Cooperative Disengagement of Fas and Intercellular Adhesion Molecule-1 Function in Neoplastic Cells Confers Enhanced Colonization Efficiency

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Abstract
Understanding the mechanisms of tumor progression is crucial toward the development of therapeutic interventions. Although the loss of sensitivity to cell death is a hallmark of neoplastic progression, it is likely one of several essential features that underlie a malignantly proficient or aggressive tumorigenic phenotype. Here, we identified intercellular adhesion molecule-1 (ICAM-1) as a molecule with expression coordinate regulated with Fas and inversely correlated with malignant phenotype between matched pairs of differentially aggressive malignant subpopulations in three mouse models. To determine whether coordinate expression of Fas and ICAM-1 regulated malignant behavior, tumor sublines were produced that expressed either lower levels of both Fas and ICAM-1, lower levels of Fas, or lower levels of ICAM-1 and then assessed for metastatic lung tumor growth. Tumor sublines rendered both Fas incompetent and ICAM-1 incompetent displayed significantly higher numbers of tumor nodules compared with tumor sublines separately expressing low levels of Fas or ICAM-1. However, all tumor sublines regardless of their Fas and ICAM-1 levels comparably infiltrated the lung, suggesting that Fas- and ICAM-1–based interactions ultimately influenced lung colonization efficiency. Overall, these data suggested that both Fas and ICAM-1 pathways cooperated to regulate tumor progression and that the coordinate down-regulation of Fas and ICAM-1 intensified malignant progression at the level of colonization. Thus, a Fas−ICAM-1− phenotype may be characteristic of at least certain advancing, immune-resistant neoplastic subpopulations. (Cancer Res 2005; 65(3): 1045-54)

Introduction
Understanding the molecular events important for metastatic formation of solid tumors (1, 2) is crucial to the design of effective therapeutic strategies. For example, loss of sensitivity to cell death is considered a hallmark of malignant progression (3). Because Fas is an important receptor that mediates apoptotic death, disruption of such a cell death pathway in neoplastic cells might confer a survival advantage for tumor escape from Fas ligand (FasL)–dependent immune and nonimmune defense mechanisms (3–5). Indeed, down-regulation of Fas has been reported as a common occurrence of an advancing neoplastic phenotype in diverse malignancies (6–8).

However, how alterations in Fas expression or function influence metastatic behavior has remained to be resolved. Earlier studies indicated that loss of Fas function alone was sufficient for tumor progression in mouse models of melanoma (6, 9). Recent findings also revealed an inverse correlation between Fas expression and metastatic phenotype in mouse models of sarcoma and mammary carcinoma (10). Furthermore, in the CMS4 sarcoma model, biologically generated Fas-resistant/refractory sublines displayed enhanced metastatic ability compared with the unselected parental population. However, if the parental tumor cell line was rendered completely Fas resistant by transfection with the virally encoded FLICE inhibitory protein (vFLIP) gene or transplanted into a Fasl-deficient (gld) host, little to no additional metastatic activity was observed (10). Similarly, the link between functional Fas status and malignant phenotype was examined using a matched pair of naturally occurring primary (Fas-sensitive) and metastatic (Fas-resistant) human colon carcinoma cell lines in both in vitro and in vivo (xenograft) settings (11, 12). As with the mouse studies, simply disrupting the Fas pathway in the Fas-sensitive primary tumor via vFLIP transfection failed to achieve the same metastatic outcome compared with the biologically selected Fas-resistant sublines or the naturally occurring metastatic tumor cell line (11). Therefore, loss of Fas function was linked to, but alone was insufficient for, full acquisition of the metastatic phenotype.

Thus, the observations that the parental or primary tumor cell lines failed to display increased metastatic ability even after molecular disruption of the Fas pathway also raised the hypothesis that other tumor-associated genetic events in combination with alterations in Fas expression or function were required for an optimally productive tumorigenic phenotype. Therefore, in this CMS4 model, we now sought to identify and functionally characterize the contribution of additional biological determinants, besides Fas status, toward enhanced malignant potential. To address this notion, we first made use of cDNA microarray analysis as a broad-based approach to potentially identify additional genetic events associated with the tumorigenic phenotype in this CMS4 sarcoma model (10). We first compared the in vitro–derived highly metastatic CMS4 subline to the parental cell line from which it was derived. Although several differences in gene expression were observed, one gene that struck as potentially important was intercellular adhesion molecule-1 (ICAM-1). ICAM-1 has been reported to exhibit several different functions, including those important for facilitating cell-to-cell adhesion and costimulation (13, 14). Cellular adhesion involves interactions between ICAM-1 and its principal counterreceptors, LFA-1 or Mac-1, expressed by various cell types, including leukocyte subpopulations. Cellular adhesion via the ICAM-1/LFA-1 pathway is important for antigen presentation between T lymphocytes and antigen-bearing, antigen-presenting cells; cell-mediated cytotoxicity between natural killer cells or CTL and their targets.
and trafficking of leukocytes (e.g., neutrophils) during inflammation (15–18). Differential expression of ICAM-1 has been also implicated in the processes of tumor growth, invasion, or metastasis (17, 19–22).

Despite its emerging association with neoplastic behavior, it remains unknown whether differential expression of ICAM-1 in conjunction with differential expression of Fas collectively contribute to alterations in malignant phenotype. The basic strategy adopted here to address that hypothesis was to compare tumor cell sublines expressing low levels of both Fas and ICAM-1 elements with tumor sublines expressing each one separately. Overall, under circumstances in which both Fas and ICAM-1 pathways were “operationally disengaged,” we showed that the magnitude of tumor burden in the lung significantly rose and more closely approached levels achieved by the in vivo–derived highly metastatic sublines. The inverse correlation between Fas and ICAM-1 levels and malignant phenotype was confirmed in three tumor models reflecting experimental and spontaneous metastatic settings. We postulate therefore that such a Fas−/ICAM-1− phenotype may be characteristic of enhanced malignant or metastatic capability.

Materials and Methods

Mice. Female BALB/c (H-2d) mice were obtained from the National Cancer Institute-Frederick Cancer Research Animal Facility (Frederick, MD). All mice were used at age >6 weeks in accordance with institutional guidelines and practices.

Cell Lines. The CMS4 sarcoma, kindly provided by A. DeLeo (University of Pittsburgh, Pittsburgh, PA), is a solid tumor of BALB/c (H-2d) origin (23), which grows aggressively in naive, syngeneic hosts following a s.c. transfection. (24). Although the parental tumor cell line forms few metastatic foci in the lungs following i.v. administration, a highly metastatic subline, termed CMS4-met, was established from lung digests of those mice as described (24). The CMS4 selv subline was selected from the parental line in vitro following six successive passages in the presence of anti-Fas stimuli as described (10). Briefly, CMS4 cells were first treated with recombinant mouse IFN-γ (100 units/mL, R&D Systems, Minneapolis, MN) and tumor necrosis factor-α (TNF-α; 100 units/mL, R&D Systems) overnight followed by culture with anti-mouse Fas monoclonal antibody (mAb; 10 μg/mL, clone Jo2, PharMingen, San Diego, CA) or FITC-conjugated anti-ICAM-1 mAb (clone 3E2, PharMingen) and analyzed by flow cytometry. For two-color immunostaining, tumor cells were incubated with phycoerythrin-conjugated anti-Fas mAb (clone Jo2) and FITC-conjugated anti-ICAM-1 mAb. Control preparations were incubated with FITC-conjugated, isotype-matched hamster IgG (clone A19-3, Pharmingen) and phycoerythrin-conjugated, isotype-matched hamster IgG (clone Ha1/8, Pharmingen). All experiments involving two-color flow cytometry were corrected for compensation between FITC and phycoerythrin overlap.

Measurement of Fas-Induced Cell Death. Cell death was measured by propidium iodide staining, albeit similar results were observed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays. Briefly, untreated or cytokine-treated cells were incubated with soluble FasL (20 ng/mL) for 20 to 24 hours. Collected cells were stained with propidium iodide for 10 minutes at room temperature according to the manufacturer’s instructions (R&D Systems). After staining, the cells were washed and immediately analyzed by flow cytometry.

Reverse Transcription-PCR Analysis. Total RNA was isolated from tumor cells using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions and used for the first-strand cDNA synthesis using the ThermoScript reverse transcription-PCR (RT-PCR) system (Invitrogen, San Diego, CA). cDNA was then used as template for PCR amplification of mouse Fas, mouse ICAM-1, and mouse β-actin. The following variables were used: 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C for 30 cycles. The PCR primers for mouse Fas were as follows: forward primer 5'-ATGCTGG-GATCTGGGTC-3' and reverse primer 5'-TACCTCCGACTTGTC-3'.

The PCR primers for mouse β-actin were as follows: forward primer 5'-GCCAATCCTCCGCTGAGT-3' and reverse primer 5'-GTACCGGAGGACGCTG-3'. The PCR primers for mouse ICAM-1 were as follows: forward primer 5'-CAGATGCCGACCCAGGAG-3' and reverse primer 5'-ACAGAATCCAACCAGAGG-3'. For detection of the mouse gp70 transcript (27) in CMS4 cells or CMS4 sublines infiltrating mouse lung, the following variables were used: 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C for 24 cycles. The PCR primers for the gp70 transcript were as follows: forward primer 5'-ACCTTGGCCGATG-3' and reverse primer 5'-GTACAGGACTGTC-3'. To quantify PCR band intensities, gel images were first captured with an Epi ChemiII Digital Image System (UVI, Upland, CA). The individual PCR-amplified DNA fragment intensities were then obtained with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). This gp70 tumor-associated antigen reflects an envelope protein encoded by an endogenous murine retrovirus uniquely expressed by tumor cells and not by normal host cells (24, 27). We used this as a surrogate biomarker to detect for evidence of tumor infiltration and persistence in the lung microenvironment.

Stable Transfection of CMS4 Cells with vFLIP. CMS4 cells were transfected with the mammalian expression plasmid pEGFPN1 (Clontech, Palo Alto, CA) containing the gene encoding the green fluorescent protein (GFP) or pEGFPN1-vFLIP containing genes encoding both vFLIP protein (GFP) or pEGFPN1-vFLIP containing genes encoding both vFLIP sequence were then linearized with AluII restriction enzyme and used for transfection. Transfections were done using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. The transfected cells were propagated in culture medium containing Genitin (Invitrogen) at a concentration of 0.75 mg/mL for 7 days, recovered, and recultured under the same conditions for two more passages before being sorted by a FACSVantage SE cell sorter (Becton Dickinson, Mountain View, CA) based on GFP intensity. The sorted cells were cultured with Genitin for another 7 days and resorted once more to ensure stable retention of GFP-positive cells. The sorted cells were then maintained and propagated under Genitin selection.

Cell Sorting for ICAM-1+ and ICAM-1− Tumor Cells. CMS4 cells were treated with recombinant mouse IFN-γ and TNF-α (100 units/mL of each cytokine) as described above. Cells were then collected, stained with FITC-conjugated anti-mouse ICAM-1 mAb, and sorted by a FACSVantage SE cell sorter based on ICAM-1 intensity. For GFP-vFLIP and GFP-vector transfected cells, cells were stained with biotinylated anti-mouse ICAM-1 mAb (also clone 3E2) followed by streptavidin-linked Cy5 (Cal-Tag, Burlingame, CA). Cells were then sorted based on the
expression intensities of both ICAM-1 and GFP. Stable sublines, maintained in culture without any further exposure to anti-ICAM-1 mAb, were used in the in vivo experiments.

Experimental Lung Metastasis Model. The different groups of CMS4 and Renca cells were suspended in HBSS (BioSource International, Camarillo, CA) and injected i.v. into the lateral tail vein (100 μL) at various concentrations. Mice were sacrificed at the indicated time points. Lungs were inflated with a 1% solution of India ink, resected, and fixed in Fekete solution as described (29). The number of lung nodules was enumerated in all four lobes in a single-blinded fashion under a dissecting microscope. Values exceeding 250 nodules were considered too numerous to count accurately and therefore were reported as >250. In the CMS4 model, the results are illustrated as a compilation of two or more separate experiments. In the Renca model, two studies were conducted in which mice received either 1 × 10⁵ and 2 × 10⁵ tumor cells. Because similar patterns of tumor growth were observed at both concentrations, the results are presented from mice receiving the higher dose. For detection of the gp70 transcript by RT-PCR in the various CMS4 sublines, three independent mice were sacrificed at the indicated time points and lungs were removed, homogenized in a PowerGen 35 homogenizer (Fisher Scientific, Pittsburgh, PA), and used for total RNA isolation as above.

Statistical Analysis. Where indicated, data were reported as the mean ± SD. Statistical analysis was determined using an unpaired, two-sided t test, with Ps < 0.05 considered statistically significant.

Results

Identification of ICAM-1 as a Potential Determinant of Malignant Phenotype. RT-PCR analysis revealed that CMS4 cells expressed both ICAM-1 and Fas transcripts at a relatively higher level compared with the in vivo–derived highly metastatic subline, CMS4-met, and an in vitro–derived anti-Fas-selected variant, CMS4.sel (Fig. 1A). Analysis by flow cytometry indicated that all three lines expressed low to undetectable levels of cell surface ICAM-1 (Fig. 1B) as well as low levels of cell surface Fas (Fig. 1B and C). Because ICAM-1 as well as Fas expression has been reported to markedly increase following exposure to proinflammatory cytokines, IFN-γ and TNF-α (6, 30, 31), we examined the three groups of CMS4 cells after treatment with both cytokotins. As observed before (10), we found that treatment of the different CMS4 sublines with IFN-γ and TNF-α enhanced Fas expression at both mRNA and protein levels (Fig. 1A and B). However, the increase was significantly higher in CMS4 cells than in the CMS4-met or CMS4.sel sublines as measured by cell surface Fas mean fluorescent intensity (MFI) values (P = 0.007 and 0.0004, respectively; Fig. 1C). Treatment of CMS4 cells with IFN-γ and TNF-α also increased ICAM-1 transcript and cell surface levels (Fig. 1A and B). In contrast, treatment of the CMS4-met or CMS4.sel sublines under these same conditions marginally enhanced ICAM-1 expression (Fig. 1A and B).

We next sought to extend the association or link between Fas and ICAM-1 expression levels in two matched pairs of cell lines (10) established from a mouse model of spontaneous primary and metastatic mammary carcinoma (26). As with the CMS4/ CMS4-met model (Fig. 1), we found a very similar pattern of expression of Fas and ICAM-1. In the primary tumor lines, termed D4387B and K6625B, both Fas and ICAM-1 transcripts were expressed at relatively higher levels compared with their metastatic counterparts, termed D4387L and K6625L (Fig. 2A), respectively. Next, we examined cell surface Fas and ICAM-1 expression by these two matched pairs of primary and metastatic tumor cell lines (Fig. 2B and C). In the D4387 cell line pair, cell surface Fas expression was observed in the primary tumor cell line but not its matched metastatic counterpart (Fig. 2B). Similarly, after cytokine treatment, cell surface Fas expression was still higher on D4387B compared with D4387L (Fig. 2B and C). In the K6625 cell line pair, cell surface Fas expression was weakly detectable on both primary and metastatic cell lines without any cytokine treatment. However, following cytokine treatment, K6625B displayed a small but reproducible increase in cell surface Fas levels compared with K6625L (Fig. 2B and C). In ICAM-1 expression, both pairs of primary and metastatic tumor cell lines exhibited low to undetectable levels of cell surface ICAM-1 (Fig. 2B). After cytokine treatment, ICAM-1 expression increased more so with D4387B compared with D4387L (Fig. 2B and C). In the K6625 cell line pair, the primary tumor displayed a very small but reproducible increase in cell surface ICAM-1 levels compared with its metastatic counterpart (Fig. 2B and C). Taken together, these observations strengthened the notion that coordinate down-regulation of Fas and ICAM-1 was associated with a more
metastatic phenotype and that the relative levels of Fas and ICAM-1 expressed by a given primary tumor line may reflect its stage of progression within the neoplastic process.

Coordinate Down-Regulation of Fas and ICAM-1 Expression Was Associated with Enhanced Malignant Proficiency in the CMS4 Model. To further investigate the correlation between Fas and ICAM-1 expression in the CMS4 model, we costained CMS4 cells with anti-Fas and anti-ICAM-1 mAb and examined Fas expression levels in both ICAM-1lo and ICAM-1 hi subpopulations of the parental (unsorted) cell line. The CMS4 cell line contained a small subpopulation (2.2%) of ICAM-1 hi cells (Fig. 3A). Analysis of Fas expression by the gated ICAM-1lo and ICAM-1 hi subpopulations revealed that the pattern of Fas expression was correlated with that of ICAM-1 expression (Fig. 3A); that is, Fas MFI in the ICAM-1lo subpopulation was significantly lower than that of the ICAM-1 hi subpopulation (P = 0.00005; Fig. 3A, bottom left). Cytokine-treated CMS4 cells also contained ICAM-1lo and ICAM-1 hi subpopulations; however, the percentage of the ICAM-1 hi subpopulation increased to as high as 55.1% (Fig. 3B). Nonetheless, a similar coordinate pattern of Fas and ICAM-1 expression was observed after cytokine treatment (Fig. 3B); that is, Fas MFI in the ICAM-1lo subpopulation was significantly lower than that of the ICAM-1 hi subpopulation (P = 0.0001; Fig. 3B, bottom left). Although IFN-γ and TNF-α generally increased both Fas and ICAM-1 expression, the changes were strongly correlated between ICAM-1lo and ICAM-1 hi subpopulations.

Next, we sought to examine the relationship between ICAM-1 expression and metastatic tumor growth in the lung. To do so, we pretreated the parental CMS4 population with IFN-γ and TNF-α to maximally up-regulate ICAM-1 expression and then sorted them into two distinct ICAM-1-expressing subpopulations and established them as stable sublines, termed ICAM-1lo and ICAM-1 hi. Here, we made use of a blocking, rather than an agonistic, anti-ICAM-1 mAb (clone 3E2) for sorting applications to avoid causing potential alterations in the biology of the sorted cells. Furthermore, we conducted additional control experiments, which revealed that this particular anti-ICAM-1 mAb clone did not trigger signal transduction events (i.e., tyrosine phosphorylation) or affect cellular proliferation in vitro (14, 17, 32). To do so, the ICAM-1 hi subline was pretreated with IFN-γ and TNF-α and then incubated with anti-ICAM-1 mAb. Western blot analysis revealed no evidence of tyrosine phosphorylation induction, whereas [3H]thymidine uptake assays showed no changes in cellular proliferation caused by exposure to this anti-ICAM-1 mAb clone (data not shown). Lastly, all in vivo experiments were done using stable sublines maintained in vitro without any further exposure to anti-ICAM-1 mAb.

Next, we verified ICAM-1 and Fas expression levels of the sorted sublines by flow cytometry before and after cytokine treatment (Fig. 3C). First, the sorted ICAM-1lo (a) and ICAM-1 hi (b) sublines maintained their respective ICAM-1 phenotype. Second, Fas MFI in
the ICAM-1lo subline was significantly lower than that of the ICAM-1hi subline, with \( P = 0.001 \) or without \( P = 0.007 \) cytokine treatment, which mirrored the patterns seen in the gated parental populations (Fig. 3A and B). Thus, both ICAM-1 and Fas expression patterns seen in the parental (unsorted) CMS4 cell line were maintained in the sorted sublines. Given the observation that ICAM-1 expression correlated with Fas expression (Fig. 3A-C), we reasoned that the ICAM-1lo subline should be less Fas sensitive compared with the ICAM-1hi subline. Consistent with that notion, functional analysis revealed that the two sublines (ICAM-1lo versus ICAM-1hi) displayed somewhat different degrees of sensitivity to Fas-mediated death, which were significantly \( P = 0.006 \) different from each other. In fact, comparison of these two sublines against CMS4 and CMS4-met reproducibly showed a hierarchical pattern of sensitivity toward Fas-mediated death in the following order (from most to least Fas sensitive): CMS4 = CMS4-ICAM-1hi > CMS4-ICAM-1lo > CMS4-met (Fig. 3D).

To examine the correlation between ICAM-1 levels and malignant phenotype, we assessed these sublines for tumor growth in the lung (Fig. 3E and F). As expected, in mice receiving CMS4

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**Figure 3.** Effects of Fas and ICAM-1 expression levels on tumorigenic phenotype in the CMS4 model. A, CMS4 cells were costained with phycoerythrin-conjugated anti-Fas and FITC-conjugated anti-ICAM-1 mAb. ICAM-1lo and ICAM-1hi subpopulations are demarcated by the horizontal line and vertical line (top left). Top right and bottom right, gated ICAM-1hi (top right quadrant in top left) and ICAM-1lo (top left quadrant in top left) subpopulations were then analyzed for Fas expression intensities. Bottom left, Fas MFI of both ICAM-1lo and ICAM-1hi subpopulations. Columns, mean of three separate experiments; bars, SD. B, as in A, except that Fas and ICAM-1 expression intensities by CMS4 cells were analyzed after overnight treatment with both IFN-\( \gamma \) and TNF-\( \alpha \). C, cell surface ICAM-1 of the ICAM-1lo (a) and ICAM-1hi (b) CMS4 sublines with (solid thick line) and without (dashed line) cytokine pretreatment. Gray area, staining with FITC-conjugated IgG isotype control antibody. Bottom, Fas MFI of both ICAM-1lo and ICAM-1hi subpopulations. Columns, mean of three separate experiments; bars, SD. D, sensitivity of the ICAM-1lo and ICAM-1hi CMS4 sublines to Fas-mediated death compared with CMS4 and CMS4-met cells. E, metastatic tumor growth of ICAM-1lo and ICAM-1hi CMS4 sublines. Representative of one of four lung lobes of the indicated tumor-bearing mouse 14 days postinjection. F, quantification of lung tumor nodules from mice receiving the indicated CMS4 subline. Columns, total lung tumor nodule counts of a single mouse. Statistical analysis of total lung tumor nodules in mice receiving CMS4 versus CMS4-met, CMS4 versus CMS4-ICAM-1lo, and CMS4 versus CMS4-ICAM-1hi: \( P = 0.000003, 0.0014, \) and 0.0019, respectively.
cells, only a few lesions were detectable, whereas in mice receiving CMS4-met a large number of nodules were visible. Interestingly, in mice receiving the CMS4-ICAM-1hi subline, a significantly ($P = 0.0014$) larger number of nodules were found compared with the unselected parental CMS4 population. In contrast, in mice receiving the CMS4-ICAM-1lo subline, very few nodules were visible, which was also significantly ($P = 0.0019$) different from that of the unselected parental CMS4 population (Fig. 3E). Overall, these data (Fig. 3) showed that ICAM-1 expression levels inversely correlated with tumorigenic phenotype (or the efficiency of tumor growth) in the lung.

**Coordinate Down-Regulation of Fas and ICAM-1 Expression Was Associated with Enhanced Malignant Proficiency in Renca Tumor Model.** Next, we sought to extend the relationship between Fas and ICAM-1 expression and malignant phenotype in another transplantable tumor model, termed Renca (25, 33). Renca has also been reported to harbor a cytokine-inducible (IFN-γ plus TNF-α) Fas-sensitive phenotype (25, 33) analogous to what we found with the CMS4 system. Renca cells expressed cell surface Fas but contained very few ICAM-1+ cells (Fig. 4A). Nonetheless, two-color staining of Renca cells with Fas- and ICAM-1-specific mAb indicated that both markers were expressed in a coordinate pattern (Fig. 4A); that is, Fas MFI in the ICAM-1hi subpopulation was significantly higher than that of the ICAM-1lo subpopulation ($P = 0.005$; Fig. 4A, bottom left). As with CMS4 cells, treatment of Renca cells with IFN-γ and TNF-α treatment increased both Fas and ICAM-1 expression intensities (Fig. 4B). Furthermore, two-color staining of cytokine-treated Renca cells with Fas- and ICAM-1-specific mAb showed that Fas expression levels were increased proportionally in both ICAM-1hi and ICAM-1lo subpopulations (Fig. 4B). Fas MFI in the ICAM-1lo subpopulation was still significantly higher than that of the ICAM-1hi subpopulation ($P = 0.004$; Fig. 4B, bottom left), demonstrating that cytokine treatment did not eliminate the differences in Fas expression maintained between both subpopulations.

To examine the correlation between ICAM-1 levels and malignant phenotype, we sorted the ICAM-1hi and ICAM-1lo subpopulations of Renca cells and established stable ICAM-1lo and ICAM-1hi sublines and injected these sublines i.v. for assessment of metastatic tumor growth in the lung (Fig. 4C and D). As with the CMS4 model, the Renca-ICAM-1lo subline displayed a significantly enhanced number of tumor nodules compared with the unsorted parental population [Fig. 4D, average of 20 versus 33 nodules, respectively (top), and average of 66 versus 97 nodules, respectively (bottom)].

**Physiologic Site of ICAM-1–Dependent Interactions.** The observations that both Fas and ICAM-1 expression levels seemed to influence the extent of tumor growth in the lung was consistent with a model in which ICAM-1-dependent interactions played an important role in cellular adhesion (15, 17), whereas functional Fas status played an important role in governing tumor cell survival after conjugate formation (7, 8, 11, 15, 34–36). However, in these tumor models, it is possible that such interactions might occur in the bloodstream, in the lung, or at both sites. To discriminate among these possibilities, we examined the lungs of CMS4 tumor-bearing mice for evidence of tumor infiltration at discrete time points. To discriminate among these possibilities, we examined the lungs of CMS4 tumor-bearing mice for evidence of tumor infiltration at discrete time.
points post-transplant. Because it would be too difficult to accurately quantitate tumor foci in the lungs during the early time points postinjection, we developed a RT-PCR assay to detect a CMS4 tumor-associated antigen, termed gp70 (24, 27), to quantify tumor cell infiltration or persistence in mouse lung under those experimental conditions.

Initial control experiments examined both specificity and sensitivity of the RT-PCR assay for detection of the gp70 transcript in the lungs. To do so, we harvested lungs from non-tumor-bearing BALB/c mice, spiked them with varying numbers of CMS4 cells in vitro, and then analyzed the total RNA of these preparations for gp70 transcript expression. We found that, in the presence but not in the absence of tumor cells, gp70 expression was detectable in normal lung cells (Fig. 5A), thus verifying the specificity of this assay. Analysis of gp70 expression by CMS4 and CMS4-met cells revealed that both populations expressed strong levels of this transcript (data not shown). To better define the sensitivity of the assay, gp70 transcript expression was analyzed from total RNA isolated from the lung-tumor admixtures. Indeed, gp70 expression was demonstrable in a dose-dependent fashion, with a limit of detection of \(~1 \times 10^4\) tumor cells per lung (Fig. 5A).

Next, we examined the lungs of the various groups of CMS4-bearing mice for detection and expression of the gp70 transcript. Four groups of CMS4-bearing mice were analyzed, reflecting differences in ICAM-1 (and Fas) levels. Lungs were harvested at different time points after tumor cell injection, and total RNA was isolated for molecular assessment of the gp70 transcript. One day after tumor injection, lungs of all four groups of mice receiving the different tumor cell populations were found to be positive for the expression of the gp70 transcript, and the gp70 levels in all groups were relatively similar (Fig. 5B and C). These data thus confirmed that all CMS4 sublines efficiently infiltrated the lungs, regardless of their ICAM-1 phenotype and/or sensitivity to Fas-mediated cell death, at least 24 hours post-tumor transplant. To better characterize the fate of these sublines, we then monitored their persistence over time (Fig. 5D and E). We showed that gp70 expression for the CMS4-met subline persisted over the 14-day period (Fig. 5D and E), which was consistent with its metastatic phenotype (Fig. 3). Similarly, gp70 expression of the CMS4-ICAM-1lo subline also persisted; however, it began to decline by days 7 to 14 post-transplant relative to the CMS4-met subline. In contrast, gp70 expression for the CMS4-ICAM-1hi subline persisted for 3 days and then diminished to nondetectable levels by days 7 to 14 post-transplant (Fig. 5D and E). Based on gp70 expression, the parental CMS4 population seemed to persist over the 14-day period but at lower levels when compared with the more metastatic sublines, CMS4-met and CMS4-ICAM-1lo. Overall, we found a hierarchical pattern of gp70 expression among these

Figure 5. Colonization efficiency of the various CMS4 sublines. A, specificity and sensitivity of detection of the gp70 transcript by RT-PCR in admixtures of CMS4 cells with lung-derived cells from non-tumor-bearing mice. Numbers on top, detection of tumor cells expressing gp70 message determined on a per lung basis. B, detection of the gp70 transcript in lungs of the indicated tumor-bearing mice. Each mouse was injected with a given tumor cell population and the lungs were harvested 24 hours later. Three mice were examined per tumor-bearing group. \(\beta\)-actin was used as normalization standard. C, gp70 and \(\beta\)-actin levels in B were quantified by analysis of the PCR band intensities as described in Materials and Methods. Relative levels of lung-associated gp70 were obtained by dividing the gp70 band intensity by the corresponding \(\beta\)-actin band intensity. Relative levels of gp70 in CMS4-bearing mouse lung were then arbitrarily set at 1 for comparison with all other sublines. Relative levels of gp70 in mouse lungs bearing all other sublines were then expressed as the ratio relative to CMS4-bearing mouse lung (i.e., normalized gp70 ratio of subline/normalized gp70 ratio of CMS4-bearing mouse lung). Columns, mean of the relative gp70 levels from three separate mice; bars, SD. D, agarose gel images showing detection of the gp70 transcript in the lungs of a representative tumor-bearing mouse at 1, 3, 7, and 14 days post-tumor transplant. In all panels, mouse \(\beta\)-actin was used as normalization standard. E, gp70 levels were quantified as in C. Relative levels of gp70 in CMS4-bearing mouse lung were arbitrarily set at 1 at each time point for comparison with each subline. Columns, mean of three separate mice; bars, SD.
Experimental Lung Metastasis Model

As observed previously, Fas-resistant (3) nodule counts, where B, from representative tumor-bearing mice. 

malignant outcome, we made use of the CMS4 model and relationship between Fas and ICAM-1 expression and the malignant phenotype or proficiency in this CMS4 experimental lung metastasis model. CMS4 cells were compared with the CMS4-vector/ICAM-1lo transfectants were rendered virtually nontransfected counterparts (as in Fig. 3). The CMS4-vFLIP sublines was similar to that of their CMS4-vector/ICAM-1 incompetent). Fas sensitivity of the CMS4-vector subline, this resulted in a subline expressing ICAM-1 incompetent, and in the CMS4-vFLIP+ subline, this resulted in a subline expressing low Fas and ICAM-1 levels versus sublines expressing low levels of each one independently. CMS4 cells were rendered Fas resistant via vFLIP transfection followed by the isolation of ICAM-1lo cells. Thus, proof of concept required the isolation of ICAM-1lo cells. Thus, proof of concept required the isolation of ICAM-1lo cells.

Four sublines, which mirrored their tumor growth behavior in the lung (Fig. 3). These data revealed that CMS4 cells, independent of ICAM-1 and Fas expression levels, entered the lungs at least as determined within 24 to 72 hours post-transplant.

Disengagement of Both Fas and ICAM-1 Pathways Jointly Influenced the Malignant Outcome. To assess the causal relationship between Fas and ICAM-1 expression and the malignant outcome, we made use of the CMS4 model and compared sublines expressing low Fas and ICAM-1 levels versus sublines expressing low levels of each one independently. CMS4 cells were rendered Fas resistant via vFLIP transfection followed by the isolation of ICAM-1lo cells. Thus, proof of concept required the disruption of the Fas and ICAM-1 pathways. 

Previously, we had produced CMS4-vFLIP and vector control sublines from the parental CMS4 population (10). Next, both sublines were sorted by flow cytometry for the recovery of the ICAM-1lo-expressing cells (as in Fig. 3). In the vector control, this resulted in a subline expressing ICAM-1lo levels only (Fas competent/ICAM-1 incompetent), and in the CMS4-vFLIP subline, this resulted in a subline expressing a defect in Fas function concomitant with low levels of ICAM-1 (Fas incompetent/ICAM-1 incompetent). Fas sensitivity of the CMS4-vector and CMS4-vector/ICAM-1lo sublines was similar to that of their nontransfected counterparts (as in Fig. 3D). The CMS4-vFLIP and CMS4-vFLIP/ICAM-1lo transfectants were rendered virtually Fas-resistant (3 ± 0.3% and 1 ± 1%, respectively).

Next, we examined the different sublines for tumor growth in our experimental lung metastasis model. As observed previously (10), rendering the parental CMS4 population Fas resistant via vFLIP transfection (CMS4-vFLIP) failed to improve tumor growth compared with the vector control (Fig. 6A), revealing that the loss of Fas function alone was insufficient to generate an overt metastatic-competent phenotype. In contrast, the loss of ICAM-1, as achieved via fractionation of the CMS4-vector/ICAM-1lo subpopulation, enhanced tumor growth compared with the CMS4-vector control (Fig. 6A). Furthermore, the observations that the ICAM-1lo subpopulations derived from the untransfected CMS4 cells (Fig. 3F) and the vector-transfected CMS4 cells (Fig. 6A and B) displayed similar degrees of tumor growth in the lungs showed that the transfection process per se had neither negative nor positive effects on the metastatic outcome. Importantly, in mice receiving the double-deficient Fas incompetent/ICAM-1 incompetent subline, CMS4-vFLIP/ICAM-1lo, a significantly ($P = 0.000084$) higher number of lung tumor nodules were observed compared with the single-deficient CMS4-vector/ICAM-1lo or CMS4-vFLIP sublines (Fig. 6A and B). Thus, these data indicated that the loss of Fas function alone was insufficient for acquiring a metastatic phenotype but that the loss of Fas function significantly enhanced lung colonization or growth of the ICAM-1lo subpopulation.

Discussion

We and others have reported that loss of Fas function is an important determinant of tumor progression perhaps as a consequence of tumor escape from Fas/FasL-dependent interactions (9–11, 37–39). In studies involving syngeneic mouse and human xenograft tumor models (10, 11), Fas-based interactions were shown to mechanistically enforce a biological selective pressure favoring the emergence of preexisting Fas-resistant neoplastic subpopulations coexpressing a metastatic-competent phenotype. This conclusion was substantiated by the observation that simply converting a Fas-sensitive tumor cell population into a Fas-resistant one, at least molecularly via vFLIP transfection, failed to significantly improve metastatic ability (10, 11). These findings suggested therefore that other tumor-associated events in concert with alterations in Fas expression were obligatory for an optimally capable malignant phenotype, which was the subject of this investigation.

We first made use of cDNA microarray analysis to potentially identify additional genetic events associated with malignant phenotype or proficiency in this CMS4 experimental lung metastasis model. CMS4 cells were compared with the CMS4-met subline because this represented a matched pair of poorly and highly metastatic subpopulations. Although several differences in gene expression were observed, one gene that struck as potentially important was ICAM-1. (The complete microarray design, protocol, and results will be deposited in the public microarray database, "Array Express.") ICAM-1 has been previously implicated in the processes of immune cell-tumor interactions, tumor escape, and progression (19, 21, 22, 40–42). However, in those studies, it remained unclear whether differential expression of ICAM-1 was causally linked to malignant progression and whether it worked in conjunction with other genetic or epigenetic alterations to influence the resultant tumorigenic phenotype.

We found that ICAM-1 was underexpressed in CMS4-met compared with the parental population. Furthermore, these observations were confirmed at both RNA and protein levels.
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(Fig. 1) and then extended to a mouse tumor model of spontaneous metastasis (Fig. 2). In fact, fractionation of the parental CMS4 line into ICAM-1lo and ICAM-1hi subpopulations revealed that it was the ICAM-1hi subline that displayed efficient tumor growth in the lung (Fig. 3). Of further interest, the intensity of ICAM-1 expression was found to directly correlate with the intensity of Fas expression, demonstrating that both markers were regulated in a coordinate manner (Fig. 3). Thus, the Fashi-ICAM-1lo subpopulation expressed significantly greater potential for tumor growth in the lung compared with the corresponding Faslo-ICAM-1hi subpopulation. Moreover, the inverse correlation between Fas and ICAM-1 expression and tumor growth capacity in the lung was shown in a second tumor model, Renca (Fig. 4), which strengthens the potential physiologic relevance of alterations of these biological markers on a malignant phenotype. It is important to point out that the relationship between Fas levels and malignant behavior in both CMS4 and Renca models was much more quantitative than qualitative (Figs. 1, 3, and 4). For example, although IFN-γ and TNF-α up-regulated cell surface Fas, the intensity of Fas enhancement was significantly higher in CMS4 compared with CMS4-met and CMS4-sel based on the Fas MFI values of single population shifts (Fig. 1C).

Although these observations suggested that ICAM-1 could represent a molecular determinant acting in concert with Fas to regulate tumor progression, the finding that both markers were expressed in a coordinate manner made it difficult to conclude whether one marker was more important than the other or whether both markers acted jointly to causally influence that malignant outcome. To examine those possibilities, we compared CMS4 sublines expressing low levels of both Fas and ICAM-1 elements (i.e., CMS4-vFLIP/ICAM-1lo) with CMS4 sublines expressing each one separately (Fig. 6). This particular strategy served to make the parental CMS4 population more efficient for tumor growth in the lung by introducing two “molecular hits.” Taken together, under circumstances in which both Fas and ICAM-1 pathways were functionally disengaged (CMS4-vFLIP/ICAM-1lo), we found that the magnitude of tumor burden in the lung significantly rose and more closely approached levels achieved by CMS4-met (Fig. 6). We postulate that such a Faslo-ICAM-1lo phenotype is characteristic of at least certain neoplastic clones within a larger, heterogeneous population that comprises a continuum of genetic traits and malignant potentials. Thus, although this study identified important contributions of both Fas- and ICAM-1-dependent events in the regulation of the malignant outcome, clearly additional molecular features were required to fully reconstitute the tumorigenic phenotype characteristic of CMS4-met, which requires further elucidation. This is supported at least in part by the observations that a large number of differentially expressed genes were identified between CMS4 and CMS4-met (by cDNA microarray) and that a Faslo-ICAM-1lo phenotype (CMS4-vFLIP/ICAM-1lo) alone did not quantitatively achieve the same malignant outcome seen with the CMS4-met subline (Figs. 3 and 6).

If Fas and ICAM-1 expression/function were mechanistically linked (inversely) with the metastatic tumor growth outcome, the next question was: where would ICAM-1 play a role in this model? Differential regulation of ICAM-1 expression may affect interactions with LFA-1-bearing leukocytes (15), for example, in at least two distinct anatomic compartments, in the bloodstream or in the lung, which may affect proliferation and colonization. We explored this issue and found that the four CMS4 sublines tested regardless of ICAM-1 (and Fas) and phenotypes comparably entered the lung as determined within 24 to 72 hours post-transplant (Fig. 5). This observation is consistent with the notion that ICAM-1 expression and function played a more prominent role in the lung compared with the bloodstream. Interestingly, gp70 expression for the parental CMS4 population, and even more so for the CMS4-ICAM-1hi subpopulation, declined after 3 days, consistent with their poorer growth or colonization efficiency in the lungs (Fig. 3). Therefore, one prediction of this model is that ICAM-1 expression may play an important role in cellular adhesion, whereas functional Fas status may play an important role in governing tumor cell survival, persistence, and colonization subsequent to conjugate formation. However, the observations that the CMS4-vFLIP subline, which expressed a Faslo-ICAM-1lo phenotype, failed to persist or colonize in the lungs, as determined by RT-PCR analysis of gp70 transcript expression (data not shown), and failed to grow efficiently in the lungs (ref. 10; Fig. 6) suggested that ICAM-1 engagement may have multiple consequences or trigger multiple effector mechanisms. Thus, tumor cell persistence or colonization in the lung microenvironment may be governed by Fas-dependent and Fas-independent events. For example, in addition to promoting cellular adhesion, engagement of ICAM-1 has been shown to initiate costimulatory signals involving tyrosine phosphorylation of cytoskeletal proteins or cell cycle regulators (14, 17, 43), which may play a role in the regulation of tumor growth in this model. Future investigations therefore are warranted to test these possibilities in detail. Moreover, the observations that all CMS4 sublines initially infiltrated the lungs implied that differential expression of ICAM-1 influenced the malignant process at the stage of colonization efficiency, considered a terminal step of the metastatic pathway for successful formation of secondary foci (1, 2, 44). However, it also remains to be fully understood whether the loss of adhesion via loss of ICAM-1 expression in the primary tumor microenvironment influences the metastatic cascade.

It is thus conceivable that several cell types may represent a source of both functional FasL and a counterreceptor for ICAM-1, including resident cells of the lung, neutrophils, macrophages, natural killer cells, and antigen-specific T cells (9, 45–48). An inflammatory response provoked during the process of tumor growth may initiate the recruitment or engagement of such host-derived cells. These FasL-bearing cells may then promote the death of Fas-responsive tumor subpopulations following antigen-independent interactions, such as LFA-1 or Mac-1/ICAM-1 pathways, thereby reducing but not necessarily eliminating the overall incidence or efficiency of tumor nodule formation. Neoplastic clones expressing a Faslo-ICAM-1lo phenotype, for example, may then emerge and contribute to tumor progression via escape from innate and adaptive elements of the immune system. Overall, these data showed that differential expression of Fas and ICAM-1 in a coordinate fashion played key roles in the generation of neoplastic subpopulations bearing a more progressive tumorigenic phenotype, which may have important implications for tumor escape and immunotherapy.

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