Adenosine Receptors Control a New Pathway of Fas-associated Death Domain Protein Expression Regulation by Secretion

Received for publication, March 21, 2008. Published, JBC Papers in Press, April 29, 2008, DOI 10.1074/jbc.M802263200

Léa Tourneur1, Sylvie Mistou1, Alain Schmitt2, and Gilles Chiocchia1,3

From the 1Inserm, U567, Institut Cochin, 75014 Paris, France, the 2Université Paris Descartes, CNRS (UMR 8104), 75014 Paris, France, and the 3Service de rhumatologie, Hôpital Ambroise Paré, 92100 Boulogne, France

FADD is the key adaptor transmitting the apoptotic signal mediated by death receptors. We have previously shown that FADD protein expression could be lost in vivo in cancerous cells, in mice and humans, and be used as prognostic factor. Furthermore, loss of FADD could contribute to tumor progression and aggressiveness. However, the mechanism accounting for the loss of FADD was unknown. Using in vitro-cultured mouse organ models, we demonstrated that loss of FADD occurred through a new regulatory pathway of FADD expression by secretion. The secretion of FADD is an active release following shedding of microvesicles derived from the plasma membrane. In our experimental settings, this phenomenon was restricted to 6 of 12 FADD-expressing organs. This process is calcium- and adenosine-dependent. Moreover, we identified the two receptors with low affinity to adenosine, namely A2B and A3 adenosine receptors, as regulators of the FADD secretion process. Furthermore, we showed that modulating A3 adenosine receptor can convert a nonsecreting organ into a FADD-secreting one. Finally, we reported that mouse FADD release occurred in vivo during tumor disease. These results demonstrate the existence of a new localization site (in microvesicles) and regulatory mechanism (by secretion) of the FADD protein, and the implication of adenosine receptors in this process. These data open a new field of investigation consisting of the possibility to regulate FADD expression via the modulation of adenosine receptors, which constitutes a therapeutic target in diseases in which FADD-mediated signaling is impaired.

Fas-associated death domain protein (FADD)2 is a common adaptor molecule for the death receptors of the tumor necrosis factor receptor superfamily (1–4). Upon engagement of a death receptor by its ligand, FADD is recruited either directly or indirectly to the receptor. FADD then binds and activates procaspase 8, the initiator of a caspase cascade that ultimately results in cell death. Because the first role described for FADD was to interact with these death receptors localized at the plasma membrane, it was thought that FADD protein was mainly located in the cell cytoplasm. However, recent reports demonstrated that human FADD protein possesses nuclear localization and export signals (5, 6). The nuclear localization of FADD has been associated with a decreased ability of this protein to induce apoptosis of T cells, suggesting that the cytoplasmic protein could be involved in cell death, whereas the nuclear protein could be implicated in survival mechanisms (5).

The cytoplasmic FADD is a multifunctional protein. It has a central role in induction of apoptosis (1, 3), survival (7), growth/proliferation (8, 9), cell cycle progression (10, 11), and inflammation (12). Additionally, FADD can act as a tumor suppressor (13, 14). Using a murine model of thyroid adenoma/adenoarcinoma (the gsp mice, Ref. 15), we have previously shown that FADD protein expression is lost in vivo during the course of thyroid tumor progression (16). Moreover, we have identified a low level or absence of the FADD protein in leukemic cells at diagnosis as a poor prognostic factor for response of acute myeloid leukemia (AML) patients to chemotherapy (17). In both types of tumors, the absence of FADD protein expression was not due to an absence of FADD mRNA. We have found that the loss of FADD protein, but not mRNA, also occurred in in vitro primary cultures of normal thyroid follicular cells (TFC), and did not involve the proteasome (16) or any mechanism of regulation of protein degradation or expression tested (including transcription, translation, protein kinase, and caspase inhibitors, addition of environmental factors (including thyroid-stimulating hormone) or cytokines).3 Thus, up to now, the mechanism implicated in this regulation of expression has stayed unclear. Here, we used in vitro organ culture to seek for the mechanism(s) that could account for the loss of FADD protein. We describe a new regulatory mechanism of FADD protein expression following adenosine receptor signaling.

EXPERIMENTAL PROCEDURES

Mice—Normal mice were from Ifa Credo, Janvier, or Charles River. P2X7R−/− mice (18) were generously provided by Dr. J. 4

1 To whom correspondence should be addressed: Institut Cochin, Département d’immunologie, Pavillon Hardy, 27 rue du fbg St-Jacques, 75014 Paris, France. Tel: 33-1-40516615; Fax: 33-1-40516641; E-mail: gilles.chiocchia@inserm.fr.

2 The abbreviations used are: FADD, Fas-associated death domain protein; AML, acute myeloid leukemia; AR, adenosine receptor; EM, electron microscopy; PS, phosphatidylserine; TFC, thyroid follicular cells; PBS, phosphate-buffered saline; WT, wild type; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DAPI, 4′,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase.

3 G. Chiocchia, unpublished data.
Adenosine Receptor-dependent FADD Release

Kanellopoulos (Pasteur Institute), and J. Mobley (Pfizer). The studies were approved by the Cochin Institute Committee on Animal Care. The agreement number to perform experiments on living animals is 75-1384 and the animal facility agreement number is A75-14-02.

In Vitro Culture of Mouse Organs—Animals were euthanized by CO₂ inhalation, and the different organs removed after intracardiac puncture. Organs were incubated in an appropriate volume (100 μl/thyroid) of RPMI 1640 medium (Invitrogen) without supplement at 37 °C in a 5% CO₂ atmosphere or in specific medium as indicated. Culture supernatants were then collected and centrifuged 1 min at 12,000 rpm to remove contaminating tissue. Alternatively, organs were incubated in the upper side of a trans-well membrane with 0.4-μm pores (Falcon), and the culture medium was collected in the lower side of the membrane.

Lactate Dehydrogenase (LDH) Assay—Organs were cultured 1 h in RPMI 1640 medium through a trans-well membrane with 0.4-μm pores. Culture medium was then collected in the bottom of the well, and the organs were lysed mechanically in water, frozen, and thawed. LDH activity in the collected culture medium (secreted LDH) and organ lysate (cellular LDH) was determined by measuring the change in absorbance at 490 nm using an LDH kit (Tox-7, Sigma-Aldrich) in a microplate reader (EL800, Bio-Tek Instruments, Inc). The percentage of LDH release was calculated as (secreted LDH/total LDH) × 100, where total LDH = secreted LDH + cellular LDH.

Reagents—All reagents were from Sigma-Aldrich, unless otherwise indicated.

Western Blot—Total proteins were extracted from organs with lysis buffer (10 mM Tris-HCl, 150 mM NaCl pH 7.8, 1% Nonidet P-40, containing a mixture of protease inhibitors (Roche Applied Sciences)), and sample concentrations were determined as previously described (16). Laemmlı buffer was added to total proteins (40 μg) or to 20 μl of collected supernatant culture, and boiled for 5 min. Then samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane (PerkinElmer Life Sciences), and probed with specific primary anti-FADD antibody (M-19, 0.2 μg/ml in TTBS 0.1% containing 5% milk) (Santa Cruz Biotechnology). Alternatively, anti-FADD antibody from Chemicon International was used. The proteins were visualized using the enhanced chemiluminescence technique (Amersham Biosciences). Bands obtained were quantified by densitometry using bioquant® and bio-profile biod1® software.

Immunohistochemistry—The thyroid lobes were immediately covered in optimal temperature medium (Tissue-Tek). Sections of 5–6 μm were cut on a cryostat at −18 °C, collected onto SuperFrostplus slides (Roth Sochiel), dried overnight, fixed in acetone, saturated in 10% normal horse serum buffer, and stained with anti-FADD (M-19, 10 μg/ml) antibody followed by biotin-conjugated secondary antibody (VECTOR). Avidin-alkaline phosphatase (Amersham Biosciences Life Science) and FastRed substrate (Acros Organics) were used to visualize specific staining. Sections were counterstained in hemalun (Fisher Scientific) and mounted in an aqueous mount (Aquatex).

Immunofluorescence—Thyroid sections were prepared as for immunohistochemistry except that sections were fixed in PBS containing 2% PFA, saturated in 2% bovine serum albumin buffer, and that Alexa Fluor® 488 conjugate streptavidin (10 μg/ml, Molecular Probes) was used to visualize specific staining. To evaluate PS exposure, sections were incubated with 5 μl of fluorescein isothiocyanate-conjugated annexin V (BD Pharmingen). Sections were mounted in VECTASHIELD® Mounting Medium with DAPI (Vector), and analyzed using a confocal fluorescence microscope (Bio-Rad MRC1024) equipped with a digital Diaphot 300 system. Digital pictures were analyzed using LaserSharp software and processed using Adobe Photoshop®.

Trypsin Digestion—Thyroid lobes were cultured in RPMI 1640 medium through a trans-well membrane with 0.4-μm pores. Following a 30-min incubation, medium was collected as previously described. 100 ng of heat-denatured control mouse IgG1 (BD Pharmingen) was added to 20 μl of collected culture medium. 2 μg of trypsin proteomics grade (Sigma-Aldrich) were then added to the mix and incubated 2 h at 37 °C. As control, mixed culture medium/IgG1 was incubated 2 h at 37 °C without trypsin. Thereafter, Laemmlı buffer was added, samples boiled for 5 min, and FADD Western blot analysis was performed as previously described. To ascertain trypsin digestion efficiency, the membrane was also probed with anti-mouse IgG1 specific antibody (Caltag).

Isolation of Annexin V-expressing Microvesicles—Thyroid lobes from eleven mice were pooled and cultured in 1 ml RPMI 1640 medium for 1 h. Thereafter, culture supernatant was collected and used directly without centrifugation. Microvesicles were isolated as described by MacKenzie et al. (19).

Electron Microscopy Processing for Ultrastructure—Tissue was fixed for 1 h with 3% glutaraldehyde. Samples were post-fixed in osmium tetroxide, 1% in 0.1 M phosphate buffer, then dehydrated in 70, 90, and finally 100% ethanol. After 10 min in a mixture of 1.2 epoxy propane and epoxy resin, the tissue was embedded in gelatin capsules with freshly prepared epoxy resin and polymerized at 60 °C for 24 h. 80-nm sections were cut with an ultramicrotome (Reichert ultracut S), stained with uranyl acetate and Reynold’s lead citrate, and observed with a transmission electron microscope (Philips CM10).

Immunogold Electron Microscopy—Thyroid lobes were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), then embedded in sucrose, and frozen in liquid nitrogen. Cryosections were made using an ultracyromicrotome (Reichert Ultracut S.), and ultrathin sections mounted on Formvar-coated nickel grids were prepared. Sections were incubated with PBS, 15% glycine (15 min), PBS, 15% glycine, 0.1% bovine serum albumin (5 min), and PBS, 15% glycine, 0.1% bovine serum albumin, 10% normal donkey serum (20 min), followed by anti-FADD antibody (M-19, 2 μg/ml, 2 h) diluted in PBS, 15% glycine, 0.1% bovine serum albumin, and 4% normal donkey serum. Alternatively, anti-FADD antibody from Calbiochem was used. After extensive rinsing, sections were incubated with gold-labeled secondary antibody with a gold particle size of 10 nm (GAM 10, British Biocell International), washed again, stained with 2% uranyl acetate (10 min), and air-dried. Sections were examined using a Philips CM 10 electron microscope.
Measurement of Released ATP—Organs were cultured for various periods of time in RPMI 1640 medium through a transwell membrane with 0.4-μm pores. Culture medium (100 μl) was then collected in the bottom of the well, and released ATP was measured using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. Luminescence was measured in a luminometer (Wallac 1420 VICTOR Luminometer, PerkinElmer Life Sciences, Inc).

**RESULTS**

**Loss of FADD Protein Expression in in Vitro-incubated Thyroid Resulted from FADD Release in the Extracellular Compartment**—In vitro incubation of entire thyroid organ induced a rapid loss of FADD protein within minutes of culture, as shown by Western blot analysis (Fig. 1A), and by confocal immunofluorescence microscopy (not shown). From 5 min after incubation and up to 60 min, FADD was either not expressed or barely detectable (Fig. 1A) with the persistence of a high molecular mass band (>120 kDa, not shown). Moreover, FADD was slightly detectable when Western blotting was performed under reducing conditions. Under these conditions, the >120-kDa molecular mass band disappeared, and a 70-kDa band was detectable (not shown). Although these data are consistent with FADD protein belonging to a large complex of proteins, the amount of FADD protein potentially present in this complex does not represent all the FADD expressed in ex vivo thyroid, demonstrating that a part of the protein is lost. Western blot analysis revealed the presence of FADD in the culture supernatants as soon as 10 s after culture of thyroid lobes (Fig. 1B). The amounts of FADD in culture supernatant accumulated progressively, and the levels were still high after 4 h of culture (not shown). These results were confirmed using two different anti-FADD antibodies and strains of mice with different genetic backgrounds including CBA/J, DBA/1, DBA/2, C57BL/6, and BALB/c (not shown).

To test whether FADD release was an active process rather than a consequence of TFC death, we compared the histological features of ex vivo and 1 h RPMI 1640-incubated in vitro-cultured thyroid lobes. We showed that ex vivo and in vitro cultured thyroid lobes presented similar histological features with no necrosis or apoptosis detected (see supplemental Fig. S1). Moreover, thyroid lobes were incubated in RPMI 1640 medium for 1 h, then immersed for 5 min in trypan blue vital colorant, and sections prepared for microscopic analysis. We observed that colorant was fully excluded from the organ (not shown), confirming that TFC were alive, and that the release of FADD protein did not result from plasma membrane permeability leading to nonspecific escape of proteins. Furthermore, no LDH, which is released upon cell lysis, was detected in the thyroid culture supernatants (Fig. 3B), indicating that the release of FADD protein did not result from plasma membrane disruption. On the other hand, electron microscopy analysis of in vitro-incubated thyroid sections showed no morphological changes characteristic of apoptosis (like chromatin condensation and membrane blebbing), and mitochondria were not altered (observation of ~60 TFC, see supplemental Fig. S2). The absence of chromatin condensation

![FIGURE 1. Loss of FADD in the in vitro thyroid lobes results from protein secretion. A, loss of FADD protein expression in cultured thyroid lobes. Upper panel, kinetic analysis of FADD protein expression in thyroid from CBA/J mice using Western blot under nonreducing conditions. One thyroid is used for each time point. Extraction of the cell proteins with a lysis buffer containing Triton or Chaps instead of Nonidet P-40 gave similar results, confirming that the absence of FADD is not due to a problem of protein solubilization (not shown). This experiment is representative of 15. Lower panel, immunohistochemistry analysis of FADD protein expression in thyroid lobes from C57BL/6 mice. The same results were obtained when thyroid lobes from CBA/J or BALB/c mice were analyzed. Bars represent 250 μm. B, kinetics of FADD detection in extracellular medium by Western blot. C, confocal microscopy analysis of DAPI staining of ex vivo and 1-h incubated thyroid lobes. Bars represent 2 μm. D, inhibition of FADD release following incubation at 4 °C. The histogram represents quantification of bands obtained by Western blot.](image-url)
Adenosine Receptor-dependent FADD Release

FIGURE 2. FADD protein secretion from thyroid lobes occurs through microvesicle shedding. A, confocal immunofluorescence microscopy analysis of ex vivo C57BL/6 thyroid lobes. The same results were obtained in three independent experiments. Controls included use of secondary (control) or isotype-matched antibodies. Bars represent 50 μm. Co, colloid; Cy, cytoplasm; F, follicle; N, nucleus. B, Western blot analysis of FADD-containing supernatant (left) or protein lysate (right) treated (+) or not (−) by trypsin, FADD (top) and control mouse IgG1 (bottom). IgG1 was added to culture medium from thyroid lobes or protein lysate from liver before trypsin digestion. Western blots were performed under reducing conditions. Anti-mouse IgG1 antibody recognizes a 50-kDa fragment of IgG1. C, confocal microscopy analysis of ex vivo thyroid stained with annexin V (top). Annexin V staining was concentrated in the colloid in close contact with TFC as indicated by arrows. The bar represents 25 μm. FADD detection in thyroid culture supernatant by Western blot after enrichment in annexin V-positive microvesicles (bottom) is shown. D, electron microscopy analysis of ex vivo thyroid. Arrow indicates the junction between two TFC (panel a) or plasma membrane-derived microvesicles (panel b); arrowheads indicate the double plasma membrane of microvesicles (panel c). Bars represent 1 μm (panel a), 500 nm (panel b), or 50 nm (panel c). E, FADD immunogold electron microscopy of ex vivo thyroid. Arrowheads indicate FADD labeling. Bars represent 200 nm. Co, colloid; Cy, cytoplasm; m, mitochondria; N, nucleus; pm, plasma membrane; TFC, thyroid follicular cell; V, vesicle; pre-V, pre-vesicle.

in cultured thyroctyes was confirmed by DAPI staining, which showed no difference between nuclei of ex vivo and 1 h in vitro-cultured thyroid cells (Fig. 1C). Finally, incubation at 4 °C delayed FADD release from thyroid lobes (77% inhibition at 5 min, 19% inhibition at 15 min, Fig. 1D). Altogether, these data demonstrated that FADD release from thyroid lobes was not the consequence of TFC death but was an active phenomenon.

In Vitro-incubated Thyroid Lobes Released FADD-containing Microvesicles Shed from the Plasma Membrane in the Extracellular Compartment—To characterize subcellular localization of FADD protein in ex vivo thyroid, confocal immunofluorescence microscopy analysis was performed. FADD protein was detected both in the cytoplasm and nucleus of TFC, and into the colloid of follicles (Fig. 2A). FADD staining appeared speckled (Fig. 2A). Such particular labeling of FADD was evocative of vesicular structures suggesting that FADD protein could be localized within vesicles and rapidly secreted in the extracellular compartment following thyroid incubation. FADD is encapsulated within vesicles, then it should be protected from enzymatic digestion. To test this hypothesis, we added trypsin to collected culture supernatant from thyroid lobes before loading on a gel. Later, FADD Western blot analysis was performed as previously described. As a control, we added mouse IgG1 in thyroid culture medium to test the efficiency of trypsin digestion. The results showed that FADD was protected from trypsin digestion, although fewer proteins were detectable compared with the amount of FADD present in untreated thyroid culture medium (Fig. 2B). In contrast, IgG1 proteins added to the samples as a control was fully degraded following trypsin digestion (Fig. 2B). Moreover, intracellular FADD obtained after membrane disruption was entirely digested by trypsin (Fig. 2B), showing that the secreted FADD protein detected after trypsin digestion did not result from incomplete trypsin digestion. These results strongly suggested that at least a part of the FADD protein was encapsulated and secreted within vesicles.

Secretion of proteins that do not contain a secretory signal sequence has previously been described and involved, among other mechanisms, secretion of exosomes (20) and shedding of microvesicles derived from the plasma membrane (21). Whatever the duration of thyroid incubation, neither MHC class I nor Gi2α molecules, which are typically overexpressed in exosomes, could be detected in thyroid culture supernatant, demonstrating that FADD release did not occur through secretion of this type of vesicle (see supplemental Fig. S3). Unlike exosomes, microvesicles shed from the
plasma membrane exhibit phosphatidylserine (PS) (21, 22). Here, we showed a positive staining with annexin V (a PS ligand), evocative of microvesicles, within the colloid of ex vivo thyroid (Fig. 2C). Moreover, we observed that annexin V staining was concentrated in the colloid in close contact with thyrocytes (Fig. 2C). These results suggested that FADD protein localized within small vesicles, which shared features with microvesicles shed from the plasma membrane. In agreement with these data, FADD protein was detected in the culture supernatant when we performed trans-well thyroid culture using 0.4-µm filtering (not shown), indicating that FADD could be released either freely or within vesicular structures that ranged up to 400 nm in diameter.

Interleukin-1β (IL-1β) is a cytokine secreted upon inflammatory conditions. This protein lacks signal peptide, and its release involves shedding of microvesicles derived from the plasma membrane. The authors hypothesized that microvesicle shedding could be a general secretory pathway for rapid secretion of proteins that do not contain a secretory signal sequence (19). Following this hypothesis, we sought for the presence of such vesicles containing FADD in the supernatant of thyroid culture. Released microvesicles were enriched with annexin V-coated beads as previously described for IL-1β (19). FADD was expressed in the annexin V-coated bead fraction (Fig. 2C), showing that FADD protein could be secreted within microvesicles that expressed PS. To further characterize these microvesicles, we performed electron microscopy (EM) analysis. We confirmed that numerous microvesicles of ~100–300 nm diameter were contained within the colloid of follicles in ex vivo thyroid from normal mice (Fig. 2D, panel a). These microvesicles appeared to derive from the plasma membrane of TFC (Fig. 2D, panel b), and possessed a double plasma membrane (Fig. 2D, panel c). Localization of these microvesicles concentrated in the colloid in close contact with thyrocytes (Fig. 2D, panel a) was in agreement with the annexin V staining (Fig. 2C), strongly suggesting that these microvesicles expressed PS. Numerous vesicular structures were also observed in the cytoplasm of thyrocytes (Fig. 2D, panel b). All these results were obtained in ex vivo thyroid immediately frozen or fixed for subsequent analysis, excluding an apoptotic mechanism as an explanation of the phenomenon. As shown by EM analysis, tissues are well conserved, and cell ultrastructure did not reveal alterations that could result from organ uptake and inclusion (see supplemental Fig. S2). Using FADD-specific immunogold EM, we confirmed that FADD was expressed in the cytoplasm (Fig. 2E, panel a) and nucleus of TFC (see supplemental Fig. S4c). In the cytoplasm, FADD was mainly detected as a free protein. It was also contained within the colloid of thyroid follicles enclosed in microvesicles that shared features of vesicles shown in Fig. 2D (Fig. 2E). FADD-containing microvesicles were ~200 nm in diameter (from 140 to 310 nm, mean 196 nm of 28 counted vesicles) (Fig. 2E, panels b and c), in agreement with the size of microvesicles shed from the plasma membrane (100–400 nm in diameter), and in contrast to exosomes that are smaller (<100 nm) (19). Thus, blebbing of TFC membrane (pre-vesicle, Fig. 2E, panel d) allowed formation of microvesicles that included free cytoplasmic FADD. FADD protein was detected in the colloid of follicles either in these vesicular structures (Fig. 2E, panels b and c) or as a free protein (Fig. 2E, panel a). Furthermore, incubation of thyroid in RPMI 1640 medium was sufficient to induce the loss of both free and vesicular FADD protein in the colloid of follicles (supplemental Fig. S4, b versus a). It also induces a strong decrease in the cytoplasmic (not shown) and nuclear (supplemental Fig. S4, d versus c) FADD protein within minutes of culture. This strong loss of thyrocyte FADD protein content was still observed after 1 h of thyroid incubation (not shown), in agreement with the Western blot results (Fig. 1). These experiments were confirmed using two different anti-FADD antibodies (not shown). Moreover, microvesicles were concomitantly detected in the culture supernatant of incubated thyroid lobes by EM analysis (not shown). However, we were not able to preserve these structures following FADD-specific immunogold EM. All these results suggested that at least a part of the endogenous FADD protein was included within microvesicles that are shed from the plasma membrane, stored in the colloid of thyroid follicles, and rapidly released in the extracellular and extracellular compartments following culture.

FADD Release Is Not Thyroid-specific—Because FADD is expressed almost ubiquitously in mice (Fig. 3A, top), we next tested whether FADD release was a phenomenon specific to thyroid or whether other in vitro-cultured organs were able to secrete this protein. FADD protein was detected in the culture medium from liver, thymus, skin, muscle, and kidney but not of salivary glands, lung, brain, eye, heart, or spleen, following 1 h of incubation in PBS-EDTA (Fig. 3B, bottom) or RPMI 1640 medium (not shown). As observed for thyroid, no LDH activity was detected in the organ culture medium, indicating that the release of FADD did not result from cell death (Fig. 3B). These data demonstrated that the extracellular release of FADD protein following in vitro culture is not a universal phenomenon, but is restricted to some tissues.

FADD Release Is Calcium-dependent but P2X7 Receptor-independent—Plasma membrane-derived microvesicle shedding was reported to require extracellular calcium (19). FADD release from thyroid was completely inhibited during the first 2 min of culture in calcium-free medium, and partially abolished thereafter (Fig. 4A). The inhibition observed after 60 min was no more significant (not shown), suggesting that calcium is necessary for secretion at early time points but dispensable thereafter. The P2X7 receptor (P2X7R) is implicated in secretion of IL-1β by microvesicle shedding (19, 23). Features of FADD-containing microvesicles and their release kinetics resembled those of IL-1β-containing microvesicles, suggesting that the P2X7R could be involved in the FADD secretion process. We used the previously described P2X7R−/− mice (18). Ex vivo P2X7R−/− organs expressed equal levels of FADD protein as ex vivo WT organs (not shown). Moreover, the amount of FADD secreted from P2X7R−/− and WT thyroids, livers, and thymus did not differ (Fig. 4B), demonstrating that release of FADD from these organs was not under the control of P2X7R.

FADD Release Is Under the Control of A2A and A3 Adenosine Receptor Activation—To further characterize the metabolic pathway implicated in FADD secretion, we tried to identify the metabolite responsible for the activation signals. ATP is a well characterized nucleotide receptor agonist released under stress...
conditions (24). We measured the concentration of ATP present in the extracellular medium from secreting (thyroid) and nonsecreting (lung) cultured organs. Following 5 min of incubation in RPMI 1640 medium (Fig. 5A, top table), ATP concentration in culture medium from thyroid lobes exceeded 100 nM. By contrast, the ATP concentration in the culture medium from lung did not reach 10 nM (Fig. 5A, bottom table). These results suggested that ATP could account for the observed FADD secretion. To test this hypothesis, we incubated thyroid lobes, liver, and thymus in the presence of apyrase, an ATPase/ADPase (10:1 ratio) enzyme. Apyrase accelerated FADD secretion from thyroid lobes (Fig. 5B), liver, and thymus (not shown), suggesting that the release of FADD could be under the control of a receptor using adenosine as a substrate.

High level of adenosine is produced and released in the extracellular space following cell stress or damage. Furthermore, adenosine can be generated directly in the extracellular compartment by degradation of released ATP (25). Because apyrase accelerated FADD secretion from different organs, we next tested the hypothesis that extracellular adenosine could act as a signaling molecule to induce FADD secretion. To test this hypothesis, we incubated thyroid lobes, liver, and thymus in the presence of adenosine receptor agonists or antagonists. Adenosine receptor agonists accelerated FADD secretion from thyroid lobes (Fig. 5C), liver, and thymus (not shown), suggesting that the release of FADD could be under the control of adenosine receptor activation.

FIGURE 3. Extracellular secretion of FADD is not restricted to thyroid and did not result from cell death. A, Western blot analysis of FADD expression in ex vivo organs and of FADD release from different organs through a transwell membrane with 0.4-μm pores after 1 h of incubation in PBS-EDTA. The same results were obtained using PBS-EDTA or RPMI 1640 as culture medium. Results were confirmed in three independent experiments. Note that the amount of FADD released in culture supernatant cannot be compared from one organ to another because the volume of medium used for organ incubation was not proportional to the organ size. B, LDH release from FADD-secreting and FADD-nonsecreting organs following 1 h of incubation in RPMI 1640 medium through a trans-well membrane with 0.4-μm pores.

FIGURE 4. The P2X7 receptor is not involved in the FADD secretion process in vivo. A, Western blot analysis of FADD expression in the culture supernatants of thyroid lobes incubated for various times in calcium-free medium (PBS containing 1 mM EDTA). Note that the inhibition observed after 10 min of incubation is variable from experiment to experiment, and that the inhibition observed following 1 h of incubation is usually no more significant (not shown). B, Western blot analysis of FADD release through a trans-well membrane in RPMI 1640 medium from thyroid lobes, liver, and thymus from WT or P2X7−/− C57BL/6 mice. The histogram represents quantification of bands normalized according to the total amount of protein per organ.
tested the implication of the adenosine receptors (AR) in the FADD secretion process. Four subtypes of AR have been identified (26) and knocked-out in mice (27–30). To evaluate the role of each AR in FADD secretion, different organs were cultured with specific AR antagonists.

Whatever the doses we tested, the A1AR inhibitor DPCPX and the A2A AR inhibitor CSC did not affect the FADD secretion from thyroid lobes (Fig. 5C and not shown). The A2BAR antagonist MRS1754 increased FADD release from thyroid (Fig. 5C) even at the low dose of 200 nm (see supplemental Fig. S5). By contrast, the A3AR antagonist MRS1191 inhibited FADD secretion at both doses tested (Fig. 5C).

As observed for thyroid, antagonizing A1AR and A2AAR did not alter the FADD secretion from liver (see supplemental Fig. S6). FADD release was inhibited by high doses of the A2BAR inhibitor MRS1754 at an early time point (supplemental Fig. S6). Moreover, the A2AR inhibitor MRS1191 enhanced FADD secretion from liver after 5 min of culture (supplemental Fig. S6), and this increase was still observed following 30 min of incubation (not shown). To confirm the involvement of the A3AR in FADD secretion from liver, we tested different doses of MRS1191. At 0.1 and 1 µM, MRS1191 increased FADD release at all time points examined (Fig. 5D). Furthermore, concomitant inhibition of A1, A2A, and A2BAR (with 10 nM DPCPX + 10 µM CSC + 3 µM MRS1754, respectively) to allow activation of A3AR only showed that activation of this receptor inhibited FADD release from liver (77 and 30.6% inhibition following 10 and 30 min of culture, respectively (not shown)). These data confirmed that the A3AR functions as an inhibitory receptor in liver. These results demonstrated that although the A3AR activated FADD release from thyroid lobes, it regulates negatively the FADD secretion process from liver.

Induction of FADD Release by Modulation of A3AR—The lung did not secrete FADD in our experimental model (Fig. 3A). Because we demonstrated that A3AR can regulate FADD secretion, we hypothesized that high expression of A3AR in the lung (31) could impair FADD secretion. Lung was cultured in the presence of different AR inhibitors. Whatever the antagonist used, we observed no FADD in the culture supernatant following 10 min of incubation (not shown). By contrast, the A3AR inhibitor MRS1191 induced FADD release from cultured lung after 30 min of treatment (Fig. 5E), and FADD was detectable thereafter (not shown). Moreover, FADD secretion induction did not result from cell death as MRS1191 treatment did not increase the LDH release from lung, even at high dose, and up to 120 min of incubation (Fig. 5E). MRS1191 treatment did not raise the ATP concentration in the culture medium (Fig. 5E). These results demonstrated that, as observed in the liver, adenosine via the A3AR regulates negatively the FADD secretion process from lung.

FADD Is Released in Vivo in the Serum from Tumor-affected Mice—We have previously shown that FADD protein was lost during the course of mouse thyroid tumor development, and that this absence of FADD protein expression in cancerous cells was not a transcriptional process (16). To test whether loss of FADD could be consequent to the secretion of the protein, we looked for FADD in the serum from gsp transgenic mice (15) at the different stages of thyroid tumor progression. Western blot analysis showed that high levels of FADD protein were detected in the serum from gsp mice with thyroid hyperplasia or adenoma/adenocarcinoma, compared with mice with nonpathological glands (Fig. 6). In contrast, no FADD was detected in the serum from healthy nontransgenic mice (not shown). These results demonstrated that FADD secretion occurred in vivo under pathological conditions.

DISCUSSION

The first role described for FADD was to interact with death receptors localized at the plasma membrane. For this reason, it was assumed that FADD protein was exclusively localized in the cytosol of the cell. However, it is now established that FADD is expressed in the nucleus of both human and mice adherent and nonadherent cell lines (5, 6, 32). Here, we confirm that FADD is expressed both in the cytoplasm and nucleus of ex vivo normal thyrocytes. Importantly, we demonstrated for the first time that FADD protein is stored in microvesicles within both TFC and the colloid of thyroid follicles and is rapidly released on AR activation. As suggested by the secretion of IL-1β (19) and IL-1 receptor antagonist (33), microvesicle shedding must be a conserved secretory pathway for release of proteins that do not contain a peptide signal. It is highly probable that FADD-containing microvesicles may contain other proteins that remain to be identified. We could not detect the Fas receptor protein in the extracellular medium from in vitro-cultured thyroid lobes (see supplemental Fig. S7A), suggesting that Fas may not be encapsulated within FADD-containing microvesicles.

Because our results were the first describing the secretion of a molecule such as FADD, it was important to ascertain that this protein release was not a passive phenomenon resulting from TFC death. We have accumulated evidence that it is not the case. First, the rapidity of the secretion process (within a few seconds) strongly suggested that the presence of FADD in the culture medium did not result from thyroid cell death. Second, if we hypothesize that FADD release is a consequence of cell death, and because loss of FADD concerned almost all thyrocytes (Fig. 1A and not shown), it would implicate that all cells died following a few seconds of culture, which does not seem reasonable and is not supported by any experimental evidence. Third, we showed using several technical approaches that TFC were alive following in vitro incubation. Using trypan blue exclusion and LDH assay, we demonstrated that no necrosis was observed in cultured thyrocytes (Fig. 3B and not shown). Moreover, in vitro-incubated thyroid cells did not present characteristic morphological changes accompanying necrosis or apoptosis (see supplemental Figs. S1 and S2, and Fig. 1C).

Fourth, for ex vivo thyroid examination, lobes were immediately covered in optimal temperature medium following uptake, strongly suggesting elimination of apoptosis as a cause of the annexin V staining we observed (Fig. 2C). Moreover, such annexin V staining of microvesicles was previously described, for example for IL-1β-containing vesicles and is a reversible
phenomenon not associated with apoptosis (19). Fifth, FADD secretion was delayed at 4 °C (Fig. 1D), confirming that it was an active process.

What then are the signals involved in FADD secretion? Although these signals are not fully elucidated, one of them involved activation of adenosine receptors. Cellular stress con-
Adenosine Receptor-dependent FADD Release

FIGURE 6. FADD protein is secreted in vivo in pathological conditions. Western blot analysis of FADD expression in the serum from gsp transgenic mice at the different stages of tumor development is shown.

FIGURE 5. FADD protein secretion is under the control of adenosine receptors. A, ATP concentration measurement in the culture medium from FADD-secreting (thorroid, top and middle tables) and nonsecreting (lung, bottom table) organs. Organs were cultured either in RPMI 1640 medium (top table) or in PBS medium (middle and bottom tables), and ATP was measured (nM) in the collected medium. All concentrations were normalized according to the weight of thyroid. Results presented in the tables are for 5 mg of tissue, the average weight of a thyroid.

through AR signaling, could modulate cell response such as apoptosis mediated by death receptors.

We have previously described an important role for the FADD molecule during the course of carcinogenesis (14). Using a murine model of thyroid tumor development (15), we previously showed that FADD protein expression was lost in thyroid adenoma/adenocarcinoma, and that the Fas signaling in the absence of FADD led to an accelerated growth of thyrocytes (16). Here, we showed the presence of the FADD protein in the serum of mice that have developed a thyroid tumor. Although requiring confirmation, our data suggested that the loss of FADD we observed in mouse cancer cells could occur through release of the protein in the extracellular compartment. Moreover, the observation that FADD was found in the serum from nonpathological gsp mice but not in the serum from healthy nontransgenic mice suggested that release of FADD could be an early marker for tumor development. Interestingly, previous report suggested that activation of the A3AR could induce apoptosis of human HL-60 leukemic cell line, and could be of potential therapeutic value in the treatment of leukemia (37) by a still unclear mechanism. Because we have previously shown that FADD protein expression was low or absent in leukemic cells of 65% of AML patients at diagnosis, and that this absence of protein was not due to an absence of FADD mRNA (17), future work is necessary to test whether leukemic cells could secrete FADD protein, and whether A3AR activation-induced apoptosis of leukemic cells occurs through regulation of FADD expression by secretion, both in vitro and in vivo.

In summary, our data demonstrated a new localization site (in microvesicles) and regulatory mechanism (by secretion) of the FADD protein, and implicate AR in this process. It is highly probable that FADD-containing microvesicles may contain other proteins that remain to be identified. On the other hand, we showed that the FADD secretion process was not determined by the embryologic origin of the tissue. Whereas some organs secreted FADD almost immediately following in vitro culture (thyroid, liver, thymus, muscle, skin, and kidney), FADD secretion did not occur spontaneously for others (lung, brain, eye, salivary gland, heart, and spleen). However, FADD secretion could be induced from lung by inhibiting the A3AR. These data open a new field of investigation consisting in the possibility to regulate FADD expression via the modulation of AR, which constitutes a therapeutic target in diseases in which FADD-mediated signaling is impaired, including cancer, inflammation, and infection.
Acknowledgments—We thank Dr. C. Hivroz and Dr. C. Thery for help with exosome preparation. We also thank Dr. J. Kanellopoulos and R. Auger for generously typing and providing P2X7R−/− mice. We are indebted to M. Garfa for excellent technical assistance with confocal fluorescence microscopy, to A. Gaston, E. Souil, and J. M. Masse for performing cryo and ultra cryo sectioning for microscopy and electron microscopy, respectively, to M. Bretou and N. Cagnard for technical assistance, and to F. Lager for help with animal care. We are grateful to Dr. C. Fournier and Dr. C. André for critical reading of the manuscript and to Prof. M. Seman for helpful discussions.

REFERENCES

1. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O’Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4961–4965
2. Chinnaiyan, A. M., O’Rourke, K., Yu, G. L., Lyons, R. H., Garg, M., Duan, D. R., Xing, L., Gentz, R., Ni, J., and Dixit, V. M. (1996) Science 274, 990–992
3. Kuang, A. A., Diehl, G. E., Zhang, J., and Winoto, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10488–10492
4. Schreiber, B. M., Gavras, H., Wagner, D. D., and Ravid, K. (2006) J. Clin. Investig. 118, 1615–1624
5. Wilson, H. L., Campbell, A. F., and Johnson, L. (1997) Science 276, 125–132
6. Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Immunity 7, 7–32
7. Hasko, G., and Cronstein, B. N. (2004) Trends Immunol. 25, 33–39
8. Jacobson, K. A., and Gao, Z. G. (2006) Nat. Rev. Drug Discov. 5, 247–264
9. Johansson, B., Hallgren, L., Dunwiddie, T. V., Masino, S. A., Poelchen, W., Gimenez-Lloret, L., Escorihuela, R. M., Fernandez-Teruel, A., Wiesenfeld-Hallin, Z., Xu, X. J., Hardemark, A., Betsholtz, C., Herlenius, E., and Fredholm, B. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9407–9412
10. Ledent, C., Vaugeois, J. M., Schiffrin, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. I., Costentin, J., Heath, J. K., Vassart, G., and Parmetier, M. (1997) Nature 388, 674–678
11. Salvatore, C. A., Tilley, S. L., Latour, A. M., Fletcher, D. S., Koller, B. H., and Jacobson, M. A. (2000) J. Biol. Chem. 275, 4429–4434
12. Yang, D., Zhang, Y., Ngyuen, H. G., Koupennova, M., Chauhan, A. K., Makitalo, M., Jones, M. R., St Hilaire, C., Seldin, D. C., Toselli, P., Lamperti, E., Schreiber, B. M., Gavras, H., Wagner, D. D., and Ravid, K. (2006) J. Clin. Investig. 116, 1913–1923
13. Salvatore, C. A., Jacobson, M. A., Taylor, H. E., Linden, J., and Johnson, R. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10365–10369
14. Shiek, S. M., and Huang, Y. (2003) Cell Cycle 2, 346–347
15. Wilson, H. L., Francis, S. E., Dower, S. K., and Crossman, D. C. (2004) J. Immunol. 173, 1202–1208
16. Sorensen, C. E., and Novak, I. (2001) J. Biol. Chem. 276, 32925–32932
17. Ma, Y., Liu, H., Tu-Rapp, H., Thiesen, H. J., Ibrahim, S. M., Cole, S. M., and Pope, R. M. (2004) Nat. Immunol. 5, 380–387
18. Mentzer, R. M., Jr, Rubio, R., and Berne, R. M. (1975) Am. J. Physiol. 229, 1625–1631
19. Kohno, Y., Sei, Y., Koshba, M., Kim, H. O., and Jacobson, K. A. (1996) Biochem. Biophys. Res. Commun. 219, 904–910