Up-regulation of Fas (CD95) expression in tumour cells in vivo

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Summary
Both the function and regulation of Fas expression in tumours is poorly understood. Our laboratory has reported that cultured, low Fas-expressing tumours undergo massive, yet reversible, up-regulation of cell surface Fas expression when injected into mice. The present study was aimed at determining what causes this enhanced Fas expression and whether the newly expressed Fas functions as a death receptor. Newly expressed Fas is indeed capable of inducing apoptosis. Based on our observation that Fas induction is reduced when tumour cells are injected into immune-deficient mice, we propose that Fas up-regulation in vivo involves the host’s immune system. Accordingly, Fas up-regulation occurs in vitro when low Fas-expressing tumour cells are cocultured with lymphoid cells. Furthermore, ascitic fluid extracted from tumour-bearing mice trigger Fas up-regulation in low Fas-expressing tumours. This last finding suggests that a soluble factor(s) mediates induction of Fas expression. The best candidate for this soluble factor is nitric oxide (NO) based on the following observations: the factor in the ascites is unstable; Fas expression is induced to a lesser degree after injection into inducible NO synthase (NOS)-deficient (iNOS−/−) mice when compared to control mice; similarly, coculture with iNOS−/− splenocytes induces Fas less effectively than coculture with control splenocytes; and finally, the NO donor SNAP induces considerable Fas up-regulation in tumours in vitro. Our model is that host lymphoid cells in response to a tumour increase NO synthesis, which in turn causes enhanced Fas expression in the tumour.

Keywords: Fas; apoptosis; tumour; nitric oxide; mice

Introduction
The death receptor Fas/APO-1 (CD95) and its ligand (FasL) are key players in the regulation of immune development (e.g. in negative selection)1,2 and in the termination of the immune response.3 Fas is considered also to play a role in tumour formation, progression, and immunity4–7 as well as in tumour responses to anticancer drugs.8,9 Fas is expressed both in lymphoid and nonlymphoid normal and malignant tissues. Its expression varies widely among, and even within, tumours of the same histology.10 The reasons behind such variation in Fas tumour expression and its precise function(s) are presently unknown. The ubiquitous nature of Fas tumour expression suggests that it is important for tumour biology and thus, the regulation of Fas tumour expression should be the subject of further study.

Fas expression can be affected by several endogenous and exogenous stimuli, including infectious agents,11 γ-irradiation,12 chemotherapy13 and cytokines such as

Abbreviations: CFSE, 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocytes; GFP, green fluorescence protein; FasL, Fas ligand; FCS, fetal calf serum; IFN, interferon; IL, interleukin; i.p., intraperitoneally; NK, natural killer; NO, nitric oxide; B2mnull, NOD/LtSz-scid β2m null mice; NOS, nitric oxide synthase; iNOS−/−, inducible NOS deficient; PEL, peritoneal exudates lymphocytes; PO, perforin-deficient; rFasl, recombinant Fasl trimer; SNAP, S-Nitroso-N-acetylpenicillamine; STS, staurosporine; TNF-α, tumour necrosis factor-α.
interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), IL-1b, IL-1 and IL-6, each by itself, or in combination with others. Another factor shown to affect Fas expression is nitric oxide (NO).

We reported previously a considerable up-regulation of Fas expression in Fas-deficient tumour cells injected into mice, a finding observed simultaneously and independently by other groups. Down-regulation of Fas and its mRNA occurred when the Fas up-regulated tumour cells were returned to culture. The present study investigates the mechanism(s) underlying activation of Fas expression in tumours in vivo and the biological consequences of this expression, specifically tumour cell susceptibility to apoptosis.

Materials and methods

Mice and tumour cells

Two-to-4-month-old mice were used, with the following strains: C57BL/6 (H-2d); DBA/2 (H-2b); BALB/c (H-2d); PO (perforin-deficient mice) (H-2b); Nos2tm1Lau/J (inducible nitric oxide synthase deficient (iNOS−/−), Jackson Laboratory, Bar Harbor, ME); NOD/LtSz-scid/scid (non-obese diabetic/severe immunodeficient (NOD/SCID)); NOD/SCID/β2-microglobulin null (B2mnull). Wild type L1210 leukaemia of DBA/2 mice and several sublines thereof including L1210–3 (selected for low Fas-expression), high Fas-expressing LF+ (transfected with a mouse Fas over-expression construct), and LF− (expressing low levels of Fas as a result of transfection with a Fas antisense construct) were employed. Tumour cells were cultured in RHM medium (RPMI-1640 containing 5% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10−5 M 2-mercaptoethanol). The C57BL/6 (H-2d) T-cell leukaemia EL4, DBA/2 (H2b) mastocytoma P815 and AKR (H-2k) leukaemia BW were also grown in RHM.

Several subclones of the C57BL/6 mouse Lewis lung carcinoma 3LL were used: D122 is a highly metastatic, low immunogenic cell line, which expresses a very low level of Fas. K562-39-5 is a non-metastatic, highly immunogenic line expressing H-2Kb major histocompatibility complex (MHC) molecules, caused by transfection of the H-2Kb gene to D122. All Lewis lung carcinoma lines were grown in Dulbecco’s modified Eagle’s minimal essential medium with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and streptomycin, 1 mM sodium pyruvate, and 1% non-essential amino acids. Another low Fas-expressing tumour used was RMA-S, which is a clone of the Rauscher virus-induced T-cell lymphoma RBL-5 from C57BL/6 mice (H-2b). It was maintained in RMA-S medium (RPMI-1640 with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin). All cell lines were tested and found free of Mycoplasma sp.

Flow cytometry

Cells (0.25 × 10⁶) were washed in staining buffer (1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 0.02% azide), centrifuged, resuspended on ice in 30 μl diluted antibody for 30 min with occasional shaking. When conjugated fluorescent antibody was used, cells were incubated as above, in the dark. After a final wash, the cells were resuspended in PBS containing 0.02% azide and analysed by FACSscan. Antibodies used were: Jo2 Cat. #15400D, PharMingen, San Diego CA; fluoroscein isothiocyanate (FITC)-goat anti-hamster F(ab’)2 (Cat. # 107-096-142 Jackson Immuno Research Laboratories, West Grove, PA); biotinylated goat anti-hamster (Cat.# 127-065-160 Jackson Immuno Research Laboratories; phycoerythrin (PE)-streptavidin (Cat.# 016-110-084, Jackson Immuno Research Laboratories); anti-mouse Fas (Cat.# 12-0951, Ebioscience, San Diego, CA).

Cell labelling

To distinguish between tumour and mouse host cells, we used either cells labelled with 5 and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) or LF− cells transfected with green fluorescent protein (LF−GFP).

CFSE labelling. Cells (5–10 × 10⁶/ml PBS) were labelled with CFSE (Molecular Probes, Eugene, OR), 75 μm, 10 min at 37°C. After two washes in PBS, the cells were re-suspended in PBS or culture medium.

Transfection of cells. LF−GFP tumour cells were produced by the Phoenix system (http://www.stanford.edu/group/nolan/protocols/pro_helper_Department.html), in which Phoenix cells (produced by the Nolan lab as a helper system) were transfected with pBabe-GFP and then used to infect LF+ cells.

Co-incubation of tumour and lymphoid cells

LF− cells were injected into mice; 3–7 days later peritoneal exudate lymphoid cells (PELs) or splenocytes were withdrawn. Immune cells, or LF− cells were labelled with CFSE (or GFP in the case of LF+ cells) before they were co-incubated in culture dishes for 3–5 days.

Ascitic fluid

Peritoneal ascitic fluid was withdrawn 3–7 days after an intraperitoneal (i.p.) injection of 25 × 10⁶ leukaemia LF− or EL4 cells into syngeneic or allogeneic mice. Peritoneal washes (ascitic fluid) from control, non-injected or injec-
ted mice were obtained by rinsing the peritoneal cavity with 3 ml PBS. Supernatant was separated by centrifugation (180 g, 10 min), diluted and incubated with low Fas-expressing tumour cells at 37°. Fas expression on cells was analysed 17–24 hr later by fluorescence-activated cell sorting (FACS).

Propidium iodide (PI) and cell cycle analysis

To induce Fas-mediated apoptosis, cells were incubated with recombinant FasL trimer (rFasL)31 (1 ml of ~0.1 µg/ml) (kindly provided by Dr D. Wallach, Weizmann Institute, Rehovot) for 2 hr at 37°, or with the mouse Fas antibody Jo2 (Cat. #15400D, PharMingen, San Diego), 1 µg/ml, for 16 hr at 37°. Alternatively, apoptosis was induced by incubating cells with staurosporine (STS) (0.3 µM), or etoposide (15 µM) for 17–24 hr at 37°.

Cells were then centrifuged (180 g, 10 min), and 3 ml of cold methanol was added to the resulting pellets. Following incubation at −20° for 20 min, and two washes with PBS, RNAase was added to a final concentration of 50 µg/ml, followed by 50 µg/ml of propidium iodide (PI). Finally, the cells were analysed for PI fluorescence, which indicates DNA fragmentation, by FACS.

Cell-mediated cytotoxicity

Cytotoxicity assays were performed as described before22 using allogeneric immune PEL as effecter cytotoxic T lymphocyte (CTL) and cognate, 51Cr-labeled tumour cells as targets. Briefly, 25 × 10⁶ tumour cells were injected into the peritoneum, 10 days later PEL were withdrawn by washing the peritoneal cavity with 10 ml of PBS, centrifuged and resuspended in RHFM; simultaneously target cells were labelled with Na₂⁵¹CrO₄ (Amer sham, Amersham, UK) for 1 hr at 37°, and washed twice with PBS–FCS before use. Lytic assays were conducted in U-shaped 96-well microtiter plates with 3–10 × 10⁴ labelled target cells per well at different effector:target cell ratios. The plates were centrifuged (180 g, 5 min) at room temperature to promote PEL–target conjugate formation, and then incubated for 6 hr at 37°. To terminate the lytic assay, the plates were re-centrifuged in the cold (750 g, 10 min), 100 µl supernatant from each well was harvested, and the radioactivity was determined in a gamma counter. The percentage of lysis was calculated according to the formula: percentage specific release = ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100.

S-Nitroso-N-acetylpenicillamine (SNAP)

SNAP (Sigma-Aldrich, St Louis, MO), an NO donor. A 25 mM stock solution (1·1 mg SNAP dissolved in 194 µl of PBS + 6 µl of 1 M of sodium hydroxide)32 was freshly prepared before each experiment, kept on ice and protected from light by aluminium foil. SNAP solution was then added to a final concentration of 10–100 µM once, or every 5–7 hr. Cells were incubated with SNAP at 37° for 17–24 hr and then analysed for Fas expression by FACS as previously described.

RT–PCR

Total RNA was extracted with of TRI-reagent (Sigma) from 5 × 10⁶ cells (using Sigma’s protocol). RT–PCR was done using the ABgene (AB-0575) or Promega Access RT–PCR kit (Cat#A1250). 2 µl of the resultant CDNAS were amplified by PCR using gene specific primers under the following condition: 30 cycles of 30 s at 94°, 1 min annealing (BCL-2, BCL-XL at 59.4°, FLIP at 58°, caspase-8 at 60°), 2 min extension at 68°, and a final extension for 7 min at 68°. Primers: β-actin- forward 5’-TCTGTGGCATCCCATGAAACTACATTCAATTCC-3’; reverse 5’-GTGAAAACGACGCTCATGTCGCTTAG-3’; bcl-2-forward 5’-CTGGCATCTTCTCCTTCCAG-3’; reverse 5’-CATCCGGTTCAGGTACTC-3’; bcl-xl-forward 5’-TGGTGTCGACTTCTCTCC-3’; reverse 5’-ACCCAGTTTACTCCATCCC-3’; Caspase-8-forward 5’-CCTAGACTGCAACCGAGGG-3’; reverse 5’-CGTCCATAGACGACACCT-3’.

Results

The activity of up-regulated Fas

As reported before22 and extended here (Table 1), most tumour cells that express Fas at low levels (LF) undergo Fas up-regulation when injected i.p into either syngeneic or allogeneic recipients. To investigate whether this newly expressed Fas is functional as a death receptor, LF tumour cells were withdrawn 3–7 days after injection into the peritoneal cavity and examined for their susceptibility to Fas-mediated apoptosis. Indeed, we found that Fas up-regulated LF cells exhibit enhanced susceptibility to Fas-mediated apoptosis (Table 2). This was true for three alternative ways of stimulating Fas: (1) the natural ligand (FasL) expressed on the surface membrane of perforin knockout (PO) cytotoxic T lymphocytes (CTLs); (2) soluble recombinant FasL trimer; and (3) Fas-specific antibody Jo2. Taken together, these data indicate that newly expressed up-regulated Fas functions as a death receptor.

Next we examined whether newly expressed Fas sensitizes tumour cells to apoptosis induced by the chemotherapy drugs etoposide and staurosporine (STS). Each of these drugs induces apoptosis primarily by a different mechanism: the former via caspas and the latter via the mitochondrial pathway.33,34 LF tumour cells were withdrawn five days after injection into three different mice backgrounds, BALB/c, B/6 or PO mice and drug-induced apoptosis assessed. Etoposide (15 µM) induced 24-2, 39-2
Table 1. Up-regulation of Fas expression on tumour cells in vivo

<table>
<thead>
<tr>
<th>Tumour cell type</th>
<th>Injected mice</th>
<th>Repeats</th>
<th>% Fas-expressing cells</th>
<th>Days from injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>In syngeneic mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>DBA/2</td>
<td>7</td>
<td>12.6 ± 9.5</td>
<td>4</td>
</tr>
<tr>
<td>L1210 wild type</td>
<td>DBA/2</td>
<td>2</td>
<td>2.4 ± 12.3</td>
<td>3</td>
</tr>
<tr>
<td>RMA-S</td>
<td>PO</td>
<td>3</td>
<td>0 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>3LL-D122</td>
<td>PO</td>
<td>8</td>
<td>3.6 ± 8.4</td>
<td>6</td>
</tr>
<tr>
<td>3LL-K39-5</td>
<td>PO</td>
<td>4</td>
<td>6.6 ± 19.8</td>
<td>4</td>
</tr>
<tr>
<td>In allogeneic mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>PO</td>
<td>8</td>
<td>12.6 ± 9.5</td>
<td>5</td>
</tr>
<tr>
<td>LF</td>
<td>C57BL/6</td>
<td>5</td>
<td>12.6 ± 9.5</td>
<td>7</td>
</tr>
<tr>
<td>L1210 wild type</td>
<td>PO</td>
<td>3</td>
<td>2.4 ± 12.3</td>
<td>3</td>
</tr>
<tr>
<td>P815</td>
<td>PO</td>
<td>6</td>
<td>15.2 ± 1.3</td>
<td>4</td>
</tr>
<tr>
<td>BW</td>
<td>PO</td>
<td>3</td>
<td>0 ± 1.8</td>
<td>5</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td></td>
<td></td>
<td>77 ± 9.0</td>
<td></td>
</tr>
</tbody>
</table>

1Tumour cells grown in vitro or in vivo (25 × 10^6 cells injected i.p. into syngeneic or allogeneic mice) were analysed for cell-surface Fas expression by FACS using the Fas-specific antibody Jo2.

Table 2. Apoptosis of Fas-up-regulated LF-tumour cells

<table>
<thead>
<tr>
<th>% Cells in apoptosis induced by:</th>
<th>% Fas up-regulation</th>
<th>Control</th>
<th>CTL</th>
<th>rFas</th>
<th>Jo2</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF in BALB/c</td>
<td>32 ± 13.9</td>
<td>2.2 ± 1.6</td>
<td>10.9 ± 6.7</td>
<td>18.6 ± 3.5</td>
<td>14.2 ± 6.7</td>
</tr>
<tr>
<td>LF in B/6</td>
<td>45 ± 12.1</td>
<td>3.6 ± 2.7</td>
<td>n.m</td>
<td>29.3 ± 10.4</td>
<td>n.m</td>
</tr>
<tr>
<td>LF in PO</td>
<td>38 ± 17.6</td>
<td>4.1 ± 1.8</td>
<td>24.5 ± 8.1</td>
<td>47.4 ± 18.1</td>
<td>46.9 ± 17.9</td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>74 ± 4</td>
<td>0</td>
<td>71.2</td>
<td>89</td>
<td>52.1</td>
</tr>
<tr>
<td>LF</td>
<td>8.2 ± 9.5</td>
<td>0</td>
<td>4.8 ± 3.1</td>
<td>1.7 ± 1.4</td>
<td>1 ± 1.8</td>
</tr>
</tbody>
</table>

1Low Fas-expressing LF− tumour cells (25 × 10^6) were injected into BALB/c, C57BL/6 (B/6) or PO mice. Each experiment was repeated 3–5 times at the same settings.
2Cells were withdrawn 5 days after tumour injection, and Fas expression was determined by FACS using the Fas-specific antibody Jo2.
3Apoptosis was induced by incubating tumour cells with CTL (PEL obtained from PO anti-LF−) for 6 h and determined by the 51Cr release assay. The effector : target cell ratios was 10 : 1.
4Apoptosis induced by recombinant FasL trimer (0.1 μg/ml, 2 h), or by the Fas antibody Jo2 (1 μg/ml, 16 h) and measured by the PI cell cycle assay. Control values represent apoptosis of cells which were not exposed to apoptotic inducers. n.m, not measured.

and 34.2% apoptosis, respectively, and STS (0.3 μM) induced 12.9, 21.1 and 6.6% apoptosis, respectively. In contrast, cultured LF− cells that have not had their Fas up-regulated exhibited 8.6% and 4.2% apoptosis when exposed to etoposide and STS, respectively. These data indicate that the Fas up-regulated LF− cells are more sensitive not only to Fas-mediated apoptosis, but also to other types of apoptosis. This raises the possibility that Fas up-regulation is accompanied by enhanced activity and/or expression of molecules that function downstream from Fas in the apoptosis pathway.

To investigate this issue, the gene expression of various apoptotic molecules were surveyed in LF− tumour cells after injection into mice. Surprisingly, we found enhanced mRNA expression of the anti-apoptotic factors FLIP, BCL-2 and BCL-X coinciding with Fas up-regulation (Fig. 1). Fas signalling has been implicated in activating non-apoptotic, counter-apoptotic and even growth-promoting pathways. Therefore, it is conceivable that these up-regulated anti-apoptotic factors account for the survival, progressive growth, and even metastasis of high Fas-expressing cells that succeed to proliferate in vivo.
despite the hostile environment created by FasL-expressing effector cells, such as CTL and natural killer (NK) cells. Nevertheless, in various assays in vitro, LF− tumour cells with up-regulated Fas display enhanced sensitivity to Fas-mediated apoptosis, confirming that the newly expressed Fas is capable of death receptor activity (Table 2).

Regulation of Fas expression

CTLs,37 NK cells38 and cytokines17,39 have all been shown to influence Fas expression in vitro. To explore whether the immune system plays a role in the Fas up-regulation observed in vivo (Table 1), we examined Fas levels in LF− tumour cells 3–6 days after i.p. injection into immunocompromised mice. Several types of immunocompromised mice were used in these experiments: mice subjected to total body X-irradiation the day before injection, immune-deficient mice (NOD)scid/scid and B2mnull) and mice that lack NK cytotoxicity (Beige). FACS analysis revealed that, in contrast to the situation when using immune competent mice, almost no induction of Fas is observed in the LF− cells recovered from immune deficient mice (Table 3). These findings support the hypothesis that the immune system is involved in the Fas up-regulation observed in tumours in vivo.

Moreover, these results allowed us to corroborate and explore further this hypothesis. For we now had an in vitro assay, where we could investigate which immune subpopulation influenced Fas up-regulation. LF− tumour cells were cocultivated with either immune peritoneal exudates lymphocytes (PEL) or with spleen cells procured from PO (H-2b) mice injected with the tumour. Prior labeling of the immune cells with CFSE facilitated their distinction from the tumour cells. We observed that both PEL and spleen cells restore enhanced Fas expression to the LF− tumour cells (Table 4), confirming that these immune cells induce Fas up-regulation. We verified, as above, that the newly expressed Fas observed in these coinoculation experiments is functional as a death receptor. Indeed,

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### Table 3. Fas up-regulation in LF− tumour cells injected into immuno-compromised mice

<table>
<thead>
<tr>
<th>Mice injected</th>
<th>% Fas expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune deficient mice</td>
<td></td>
</tr>
<tr>
<td>950 rad irradiated PO mice</td>
<td>14</td>
</tr>
<tr>
<td>NOD SCID/SCID</td>
<td>7 ± 12</td>
</tr>
<tr>
<td>B2mnull</td>
<td>11 ± 13</td>
</tr>
<tr>
<td>Beige</td>
<td>7 ± 9</td>
</tr>
<tr>
<td>Normal mice</td>
<td></td>
</tr>
<tr>
<td>PO</td>
<td>51 ± 16</td>
</tr>
<tr>
<td>BALB/c</td>
<td>65 ± 12</td>
</tr>
<tr>
<td>B/6</td>
<td>64 ± 15</td>
</tr>
<tr>
<td>LF−</td>
<td>15 ± 8</td>
</tr>
</tbody>
</table>

1 Low Fas-expressing tumour cells (LF) (25 × 10⁶ cells) were injected i.p. into mice. Three days later, surface Fas expression was determined by FACS using the Fas antibody JO2. The results represent averages of four similar experiments.

2 Tumour cells were injected into X-irradiated mice, one day after irradiation.

3 The difference between Fas expressed on tumour cells grown in-vivo and in-vitro.

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### Table 4. Induction of Fas expression by lymphoid cells

<table>
<thead>
<tr>
<th>Source of lymphoid cells</th>
<th>Δ% Fas-expressing cells after coculture with ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>Immunized mice ³</td>
<td>0</td>
</tr>
<tr>
<td>Naive mice ²</td>
<td>0</td>
</tr>
</tbody>
</table>

¹LF− tumour cells (25 × 10⁶) were injected into primed PO anti-LF mice. Four days later, peritoneal cells and splenocytes were withdrawn and labelled with CFSE.

²Splenocytes were prepared from naive PO mice and labelled with CFSE.

³Fas expression on tumour cells (non-CFSE labelled) cocultured for 3 days with CFSE-labelled lymphoid cells was determined by FACS using the anti-mouse Fas antibody JO2 PE-labelled second antibody.

⁴Peritoneal cells were collected by washing the peritoneal cavity with 3 ml PBS.
whether the splenocytes were sourced from BALB/c (quasi-syngeneic) or PO (allogeneic) mice, exposure to rFasL induced 36% more apoptosis in the Fas up-regulated LF\(^+\) cells than in control LF\(^-\) cells (data not shown). It is noteworthy that splenocytes from naïve and immunized mice induce comparable Fas up-regulation, which supports the involvement of innate immune system cells in this process. Such a role for the innate immune system has been implied by another of our findings that RMA-S tumour cells, which express low levels of both MHC class I and Fas\(^+\) up-regulate Fas when injected into mice (Table 1).

Fas expression could be induced as a result of direct contact between tumour and immune cells\(^{41}\) mediated by cell surface molecules such as CD40\(^{42}\) and CD44.\(^{43}\) Alternatively, Fas induction could be mediated by soluble factor(s) produced by immune cells in response to the tumour, such as one or more of the cytokines.\(^{14,16,17}\) To differentiate between these possibilities, we tested whether the i.p. ascitic fluids that surround the LF\(^+\) tumour cells accumulate any Fas up-regulatory activity. Three to 7 days after i.p. injection of LF\(^+\) tumour cells into immune competent mice, peritoneal ascitic fluids were withdrawn, diluted in medium, and then tested for their ability to induce Fas up-regulation in naïve LF\(^+\) tumour cells. Ascitic fluid collected from either syngeneic or allogeneic tumour-bearing mice induces moderate Fas up-regulation (Table 5); this is in contrast to peritoneal washes from non-tumour bearing mice, which have no detectable affect on Fas expression (data not shown). Notably, Fas expression induced by ascitic fluids exhibits no tumour specificity, i.e. ascitic fluids collected from one tumour up-regulates Fas expression in non-related tumour cells (Table 5), which implies that antibodies are unlikely to be the soluble molecules mediating Fas up-regulation.

The above data notwithstanding, the finding that ascitic fluids induce Fas up-regulation is difficult to reproduce and the activity of the ascitic fluids is often lost upon freezing and thawing, implicating that a meta-stable factor(s) constitutes this activity. One candidate unstable factor produced by cells of the immune system, which has been shown to induce Fas expression \textit{in vitro}, is nitric oxide (NO).\(^{18,19,21,44}\) As a first step to exploring whether NO is involved in Fas up-regulation of LF\(^+\) tumour cells, we examined the effects of the NO-donor SNAP on Fas expression in RMA-S cells (tumour cells that undergo considerable Fas up-regulation \textit{in vivo} (Table 1)). The SNAP concentrations employed were based on previous reports that measured the amounts of NO in sera of control and immune-activated mice as 20–50 \(\mu\)M and 60–80 \(\mu\)M, respectively.\(^{45,46}\) Addition of SNAP (0–100 \(\mu\)M) to cultures induces Fas expression in RMA-S cells (Fig. 2).

![Figure 2](image-url)  
**Figure 2.** The effect of the NO donor SNAP on Fas expression \textit{in vitro}. RMA-S tumour cells were incubated in the presence of SNAP (0–100 \(\mu\)M), added either once (solid bare), or four times every 5–10 hr (striped). Fas expression was determined after 23 hr. Each column is the average Fas expressed on RMA-S obtained in three to five similar treatments. \(* P < 0.05; \**P < 0.01.**
Because the half-life of SNAP is about 5 hr\(^4\) its continuous presence was ensured by adding fresh SNAP every 5–7 hr. Fas expression was considerably higher when SNAP was supplemented continuously to the cultures than if SNAP was added only once (Fig. 2).

In order to investigate directly whether NO is involved in Fas up-regulation \textit{in vivo}, we performed our ‘\textit{in vivo} assay’ using mice that lack the inducible NO synthase (iNOS) enzyme. LF\(^{–/–}\) tumour cells (in these experiments LF\(^{–/–}\) labelled with green fluorescence (LF\(_{\text{GFP}}^{–/–}\)) were injected into mice and then Fas expression assessed. When iNOS\(^{+/+}\), B/6, mice were used Fas was up-regulated by 51 ± 4.6%, however, when iNOS\(^{–/–}\) mice were employed then Fas expression increased only by 42 ± 5.9% (\(P = 0.05\)). These data support the premise that NO is involved in Fas up-regulation and furthermore, imply that the NO is produced by the host’s immune cells. Similar results were obtained when using an alternative experimental protocol. LF\(^{–/–}\) tumour cells were injected i.p. into iNOS\(^{–/–}\) or C57BL/6 (B/6) mice, after 4 days splenocytes were withdrawn and cocultured with naive LF\(_{\text{GFP}}\) tumour cells for three days before assessment of Fas expression (Fig. 3). Fas is up-regulated less effectively (by 11-8%; \(P = 0.05\)) when cocultured with iNOS\(^{–/–}\) splenocytes. This difference in Fas expression correlates with a change in susceptibility to rFasL-induced apoptosis. LF\(_{\text{GFP}}\) cells are 12.7% more susceptible to Fas-mediated apoptosis after coincubation with control splenocytes than after coincubation with iNOS\(^{–/–}\) splenocytes (\(P = 0.05\)) (Fig. 3). The reduced Fas expression observed in LF\(_{\text{GFP}}\) tumour cells exposed to iNOS\(^{–/–}\) immune cells supports the hypothesis that NO produced by immune cells is involved in Fas up-regulation. However, because a significant amount of Fas up-regulation is still observed under these conditions, it seems that NO cannot be the sole factor mediating Fas induction. Other candidate soluble mediators are IFN-\(\gamma\) and TNF-\(\alpha\)\(^{14,48}\) both reported to induce Fas up-regulation \textit{in vitro}. Notably, we find that in the absence of the NO-donor SNAP, neither of these cytokines enhances Fas expression \textit{in vitro} within a 17 hr incubation period (data not shown). Future studies will be required to delineate what factors in combination with NO mediate Fas-regulation \textit{in vivo}.

**Discussion**

Normal and malignant cells express the death receptor Fas, both internally and on their surface membrane. Its expression can be affected by endogenous and exogenous stimuli. These include stress, infectious agents,\(^2,11\) drugs, and cytokines such as IFN-\(\gamma\), TNF-\(\alpha\),\(^14\) IL-7,\(^15\) IL-1\(\beta\),\(^16\) IL-1, and IL-6\(^17\) each in itself or in combination with others. Another factor shown to affect Fas expression is nitric oxide (NO).\(^12–19\) NO is produced by a wide range of mammalian cells including neurons, endothelial and immune cells (the latter includes macrophages that secrete high levels of NO).\(^49\) NO is a secondary messenger in some systems (e.g. a neural messenger)\(^50\) and an apoptosis inducer\(^51\) in others. It is a diffusible, highly reactive molecule and one of the smallest bio-reactive molecules found in nature. NO is synthesized from \(\text{L-arginine}\) by the enzyme NO synthase (NOS). Garban and Bonavida\(^18\) and Vekemans et al.\(^21\) reported that supplementation of IFN-\(\gamma\) to human ovarian and colon carcinoma cell lines \textit{in vitro} induces NO synthesis and Fas expression, thereby increasing their sensitivity to Fas-mediated apoptosis. Although these and many other studies have reported several factors capable of affecting Fas expression \textit{in vitro}, no factors have been shown yet to be involved in the regulation of Fas expression and function(s) \textit{in vivo}.

In an attempt to ascertain the significance and elucidate the underlying mechanism of Fas up-regulation \textit{in vivo}, the present study extends and substantiates earlier observations\(^2\) that most (17/23 mouse tumours tested to date) low Fas-expressing (LF\(^{–/–}\)) tumours up-regulate their surface Fas expression when injected into either syngeneic or allogeneic recipient mice (Table 1). Based on the classical characterization of Fas as a death receptor, it was reasonable to assume that its up-regulation would increase susceptibility to apoptosis induced by the Fas natural ligand expressed on CTL\(^37\) and NK cells.\(^38\) Indeed, we found that the newly expressed Fas is functional and increases susceptibility to apoptosis induced by CTL, Fas antibody Jo2, and soluble recombinant FasL (Table 2). This enhanced susceptibility rules out the possibility that an isoform of Fas, non-functional Fas, is up-regulated in the LF\(^{–/–}\) tumour cells.\(^32\) Fas up-regulation, and the consequent increased susceptibility to apoptosis, likely represents the transitory response of the tumour to a defence mechan-
ism instigated by the host against the tumour, although it does not curtail the ability of such a tumour to proliferate and eventually kill the host.

We observe that Fas up-regulation is concomitant with enhanced susceptibility to other apoptosis-inducing agents, such as etoposide and STS. Hence, Fas up-regulated cells appear to manifest a greater sensitivity to apoptotic signals mediated not only by a death receptor, but also by both the caspase and mitochondrial apoptosis pathways. Using RT–PCR we examined whether this broad apoptotic sensitivity reflected de novo synthesis of apoptotic factors. Unexpectedly, we detected increased expression of anti-apoptotic factors and decreased expression of caspase 8 (Fig. 1). Although it is surprising that Fas up-regulation is associated with changes in the mRNA expression of anti-apoptotic genes, these findings likely expose the delicate balance that exists in cells between pro-apoptotic and anti-apoptotic factors, which is constantly being fine-tuned in response to the complex mix of signals received. The critical outcome of this balancing act is either tumour death or tumour survival and growth. Our findings are in accord with those of Mitsiades et al. who showed that in thyroid carcinomas, the proteolytic cleavage and activation of caspase-8 depends on the relative expression levels of procaspase-8 and FLIP. Furthermore, previous reports have demonstrated that in response to Fas stimulation the expression of certain genes, not associated with apoptosis, are induced even and even that cell proliferation is promoted.

We propose that the immune system is involved in Fas up-regulation in vivo based on our present findings. Fas expression is not up-regulated in LF– tumour cells injected into X-irradiated or otherwise immune-deficient mice (Table 3). However, coinjection of LF– tumour cells with splenocytes or PELs derived from normal mice restores Fas up-regulation in this assay (Table 4). Moreover, our data suggest that Fas up-regulation involves cells of the innate immune system, since splenocytes from immunized and non-immunized mice up-regulate Fas to nearly the same extent (Table 4) and Fas up-regulation occurs in RMA-S tumour cells (low Fas, low MHC) in vivo (Table 1). This conclusion is in line with Screpanti et al. who showed that cells of the innate immune system, in particular NK cells, are involved in Fas up-regulation in tumours, a phenomenon first reported by this lab.

The present study suggests that Fas up-regulation is induced by one or more soluble factors secreted within the tumour microenvironment, which is the peritoneal cavity in the case of intraperitoneal ascites tumours. Accordingly, ascitic fluid derived from the peritoneal cavities of tumour-bearing mice was found to stimulate Fas expression in LF– tumour cells (Table 5). Candidates for soluble factor(s) include cytokines and NO, which have been shown to affect Fas expression in vitro. Our experiments indicate the presence of an unstable mediator in the ascitic fluid, which led us to focus on NO. The hypothesis that NO is involved in Fas up-regulation in vivo is supported by experiments with the NO donor SNAP, which induces Fas up-regulation when added to the culture medium of RMA-S tumour cells (Fig. 2), but is evidenced more directly by experiments using iNOS-deficient mice and their immune cells. Both LF–GFP cells injected into iNOS+/– mice and LF–GFP cells coincubated with spleen cells from iNOS–/– mice exhibit elevated Fas expression. This Fas up-regulation is lower than that induced by control mice or control splenocytes (Fig. 3). Taken together, these data suggest that NO produced by the host’s immune cells is responsible for Fas up-regulation, at least in part.

In vitro studies report that increased NO secretion is caused by elevated NOS activity, triggered by cytokines such as IL-1, IFN-γ and TNF-α. It is likely that in vivo such cytokines are secreted by activated CTL and/or NK and dendritic cells in response to the tumour. In addition to influencing Fas expression via NO, these cytokines can also, in a less direct manner, activate transcription factors that induce Fas expression. Arnold et al. have shown that Fas activity is induced 48 hr after exposure to IFN-γ and TNF-α. This longer timescale is likely a reflection of the less direct mechanism of Fas induction, for in our experiments where NO is mediating Fas induction, at least in part, an effect on Fas expression is detected as early as 24–36 hr (Table 5; Fig. 2).

The experiments in this study do not address directly the mechanism by which NO induces Fas up-regulation. It has been shown that NO can induce Fas up-regulation in vitro via its interaction with the zinc finger protein Yin-yang 1 (YY1). The interaction of NO with YY1 results in release of YY1 from the Fas gene and relief from transcriptional repression. Transcription factors such as p53, nuclear factor-κB or STAT-3 and c-Jun, that each bind to the Fas promoter and trans-activate the Fas gene may also be targets for NO regulation. In summary, although we do not exclude the possibility that factors other than NO contribute to Fas up-regulation, particularly at later times, we propose that NO is an important early feature of the mechanism of Fas up-regulation in tumours in vivo.

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