Nuclear Localization of Apoptosis Protease Activating Factor-1 Predicts Survival after Tumor Resection in Early-Stage Non–Small Cell Lung Cancer

Benjamin Besse, Céline Candé, Jean-Philippe Spano, Antoine Martin, David Khayat, Thierry Le Chevalier, Thomas Tursz, Laure Sabatier, Jean-Charles Soria, and Guido Kroemer

ABSTRACT

The proapoptotic protein apoptosis protein activating factor-1 (Apaf-1), which is normally located in the cytoplasm, can translocate to the nucleus before non-small cell lung carcinoma (NSCLC) cells manifest signs of apoptosis such as mitochondrial damage, caspase activation, or chromatin condensation. This may indicate a stage of imminent apoptosis. Importantly, we found that 24% (15 of 62) of resected stage I NSCLC (T1 N0 M0 or T2 N0 M0), manifested a marked nuclear localization of Apaf-1 (Apaf-1Nuc), as compared with the mostly cytoplasmic localization of Apaf-1 found in the remaining tumors (Apaf-1Cyt). After a median follow-up of 6.31 years, the actuarial 5-year overall survival rates were 89% (56–98%) in the Apaf-1Nuc group and 54% (36–71%) in the Apaf-1Cyt group (P = 0.039). No correlation between the subcellular localization of Apaf-1 and that of p53 and Hsp70 could be established. Thus, the subcellular location of Apaf-1 (but not that of p53 or Hsp70) constitutes an accurate prognostic factor for overall survival in NSCLC.

INTRODUCTION

Oncogenesis is linked to a notable degree of apoptosis resistance (1–3) and, at least in some tumors, the expression level of apoptosis-regulatory proteins does have a prognostic impact. This applies also for apoptosis protein activating factor-1 (Apaf-1), a caspase-activatory protein with a promotor that is hypermethylated and hence silenced in some tumors, in particular in melanosomes (4) and leukemias (5). Moreover, frameshift mutations of Apaf-1 have been observed in gastrointestinal cancers (6), and endogenous inhibitors of Apaf-1 have been detected in ovarian carcinoma (7, 8). Altogether, these findings suggest that Apaf-1 can act as a tumor suppressor. Although Apaf-1 is not universally required for cell death execution (9) and certainly does not act as a general tumor suppressor (10), it is well possible that it acts as a cell type-specific antioncogene (11).

Apaf-1, a relatively large (approximately 130 kD) protein, is an obligatory constituent of the apoptosome caspase activation complex, a polypeptide complex formed by the congregation of Apaf-1, cytochrome c, and caspase-9 (12, 13). After apoptosis induction, cytochrome c is released from mitochondria, and this constitutes the signal for apoptosisome. Cytochrome c interacts with the COOH terminus of the Apaf-1 molecule (the so-called tryptophan asparagine (WD) repeats), leading to a conformational change of Apaf-1. In the presence of ATP, a hetero-heptamerer complex involving the equimolar contribution of Apaf-1 and pro-caspase-9 is formed through CARD-CARD (caspase recruitment domain) interactions. This leads to the allosteric activation and ultimately to the proteolytic maturation of caspase-9 (the initiator caspase). Caspase-9 in turn cleaves and activates caspase-3 (the effector caspase), which then contributes to the ordered dismantling of cellular structures. The expression of Apaf-1 thus is required for caspase activation through the so-called intrinsic (or mitochondrial) pathway of cell death induction formation (12, 14). Retransfection of Apaf-1 into Apaf-1-deficient cells can reconstitute their apoptosis susceptibility (4, 15). In addition, some compounds promoting the protein-protein association of Apaf-1 into the functional apoptosome reportedly have a potent antitumor activity (16).

CED4 is the Caenorhabditis elegans ortholog of Apaf-1 (17). CED4 lacks the Apaf-1 COOH-terminus (and hence cannot bind cytochrome c) and is normally tethered to CED9 (the Bcl-2 homologue) present on mitochondrial membranes (18). After apoptosis induction, a proapoptotic BH3-only protein from the Bcl-2 family can displace CED4 from CED9, making CED4 available for caspase activation and causing its relocation from mitochondria to (peri)nuclear membranes (18, 19). The nuclear localization of CED4 may be stimulated by CED9, at least in yeast and mammalian cells (20). It has also been reported that overexpression of Bcl-2 can induce the perinuclear...
aggregation of Apaf-1 in murine B cell lymphoma cells (21). Despite the initial description of an Apaf-1/Bcl-XL/caspase-3 complex in mammalian cells (22, 23), a number of studies indicated that, in human cells, Apaf-1 is not tightly bound to mitochondria and rather is a cytosolic protein (12, 14, 24).

The nuclear envelope provides a level of regulation in tumor pathways that could be underestimated. Numerous proteins follow a precise nuclear trafficking in response to cell cycle progression, growth signals, or environmental stimuli. Such proteins may be transcription factors like p53 but also protein kinases such as Akt/PKB or ERK (25). Subcellular localization may have a major impact on the function of proteins, because this has been documented for cyclin B1, which stimulates mitosis in the nucleus and regulates cell mobility in the cytoplasm (26). Apaf-1 has been reported to display a nuclear distribution in HeLa cells exposed to cisplatin (27). However, such a relocalization pattern has not been addressed in tumor samples, although the total Apaf-1 content may be reduced in melanoma (28) and glioblastoma (29).

In view of the plausible role of Apaf-1 as a tumor suppressor protein, we determined its role in the apoptotic demise of non-small cell lung carcinoma (NSCLC). As described here, we found that Apaf-1 can translocate into the nucleus of such cells as a preapoptotic event. The nuclear presence of Apaf-1 constituted a positive prognostic feature of resectable NSCLC.

MATERIALS AND METHODS

Apoptosis Induction In vitro. Two lung carcinoma cell lines, A549 and H460 (American Type Culture Collection, Manassas, VA), were treated with cisplatin and/or actinomycin D (Sigma, St. Louis, MO) in F12-K medium (A549) or DMEM (H460) complemented with 10% fetal bovine serum. Cells cultured on cover slips were fixed with 4% paraformaldehyde, permeabilized with 0.1% SDS, blocked in PBS supplemented with 10% fetal bovine serum, and stained with an anti-Apaf-1 antibody (Novocastra) and an anticytochrome c antibody (PharMingen, San Diego, CA; ref. 30). Cells were counterstained with Hoechst 33342 (0.1 μg/ml in PBS for 15 minutes). Sense and antisense oligonucleotides (Proligo France SAS, Paris, France) composing an RNA heteroduplex targeting the Apaf-1 mRNA (5′-AATTGGBTGCACCTTGACGTA3′; ref. 31) were transfected into A549 cells by oligofectamine (Invitrogen, Carlsbad, CA) 48 hours before the addition of cytotoxic drugs. Subcellular fractionations were performed as described previously (32).

Patient Characteristics. Surgical specimens were obtained from a total of 62 consecutive patients with pathological stage I NSCLC (49% pT1N0M0 and 51% pT2N0M0; 50% adenocarcinoma, 39% squamous cell carcinoma, 5%, mixed tumors others 6%) who underwent surgical resection at Avicenne Hospital (AP-HP), France, between 1987 and 1999. None of the patients had received neo-adjuvant therapy at the time of surgery. Mean age at diagnosis was 61.4 years; 80% of the patients were males; 88% were tobacco users, and mean tobacco consumption was 38 pack years. All patients signed a consent form, in compliance with the national legislation and the local ethics committee.

Immunohistochemistry. Immunohistochemical staining of p53 protein, Apaf-1, and Hsp 70 was performed using the avidin-biotin peroxidase method, with modifications (33). Briefly, sections were deparaffinized in toluene and rehydrated through a graded alcohol series. To retrieve the antigenicity, the tissue sections were microwaved (650W) in 10 mmol/L citrate buffer (pH 6.0) for 12 minutes, treated with 0.3% hydrogen peroxide in methanol for 20 minutes (to block endogenous peroxidase activity), and incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with one of the following primary antibodies: Apaf-1 (1:40, Novocaztra, Newcastle upon Tyne, United Kingdom), p53 (clone DO7, 1:1,000, Abcam, Cambridge, United Kingdom), or Hsp 70 (Stress Gene, Victoria, Canada, 1:200). The sections were then processed using standard avidin-biotin immunohistochemical techniques according to the manufacturer’s recommendations (Vectastain kit, Vector Laboratories, Burlingame, CA). Diaminobenzidine was applied for 3 minutes as a chromogen, and commercial hematoxylin was used for counterstaining. Adjacent normal-appearing epithelium within the tissue sections served as a positive internal control. Representative areas of each tissue section were selected, and a minimum of 600 cells were counted in at least two fields (×200). On the basis of the results of the immunohistochemical staining, specimens were scored and classified into two groups: nuclear or perinuclear staining in at least 20% of cell (nuclear) and <20% or absence of nuclear staining (cytoplasmic). This cutoff is generally chosen for p53 (33, 34). All of the slides were analyzed in a blinded fashion and scored independently by two investigators (B. B. and J.-C. S.). The level of concordance, expressed as the percentage of agreement between the observers, was 95.1% (59 of 62 specimens). In the remaining specimens, scores were obtained after collegial revision and agreement.

Statistics. Fisher’s exact test or the χ2 test was used to analyze the association between two categorical variables. Student’s t test was used to compare mean values. Overall survival rates were calculated using the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. All survival times were calculated from the date of surgery. The overall survival accounted for all deaths (cancer related or not). Cox proportional hazards were used for univariate analysis to evaluate the association between survival time and risk factors. P values <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Relocalization of Apaf-1 to the Nucleus in Stressed NSCLC Cells. The 549 NSCLC cell line, which normally does express Apaf-1, failed to undergo cis-diaminedichloroplatinum (cisplatin, CDDP)-induced apoptotic chromatin condensation when Apaf-1 was depleted by small interfering RNA, even when using high cisplatin dose (200 μmol/L; Fig. 1A). When treated with 100 μmol/L cisplatin in vitro, A549 cells manifested the translocation of Apaf-1 from the cytoplasm to the nucleus, as detectable by subcellular fractionation (Fig. 1B). Confocal immunofluorescence revealed that the nuclear relocalization of Apaf-1 (Apaf-1(N)) occurred before mitochondrial released cytochrome c (and apoptosis inducing factor, not
shown) and even in conditions in which caspases were inhibited by addition of the pan-caspase antagonist Z-VAD.fmk (Fig. 1C for A549). H460 cells displayed the same subcellular localization of Apaf-1, cytochrome c and apoptosis inducing factor when treated with the same cisplatin dose (not shown). In both cell lines, the CDDP-induced nuclear redistribution of Apaf-1 was not affected by actinomycin D, a general inhibitor of transcription (not shown), indicating that pre-existing (rather than de novo synthesized) Apaf-1 moved into the nucleus. Moreover, there was no apparent shift in the electrophoretic mobility of Apaf-1 (Fig. 1B). Thus, Apaf-1Nuc constitutes an early marker of imminent apoptosis (preapoptosis) that is detectable before the hallmarks of apoptotic cell death (mitochondrial membrane permeabilization with cytochrome c release, caspase activation, chromatin condensation) become manifest.

Relocalization of Apaf-1 to the Nucleus in a Subset of Operable NSCLC. We wondered whether this sign of early apoptosis (preapoptosis) would be detectable in vivo, in NSCLC specimen, obtained from surgically treated patients. This single institution series was handled with a uniform therapeutic strategy and follow-up; however, it contained a sufficient number of patients to explore new biomarkers, before validating them in a large multicenter series. All NSCLC samples (n = 62) were positive for Apaf-1, with a staining intensity comparable to that of surrounding normal tissue. Most surgically removed stage I NSCLC (T1,N0,M0), contained Apaf-1 in an orthotopic, exclu-

<table>
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<th>No. of patients (n = 62)</th>
<th>Apaf-1 localisation</th>
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* P was calculated comparing smoking versus non-smoking patients.
† Squamous versus adenocarcinoma.
sively cytoplasmic (non-nuclear) localization, as detectable by immunohistochemistry (Fig. 2A). However, independently of the histologic type (adenocarcinoma, squamous cell carcinoma, or undifferentiated carcinoma), a fraction of the tumors (24%) manifested a dominant nuclear (Fig. 2B) or perinuclear (Fig. 2C) localization of Apaf-1 in cells that had a normal nuclear morphology (Fig. 2B and C) and were Tunel-negative (not shown), meaning that they were not apoptotic. Fifteen of 62 of the tumors (24%) contained \( \text{Apaf-1}^{\text{nuc}} \) cells with nuclear or perinuclear localization of Apaf-1 (20–30% cells in one case, 30–50% in four cases, and 50–100% in 11 cases). We defined this group as \( \text{Apaf-1}^{\text{nuc}} \) as opposed to the \( \text{Apaf-1}^{\text{cyt}} \) tumors composed of 38 cases with 0–10% cells with nuclear or perinuclear Apaf-1 and eight cases with 10–20% cells in this setting.

**Nuclear Localization of Apaf-1 as a Positive Prognostic Marker.** The \( \text{Apaf-1}^{\text{nuc}} \) (\( n = 15 \)) and \( \text{Apaf-1}^{\text{cyt}} \) (\( n = 47 \)) patient groups did not differ in their sex ratio (m:f 4:2.2:1 and 4:1, respectively), \( T_1 \) to \( T_2 \) ratio (\( T_1:T_2 = 1.13:1 \) and 1:1.5, respectively), mean age at primary diagnosis (61.34 ± 10.34 and 60.87 ± 10.96 years), histologic type, or smoking habits (30.20 ± 25.08 and 40 ± 31.11 package-years, respectively; Table 1). However, the two cohorts dramatically differed in their survival. After a median follow-up of 6.31 years, the actuarial 5-year overall survival rates were 89% (56–98%) in the \( \text{Apaf-1}^{\text{nuc}} \) group and 54% (36–71%) in the \( \text{Apaf-1}^{\text{cyt}} \) group (\( P = 0.039 \); Fig. 3A). In contrast, there was no statistically significant difference in the disease-free survival, with metastatic or local recurrences being observed in both the \( \text{Apaf-1}^{\text{nuc}} \) and \( \text{Apaf-1}^{\text{cyt}} \) groups, despite a clear trend for a better disease-free survival in the \( \text{Apaf-1}^{\text{nuc}} \) group (\( P = 0.078 \); Fig. 3B). Moreover, we did not find any significant correlation between the nuclear presence of Apaf-1 and that of two other antigens, p53 (Hsp70) and heat shock protein 70 (Fig. 3C and D). Both of them are functionally related to Apaf-1, p53 as a transcription factor that stimulates Apaf-1 expression at the mRNA level (35) and Hsp70 as an Apaf-1-binding inhibitor of apoptosome formation (36–38). Although the subcellular localization of p53 and Hsp70 clearly varied among different tumors, the nuclear versus cytoplasmic localization of p53 or Hsp70 did not correlate with that of Apaf-1 (inserts in Fig. 3C and D) and had no prognostic value (Fig. 3C and D).

**Concluding Remarks.** Altogether, these data lead to the identification of \( \text{Apaf-1}^{\text{nuc}} \) as a novel marker of preapoptosis in NSCLC cells and as an independent prognostic factor for NSCLC.

![Fig. 3](kabm.png) **Kaplan–Meier curve survival plots of patients with NSCLC classified according to the subcellular localization of Apaf-1, p53, and Hsp70.** A, survival of individuals bearing \( \text{Apaf-1}^{\text{nuc}} \) (\( n = 15 \)) versus \( \text{Apaf-1}^{\text{cyt}} \) NSCLC (\( n = 47 \)). \( \text{Apaf-1}^{\text{nuc}} \) (24% of the cases), as defined in the text, was significantly associated with overall survival (\( P = 0.039 \); median follow-up time, 6.31 years). B, disease-free survival of \( \text{Apaf-1}^{\text{nuc}} \) versus \( \text{Apaf-1}^{\text{cyt}} \) individuals (same patients as in A). C and D, nuclear p53 and Hsp70 were not associated with overall survival in 61 among the 62 NSCLC (median follow-up time, 6.41 years). Immunohistochemistry was used to determine the intracellular distribution of p53 and Hsp70, as well as for Apaf-1 (Fig. 2A-C), with the difference that primary antibodies specific for p53 or Hsp70 were used. Again “nuclear” refers to tumors in which >20% of cells contain nuclear p53 or Hsp70. The correlation between the subcellular localization of Apaf-1 and p53 or Hsp70, respectively (inserts) is not significant, according to Fisher’s exact test. Cox proportional hazards were used for univariate analysis to evaluate the association between survival time and risk factors.
It is important to state, however, that the number of patients included in this study was relatively small and that a larger study will have to confirm the general conclusions obtained in this paper. Although adjuvant chemotherapy tends to become a standard approach, it is a subject of debate which fraction of patients with stage I lung cancer would benefit the most from such treatment (39). If confirmed, our data indicate that Apaf-1Nuc and Apaf-1Cyt NSCLC are likely to differ in their biological response to apoptogenic anticancer agents. At present, it is difficult to understand the biological mechanisms of the cell type-specific nuclear recolorization of Apaf-1. Although the nuclear presence of Apaf-1 clearly is a marker of cellular stress (Fig. 1A and C) and Apaf-1 is required for CDDP-induced apoptosis of NSCLC cells (Fig. 1A), it is unclear whether the translocation of Apaf-1 itself constitutes a mechanism of incipient apoptosis. Nonetheless, it is tempting to speculate that those tumor cells that contain Apaf-1 in the nucleus either are engaged in a pathway that leads to cell death or are particularly apoptosis-prone. This would explain the positive prognostic impact of Apaf-1Nuc.

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