQuest software. All mAbs were purchased from BD Pharmingen.

Cytotoxicity Assays. Target cells that had been incubated overnight in the presence or absence of IFN-γ (500 units/ml) and TNF-α (400 units/ml) were labeled with [111In]Ox (Medi-Physics, Silver Spring, MD) as described previously (19). Briefly, 1 × 105 target cells were incubated with 10 μCi of [111In]Ox for 30 min at room temperature. Cells were then washed twice in complete medium, and labeled cells (1 × 105) were then incubated with effector cells at various E:T ratios for 16–18 h at 37°C in a final volume of 200 μl. To evaluate the sensitivities of tumor cell lines to FasL or TRAIL, d11S or mTRAIL/2PK-3 cells were used as effector cells. Alternatively, recombinant human FasL was added in the culture to evaluate susceptibility to Fas-induced apoptosis. After incubation, supernatants were harvested and counted on a gamma counter. Specific killing (percentage of cytotoxicity) was calculated as follows: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100. All groups were run in triplicate, and all experiments were performed three or more times with similar findings.

Animals (NIH Publication No. 86-23, 1985). Used between 6 and 12 weeks of age. Animal care was provided in accordance with the standard instructions, and the Charles River Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-38, 1985).
RESULTS

Susceptibility to Death Receptor-mediated Apoptosis of Renca and R331. Renca, a murine renal cancer cell line, was subjected to a standard limiting dilution method in vitro to establish cloned cell lines. Some of these established clones exhibited some biological characteristics quite different from those of the parental Renca cells. In particular, some of the clones had a much greater susceptibility to death receptor-mediated apoptosis in vitro than the parental Renca cells, and one of these clones, R331, was selected for further study (Fig. 1A). Renca was not killed by the FasL-expressing hybridoma cell line d11S, whereas R331 displayed some sensitivity. Renca expressed small amounts of Fas on the cell surface, whereas R331 expressed more Fas constitutively as detected by fluorescence-activated cell-sorting analysis (Fig. 1B). Cytokine stimulation with the combination of IFN-γ and TNF-α increased Fas expression on both Renca and R331 cells, although R331 cells still expressed significantly more Fas than Renca cells. These cytokine pretreatments sensitized Renca cells and enhanced the susceptibility of R331 cells to FasL-mediated apoptosis by d11S cells (Fig. 1A). After cytokine treatment, R331 was still more sensitive to d11S effector cells than Renca, suggesting that Fasl sensitivity was at least partially dependent on the expression level of Fas on the tumor cell surface. R331 was also more sensitive to TRAIL-mediated apoptosis than Renca, although both Renca and R331 were killed by the murine TRAIL-transfected cell line mTRAIL/2PK-3. In contrast, TRAIL-mediated killing was not enhanced by cytokine pretreatment. TRAIL-R2/DR5 was detected constitutively at similar levels on both Renca and R331 cells by Western blot analysis, and cytokine treatment of these cells did not enhance TRAIL-R2 expression (Fig. 1C). These data were also confirmed by flow cytometric analysis of surface TRAIL-R2 expression (data not shown). Nonetheless, these findings clearly showed that R331 was much more susceptible to death receptor-mediated apoptosis. The differences in TRAIL sensitivity between Renca and R331 could not be explained by TRAIL-R2 expression level. Therefore, other downstream signaling events in Renca and R331 may account for differences in TRAIL sensitivity. Further study of Renca and R331 tumor cells will be required to address this issue.

In Vitro and in Vivo Growth of Renca and R331. The proliferation rate of R331 in vitro was always slightly faster than that of Renca, although these differences were not statistically significant (data not shown). However, R331 developed both larger and more numerous (2–4-fold higher) colonies in soft agar colony assays (Fig. 2), indicating that R331 may have more tumorigenic potential in vivo. After s.c. injection, R331 developed larger tumors at a more rapid rate than parental Renca (Fig. 3). In the renal capsule, the growth rates of Renca and R331 were not significantly different (data not shown). Next, these tumor cells were compared for their ability to form metastases in vivo. Four different doses of Renca cells and R331 cells were inoculated i.s. (Fig. 4), and four doses were injected i.v. (Fig. 4B), and after 2 weeks, liver or lung metastases were quantified, respectively. Surprisingly, in contrast to the local growth in vivo (Fig. 3) and in vitro data shown in Fig. 2, Renca developed many more metastases than R331 in both the liver and lungs ($P < 0.01$).

Involvement of TRAIL in Antimetastatic Effects in Vivo. Because R331 is susceptible to both FasL- and TRAIL-mediated lysis in vitro, we next investigated which of these death ligands played a dominant role in controlling R331 metastasis in vivo. The liver metastatic activity of R331 was significantly increased in WT mice treated with a neutralizing mAb to mouse TRAIL ($P < 0.01$; Fig. 5A). In contrast, a mAb reactive with FasL did not significantly increase the number of liver metastases. These findings are consistent with TRAIL, and not FasL, being the key proapoptotic protein that con-
trolled R331 metastases in the liver. For a more detailed and comparative analysis of antitumor effector functions, perforin-deficient (Pfp⁻/⁻) and TRAIL-deficient (TRAIL⁻/⁻) mice were used. Perforin is a major effector mechanism used by both NK cells and T cells to kill tumor target cells, and it plays an important tumor surveillance role in a number of mouse tumor models in vivo (16, 20, 21). The number of Renca liver metastases was increased in pfp⁻/⁻ (P < 0.05) or TRAIL⁻/⁻ (P < 0.05) mice compared with WT mice as reported previously (22). The Renca tumor displayed a similar number of liver metastases in WT mice injected with anti-FasL mAb or control Ab (Fig. 5B), suggesting that FasL did not play a major role in limiting liver metastases of either Renca or R331. Much more strikingly, TRAIL⁻/⁻ mice developed 15 times more R331 liver metastases than WT mice (P < 0.01). Pfp⁻/⁻ mice and WT mice treated with anti-FasL mAb showed minor increases in R331 liver metastases as compared with the control WT mice, but these differences were not statistically significant (Fig. 5B). Furthermore, there was no significant difference in the number of liver metastases of R331 between WT mice and gld mice that possess a nonfunctional FasL (data not shown). These findings clearly demonstrate that TRAIL, rather than either perforin or FasL, is the major factor controlling R331 metastases in the liver.

When compared with Renca tumor cells, R331 cells also displayed a reduced number of metastases in the lung (Fig. 5C). Interestingly, the number of R331 lung metastases was not significantly increased in pfp⁻/⁻ mice, TRAIL⁻/⁻ mice, or WT mice treated with anti-FasL mAb. This suggests that the number of lung metastases of R331 is not influenced by local levels of perforin, FasL, or TRAIL. By contrast, the number of Renca lung metastases was significantly increased in pfp⁻/⁻ mice, confirming that perforin was an important regulator of Renca metastases in the lung (Fig. 5C). Overall, these data indicated that TRAIL was not an important effector molecule against Renca or R331 tumor cell metastasis to the lung, despite its obvious activity in the liver. It remains to be determined whether any other immune effector mechanism can be detected against R331 tumor cells in the lung.

Fig. 3. Tumor growth at primary sites in vivo. WT BALB/c mice were inoculated s.c. with Renca or R331 tumor cells at the indicated doses on day 0. Tumor sizes were monitored for up to 27 days after tumor inoculations. Mean ± SE of five mice in each group are shown. †, monitoring was terminated on day 20 due to tumor necrosis. Tumor sizes of Renca and R331 were compared at various time points, and Ps are denoted as follows: **, P < 0.01 on day 17, and P < 0.05 on day 20 (1 × 10⁴); †, P < 0.01 on days 10, 13, and 17 (1 × 10⁴).

Fig. 4. Metastatic abilities of Renca and R331 in vivo. BALB/c WT mice were inoculated i.s. (A) or i.v. (B) with Renca or R331 cells at the indicated doses. Mice were killed 14 days after tumor inoculation, and liver (A) or lung (B) metastases were quantified. Data are presented as the mean ± SE of 5–15 mice in each group. *, P < 0.05; **, P < 0.01 (Renca versus R331). Data are representative of three independent experiments with similar results.

Fig. 5. Involvement of TRAIL in prevention of R331 liver metastasis. A, WT mice were inoculated i.s. with R331 cells (5 × 10⁴) on day 0. Mice received i.p. administration of anti-TRAIL, anti-FasL mAb, control rat IgG2a (250 μg/day), or vehicle alone on days 0, 1, and 7. Mice were killed 14 days after tumor inoculation, and liver metastases were quantified. Data represent the mean ± SE of five mice in each group. *, P < 0.01 compared with control or Rat IgG. Data are representative of three independent experiments with similar results. B and C, Renca and R331 cells were injected i.s. (B, 5 × 10⁴) or i.v. (C, 1 × 10⁴) to WT, Pfp⁻/⁻, and TRAIL⁻/⁻ mice on day 0. Half of the WT mice were given anti-FasL mAb i.p. on days 0, 1, and 7. On day 14, liver (B) or lung (C) metastases were quantified. Data are presented as the mean ± SE of 5–15 mice in each group. *, P < 0.05; **, P < 0.01 (compared with WT).
TRAIL stimulates (1000 ng/ml, 4 h) using a fluorometric method. R331, R331-TC, and R331-FLIP.1 cells were measured with or without recombinant metastases of R331 but did not confirm whether the major effect of the preceding data demonstrated that TRAIL could limit liver ses.

IFN-pretreatment with IFN- was due to the apoptotic destruction of bers of liver metastases in the presence or absence of TRAIL were (23). However, in the R331 model system, differences between num-

DISCUSSION

Using the R331 clone of Renca, which is highly sensitive to death receptor-mediated apoptosis, we have shown that TRAIL plays an important role in limiting metastatic tumor burden in the liver, but not in the lung. In previous studies using a variety of tumor cell lines, TRAIL was demonstrated to partially prevent the development of liver metastases. Previous studies on liver metastases in the parental Renca model have also indicated that NK cells played a major role in liver metastases. Previous studies on liver metastases in the parental (22). We confirmed herein that control of Renca liver metastases was dependent on both perforin and TRAIL (23). However, in the R331 model system, differences between numbers of liver metastases in the presence or absence of TRAIL were very dramatic. In contrast to parental Renca, R331 liver metastases were exclusively limited by TRAIL, and TRAIL sensitivity was responsible for the low efficiency of R331 metastasis to the liver. Interestingly, R331 cells were not controlled by perforin in TRAIL−/− mice, and additional in vitro studies indicated that R331 cells were more resistant to granule-mediated cytolysis than Renca.4 These data were further supported by the large number of R331-FLIP tumor metastases that developed in WT mice. Consequently, the

![Image](47x411 to 293x748)

Fig. 6. Characteristics of FLAG-tagged cFLIP-transfected R331 clones. A. expression of FLIP was tested by Western blot with mAb against FLIP. Furthermore, FLAG-tagged cFLIP expression was determined by Western blot with anti-FLIP mAb after immunoprecipitation with anti-FLAG Ab. B. sensitivities to FasL- or TRAIL-mediated lysis of Renca, R331, R331-TC (transfection control), and R331-FLIP.1 cells were tested after pretreatment with IFN-γ and TNF-α by 16 h [111In]Ox release assay. Recombinant FasL or mTRAIL/2PK-3 cells were used as effectors at three different doses. Data of percentage of lysis are presented as the mean ± SD of triplicate samples. C. caspase-3 activities of R331, R331-TC, and R331-FLIP.1 cells were measured with or without recombinant TRAIL stimulation (1000 ng/ml, 4 h) using a fluorometric method.

Transfection of R331 with c-FLIP Reduces Death Receptor-mediated Apoptosis and Increases the Number of Liver Metastases. The preceding data demonstrated that TRAIL could limit liver metastases of R331 but did not confirm whether the major effect of TRAIL expression in vivo was due to the apoptotic destruction of tumor cells. In recent years, several mechanisms have been proposed to interfere with death receptor-induced apoptosis. The antiapoptotic protein c-FLIP protects cells from Fas- and TRAIL-induced apoptosis (10). To determine whether death receptor-induced apoptosis was directly relevant for the development of experimental metastases, c-FLIP-transfected Renca and R331 clones were generated. Both Renca and R331 cells expressed endogenous c-FLIP at low but detectable levels on Western blot analysis (Fig. 6A). Transfectants of Renca and R331 were generated that expressed high levels of c-FLIP (Renca-FLIP.1 and R331-FLIP.1), some of which could be immunoprecipitated using anti-FLAG Abs. In contrast, R331 and Renca cells had undetectable levels of FLAG-tagged c-FLIP (Fig. 6A). The sensitivity of the R331 transfectants to FasL and TRAIL was tested after IFN-γ and TNF-α treatment in cytotoxicity assays with recombinant FasL and mTRAIL/2PK-3 cells (Fig. 6B). Apoptosis of R331-TC (transfection controls) was similar to that of R331, whereas R331-FLIP.1 was almost completely resistant to TRAIL-mediated apoptosis. Although cytokine-pretreated R331-FLIP.1 showed some weak sensitivity to recombinant FasL at the highest dose tested, apoptosis of untreated R331-FLIP.1 was completely abrogated (data not shown). After TRAIL treatment, there was a large difference in caspase-3 activation between R331-FLIP.1 and R331 or R331-TC (Fig. 6C). R331 and R331-TC showed much higher caspase-3 activity after exposure to TRAIL than R331-FLIP.1. R331, R331-TC, and R331-FLIP.1 all expressed identical amounts of Fas and MHC class I by fluorescence-activated cell-sorting analysis, and growth rates of these cells were also identical in vitro (data not shown).

The R331-FLIP.1 cells and their respective parental and transfection controls were injected i.s., and liver metastases were then counted (Fig. 7, A and B). R331-FLIP.1 cells developed much larger numbers of liver metastases than R331 (P < 0.01 with 1 × 10⁵ cells as initial dose and P < 0.0001 with 5 × 10⁴ cells as initial dose; Fig. 7, A and C), whereas the number of metastases after injection of R331-TC was similar to that seen after injection of R331. Furthermore, Abs to TRAIL enhanced the number of R331 liver metastases but had little effect on the number of R331-FLIP.1 metastases (Fig. 7C). Several other independent R331 clones that expressed FLIP at high levels (R331-FLIP.2 and R331-FLIP.3) also produced larger numbers of metastases after i.s. injection (Fig. 7A). With regard to the parental Renca cell line, a similar (albeit more modest) increase in the number of Renca-FLIP.1 and Renca-FLIP.2 liver metastases over Renca and control transfectant Renca-TC cells was observed (Fig. 7D). Renca-FLIP transfectants also expressed elevated levels of FLIP and were much less sensitive to TRAIL-mediated apoptosis than controls (data not shown). Interestingly, the number of metastases of R331-FLIP clones was even significantly greater than that of parental Renca (P < 0.05 with 1 × 10⁵ cells as initial dose and P < 0.0001 with 5 × 10⁴ cells as initial dose). In addition, the FasL- and TRAIL-resistant clone R331-FLIP.1 produced a larger number of liver metastases in WT, pfp−/−, TRAIL−/−, and WT mice treated with anti-FasL mAb, whereas R331-TC showed a pattern of metastasis development similar to that of R331 in these mice (Fig. 8). These findings clearly demonstrate that sensitivity of tumor cells to TRAIL-induced apoptosis can dramatically influence the development of liver metastasis in vivo, and TRAIL sensitivity is the dominant mechanism that restricts R331 liver metastases in vivo.
TRAIL controls liver metastases

Fig. 7. Increased metastatic potential of R331-FLIP cells. A, BALB/c WT mice were inoculated i.s. with R331, R331-TC, or R331-FLIP.1 cells at a dose of 1 or 5 × 10^4 cells on day 0. In an additional experiment, 5 × 10^4 cells of R331-FLIP.2 and R331-FLIP.3 clones were also inoculated i.s. On day 14, the number of liver metastases was counted with or without anti-TRAIL mAb administrations (on days 0, 1, and 7; 250 μg/day). On day 14, liver metastases were quantified. Data are presented as the mean ± SE of 5–10 mice in each group. *, P < 0.001; †, P < 0.01; ‡, P < 0.001; ††, P < 0.0001 (compared with WT). B, livers of two mice injected with R331 and R331-FLIP.1 are shown. C, the number of liver metastases of R331 and R331-FLIP.1 in WT mice was counted with or without anti-TRAIL mAb administrations (on days 0, 1, and 7; 250 μg/day). *, P < 0.01 compared with control. Data are representative of three independent experiments with similar results. D, liver metastasis of Renca, Renca-TC, Renca-FLIP.1, and Renca-FLIP.2 clones was tested in the identical method. *, P < 0.01; †, P < 0.001; ‡, P < 0.0001 (compared with Renca or Renca-TC).

Fig. 8. c-FLIP transfectants overcome the antimitastatic effects of TRAIL. WT, Pfp^−/−, and TRAIL^−/− mice were inoculated i.s. with 5 × 10^4 R331-TC or R331-FLIP.1 cells on day 0. Half of WT mice were given i.p. anti-FasL mAb on days 0, 1, and 7 (250 μg/day). On day 14, liver metastases were quantified. Data are presented as the mean ± SE of 5–10 mice in each group. *, P < 0.05; †, P < 0.01; ‡, P < 0.001 (compared with WT).
tions, it is known that they do not constitutively express surface TRAIL (22), and therefore R331 and Renca lung metastases may not be subjected to TRAIL-expressing effector cells.

With regard to the rational application of TRAIL for antitumor therapy of R331 metastases in this model, a number of important issues remain. Firstly, R331 cells can grow efficiently at several tissue sites tested, including s.c. and under the kidney capsule. This indicates that the number of TRAIL-7 cells in these locations is too low to influence tumor growth. It would therefore be interesting to determine whether systemic administration of TRAIL would indeed result in reduction of R331 tumor growth at these sites, or whether TRAIL-expressing effector cells can be mobilized at these sites by immune activation using cytokines and other biological response modifiers. It is likely that the relative susceptibility of some individual tumor cells (such as R331 or Renca cells) to death ligands may be determined by an “apoptotic threshold.” It remains unclear why R331 tumor cells are more sensitive to TRAIL than Renca cells, because both express equivalent levels of TRAIL-R2/TRD5. Also, although apoptotic sensitivity and caspase activation of R331 cells can be blocked by overexpression of the antiapoptotic c-FLIP, endogenous levels of this protein in Renca and R331 cells were not dramatically different.

Rencsa cells can be sensitized to FasL-mediated lysis by prior treatments with the cytokines IFN-γ and TNF-α, which enhance expression of the Fas receptor (29). With regard to the sensitivity of cells to FasL-mediated apoptosis, it has been proposed that two types of cells exist (30, 31). In type I cells, caspase activation occurs in the absence of mitochondrial involvement due to a robust activation of the signaling cascade-8. By contrast, in type II cells, caspase-8 activation is relatively weak, and therefore amplification of the apoptotic signal via mitochondrial disruption is required. We do not know at present which apoptotic pathway(s) is (are) predominant in Renca and R331 tumor cells.

In conclusion, a high sensitivity to TRAIL-mediated apoptosis can severely restrict a tumor’s ability to form liver metastases. TRAIL appeared to be a more important natural regulator of tumor growth in the liver than FasL; however, neither effector pathway regulated the same tumors metastasizing to the lung. A more detailed knowledge of the crucial molecular events involved in apoptotic signaling in individual tumor cells may provide for the identification of appropriate molecular targets, thus allowing for amplification of tumor cell apoptosis in response to TRAIL. The organ specificity of effector molecules is clearly a key concern when developing effective immunotherapies for reduction or prevention of metastatic tumor burden.

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