FADD protein release mirrors the development and aggressiveness of human non-small cell lung cancer

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BACKGROUND: The need to unfold the underlying mechanisms of lung cancer aggressiveness, the deadliest cancer in the world, is of prime importance. Because Fas-associated death domain protein (FADD) is the key adaptor molecule transmitting the apoptotic signal delivered by death receptors, we studied the presence and correlation of intra- and extracellular FADD protein with development and aggressiveness of non-small cell lung cancer (NSCLC).

METHODS: Fifty NSCLC patients were enrolled in this prospective study. Intracellular FADD was detected in patients’ tissue by immunohistochemistry. Tumours and distant non-tumoural lung biopsies were cultured through trans-well membrane in order to analyse extracellular FADD. Correlation between different clinical/histological parameters with level/localisation of FADD protein has been investigated.

RESULTS: Fas-associated death domain protein could be specifically downregulated in tumoural cells and FADD loss correlated with the presence of extracellular FADD. Indeed, human NSCLC released FADD protein, and tumoural samples released significantly more FADD than non-tumoural (NT) tissue (P = 0.000003). The release of FADD by both tumoural and NT tissue increased significantly with the cancer stage, and was correlated with both early and late steps of the metastasis process.

CONCLUSION: The release of FADD by human NSCLC could be a new marker of poor prognosis as it correlates positively with both tumour progression and aggressiveness.

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Lung cancer is the deadliest cancer in the world. Non-small cell lung cancer (NSCLC), which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC), represent ~80% of the lung cancer. The usual therapy of NSCLC consists of surgical resection of the tumour possibly preceded or followed by chemotherapy or radiotherapy. Nevertheless, most patients have poor clinical outcome in spite of these treatments, in view of the tumour-node–metastasis (TNM; T = primary tumour, N = regional lymph nodes, M = distant metastasis) status (Mountain, 1997) of the patients on the day of surgery. Although the 5-year post-surgery survival rate is >70% for T1N0M0 patients, it decreases to 10–30% when lymph nodes are invaded by tumour cells (N1–N2), and declines to <5% in distant metastasis (M1). For these reasons, the need to unfold the underlying mechanisms of lung cancer aggressiveness is of prime importance.

It has been observed that functional Fasl was expressed in most resected NSCLC cells (Niehans et al., 1997). Thus, NSCLC might have developed Fas-mediated apoptosis resistance mechanisms. Because FADD (Fas-associated death domain protein) is the key adaptor molecule transducing the apoptotic signal mediated by all death receptors (DRs) of the TNF receptor superfamily (Chinnaiyan et al., 1996; Kuang et al., 2000), its regulation of expression was a good candidate. Indeed, the absence of FADD can confer numerous advantages to tumour cells including multiple resistance to DRs and some anticancer drugs (Micheau et al., 1997), acquired ability to coexpress DRs and ligands, and a proliferative advantage (Tourneur et al., 2003). Furthermore, we previously confirmed that FADD can also act as a tumour suppressor (Newton et al., 2000; Tourneur et al., 2005) by showing that (1) FADD protein could be lost in vivo in mouse thyroid adenoma/ADC cells and human acute myeloid leukaemia cells, (2) Fas signalling, in absence of FADD, led to an accelerated growth of thyrocytes, and (3) low level or absence of FADD in leukaemia cells at diagnosis was of poor prognosis in patients’ chemotherapy response (Tourneur et al., 2003; Tourneur et al., 2004).

The FADD protein is located both in the cytoplasm and nucleus of most cells (Gomez-Angelats and Cidlowski, 2003; Sreenan et al., 2003; Tourneur et al., 2009). Recently, we and others ascertained a new localisation site and regulatory mechanism for the protein (Tourneur et al., 2008),(Kriebardis et al., 2008). We demonstrated that FADD could be held within plasma membrane-derived...
microvesicles, and rapidly released in the extracellular compartment following shedding of these vesicles. Moreover, we showed that adenosine receptors (AR) are implicated in this process. We showed that treatment with A3AR inhibitor but not A1AR, A2aAR, and A2bAR inhibitors triggers FADD release from mouse lung (Tourneur et al, 2008), demonstrating that in the lung FADD secretion is a regulated process.

Herein, we tested the hypothesis that FADD release could occur during lung cancer and be a useful marker in NSCLC. To do so, we analysed a prospective cohort of 50 NSCLC patients.

**PATIENTS AND METHODS**

**Patients**

Human primary lung tumour biopsies from 50 successive NSCLC patients were collected at the Hôpital-Dieu hospital and the Institut Mutualiste Montsouris between April 2008 and 2009, during the course of complete surgical resection of their tumours (stages I–III), including multilevel lymph node sampling or lymphadenectomy. For each patient, distant lung tissue (taken at 8–10 cm from the primary tumour) was used as non–tumoural (NT) tissue. Early metastatic invasion was defined by the presence of blood and/or lymphatic vascular emboli (BVE/LVE). The TNM stage and BVE/LVE status of the tumours were determined from the histopathological reports obtained at the time of resection. Histopathological and clinical findings were scored according to staging system of the American Joint Committee for Cancer Staging and End Results Reporting, and the TNM staging system of the Union Internationale Contre le Cancer (Mountain, 1997). The main clinical and pathological features of the patients are presented in Table 1. All patients’ identities were coded to protect confidentiality. Consent was obtained from all patients and the study was approved by the local ethical committee (Agreement 2007-A00845-48).

**Table 1** Patients characteristics

| Category               | Subcategory | All patients with NSCLC (n = 50) |
|------------------------|-------------|---------------------------------|
| Surgery, n             |             |                                 |
| HDH                    |             | 28                              |
| IMM                    |             | 22                              |
| Sex, n (%)             |             |                                 |
| Male                   |             | 32 (64%)                        |
| Female                 |             | 18 (36%)                        |
| Age, year              |             |                                 |
| Mean                   |             | 63.4                            |
| Range                  |             | 44–82                           |
| Smoking history, n     |             |                                 |
| Never smoker           |             | 21                              |
| Passive smoker         |             | 1                               |
| Smoker                 |             | 28 (56%)                        |
| Pack/year              |             | 40 (mean) 5–80 (range)          |
| Preoperative chemotherapy, n (%) |         | 8 (16%)                         |
| Histological type, n (%)|           | 29 (58%)                        |
| ADC                    |             | 12 (24%)                        |
| SCC                    |             | 8 (16%)                         |
| Stage, n (%)           |             |                                 |
| I                      |             | 19 (38%)                        |
| IA                     |             | 6                               |
| IB                     |             | 13                              |
| II                     |             | 11 (22%)                        |
| III                    |             | 20 (40%)                        |
| IV                     |             | 15                              |
| V                      |             | 3                               |

### Abbreviations:
- NSCLC = non-small cell lung cancer
- ADC = adenocarcinoma
- SCC = squamous cell carcinoma
- LCC = large cell carcinoma
- HDH = Hôpital-Dieu Hospital
- IMM = Institut Mutualiste Montsouris
- TNM = pathologic TNM stage
- Neoadjuvant chemotherapy was based on platinum salt regimens.

**In vitro culture of human lung biopsies**

All tumours and distant normal lung tissues were obtained immediately at the time of surgery and transported on ice to the laboratory in RPMI 1640 medium. Lung biopsies were stored at 4°C and treated 1 day after surgery, after we have checked that storage did not modify the FADD release process. Small lung samples (1–5 mm³) were incubated in 200 μl PBS medium in the upper side of a trans-well membrane with 0.4 μm pores (BD353495, Becton Dickinson, Franklin Lakes, NJ, USA) to avoid red blood cells contamination. Lower side of the trans-well initially contained 100 μl PBS medium. After 1 h incubation at room temperature, culture mediums were collected in the lower side of the membrane and their volume measured. Lung culture mediums were kept at −20°C until FADD detection was performed. One hour-incubated lung samples were removed and kept at −20°C until protein extraction was performed.

**Protein extraction and dosage**

Total cytoplasmic proteins were extracted from lung samples using lysis buffer (10 mM Tris–HCl, 50 mM NaCl pH 7.4, 1% Triton X100, containing a cocktail of protease inhibitors) and tissue homogeniser MP FastPrep-24 (MP Biomedicals SARL, Illkirch, France). Protein extract volume was measured and sample concentration was determined using the Bradford protein assay. We then calculated the total cytoplasmic proteins quantity in each lung sample.

**Fas-associated death domain protein-specific quantitative sandwich ELISA**

To detect soluble FADD, 2.5 μl of PBS containing 1% Tween 20 were added to 50 μl of human lung culture mediums that were then heated for 5 min at 100°C. The ELISA analysis was conducted using anti-hFADD antibody clone A66–2 (1 μg/ml−1 in PBS/0.05% Tween 20/2%/BSA; BD Pharmingen, San Diego, CA, USA) for detection, and peroxidase-conjugated anti-mouse IgG1 antibody (1:8000 in PBS/0.05% Tween 20/2%/BSA; Caltag, Burlingame, CA, USA) for revelation. The colorimetric reaction was revealed by adding 3,3′,5,5′-tetramethyl-benzidine. Reaction was stopped with 2 N sulphuric acid and the plates were read at 450 nm with a Titertek multiscan spectrophotometer (MR5000, Dynatec, Mettmann, Germany). Recombinant human FADD protein (Sigma-Aldrich) was used to perform standard range. As lung biopsies’ size varied among the patients, for each lung sample the released FADD protein quantity (in ng) was reported to the total cytoplasmic proteins quantity contained within the sample (in mg), which reflected the biopsy size.

**Immunohistochemistry**

Immunostaining with FADD antibody was performed on paraffin sections of lung tumour and distant non-tumour tissues. After antigen retrieval, FADD antibody (mouse monoclonal anti-hFADD clone 64A6, Abcam, Cambridge, UK), 1:50 dilution) was applied and revealed with a streptavidin-biotin-peroxidase kit (BiOSPA, Abcys, Paris, France). Peroxidase activity was revealed using diaminobenzidine (Dako Cytomation, Glostrup, Denmark). Tissue sections were then counterstained with Harris haematoxylin (Sigma-Aldrich chimie SARI, Saint Quentin Fallavier, France), and slides were mounted with Glycergel Mounting Medium (Dako Cytomation) and visualised by light microscopy (Axiovert II, Leica, Solms, Germany). Blinded examination of sections was done by an experienced lung pathologist (DD).

**Extracellular FADD as a new prognostic marker in NSCLC**

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FADD was mainly expressed in tumoural cells located at the periphery of the tumour nests (data not shown). Whereas tumoural cells from stage I patients expressed FADD, the expression in tumoural cells from stage II patients was very low to barely detectable (Table 2). However, once the cancer has fully fixed itself (stage III), FADD expression did not decrease anymore. In all cases, we detected FADD expression exclusively in cytoplasm (Figure 1C).

Tumour samples are heterogeneous tissues containing tumoural cells, potentially hyperplastic cells, normal lung cells and immune cells. In light of that, we investigated the release's potential of different areas of a same tumour biopsy. Distant NT tissue that is postulated to contain only normal lung cells was used as control. We detected the FADD protein in the culture medium from both T and NT NSCLC biopsies (Figure 2A). These results argued for FADD release by human cells in vitro. As expected, FADD release by NT tissues was quite homogeneous for different samples from the same biopsy (mean s.d. = 7.4 ± 2.9 ng FADD per mg proteins in the tissue (PT), Figure 2A). In contrast, the amount of FADD released by different areas from the same T biopsy varied (mean s.d. = 12.3 ± 3.8 ng mg⁻¹ PT, Figure 2A).

We then investigated FADD release by T and NT tissues from a 50 NSCLC patients prospective cohort. We detected extracellular FADD protein in the culture medium from both T and NT NSCLC biopsies and showed that T tissue released significantly more FADD than distant NT tissue (Figure 2B, P = 0.000003). Results obtained by ELISA were confirmed by western blot analysis (Supplementary Figures 1–3). We observed no correlation between

**Table 2** Loss of FADD in ex vivo NSCLC

| Patients | Stage | Tumour | Alveolar EC | Bronchial EC |
|----------|-------|--------|-------------|--------------|
| 35       | I     | 3      | np          | np           |
| 31       | I     | 3      | 1           | 3            |
| 77       | I     | 1.5    | 0 and 1 near T | 3            |
| 76       | I     | 2      | 0.5         | 3            |
| 69       | I     | 1.5    | 0           | 3            |
| Mean stage I | 2.2 | 0.5   | 3           |
| 44       | II    | 0      | 0 and 1 Hyp | 3            |
| 70       | II    | 0.5    | 0           | np           |
| 30₁     | II    | 3/0 LNM | 2/0         | 3/2          |
| 55₁     | II    | 0.5/np | 0/np        | 2/3          |
| 50      | II    | 0.5    | 0           | np           |
| 48₁     | II    | 1/np   | 0.5/0       | 1/1          |
| Mean stage II | 0.8 | 0.4   | 2.1         |
| 37       | III   | 2      | 0           | np           |
| 33       | III   | 3      | 1           | 3            |
| 49      | III   | 2      | 0           | 3            |
| 86₁     | III   | 3/3    | 0.5/np      | 2/3          |
| 83      | III   | 0 and 2 VE | 0.5        | 3            |
| 82₁     | III   | 2/np   | 2/0         | 2.5/2        |
| 68₁     | III   | 2/np   | np/np       | np/np        |
| 61₁     | III   | 1.5/np | 0/0         | 3/1          |
| 87      | III   | np     | 0           | 3            |
| 88₁     | III   | np/2   | 2/0         | 3/3          |
| 59₁     | III   | np/0.5 | 0 and 2 Hyp/1 | 3/3          |
| 46      | III   | 1      | 1.5         | 2            |
| 26₁     | III   | 1/np   | 0/0         | np/np        |
| Mean stage III | 1.8 | 0.6   | 2.6         |

Abbreviations: EC = epithelial cells; FADD = Fas-associated death domain protein; np = structure not present in the section; Hyp = hyperplasia; LNM = lymph node metastasis; VE = vascular emboli; T = tumour. Correlation between patients' cancer stage and FADD expression in T area determined by IHC. All patients were treated at the Hôpital-Dieu hospital. *0: neg 0.5, +: 1, +: 1.5, ++: 2, +: 2.5, ++: 3, +++: 3. + indicates that two samples of a same biopsy were embedded in distinct paraffin blocks that were sectioned and analysed.

**RESULTS**

**Fas-associated death domain protein loss by human NSCLC**

We investigated FADD protein expression in NSCLC by immunohistochemistry. In distant NT (n = 6) lung tissue, FADD protein expression was restricted to bronchial epithelial cells and was never found in normal alveolar epithelial cells (pneumocytes) (Figure 1A). Similarly, in the tumour (T, n = 24) area, high and low FADD expression was observed in bronchial and alveolar cells, respectively (Table 2). We found a variable patient-dependent FADD protein expression in tumoural cells (Table 2 and Figure 1B), with sometimes coexistence of both FADD-positive and -negative tumoural cells within the same T area. In such cases,
FADD release by lung biopsies and patient’s hospital, sex, age, chemotherapy, tobacco consumption, or tumour necrosis state (Spearman’s correlation test, Figure 2C).

Presence of extracellular FADD in human NSCLC culture depends on the histological type

We investigated whether FADD release differed according to histological type. We observed that T biopsies released significantly more FADD than NT biopsies, and this independently of the histological type (Figure 2D). The NT biopsies from ADC, SCC, and LCC patients released similar level of FADD (Figure 2E). Despite being a morphologically and clinically heterogeneous disease, ADC was the NSCLC with the most reproducible extracellular FADD level, demonstrating that the differentiation state of ADC did not influence the FADD release process (Figure 2D). Moreover, T ADC was the histological type releasing significantly the least FADD, whereas T LCC was the one releasing the most \( (P = 0.01, \text{Figure 2E}) \).

Presence of extracellular FADD in human NSCLC culture is correlated with patients’ cancer stage

To investigate whether FADD release could be involved in tumour progression and aggressiveness, we looked for a correlation between extracellular FADD level and patients’ cancer stage. We showed that FADD release by both T and NT biopsies increased with the cancer stage (Figure 3A and B), suggesting that extracellular FADD could be a poor prognostic marker. Fas-associated death domain protein increase was significant for...
Figure 3  Fas-associated death domain protein release is correlated with cancer development. (A–C) Fas-associated death domain protein release is correlated with patients’ cancer stage. Levels of released FADD determined in Figure 2B were reanalysed after the 50 NSCLC patients were classified according to their cancer stage. Each diamond represents a patient biopsy (T in black, NT in white). Bars represent the mean value.

Presence of extracellular FADD in human NSCLC culture is correlated with early and late steps of the metastasis process

Early steps of the metastasis process include blood and lymphatic vessel emboli that can arise within the tumour or at its periphery. The T biopsies from patients with lung vascular emboli released a higher level of FADD than those of unaffected counterparts (Figure 4A, \( P = 0.04 \)). Whatever its type (BVE/LVE) and localisation (intra/peri-tumour), the emboli was always correlated with an increased amount of extracellular FADD (Supplementary Tables 1 and 2). Distant NT biopsies from NSCLC patients with lung emboli released amount of FADD equivalent to T biopsies from patients without emboli (T (A) vs NT (P)). These results confirmed that invasive/aggressive tumour (i.e., able to form distant metastasis) affected distant ‘non-tumour’ lung tissue that behaved like T tissue regarding FADD release. These results suggested that FADD release by T/NT biopsies and vascular emboli formation are positively correlated.

DISCUSSION

The overall data, obtained in a prospective cohort of patients, showed that FADD release by both T and NT biopsies was positively correlated with the patients’ cancer stage, the early and late steps of the metastasis process, and the histological type (Figure 2D, E, 3 and 4). Moreover, the data described herein brings an explanation for intracellular FADD loss by human NSCLC tissues that could result from FADD release. As patient’s TNM stage, presence of tumour vascular emboli, lymph node metastasis, and histological type are poor prognostic factors, the ability of lung tissue to release FADD appeared as a poor prognostic marker.
As we performed a prospective study, all patients were alive at the time of the study. Consequently, at the present time we could not correlate presence of extracellular FADD and patients’ overall survival.

We first investigated FADD protein expression in NSCLC and found a variable cancer stage-dependent FADD protein expression in tumoural cells, with a very low expression in tumoural cells from stage II patients. The presence of hyperplastic cells observed in some NT biopsies (not shown) could support the latter hypothesis. Data showing that FADD release by NT biopsies evolved similarly to FADD release by T tissue compared with NT tissue suggests that FADD release could be a regulated process preferentially occurring in pathological conditions, and thus potentially contributing to cancer. These results obtained in human NSCLC are in accordance with those obtained in mice showing that FADD secretion does not occur spontaneously but rather in a controlled manner in the lung (Tourneur et al., 2008). The fact that human biopsies are not taken from healthy lungs but at a distance from the tumour could explain the discrepancy between the lack of secretion by normal mouse tissue and the observed release by human NT lungs. One other possibility is a differential regulation of such process in humans and mice. It is also possible that the tumour environment either influences, modifies, or even enables the NT tissue to release FADD. The presence of hyperplastic cells observed in some NT biopsies (not shown) could support the latter hypothesis.

Several mechanisms could explain the presence of extracellular FADD in cultured NSCLC. We primarily hypothesised that FADD release was the result of cells dying within a tumour. However, the release of FADD started as soon as 5 min of biopsies’ incubation (Supplementary Figure 4), and in vitro incubation of lung biopsies never exceeded 1 h, which did not seem sufficient to induce a massive cell death. In accordance with this assumption, and independently of the level of FADD released, we observed only a slight activation of caspase 8 during FADD release, and no LDH (which is released upon cell lysis) could be detected in the culture medium from 1-h incubated NSCLC tumour biopsies (Supplementary Figure 5). Moreover, FADD release was not correlated with the necrosis state of the tumour (Figure 2C). Then, considering the results previously obtained in mouse normal lungs (Tourneur et al., 2008), we hypothesised that FADD release from human NSCLC could occur by the means of secretion. In accordance with this hypothesis, we found that FADD could be specifically lost in tumoural cells (Table 2), and the highest level of FADD was released by stage II’ T biopsies, which corresponds to the stage where the lowest cellular FADD expression was observed (Figure 1D). These data showed that, in tumour tissues, released and cellular FADD amounts were negatively correlated, suggesting that the release of FADD could much more be a mechanism accounting for cytoplasmic FADD loss than any type of cell death affecting specifically and in a stage-dependent manner the lung cancer cells.
that the level of released FADD by T biopsies and the patients 5-year survival rate were inversely correlated, confirming that FADD release may have an important role in tumour aggressiveness and may be used as a poor prognostic marker.

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Conflict of interest

The authors declare no conflict of interest.

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