Granulocytes mediate the Fas-L-associated apoptosis during lung metastasis of melanoma that determines the metastatic behaviour.

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The survival of tumour cells in a new tissue environment is crucial for tumour metastasis. Factors contributing to the death of tumour cells during metastasis are not completely understood. In murine melanoma model, activation of Fas (CD95, APO-1) signal in tumour cells reduces their lung metastasis potential, which may be associated with an induction of apoptosis in tumours. To elucidate the cellular mechanism, we used a Fas-ligand (Fas-L) specific ribozyme (Fas-L ribozyme) to suppress the expression of Fas-L but not Fas or TNF-α in B16F10 melanoma cells. The Fas-L ribozyme-carrying cells grew slightly faster in vitro with better viability than controls. Suppression of Fas-L in B16F10 melanoma cells by Fas-L ribozyme enhanced lung metastasis of the cells in C57BL/6 mice, and that was correlated with reductions in both apoptotic tumour cells and granulocytic infiltration. Mice depleted of granulocytes, but not CD4+ and CD8+ cells, showed a greatly elevated susceptibility to lung metastasis. Moreover, apoptosis in tumour cells was significantly reduced in granulocyte-depleted mice during the course of tumour formation. Taken together, our findings indicate that Fas-L-associated apoptosis in tumour cells determines the metastasis behaviour of melanoma in the lung and this apoptosis is primarily mediated by the cytotoxicity of recruited granulocytes.

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Apoptosis which occurs in tumour cells during or just after extravasation from blood vessel into a new tissue environment is a crucial step in metastasis (Walsh and Sata, 1999). Apoptotic factors of tumours contributing to metastatic tumour behaviour are not completely understood. Among the candidate apoptotic signals, the Fas (also named as CD95, APO-1)/Fas-ligand (Fas-L) system plays important roles in organ homeostasis and immune surveillance against tumours (Walker et al., 1998). Fas-L is a member of the tumour necrosis factor receptor superfamily and triggers a death signal into Fas-bearing cells after engagement with Fas molecule (Itoh et al., 1994; Takahashi et al., 1994). Functional expression of Fas-L in melanoma has been demonstrated in vitro (Hahne et al., 1996). During the progression of human melanoma, tumour Fas-L increases gradually (Terheyden et al., 1999; Soubbrane et al., 2000). However, the finding that the Fas/Fas-L interaction suppressed lung metastasis of melanoma in murine models argued against a major contribution of tumour Fas-L in escape from immune surveillance (Owen-Schaub et al., 1998; Rivoltini et al., 1998; Sprecher et al., 1999) and suggested a negative role of Fas-associated apoptosis in metastasis. In addition, ectopic expression of Fas-L in transgenic animals or tumours revealed that Fas-L enhanced neutrophil recruitment and mediated destruction of certain Fas-L-positive cells (Arai et al., 1997; Chervonsky et al., 1997; Seino et al., 1997; O’Flaherty et al., 1998). At moment, it is not known whether the Fas-L-associated suppression in metastasis is mediated by triggering the suicidal apoptotic Fas signal in tumour cells or by an indirect action through the recruited inflammatory cells.

In this study, we investigated the contribution of immune cells to Fas-associated apoptosis in tumours. We used a hammerhead Fas-L-specific ribozyme (Fas-L ribozyme) to suppress the Fas-L gene (Yang et al., 1999; Chio et al., 2001). The Fas-L ribozyme effectively inhibited the expression of Fas-L in melanoma cells at both mRNA and protein levels. Stable transfectants carrying Fas-L ribozyme were established to evaluate the contribution of tumour Fas-L to cell growth, apoptosis, and lung metastasis. The involvement of immune cells in the processes of lung metastasis was explored by cell depletions using antibodies for CD4+ , CD8+ cells or granulocytes. Our results clearly demonstrate that granulocytes play a crucial role in the Fas-L-associated apoptosis during lung metastasis.

MATERIALS AND METHODS

Cell culture

The melanoma cell line B16F10, kindly provided by Dr Shiau Al, NCKU, is derived from a spontaneous melanoma in C57BL/6 mouse and has lung metastasis ability (Mackensen et al., 1993). Tumour cells were cultured in DMEM medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) and 2 mM l-glutamine at 5% CO2/37°C in a humidified atmosphere. Cell growth rate was determined. In brief, 2.5 × 10⁴ of cells in a 60 mm-dish were at first starved in 0.1% FCS/DMEM for 24 h, and then re-grew in a regular 10% FCS/DMEM. Cells were harvested at intervals to determine the number of viable cells by Trypan blue exclusion method.

DNA transfection and selection of stable cells

The sequences of the oligonucleotides used to construct Fas-L ribozyme were as follow: sense sequence: 5’-ATGAATTCCTCCGGAAGTACTGTAGTGATCGTGATACGA-3’;
Antisense sequence: 5'-TCGGGATCCAAAGTTTCGGGTATCAC- 
GACTCTACTGTTCCGGGAAATTGAT-3'. The ribozyme was 
directly linked to upstream of the EGFP gene in pEGFP-N1 plas-
mid (CLONTEC, Palo Alto, CA, USA) to form a fusion 
transcript (Chio et al, 2001). Plasmid DNA was delivered into cells 
using the lipofection method with a ratio of 5 µg DNA per 30 µl 
lipofectamine (Qiagen, Hilden, Germany). Cells transfected with 
pEGFP-N1 plasmid served as the vector control. After DNA trans-
fection, cells were grown in regular 10% FCS/DMEM for 48 h and 
then selected with geneticin (G418 sulphate, Gibco, Darmstadt, 
Germany) at an effective concentration of 1.5 mg ml⁻¹. Bulk 
culture or stable clones were established for at least 3 months 
before they were subjected to further study.

Semi-quantitative reversed transcription-polymerase chain 
reaction (RT – PCR)

Total RNA was purified using the RNeasy Kit according to 
the manufacturer’s instruction (Qiagen) and converted to cDNA by 
StrataScrip™-H reverse transcriptase with oligo-dT primer in the 
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Western blot

Cells were extracted with a buffer containing 20 mM Tris, 150 mM 
NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF and 
0.1 U ml⁻¹ leupeptin. Proteins were separated in SDS-polyacryla-
mide gel and electroblotted onto polyvinyl difluoride membrane 
(MSI, Westboro, MO, USA). The proteins bounded on the 
membrane were probed with the mouse antibody (Ab) -recognising 
Fas-L (clone33; Transduction Laboratories, Lexington, KY, USA) 
followed by a sheep anti-mouse IgG conjugated with horseradish 
peroxidase (Dako Corp., Carpinteria, CA, USA). The Fas-L band 
was made visible by fluorography with enhanced chemiluminescence 
detection kit (Amersham/Pharmacia Biotech., UK). Duplicate blot 
was probed with a-tubulin-specific Ab (clone DM1A; NeoMarker, 
Fremont, CA, USA) and served as protein-loading control.

Tumour formation and immunohistochemical staining

Eight-week-old C57BL/6 mice (H2b) were purchased from the 
National Laboratory Animal Breeding and Research Center, Taiwan, 
R.O.C. and maintained under specific pathogen-free condition. All 
animal experiments have been carried out with approval of the ethi-
cal committee of Animal Research Center, National Cheng-Kung 
University. The ethical guidelines that were followed meet the stan-
dards required by the UKCCCR guidelines (1996). To investigate 
lung metastasis, mice received 0.5 – 5 × 10⁵ of tumour 
cells in 0.1 ml PBS via the tail vein injection (i.v. injection). Meta-
static lung tumours in mice were assessed under a dissecting 
microscopy. Organs were surgically obtained, fixed in 10% buffered 
formalin solution for paraffin block preparation or for flash-frozen 
in O.C.T. embedding medium (Miles Inc., Elkhart, IN, USA). Five-
µm tissue sections were placed on poly-L-lysine-coated glass slides, 
fixed with 3.7% paraformaldehyde, and treated with 3% H₂O₂. Cells 
were stained with rat anti-NK mAb (DXS), rat anti-CD4 mAb 
(H129.19), rat anti-CD8 mAb (53-6.7) (PharMingen, San Diego, 
CA, USA), rat anti-granulocyte mAb (RB6-8C5), rabbit anti-Fas 
Ab (M-20) or rabbit anti-Fas ligand Ab (N-20) (Santa Cruz Biotech-
nology, CA, USA). Appropriate sheep anti-rat IgG or goat anti-
rabbit IgG conjugated with peroxidase (Boehringer Mannheim 
GmbH, Mannheim, Germany) was used as secondary antibodies. 

Depletion of CD4⁺, CD8⁺ cells and granulocytes

Asctic fluids were generated from hybridomas GK1.5, 2.43 and 
RB6-8C5 secreting rat monoclonal antibodies for antibodies against 
mouse CD4, CD8 and granulocyte marker (Ly-6G), respectively 
(Staats et al, 1991; Tumpey et al, 1996). The CD4⁺ and CD8⁺-specific 
Abs were further purified by affinity chromatography on a protein 
G-sepharose column (Pharmacia, LKB Biotechnology, Piscataway, 
NJ, USA) and adjusted to a final concentration of 3 mg ml⁻¹. 
Protocols modified from Staats et al (1991) was used to deplete 
CD4⁺ or CD8⁺ cells, so mice were given 100 µg anti-CD4 or anti-
CD8 Ab by intraperitoneal (i.p.) injection on day –2. Booster 
anti-CD4 or anti-CD8 Ab was given twice on days 7 and 14. Deple-
tion of granulocytes was achieved by a serial of i.p. injections with 
anti-granulocyte Ab according to a modified protocol as previously 
reported (Tumpey et al, 1996) as follows: 100 µl on −5 h; 100 µl on 
day 3; 150 µl on day 5; 200 µl on days 7 and 9; 250 µl on days 12 
and 15. Control mice received purified rat IgG (ICN, Pharmaceuti-
cals Inc. Cappel, OH, USA) following similar protocol. Spleen 
cells were stained with anti-CD4 (H129.19, FITC-conjugated; PharMin-
gen) or anti-CD8 (53-6.7, PE conjugated; PharMingen) on days 6 or 
or 18 to determine the extent of T cell depletion. Reduced number 
of granulocytes in peripheral blood in granulocyte-depleted mice 
was also confirmed on days 6 or 18 by flow cytometric analysis using 
FITC-conjugated antibody (RB6-8C5; PharMingen).

Statistic analysis

Results were analysed by Student’s t-test. Differences with 
P < 0.05 were judged as significant.

RESULTS

Fas-L ribozyme - carrying cells

After DNA transfection followed by antibiotic selection, bulk 
cultures and randomly chosen cell clones were established. Most 
of those stable clones emitted green fluorescence under UV light.
and showed similar morphology as parental B16F10 cells. Cells carrying pEGFP-N1 plasmid were named V for bulk culture and Vn for stable clones; cells carrying Fas-L ribozyme plasmid were named R for bulk culture and Rn for stable clones. Rn, Fas-L ribozyme-carrying clones, showed reduced expression of Fas-L to various extents both at transcriptional and translational levels (Figure 1a,b). Fas-L ribozyme did not affect the expressions of Fas and TNF-α in melanoma cells. Along with a reduction in Fas-L protein, most of those Rn cells grew slightly faster in vitro than Vn, cells carrying pEGFP-N1 control plasmid, did (Figure 2). We further measured the spontaneous apoptosis in 3-day cultures, which presumably had more Fas/Fas-L engagement due to cell–cell contact in relatively confluent growth. In comparison with the enhanced growth rate, less apoptosis was observed in Rn than Vn as detected by MC-540 staining (Figure 3).

**Effect of Fas-L ribozyme on lung metastasis**

Bulk cultures and several established cell clones have been used to evaluate the contribution of tumour Fas-L to lung metastasis in C57BL/6 mice. Mice began to develop grossly observable tumour nodules in the lung around 14–18 days after inoculation with $1 \times 10^5$ of parental B16F10 cells. Similarly, metastatic tumour nodules were observed in mice who received stable cells of Vn, bulk culture or clones, at day 14 post-inoculation (Table 1). Rn produced more lung tumour nodules in mice than Vn did after day 14 post-inoculation (Figure 4). Tumour cells accumulated first in alveoli (day 7) and then expanded to alveolar sacs or near blood vessels (after 14 days).

**CD4⁺, CD8⁺ cells- and granulocyte-depletion**

To explore the anti-tumour effect of immune cells in vivo, CD4⁺, CD8⁺ cells and granulocytes in mice were depleted by antibodies. The extents of depletions for CD4⁺ and CD8⁺ cells in the spleen...
were more than 90% (Figure 5A,B). After depletion by RB6-8C5 antibody, the number of granulocytes in peripheral blood reduced to about 30% (Figure 5C). Figure 6 summarised the effects of CD4+-, CD8+ cells- or granulocyte-depletion on lung metastasis. Mice depleted of granulocytes showed an elevated susceptibility to lung metastasis (Figure 6). Particularly, the incidence of lung metastasis of controls in granulocytes-depleted mice at day 18 post-inoculation reached a degree similar to that of Rn in immune-competent mice. CD4+- or CD8+ cells-depletion did not affect, or even slightly suppress, lung metastasis of vector controls. Among the Fas-L ribozyme-carrying clones, R4 cells generated the highest number of lung tumour nodules in this study and its metastatic ability was not further increased in mice receiving antibodies against CD4+- CD8+ cells- or granulocytes.

**Immunohistochemical studies on the expression of Fas-L, tumour infiltrating cells and apoptosis in tumours**

A reduced expression of Fas-L was immunohistochemically detected in metastatic lung tumours of Rn compared to that of Vn (Figure 7). Histological examination revealed that small tumour cell mass was formed in the lung 96 h post-inoculation of tumour cells. Parental and control melanoma cells accumulated around terminal bronchioles, alveolar ducts or alveolar sacs. Rn accumulated around pulmonary veins and bronchioles. Similar to parental cells, the cell lines established in this study did not develop tumours in organs including liver, kidney, and spleen by i.v. inoculation at day 18 post-inoculation (data not shown).

Granulocytes accumulated in the lung very soon, within 48–96 h (Table 2). Intensive granulocyte infiltration in the bronchioles, alveoli and alveolar sacs was observed in mice inoculated by Vn (Figure 8A). In tumour nodules formed after 14–18 days post-inoculation, cell infiltration was reduced. A small number of infiltrated cells mostly comprised granulocytes were also seen (Figure 8C), although with low frequency. Fas-L ribozyme reduced significantly the number of infiltrating granulocytes in tumour nodules of 96 h and 18 days after cell inoculation (Figure 8B,D), but not the numbers of CD4+, CD8+ and NK cells.

Massive apoptotic cells were observed in the lung of mice received Vn cells in 48–96 h (Figure 9A) post-inoculation. Apoptosis still occurred frequently in tumour nodules formed by Vn.

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**Table 1** Metastatic tumour nodules in the lung after i.v. injection

| Groups        | Average number of gross tumour nodules/Nth day |
|---------------|-----------------------------------------------|
| Exp 1 (5 x 10^5 cells) | 14th day V3 8 ± 2 (3/3) R1 223 ± 34 (3/3) |
| Exp 2 (5 x 10^4 cells) | 14th day V1 ND* (0/4) R5 11 ± 4 (4/4) |
| Exp 3 (5 x 10^4 cells) | 18th day V 3.3 ± 1.5 (3/3) V4 15.0 ± 4.4 (3/3) R 65.8 ± 2.4 (3/3) R6 85.7 ± 7.0 (3/3) |
| Exp 4 (5 x 10^4 cells) | 14th day V2 1 ± 1 (3/3) R4 112 ± 55 (2/2) R2 137.4 ± 75.6 (5/5) |

*Number in paragraph is the number of tumour-bearing mice over total mice. *ND: Although lung tumour nodules were not grossly observed, some tiny tumour cell mass was seen in tissue section under light microscopy.
after 14 days post-inoculation (Figure 9C). Furthermore, a few death cells in tumour nodules were morphologically tumour origin and TUNEL-staining positive. Apoptosis was ameliorated significantly in tumour nodules of Rn (Figure 9B,D). We further evaluated the apoptosis in granulocyte-depleted mice. Few cells were TUNEL-positive in the tumour nodules obtained from granulocyte-depleted mice inoculated with Rn or Vn for 96 h (Figure 9E,F).

DISCUSSION

The application of Fas-L ribozyme effectively and specifically suppressed the expression of Fas-L in B16F10 cells as revealed by RT–PCR, Western blot and immunohistochemistry. Transfectants carrying Fas-L ribozyme grew slightly faster in vitro with better viability than vector controls. Since Fas-L ribozyme did not alter the expressions of Fas and TNF-α, a suppression of the Fas-L by Fas-L ribozyme resulting in less Fas/Fas-L ligation should account for the reduced apoptosis in vitro. The suicidal destruction of tumour cells has been reported in Fas signal-sensitive tumours after transfer of Fas-L gene (Arai et al., 1997; Walker et al., 1998). In addition, delivering Fas-L ribozyme into human glioma or Ras-activated NIH3T3 malignant cell lines also reduced apoptosis in those cells (Chio et al., 2001, our unpublished data). It seems that the Fas/Fas-L suicidal effect could occur widely in different malignant cells. Although a growth-promoting reverse signalling for lymphocytes through Fas-L has been suspected (Suzuki and Fink, 1998), down-regulation of Fas-L in melanoma cells caused an increase in growth rate in vitro indicating that Fas-L did not elicit such reverse signalling in melanoma.

We then further evaluated the lung metastatic potential of Fas-L ribozyme transfectants in C57BL6 mice. Down regulation of Fas-L by Fas-L ribozyme drastically enhanced lung metastasis that was correlated with reductions in both apoptotic tumour cells and granulocytic infiltration. The recruitment of granulocytes in the Fas-L-positive tumours can be attributed to the proinflammatory effect of Fas-L that has been observed in several studies using ectopic Fas-L expressing cells (Seino et al., 1997; Chen et al., 1998). Alternatively, apoptotic body generated due to suicidal destruction by itself has also been demonstrated to be a potent chemotactic agent for cell recruitment (Horino et al., 1998). When phagocytes

| Time       | Vector (V13) | Ribozyme (R6) |
|------------|--------------|---------------|
| Lung sections |              |               |
| 48 h       | 9.9 ± 5.9    | 1.8 ± 1.1     |
| 96 h       | 8.7 ± 3.6    | 2.0 ± 1.1     |
| Tumour nodules |            |               |
| 18th day   | 10.7 ± 8.6   | 1.3 ± 2.0     |

*Granulocytes stained by anti-Ly-6G antibody in 30 random observation fields in the lung. **Cells in 40 lung tumour nodules, that contained around 45 – 55 tumour cells per nodule. Shown are mean ± s.d.**

Figure 6 The formation of metastatic lung tumours in mice depleted for CD4+-, CD8+-cells or granulocytes. Vn: vector controls; Rn: Fas-L ribozyme carrying cells. Values shown are average of three independent experiments. The numbers shown in parentheses over bars indicated the original total number of lung tumour nodule. *P<0.05; **P<0.01.

Figure 7 Representative immunohistochemical staining of Fas-L protein on tumour nodules. Lung tumour nodules of vector control (A) or Fas-L ribozyme-carrying cells (B) were surgically obtained at 18 days post-inoculation and fixed in 4% paraformaldehyde. Cryosection was immunostained with antibody specific for Fas-L. Fas-L-positive cells show a reddish-brown colour. (C) Lung samples of mice 96 h post-inoculation; (D) lung nodules of 18 days post-inoculation.

Figure 8 Tumour infiltrating granulocytes. Tumour formation and tissue preparation were performed as the procedures described in Figure 4. Cryosections were immunostained with RB6-8C5 antibody recognising granulocytes. Positive cells show a reddish-brown colour. (A) and (C) vector control; (B and D) Fas-L ribozyme-carrying cells. (A and B) lung samples of mice 96 h post-inoculation; (C and D) tumour nodules of 18 days post-inoculation.

Table 2 Granulocyte infiltration in the lung and tumour nodules
highest number of tumour nodules among all clones tested and that could not be further enhanced by granulocyte depletion. Besides, other uncharacterised factors may also contribute to tumour metastasis, which would attenuate minor differences in tumorigenicity between clones having litter difference in Fas-L expression and diminish the expected dose effect. As mentioned earlier, loss function of Fas has been linked to metastatic progression (Owen-Schaub et al, 1998). Similarly, we observed in this study that lung metastasis was negatively correlated with the Fas/Fas-L suicidal destruction occurred in melanoma cells in vitro and the appearance of apoptotic cells in lung tumour nodules. However, several lines of evidence showed that the autocrine suicidal Fas/Fas-L interaction was not the major limiting factor, at best acted only as an initiator, for the Fas-L associated apoptosis in vivo and for the reduced potential in metastasis. First, in the absence of granulocytes, the expression of Fas-L on tumour did not inhibit tumour metastasis. High Fas-L expressing cells, though showing high apoptosis in vitro, could efficiently develop lung tumours in granulocytes-depleted mice to a degree as Fas-L ribozyme-carrying cells did. Second, the apoptosis in tumour nodules obtained from granulocyte-depleted mice was drastically reduced, evidence of a granulocyte-dependant apoptosis in tumour. In these experiments, the suicidal Fas/Fas-L signal in cells was not altered. Therefore, tumour cell death is indeed a restriction factor for lung metastasis. However, the death signal is not directly due to the suicidal Fas/Fas-L signal. In addition, neutrophils isolated from peritoneal do kill tumour cells in vitro, though with different efficiency depending on whether they carrying Fas-L ribozyme or not (our unpublished data). Thus, the recruited granulocytes mediate primarily the destruction of metastatic tumour cells in the lung.

A pivotal role of cells of innate immunity in tumour combat has previously been recognised in several tumours. The action of tumour-suppressive Th1 cells through CpG DNA is granulocyte-dependent (Egeter et al, 2000). Neutrophils, but not T cells, mediate the primary rejection of Fas-L-overexpressing, Fas-negative tumour cells in vivo (Seino et al, 1997). Our study provides firm evidence for the anti-tumour effect of granulocytes in lung metastasis. During the lung metastasis, T cells have few impacts on tumour control. Depletions of CD4⁺ or CD8⁺ cells did not affect (clone V3) or even slightly inhibit (clone V4) the progression of tumours having high Fas-L. Recent studies have also demonstrated that T cells specific for tumour antigens can become actively tolerated during progression of tumours (Mackensen et al, 1993; Adler et al, 1998). It is possible that altered T cells may produce a distinct profile of cytokine productions, which in turn stimulate tumour formation (Medvedev et al, 1997; Mori et al, 1997).

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Granulocytes and Fas-L-associated tumour metastasis

Y-L Chen et al

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