Cutting Edge: The Tumor Counterattack Hypothesis Revisited: Colon Cancer Cells Do Not Induce T Cell Apoptosis Via the Fas (CD95, APO-1) Pathway[1]

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The counterattack hypothesis, suggesting that cancer cells express Fas ligand (FasL) and are able to kill Fas-expressing tumor-infiltrating activated T cells, was supported by reports of the killing of Jurkat cells by FasL-expressing human colon cancer cell lines. Through the use of an improved cytotoxic assay in which soluble FasL and FasL-transfected KFL9 cells were used as positive controls, we show that none of seven human colon cancer cell lines induce apoptosis of two Fas-expressing target cell lines, Jurkat and L1210-Fas cells. Moreover, in coculture experiments, cancer cell monolayers do not inhibit the growth of Fas-expressing lymphoid cells. Although FasL mRNA and protein were detected in the extracts of the colon cancer cell lines, flow cytometry and confocal microscopy failed to detect the protein on the surface of tumor cells. These results suggest that the counterattack of tumor-infiltrating T lymphocytes by cancer cells may not account for immune tolerance toward tumor cells. ***The Journal of Immunology*****, 2000, 164: 5023–5027.**

Fas (CD95/Apo-1) is a plasma membrane receptor that belongs to the TNF receptor family. Interaction of Fas receptor with its natural ligand can trigger an apoptotic signal (for review, see Ref. 1). Activated T cells express both Fas and Fas ligand (FasL)[3] and the Fas/FasL death pathway is a crucial mechanism for immune response down-regulation (2). The expression of FasL by other cell types, such as Sertoli cells in the testis, was suggested to account for the immune privilege observed in specific tissues (3).

The ability of tumor cells to trigger a specific immune tolerance is a central event in tumor development. It was reported that tumor cells from various origins could express FasL and induce apoptosis of Fas-expressing T cells (4–10). From these observations, it was proposed that tumor cells could counterattack Fas-expressing activated tumor-infiltrating lymphocytes and escape rejection by the immune system. This Fas-mediated depletion of tumor-infiltrating lymphocytes was suggested to be an essential factor in the inhibition of anti-tumor immune response and in the induction of a specific tolerance to tumor Ags (11). Since these reports have been published, several of the anti-FasL Abs used have been shown to cross-react with unrelated molecules (12, 13). The conditions of the cytotoxicity assays, e.g., the importance of repeating washings in the JAM test, were also demonstrated to influence the results (14). Taking into account these recent developments, we revisited the tumor counterattack hypothesis. Since the role of FasL expression by tumor cells in immune tolerance to tumors was first suggested by studies bearing on human colon cancer cell lines, particularly SW480 and SW620 (4, 8), we examined these cell lines, as well as five other colon cancer cell lines, for their capacity to both express FasL and to kill Fas-sensitive target cells.

Materials and Methods

Established cell lines

Colon cancer cell lines SW480, SW620, HT29, Caco-2, HCT116, HCT15, HCT8, and Jurkat T cells were obtained from the American Type Culture Collection (Manassas, VA). The FasL-transfected and mock-transfected Neuro-2a cell lines (15) were a gift from Dr. A. Fontana (Zurich, Switzerland). Soluble FasL was obtained as described previously (15–17). L1210 cells and its murine Fas transfectant, L1210-Fas cells (kindly given by Dr. P. Goldstein, Marseilles, France), as well as the KFL9 cell line, a human FasL transfectant of human myeloid leukemia K562 cells (a gift from Dr. D. Kaplan, Cleveland, OH) (12), were used.

Modified JAM test

DNA fragmentation was measured using the modified JAM test (14, 18). Target cells were labeled with 2.5 μCi/ml [^{3}H]thymidine (Isotopchim, Ganagobie-Peynius, France) for 20 h at 37°C. The target and effector cells were incubated for 5 h at 37°C at the indicated E:T ratios. The target cells were harvested by five washings and collected onto glass fiber filters (Skatron, Suffolk, U.K.). Each filter was counted in a liquid scintillation beta counter (Wallac, Turku, Finland). Specific cell killing was calculated using

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[3] Abbreviation used in this paper: FasL, Fas ligand.

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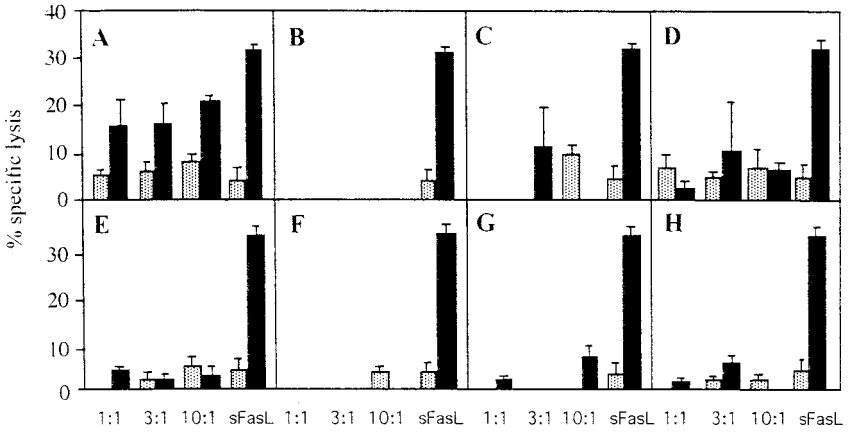

FIGURE 1. Measurement of Fas-expressing target cell lysis induced by coculture with colon cancer cell lines (JAM test). [^{3}H]Thymidine-labeled L1210 cells (▨) and L1210-Fas cells (■) were cultured with KFL9 cells (*A*) and the colon cancer cell lines SW480 (*B*), SW620 (*C*), HCT116 (*D*), HCT15 (*E*), HCT8 (*F*), HT-29 (*G*), and Caco-2 (*H*) for 5 h. Target cell death was determined by measuring fragmentation of the [^{3}H]thymidine-labeled target cell DNA. E:T ratios of 1:1, 3:1, and 10:1 were used. The effect of soluble FasL (sFasL, supernatant of FasL-transfected Neuro-2a cells, diluted 1:2) was tested as a positive control. Specific lysis was calculated relative to spontaneous cell death occurring when target cells were cultured alone. Mean values of triplicate coculture ± SD are reported.

the following equation: % specific killing = $(S - E/S) \times 100$, where E and S are the cpm of retained DNA, respectively, in the presence or the absence of effector cells.

Fas-sensitive cell growth during mixed culture

Jurkat, L1210, or L1210-Fas cells (5×10^2 cells/well) were seeded alone or mixed with SW480, SW620, or HT29 cells (5×10^5 cells/well) in a 24-well plate and cultured for 5 days before photographing the mixed culture and counting the floating lymphocytic cells.

Detection of FasL mRNA expression

Total RNA was isolated from colon cancer cell lines using the RNAble kit (Eurobio, Les Ulis, France). PCR was performed on the cDNA using intron-spanning primers (19). RT-PCR products were hybridized with the human full-length FasL DNA probe (Dr. S. Nagata, Osaka Bioscience Institute, Osaka, Japan) which was radiolabeled with the random primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

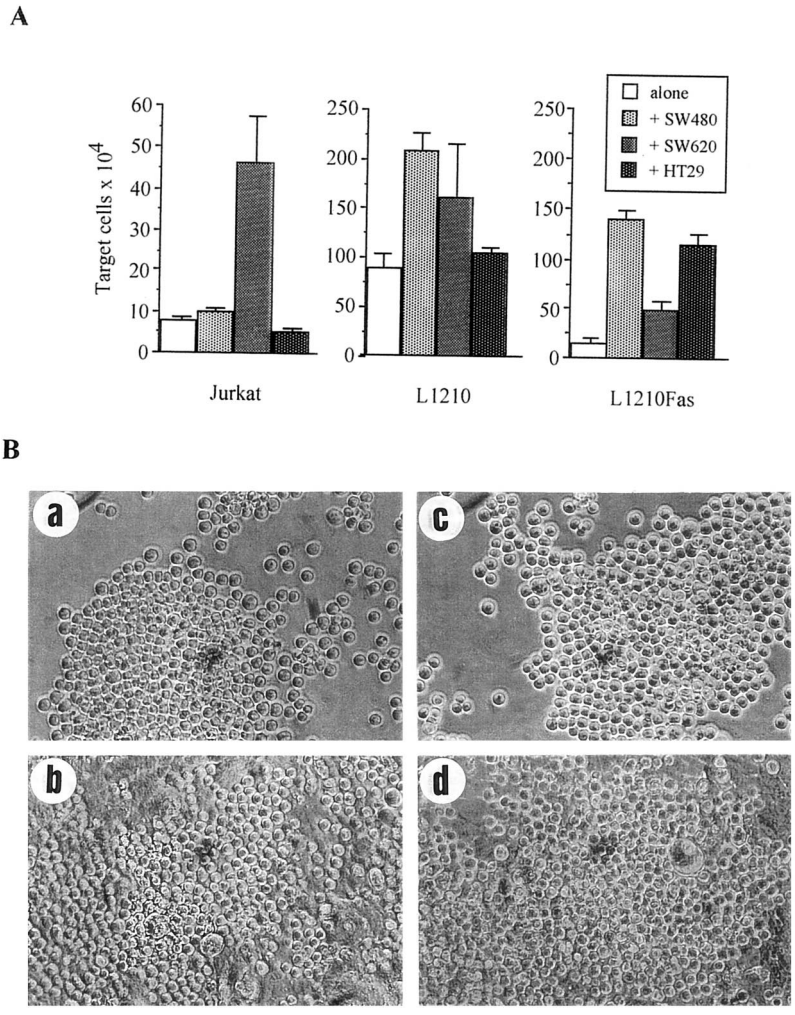

FIGURE 2. Measurement of Fas-expressing cell growth in coculture with colon cancer cell lines. *A*, Jurkat, L1210 or L1210-Fas target cells (500 cells/well) were seeded alone or with the colon cancer cell lines SW480, SW620, or HT-29 (5×10^5 cells/well; E:T ratio, 1000:1). The number of floating Jurkat, L1210, and L1210-Fas cells that excluded trypan blue was measured after 5 days. *B*, L1210 (*a* and *b*) or L1210Fas (*c* and *d*) target cells (500 cells/well) were seeded either alone (*a* and *c*) or in coculture with the colon cancer cell line SW480 (*b* and *d*) (5×10^5 cells/well; E:T ratio, 1000:1). Cell cultures were examined using phase-contrast microscopy.

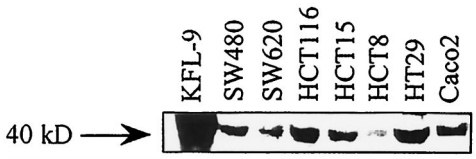

FIGURE 3. Detection of FasL protein expression. Western blotting analysis of FasL expression was performed using G247-4 mAb in the indicated colon cancer cell lines and KFL9 cells were used as a positive control.

Immunoblotting analysis and immunoprecipitation

Subconfluent cultured cells were lysed in lysis buffer. Equal amounts of protein (80 μg) were separated by SDS-PAGE using a 10% polyacrylamide gel and electroblotted to polyvinylidene difluoride membranes (Bio-Rad, Ivry sur Seine, France). FasL was detected with G247-4 anti-human FasL mAb (PharMingen, San Diego, CA) and peroxidase-coupled anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) using the enhanced chemiluminescence reaction system (Amersham, Arlington Heights, IL). The specificity of the results was verified by immunoprecipitation with the NOK-1 anti-human FasL (PharMingen) and protein A-Sepharose before immunoblotting analysis.

Confocal laser scanning microscopy analysis

Nonpermeabilized cells were incubated with NOK-1 mAb for 45 min at 4°C and then with secondary anti-mouse biotin-conjugated Ig for 45 min. After addition of FITC-conjugated streptavidin, cells were fixed in 2% paraformaldehyde for 20 min and then analyzed with a confocal laser scanning microscope as reported previously (20). A nonrelevant isotype-matching murine mAb was used as a negative staining control.

Cytofluorometric analysis

Cells were detached with 1 mM EDTA in PBS supplemented with 10 mM HEPES, adjusted to 1×10^6 cells/100 μl, and incubated with mouse anti-FasL mAb NOK-1 (PharMingen) for 45 min at 4°C. After washing, the cells were incubated with FITC-conjugated anti-mouse Ig (Amersham) for 30 min at 4°C. A total of 10,000 events was analyzed for each sample with a FACScan (Becton Dickinson, Grenoble, France).

Results and Discussion

Colon cancer cell lines do not induce apoptosis of Fas-sensitive target cells

Colon cancer cell lines were previously shown to induce apoptosis of Fas-sensitive target cells such as Jurkat T cells by using the JAM test (4, 8). We repeated these experiments with Jurkat and L1210-Fas cells as target cells and parental L1210 cells as the negative control. However, according to the recommendations of Böhm et al. (14), we multiplied the washes to completely detach labeled target cells from tumor cell monolayer. The JAM test was initially designed for measuring apoptosis induced by floating killer cells (18). When adherent tumor cells are tested as effector cells, a part of radiolabeled target cells attaches to the tumor cell monolayer. These cells are not recovered on filters and can be falsely considered as undergoing cell death (14). When repeated washes were performed, soluble FasL (16), as well as FasL-expressing KFL9 cells, induced a significant and dose-dependent cytotoxic effect on L1210-Fas cells without demonstrating any effect on the Fas-negative parental L1210 cells. In contrast, none of the seven tested colon cancer cell lines induced significant apoptosis of Fas-expressing target cells (Fig. 1). The same result was obtained with Jurkat cells (data not shown). Preincubation of colon cancer cell lines with 10 μM of the metalloproteinase inhibitor KB8301 (PharMingen), which was used to prevent potential FasL cleavage by cellular metalloproteinase (21), had no influence on the ability of colon cancer cells to trigger apoptosis of Fas-expressing target cells (data not shown).

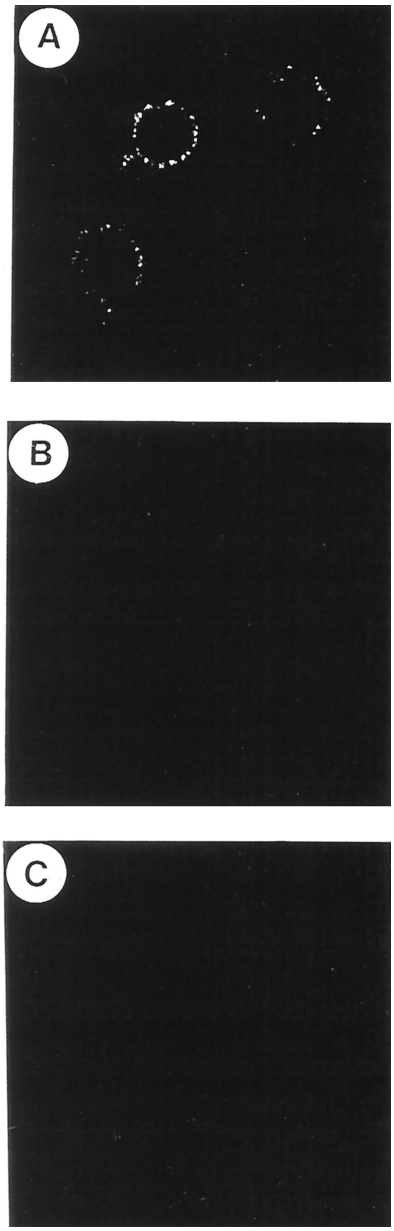

FIGURE 4. Detection of FasL by confocal microscopy. SW480 cells (*B*) and SW620 cells (*C*) were seeded into tissue culture chambers for 48 h before labeling with NOK-1 mAb as attached cell monolayers. KFL9 cells (*A*) were labeled with NOK-1 mAb in suspension. Confocal analysis was performed after letting KFL9 cells attach on gelatin-covered glass slides. A nonrelevant isotype-matching mAb was used as a negative staining control. Three cells (*A* and *B*) and four cells (*C*) were photographed using a confocal microscope under ×40 magnification.

Colon cancer cell lines do not inhibit the growth of Fas-sensitive target cells

When Jurkat cells and L1210-Fas cells were cultured in the presence of colon cancer cell monolayers, these cells proliferated vigorously, even at an initial ratio as high as 1×10^3 cancer cells for one Fas-expressing cell. Cell count demonstrated that coculture with SW480 and SW620 cancer cells did not inhibit and sometimes even enhanced the growth of Jurkat and L1210-Fas cells (Fig. 2*A*) as compared with culture growth in the absence of a colon cancer cell monolayer. Colonies of apparently unaltered Jurkat, L1210, and L1210-Fas cells did appear above the monolayers of SW480 or SW620 cells, as they appeared when leukemic

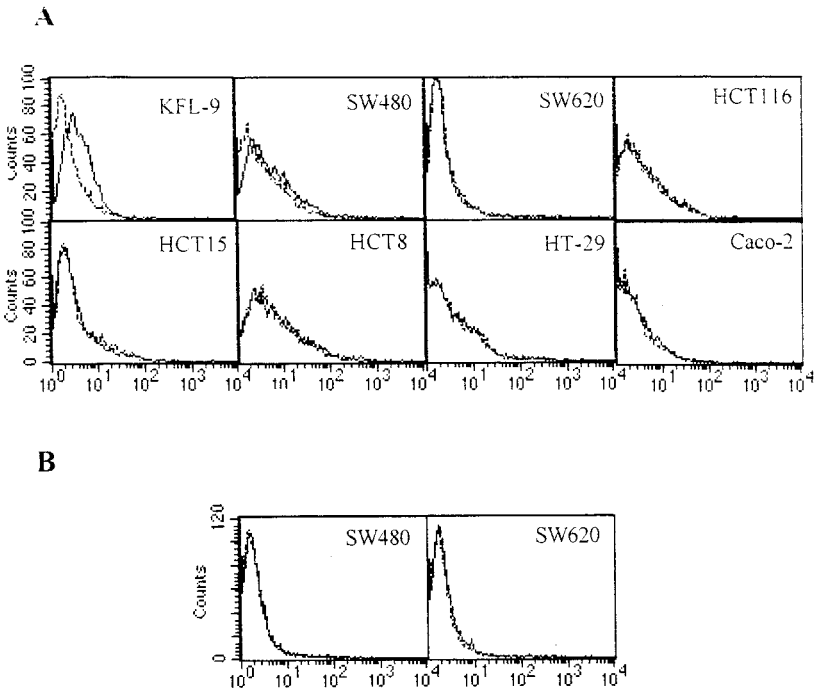

FIGURE 5. Detection of FasL surface expression by flow cytometric analysis. *A*, Flow cytometric analysis was performed on nonpermeabilized colon cancer cell lines and KFL9 cells using NOK-1 mAb (solid line). A nonrelevant isotype-matching mAb was used as a negative staining control (dotted line). A total of 10,000 events was analyzed for each sample with a FACScan (Becton Dickinson). *B*, Flow cytometric analysis was performed on nonpermeabilized SW480 and SW620 cells using NOK-1 mAb after treatment with the matrix metalloproteinase inhibitor KB8301 (solid line). A nonrelevant isotype-matching mAb was used as a negative staining control (dotted line).

T cells were cultured alone (Fig. 2*B*). When added at the end of this assay, soluble FasL induced apoptosis of virtually all Jurkat and L1210-Fas cells, whether these cells were cultured alone or in the presence of colon cancer cells (data not shown). Thus, coculture of Fas-expressing cells with cancer cells did not select Fas-resistant cell variants.

Colon cancer cell lines express FasL mRNA and protein

RT-PCR experiments initially suggested that melanoma cells expressed FasL mRNA (5). However, these results were not confirmed when intron-spanning primers were used to avoid amplification of contaminating genomic DNA (22). We used such intron-spanning primers to detect the expression of FasL mRNA in the studied colon cancer cells. A cDNA signal of expected size (234 bp) was amplified in each of the tested colon cancer cell lines (data not shown). The specificity of the signal detected in the five other cell lines was confirmed by hybridization with a FasL cDNA probe. These results confirmed previous data (22) showing that colon cancer cell lines do express FasL mRNA. Immunoprecipitation with NOK-1 and immunoblotting with G247-4 of cell extracts of KFL9 cells and the studied colon cancer cells identified the protein as a 40-kDa band (Fig. 3). These results demonstrate that FasL mRNA and protein are expressed in colon cancer cell extracts. However, expression levels were low when compared with those obtained with the FasL-transfected KFL9 cell line.

FasL protein was not detected on the surface of colon cancer cells

Differing from professional cytotoxic cells, such as T lymphocytes and NK cells, tumor cells have no capacity to store FasL in secretory lysosomes which undergo a polarized delivery to the interface between the cytotoxic cells and its target cell (23). For inducing apoptosis, FasL has to be constitutively expressed on the tumor cell surface where it binds its receptor Fas, which is located on the surface of target cells. The choice of the Ab is critical, as only a few anti-FasL mAbs, including NOK-1, were shown to specifically label FasL on the surface of nonpermeabilized cells (12, 13). For example, the C-20 polyclonal Ab raised against peptides from the extracellular domain of FasL was shown to label the surface of SW480 cells (8), but this Ab is now known to be nonspecific (12, 13). Using NOK-1 mAb, confocal microscopy (Fig. 4) and flow cytometry (Fig. 5*A*) easily identified FasL protein on the surface of KFL9 cells. Under these conditions, no protein could be identified on the surface of any of the studied colon cancer cell lines (Fig. 5*A*), even after pretreatment of the cells with the metalloproteinase inhibitor KB8301 (Fig. 5*B*). KFL9 cells were transfected to express relatively large amounts of FasL (Fig. 3). Consequently, the failure of the colon carcinoma cell lines to express FasL may be a representation of the poor sensitivity of the techniques, especially in comparison to the sensitivity of the apoptosis assay.

Conclusions

It has been reported that FasL-expressing nonhematopoietic cells could kill activated T lymphocytes, a mechanism accounting for the immune privilege observed in some tissues (3). However, these results were not reproduced by other investigators, and FasL-expressing tissues were shown to be rejected by allogeneic hosts as efficiently as FasL-negative tissues (24) or, in some cases, even more rapidly (25). Similarly, the counterattack hypothesis proposed that cancer cells expressed FasL at their surface and consequently induced apoptosis of activated T cells infiltrating the tumor (4–8). Such a mechanism could have been of considerable importance in explaining how cancer cells escape the immune response. However, it was recently shown that melanoma cells initially reported as being able to kill Fas-sensitive T cells through FasL (5) do not express FasL mRNA (22), and the present study demonstrates that colon cancer cell lines, in which FasL mRNA and protein can actually be detected, do not express FasL on their plasma membrane and are not capable of killing Fas-sensitive T cells. Thus, mechanisms other than the Fas-dependent killing of tumor-immune T lymphocytes by FasL-expressing tumor cells may account for immune tolerance to cancer. For example, following continued activation by the tumor, tumor-specific T cells,

like any activated T cell, could coexpress Fas and FasL and undergo suicidal or fratricidal apoptosis according to the well-known process of the activation-induced cell death (2), independently of any FasL expression on the tumor cell (26).

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