K-T gap, rarefaction analysis indicates that anywhere from 3 to all 10 of the missing ammonites may have become extinct before the last 1.5 m of the Cretaceous ($P = 0.05$). We are unable to distinguish between these two end-member possibilities. Thus, there was at least a minor biological change, and perhaps a fairly large extinction event, associated with the regression that peaked shortly before the K-T boundary. The fossil record from outside the Bay of Biscay basin does not help distinguish between these possibilities, as only one of the 10 missing species is known to have survived to the K-T boundary elsewhere [*Hoploscaphites constrictus* in Denmark (*29*)].

The ammonites in the Bay of Biscay are preserved as impressions only and hence can only be seen on bedding surfaces. Within the marly pre–K-T gap there are essentially no bedding surfaces exposed. Hence, it is difficult to confirm that there was a drop in abundance, if not diversity, during the interval, or whether their absence in the pre–K-T gap simply reflects collection failure (*30*). However, near Hendaye in southern France, it is relatively easy to excavate bedding surfaces in the top 2 m of the pre–K-T gap. Quantitative analysis of collecting intensity at this locality does indeed support the hypothesis that, minimally, the abundance of ammonites within the pre–K-T gap dropped in comparison with the last 1.5 m of the Cretaceous (*31*).

Of the 13 molluscan species known from the last 1.5 m of the Cretaceous, only six are known from two or more fossils: five ammonites and *T. argentea*. Statistical analysis of the fossil records of these six species failed to reject the null hypothesis that they became extinct at the K-T boundary (Fig. 4). Thus, using the results of the six species as a proxy for all 13 found in the last 1.5 m of the Cretaceous, as well as any of the 10 missing ammonite species that may have survived the pre–K-T gap, we cannot reject the hypothesis that all these species became extinct as a result of the impact. However, the data are also consistent with a range of gradual extinction scenarios (*12*) not enumerated here.

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19. Based on graphic correlation of the major lithological boundaries.
20. Some of the echinoid species present in the Bay of Biscay Cretaceous sections are also known from the Tertiary (A. B. Smith, personal communication).
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22. Forty-two diagnosable ammonite fossils are known from the interval (about 30 fossils per meter of section), compared with a recovery of 1.5 diagnosable ammonite fossils per meter over the preceding 200 m of section.
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30. The absence of ammonites in the marls of Member V is unlikely to be due to preservation failure, given that ammonites are found in the similar marls of Member III, which occurs lower in the section.
31. Ammonite fossils have been found, on average, once every 18 m^2 of outcrop in the top 1.3 m of the Cretaceous at Hendaye, France. Echinoids are recovered at a comparable rate of one specimen per 19 m^2 of outcrop. At least one echinoid per collecting season, but no ammonites over seven seasons, has been recovered in about 50 m^2 of outcrop excavated within the pre–K-T gap (1.3 to 3.0 m below the K-T boundary). At these recovery rates, the probability that ammonites were as abundant in the excavated interval as they were above the pre–K-T gap is only 0.06; however, the probability that the ammonites were a third as abundant in the excavated interval as they were above the pre–K-T gap is 0.39.

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Melanoma Cell Expression of Fas(Apo-1/CD95) Ligand: Implications for Tumor Immune Escape

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Malignant melanoma accounts for most of the increasing mortality from skin cancer. Melanoma cells were found to express Fas (also called Apo-1 or CD95) ligand (FasL). In metastatic lesions, Fas-expressing T cell infiltrates were proximal to $FasL^+$ tumor cells. In vitro, apoptosis of Fas-sensitive target cells occurred upon incubation with melanoma tumor cells; and in vivo, injection of $FasL^+$ mouse melanoma cells in mice led to rapid tumor formation. In contrast, tumorigenesis was delayed in Fas-deficient *lpr* mutant mice in which immune effector cells cannot be killed by FasL. Thus, FasL may contribute to the immune privilege of tumors.

Membrane-bound FasL (mFasL) induces rapid cell death of Fas-sensitive cells (*1*). FasL is not only one of three major cytolytic pathways used by cytolytic T cells to kill target cells (*2–4*), but is also a key element in the elimination of activated T cells during the downregulation of the immune response (*5, 6*). Similar to the structurally homologous tumor necrosis factor α (TNFα) (*7, 8*), processing of the membrane-bound form of FasL by metalloproteases results in shedding of the extracellular portion (sFasL) (*9–11*). Patients with diseases characterized by pathological cell death, such as alcoholic hepatitis, contain high concentrations of sFasL in their serum (*12*). In the process of screening serum samples from patients with other diseases, elevated concentrations of sFasL

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were also observed in sera from 18 of 35 patients with malignant melanoma (Fig. 1A).

Although FasL expression was initially thought to be restricted to activated T lymphocytes (*13*), the ligand is also expressed in nonlymphoid cells such as certain epithelial cells, Sertoli cells, and neurons (*14–16*). We therefore considered the possibility that sFasL detected in these sera was derived from cleavage of melanoma mFasL and not exclusively from activated T cells directed against the tumor (*17, 18*). Substantial quantities of FasL were found in lysates of a series of human melanoma cell lines (Fig. 1B). As in Jurkat T cells, two molecular species, one corresponding to mFasL (40 kD) and the other to sFasL (27 kD), were detected in some melanoma cells by Western blot analysis, indicating that cellular FasL can consist of homotrimeric ($mFasL_3$) as well as partly processed heterotrimeric complexes ($mFasL_2$-$sFasL$ or $mFasL_1$-$sFasL_2$). FasL expression by the human melanoma cell lines was confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) (*19*). Melanoma-expressed FasL was active, as Fas-sensitive A20 B lymphoma cells, but not the FasL-resistant A20R cells (*20*), underwent apoptosis when cultured with melanoma cells (Fig. 1C). Little or no Fas was expressed by these melanoma cells (*19*); they were also resistant to apoptosis mediated by recombinant FasL (Fig. 1D).

To exclude the possibility that FasL expression in melanoma cell lines was simply a consequence of prolonged in vitro culture conditions, FasL expression was studied by immunohistochemistry in sections of metastatic melanoma lesions from seven individuals. In all patients, tumor cells expressed FasL but not Fas (Fig. 2, A to F). In contrast, the majority of cells infiltrating the tumor mass were Fas-positive (Fig. 2C). No FasL was found in normal melanocytes of the skin, indicating that FasL upregulation probably occurs during tumorigenesis (Fig. 2, G and H). However, the limited number of patients studied does not yet permit us to correlate FasL expression on melanoma with the severity of the disease.

The expression of FasL expression may help to maintain the integrity of immune privileged sites. In many of these sites, including the eye, inner ear, testis, or brain, constitutive FasL expression is observed (*14, 16*), and $FasL^+$ cells may kill Fas-sensitive T cells entering such sites (*15, 16*). In this context, FasL-expressing melanoma cells might induce apoptosis of Fas-sensitive, tumor-infiltrating cells. With the use of the terminal deoxytransferase–mediated deoxyuridine 5′-triphosphate nick end labeling (TUNEL) technique that detects single strand DNA breaks (*21*), apoptotic cells were identified in regions where tumor-infiltrating T lymphocytes were found (Fig. 3, A and B).

If FasL-expressing melanomas could kill infiltrating immune effector cells through FasL-Fas interaction, cells from *lpr* mice that express little or no Fas (*1*) would be expected to be less sensitive to FasL-dependent apoptosis and, consequently, be more efficient in the elimination of tumor cells. We tested this hypothesis by subcutaneously injecting B16-F10 (*22*) mouse melanoma cells that express substantial quantities of FasL protein (Fig. 4A) and message (Fig. 4B) into young syngeneic C57BL/6 mice carrying the *lpr* mutation in the *fas* gene. Tumor growth in mutant mice was compared to that in wild-type mice (Fig. 4C). In a representative experiment, 4 of 10 wild-type mice carried a palpable tumor on day 4 after injection, but only 1 of 10 *lpr* mice was affected. At day 6 after injection, all wild-type mice had a palpable tumor, whereas half of the lpr mice were still devoid of tumors. Tumor size differences became smaller thereafter. To ascertain that the increased rejection of melanoma cells was indeed due to the absence of Fas and not due to the hyperactive immune system found in *lpr* mice (*1*), melanoma cells were also injected into *gld* mutant mice that lack functional FasL (*1*). Unlike in *lpr* mice, tumor growth in *gld* mice was rapid, and comparable to that of wild-type mice (Fig. 4C), thus supporting a crucial involvement of the Fas system in tumor rejection. The differences in tumor establishment were even more pronounced when B16-F10 cells ($H2^b$) were injected into allogeneic MRL mice ($H2^k$). At the high cell numbers injected, MRL mice did not reject the allogeneic tumor cells during the observation period (mice were killed after 12 days), with tumor growth rates comparable to syngeneic C57BL/6 mice (Fig. 4D). Five days after injection, all MRL mice had growing tumors whereas 70% of MRL-*lpr* mice were still tumor-free

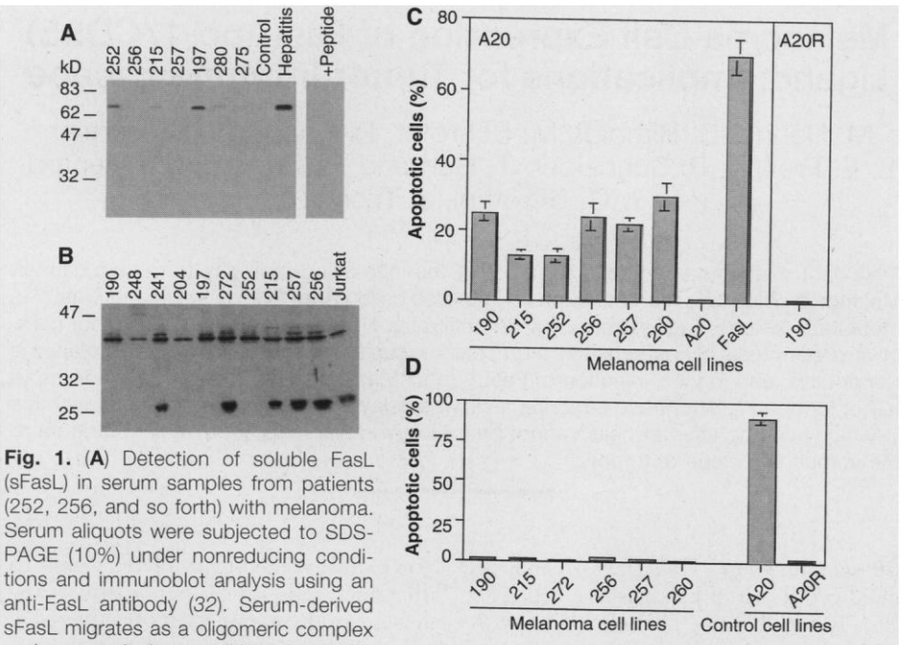

Fig. 1. (**A**) Detection of soluble FasL (sFasL) in serum samples from patients (252, 256, and so forth) with melanoma. Serum aliquots were subjected to SDS-PAGE (10%) under nonreducing conditions and immunoblot analysis using an anti-FasL antibody (*32*). Serum-derived sFasL migrates as a oligomeric complex under nonreducing conditions (~70 kD). The control lanes correspond to serum samples from a normal donor with no detectable sFasL (Control) and from a patient with alcoholic hepatitis with very high levels of FasL (Hepatitis), respectively (*12*). Immunodetection of sFasL was inhibited in the presence of peptide (100 μg/ml) corresponding to the epitope recognized by the antibody (*33*). (**B**) FasL expression in melanoma cell lines derived from patients with malignant melanoma (*32*). Triton extracts were analyzed as in (A). The transmembrane form of FasL is 40 kD; some cells show the proteolytically cleaved sFasL product (27 kD). (**C**) In vitro killing of Fas-sensitive cells by melanoma cells. Fas-bearing A20 B lymphoma cells were cultured with melanoma cell lines derived from various patients (190, 248, and so forth). Cell death was Fas-dependent, since only the FasL sensitive A20 lymphoma cell line but not the FasL-resistant variant cell line A20R was killed (*34*). Controls: Melanoma effector cells are replaced by A20 cells and FasL, respectively. (**D**) Melanoma cell lines resist killing by FasL. Various cell lines were incubated with recombinant FasL. Controls: A20 cells and mutant A20R cells were used as target cells.

(Fig. 4D). Melanoma cells freshly isolated from these tumors were cytolytic and killed target cells via a Fas-dependent lytic pathway (Fig. 4E).

Ample evidence exists showing that melanomas can be immunogenic: some tumors spontaneously regress, and they often have large lymphocytic infiltrates (*18*). Melanoma-specific cytolytic T lymphocytes (CTLs) have been isolated from tumor infiltrating lymphocytes and peripheral blood from many melanoma patients (*23–25*). Nearly a dozen CTL-defined melanoma peptide antigens have been identified (*26*). Yet, melanoma cells, like most other cancer cells, are able to avoid immune destruction in most instances. The mechanisms responsible for such an immune privilege could include the secretion of soluble inhibitory factors by tumor cells, such as transforming growth factor–β (TGF-β) or interleukin-10 (*27*, *28*), downregulation of MHC molecules expressed by tumor cells (*29*), or abnormal T lymphocyte signal transduction (*30*). Combining the results presented here with the proposed role of FasL in creating immune privileged sites (*15*, *16*), an additional mechanism may be considered: FasL-expressing melanoma cells may protect themselves against tumor-infiltrating Fas-sensitive immune effector cells. The delay in tumor formation in *lpr* mice is in agreement with this premise, attributing an important role to the Fas system in the modulation of the anti-tumor immune response. These data may also explain a previous finding showing that the immunosuppressive state induced by melanoma cells is TGF-β–independent and requires direct contact between tumor cell and T cell (*28*). The molecular basis of this observation remains unknown and may be complex, but may be similar to the one protecting allogeneic islets from immune rejection, when these are cotransplanted with myoblasts ectopically expressing FasL (*31*). As proposed for FasL-expressing testis grafts or stromal cells of the anterior chamber of the eye, FasL-expressing melanoma cells may kill Fas-sensitive activated T lymphocytes (*15*, *16*). However, other explanations, including the downregulation of an early unspecific response by

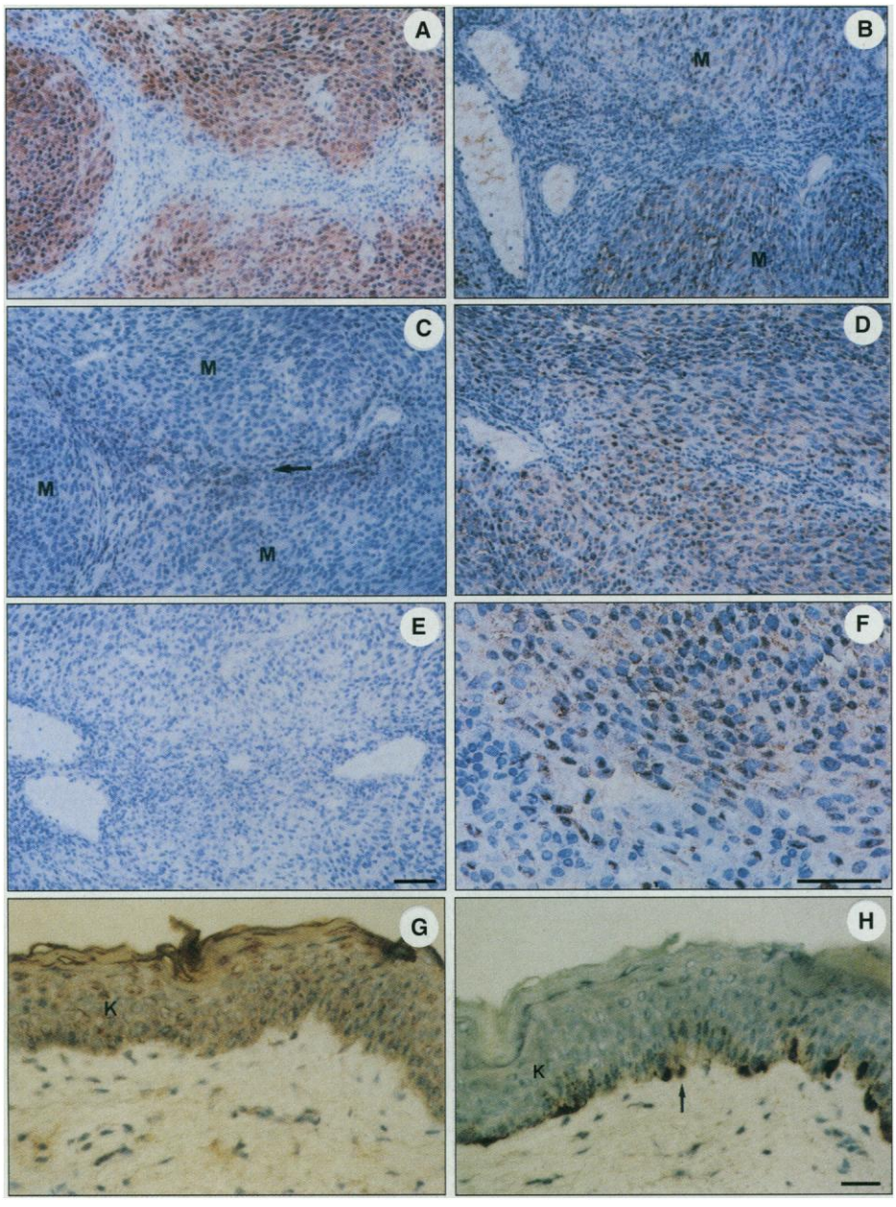

Fig. 2. Expression of FasL in human malignant melanoma but not in melanocytes (**A**) Melanomas are positive for the MAGE antigen (*35*). (**B**) Expression of FasL in malignant melanoma (M) from patients 190 (B) and 225 (**D**), respectively. Higher magnification (**F**) suggests that FasL can be retained in the cytoplasm, in agreement with its subcellular localization in activated T cells (*36*). (**C**) Cells infiltrating the tumor mass (marked with M). A substantial portion of the infiltrating cells are Fas-positive (arrow). (**E**) Control: The antibody to FasL, A11, was replaced by rat Ig. Normal human skin was stained for FasL in (**G**) and for the melanocyte-specific antigen HMB45 (arrow) in (**H**). Keratinocytes (K) express low amounts of FasL and melanocytes expressed no detectable FasL. Scale bars are each 20 μm: bar in (E) applies to (A) through (E); bar in (H) also applies to (G).

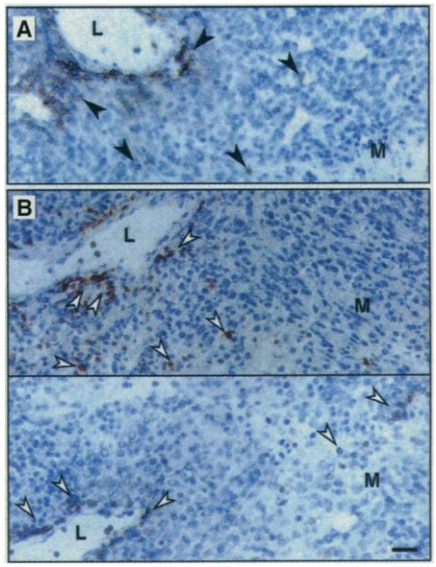

Fig. 3. (**A**) In situ detection of T cells infiltrating a melanoma lesion (M). T cells extravasating a blood vessel (L, lumen) were stained using an antibody-CD3 specific antibody (closed arrow). (**B**) In situ detection of apoptotic cells (open arrow) in the tumor. Apoptotic cells were detected through the staining of DNA fragments with the TUNEL assay (*37*). Scale bar, 20 μm.

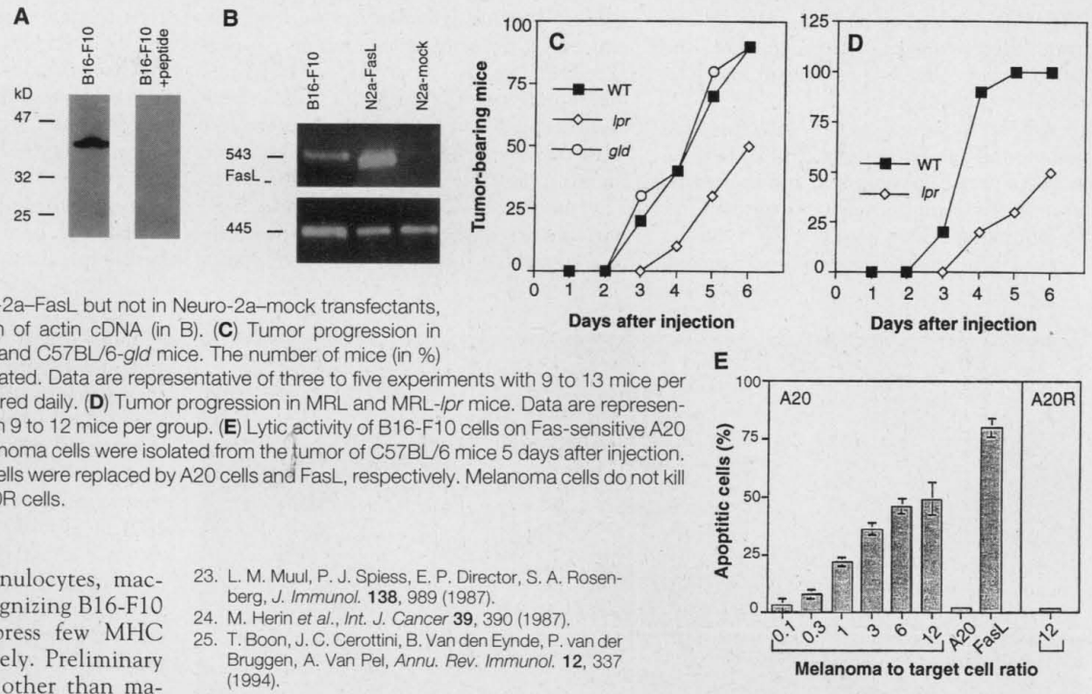

Fig. 4. Consequences of Fas-deficiency on tumor growth (*38*). (**A**) Expression of FasL in melanoma B16-F10 cell lines as detected by immunoblots and (**B**) by RT-PCR. Controls: In (A) inclusion of peptide (100 μg/ml) corresponding to the epitope detected by the antibody to FasL (+peptide), and in (B) amplification of FasL cDNA (543 bp) in Neuro-2a–FasL but not in Neuro-2a–mock transfectants, respectively, and amplification of actin cDNA (in B). (**C**) Tumor progression in C57BL/6 (WT), C57BL/6-*lpr*, and C57BL/6-*gld* mice. The number of mice (in %) with palpable tumors are indicated. Data are representative of three to five experiments with 9 to 13 mice per group. Tumor size was measured daily. (**D**) Tumor progression in MRL and MRL-*lpr* mice. Data are representative of three experiments with 9 to 12 mice per group. (**E**) Lytic activity of B16-F10 cells on Fas-sensitive A20 B lymphoma target cells. Melanoma cells were isolated from the tumor of C57BL/6 mice 5 days after injection. Controls: Melanoma effector cells were replaced by A20 cells and FasL, respectively. Melanoma cells do not kill the variant, FasL-resistant A20R cells.

killing Fas-expressing granulocytes, macrophages, or NK-cells recognizing B16-F10 melanoma cells that express few MHC molecules (*19*), are as likely. Preliminary results show that tumors other than malignant melanoma express FasL, suggesting that FasL expression may be a more general strategy used by tumor cells to escape immune rejection. Thus, pharmacological products that render infiltrating T cells insensitive to FasL-induced killing may help break the immunological unresponsiveness to melanoma and, possibly, provide a complementary approach in the therapy of malignant melanoma.

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32. Serum aliquots (1 μl) or cellular NP-40 (1%) extracts from 1×10^6 melanoma cells were separated by SDS-PAGE (10%) under nonreducing conditions and immunoblotted (ECL system, Amersham) using the affinity-purified rabbit anti-FasL antibody PE62 (*33*).
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34. Cell lines were derived from tumor samples obtained from melanoma patients by surgical excision. Samples were excised from lymph node (patients 215, 252, and 256) or subcutaneous metastases (all other patients). The killing activity was assessed by incubating 1×10^5 melanoma cells with 1×10^5 ^{125}I-UdR labeled target cells [A20 (*20*) and A20R, respectively] in 96-well plates 2 days after seeding (*3*). The FasL-resistant variant cell line A20R was generated by continuous culture in medium containing recombinant FasL (*39*). The release of ^{125}I-UdR was then determined after a 16 hour co-incubation. Fas sensitivity of various cells was determined by adding 5 μl of supernatant from recombinant FasL producing Neuro-2a cells (*39*) to 1×10^5 labeled cells in a total volume of 200 μl.
35. The metastatic melanoma of patient 190 was removed by surgery and snap-frozen in liquid nitrogen cooled isopentane. Ten-micrometer sections were cut and mounted on gelatin-coated slides. After fixation in 4% paraformaldehyde for 10 min, sections were rinsed with phosphate-buffered saline (PBS) and endogenous peroxidase quenched with 0.3% H_2O_2 and 0.1% azide. Supernatants (1:1 dilution) of the monoclonal antibody (mAb) to FasL, A11 (*33*) (Alexis Corp., San Diego, CA) was added to the section for 1 hour, and after rinsing in PBS, peroxidase-conjugated goat antibody to rat Ig (TAGO, Burlingame, CA) was added for 0.5 hour. After rinsing, antibody location was revealed by the AEC chromogen. For the detection of melanoma cells, a mouse mAb to MAGE-1, detecting a subpopulation of melanoma cells (*40*), was used (20 μg/ml), and detected with a biotinylated goat anti-mouse Ig and avidin-peroxidase-conjugate (TAGO, Burlingame, CA). Fas antigen was detected with a mouse mAb to human Fas (10 μg/ml). In the negative control, the FasL antibody was replaced with rat Ig. The mAb to human melanoma (HMB45) was from ENZO Diagnostics Inc., New York, USA.
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37. Frozen sections were stained as in Fig. 2. T cells were detected using the anti-CD3 specific antibody Leu-4 (Becton-Dickinson, Oxnard, CA). TUNEL staining was done according to kit instructions from Boehringer, Mannheim, Germany.
38. FasL was detected by Western blot analysis as in Fig. 1. Total RNA was isolated from the B16-F10 cell line and reverse transcribed to cDNA (Pharmacia, Zürich, Switzerland), and amplified by PCR generating a 543-bp fragment of FasL (forward: 5′-CACTCAAGGTCCATCCCTCTG-3′; reverse primer: 5′-TAGCTGACCTGTTGGACCTTGC-3′) or a 445-bp fragment of actin (forward: 5′-ATCAAGATCCTGACCGAGCG-3′; reverse: 5′-TACTTGCGCTCAGGAGGAGC-3′). The following conditions were used: 1 cycle at 94° for 5 min, then 30 cycles at 94° for 1 min, 55° for 1 min, and 72° for 2 min. The products were resolved on a 2% agarose gel. The lytic activity of freshly isolated tumor cells was assayed as in Fig. 1. B16-F10 melanoma cells (1×10^5, American Type Tissue Culture) were injected subcutaneously into 4-week old C57BL/6, C57BL/6-*lpr*, C57BL/6-*gld* (Jackson Laboratory, Bar Harbor, ME), MRL and MRL-*lpr* mice (Harlan, Zeist, Nederland).
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