SMAC is expressed de novo in a subset of cervical cancer tumors
Magali Espinosa, David Cantu, Carlos M Lopez, Jaime G De la Garza, Vilma A Maldonado* and Jorge Melendez-Zajgla*

Address: Subdirección de Investigación Básica. Instituto Nacional de Cancerología. Av. San Fernando # 22. Tlalpan 14080 México, D.F. MEXICO

Email: Magali Espinosa - maggiec73@hotmail.com; David Cantu - dcantu3@excite.com; Carlos M Lopez - carlos2724mx@yahoo.com.mx; Jaime G De la Garza - jgdelagarza@starnet.net.mx; Vilma A Maldonado* - vilmaml@yahoo.com; Jorge Melendez-Zajgla* - jorgezajgla@ssa.gob.mx

* Corresponding authors

Abstract

Background: Smac/Diablo is a recently identified protein that is released from mitochondria after apoptotic stimuli. It binds IAPs, allowing caspase activation and cell death. In view of its activity it might participate in carcinogenesis. In the present study, we analyzed Smac expression in a panel of cervical cancer patients.

Methods: We performed semi quantitative RT-PCR on 41 cervical tumor and 6 normal tissue samples. The study included 8 stage I cases; 16 stage II; 17 stage III; and a control group of 6 samples of normal cervical squamous epithelial tissue.

Results: Smac mRNA expression was below the detection limit in the normal cervical tissue samples. In contrast, 13 (31.7%) of the 41 cervical cancer biopsies showed detectable levels of this transcript. The samples expressing Smac were distributed equally among the stages (5 in stage I, 4 in stage II and 4 in stage III) with similar expression levels. We found no correlation between the presence of Smac mRNA and histology, menopause, WHO stage or disease status.

Conclusions: Smac is expressed de novo in a subset of cervical cancer patients, reflecting a possible heterogeneity in the pathways leading to cervical cancer. There was no correlation with any clinical variable.

Background

Apoptosis is an evolutionarily conserved biological process that plays a fundamental role in development and tissue homeostasis in metazoans [1]. This type of cell death is executed by a family of proteases known as caspases [2]. There are two well-characterized apoptotic pathways that converge in caspase activation: the death receptor pathway and the mitochondrial pathway [3]. Inhibitors of Apoptosis Proteins (IAPs) are the most important regulators of caspases. These proteins inhibit caspase activation, thus preventing the induction of apoptosis [4]. In cells undergoing apoptosis, IAPs are inactivated by interaction with proteins containing the so-called IBM (IAP-binding motif) [4,5].

One IBM protein is the recently identified Smac/DIABLO [6,7]. Smac resides in the mitochondrial intermembrane space in healthy cells but is released into the cytosol during apoptosis, where it interacts with IAPs and disrupts their ability to bind caspases [8]. Smac is expressed ubiquitously, with high expression in adult testis, heart, liver, kidney, spleen, prostate and ovary and low expression in...
brain, lung, thymus, and peripheral blood leukocytes [9].
It is encoded in a nuclear gene and is post-translationally
imported into the mitochondria via a targeting sequence
in its amino terminus. Removal of this signal generates a
mature polypeptide with the IBM at the amino terminal
end [10]. Smac interacts with all mammalian IAPs exam-
ined so far: XIAP, cIAP-1, cIAP-2, survivin and ML-IAP
[6,7,11,12]. The structure of the Smac-XIAP complex has
been studied by X-ray crystallography [13] and high-reso-
lution NMR [14]; it appears that the tetrapeptide AVPI is
indispensable for the formation of this complex.

IAPs are highly expressed in human tumor cells [15-17],
contributing to the intrinsic resistance of these cells to

| Sample | Age | Stage | Histology of tumor | Menopause | Current status | Smac/GAPDH |
|--------|-----|-------|--------------------|-----------|----------------|------------|
| Control | 42 | - | Menopause | Disease-free | 0 |
| Control | 33 | - | Menopause | Disease-free | 0 |
| Control | 28 | - | Pre-menopause | Disease-free | 0 |
| Control | 44 | - | Menopause | Disease-free | 0 |
| Control | 47 | - | Menopause | Disease-free | 0 |
| Control | 35 | - | Pre-menopause | Disease-free | 0 |
| 1 | 46 | I | Adenocarcinoma | Menopause | Disease-free | 0 |
| 2 | 52 | I | Squamous cell | Post-menopause | Disease-free | 76.01 |
| 3 | 49 | I | Squamous cell | Menopause | Disease | 203.36 |
| 4 | 44 | I | Squamous cell | Pre-menopause | Disease-free | 0 |
| 5 | 65 | I | Squamous cell | Post-menopause | Disease-free | 0 |
| 6 | 67 | I | Squamous cell | Post-menopause | Disease-free | 86.53 |
| 7 | 47 | I | Squamous cell | Menopause | Disease-free | 65.3 |
| 8 | 34 | I | Squamous cell | Pre-menopause | Disease-free | 66.45 |
| 9 | 49 | II | Squamous cell | Menopause | Disease-free | 78.44 |
| 10 | 63 | II | Squamous cell | Post-menopause | Disease-free | 0 |
| 11 | 38 | II | Squamous cell | Menopause | Disease-free | 0 |
| 12 | 48 | II | Squamous cell | Menopause | Disease-free | 0 |
| 13 | 55 | II | Squamous cell | Post-menopause | Disease-free | 0 |
| 14 | 35 | II | Squamous cell | Pre-menopause | Disease-free | 166.25 |
| 15 | 66 | II | Squamous cell | Post-menopause | Disease | 0 |
| 16 | 52 | II | Squamous cell | Post-menopause | Disease | 0 |
| 17 | 80 | II | Squamous cell | Post-menopause | Disease | 0 |
| 18 | 70 | II | Squamous cell | Post-menopause | Disease-free | 0 |
| 19 | 65 | II | Squamous cell | Post-menopause | Disease | 0 |
| 20 | 39 | II | Squamous cell | Pre-menopause | Disease | 70.42 |
| 21 | 57 | II | Squamous cell | Post-menopause | Disease-free | 55.45 |
| 22 | 37 | II | Squamous cell | Pre-menopause | Disease-free | 0 |
| 23 | 59 | II | Squamous cell | Post-menopause | Disease | 0 |
| 24 | 36 | II | Squamous cell | Pre-menopause | Disease | 0 |
| 25 | 33 | III | Adenocarcinoma | Pre-menopause | Dead | 0 |
| 26 | 50 | III | Adenocarcinoma | Post-menopause | Dead | 0 |
| 27 | 60 | III | Squamous cell | Post-menopause | Disease-free | 0 |
| 28 | 64 | III | Adenocarcinoma | Post-menopause | Dead | 0 |
| 29 | 80 | III | Squamous cell | Post-menopause | Dead | 0 |
| 30 | 52 | III | Squamous cell | Post-menopause | Disease-free | 173.96 |
| 31 | 56 | III | Squamous cell | Post-menopause | Disease-free | 0 |
| 32 | 70 | III | Squamous cell | Post-menopause | Disease-free | 81.5 |
| 33 | 72 | III | Adenosquamous | Post-menopause | Dead | 0 |
| 34 | 33 | III | Squamous cell | Pre-menopause | Disease | 0 |
| 35 | 82 | III | Adenocarcinoma | Post-menopause | Disease | 0 |
| 36 | 48 | III | Squamous cell | Menopause | Disease-free | 0 |
| 37 | 32 | III | Squamous cell | Pre-menopause | Disease | 98.59 |
| 38 | 48 | III | Squamous cell | Menopause | Disease | 0 |
| 39 | 36 | III | Adenosquamous | Pre-menopause | Disease | 0 |
| 40 | 52 | III | Squamous cell | Post-menopause | Disease-free | 0 |
| 41 | 67 | III | Squamous cell | Post-menopause | Disease-free | 88.95 |
endogenous death receptor-induced apoptosis and consequently to chemotherapy [18]. For this reason, peptides mimicking the action of Smac have been generated and analyzed. Four publications to date have shown promising effects of these Smac peptides in vitro and in vivo; however, further studies are required prior to clinical testing [19-22].

Recently, Sekimura and colleagues found that Smac expression was significantly lower in primary lung cancers than in normal tissue [23]; patients with lower Smac mRNA levels had worse prognoses. These results indicate that Smac expression may play a role in the progression of primary lung cancer and may be useful for prognosis [23]. However, Smac expression has not been analyzed in other tumors. In view of the possible role of Smac in cervical carcinogenesis and its potential as a therapeutic target, we have investigated the expression of this apoptotic protein in cervical cancer patients.

**Methods**

**Cell lines and tumor samples**

Cervical cancer cell lines (HeLa, SiHa, CaSki and CaLo) were obtained from ATCC and cultured as monolayers in Dulbecco Modified Eagle's Medium (DMEM) containing 10% (V/V) fetal bovine serum (GIBCO, Bethesda, MD, USA) at 37°C in a humidified atmosphere of 5% (V/V) CO₂.

Forty-one cervical cancer samples were obtained from the Instituto Nacional de Cancerologia of Mexico. Written consent was obtained from patients before the samples were collected. Tumors were staged according to the International Gynecology and Obstetric Federation (FIGO) system. The samples comprised 8 at stage IB, 16 at stage IIB and 17 at stage IIIB; and a control group comprising 6 samples of normal cervical squamous epithelial tissue (Table 1). The control samples were derived from hysterectomy specimens from patients with uterine myomatosi. Only samples with normal pathological reports were included.

**Histology**

Histopathological grading was done according to the WHO (World Health Organization) classification system (Table 1).

**RNA isolation and RT-PCR**

RNA extraction and RT-PCR analysis were performed as described previously [24]. Briefly, total RNA was extracted from cultured cells, tumors and non-neoplastic tissue samples with Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA purity was confirmed by the 260/280 nm absorbance ratio and its integrity was established with agarose gels. Total RNA (2 µg) was reverse-transcribed in a final 20 µl reaction volume using 15 U ThermoScript reverse transcriptase, 2.5 x RT Buffer and random hexamers (ThermoScript RT-PCR, Invitrogen). The RT-PCR steps were 25°C for 10 min, 50°C for 50 min and 85°C for 5 min. Smac and GAPDH mRNA PCR reactions contained 0.25 µl Amplitaq gold polymerase (Applied Biosystems, ROCHE), 2.5 µl 10 × reaction buffer, 0.5 µl dNTP mix 10 mM, 1 µl sense primer 10 µM, 1 µl anti-sense primer 10 µM and 1 µl cDNA in 25 µl final volume. The Smac primers were: sense 5’ GCGCGGATCCATGGCGGCTCTGAAGAGTTG 3’ and anti-sense 5’ AGCTCTCTAGACTCAGGCCCTCAATCCTCA 3’. The GAPDH primers were: sense 5’ GCCGGGATCCATGGCGGCTCTGAAAGCTTG 3’ and anti-sense 5’ AGCTCTCTAGACTCAGGCCCTCAATCCTCA 3’. The PCR cycle parameters for Smac were: 10 min enzyme activation at 95°C followed by 3 cycles of 30 s at 95°C and 2 min at 72°C, then 30 cycles of 30 s at 95°C and 30 s at 68°C, and finally 5 min at 72°C. The corresponding parameters for GAPDH were: 10 min enzyme activation at 95°C followed by 25 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. The products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Smac mRNA data were expressed as ratios between the densitometric values (Scion Image software) of Smac gene.
expression. The PCR products were normalized to the amplified GAPDH, the internal reference gene. Gene expression measurements were repeated at least twice.

**Statistical analysis**

To detect a correlation between pathological tumor parameters and normalized Smac expression we used ANOVA (stage, current disease and menopause status) and chi square tests (stage, histology of tumors, menopause and current status). Kaplan-Meier curves for status were generated and log rank was used to test for differences. The mean follow-up was 14.7 months. The statistical package Intercooled Stata 7.0 was used for analyses and statistical significance was accepted when the p value was less than 0.05.

**Results**

To ascertain whether Smac is expressed in cervical cancer we performed semiquantitative RT-PCR analyses on a panel of cervical cancer lines, including HeLa, SiHa, CasKi and CaLo cells. As shown in Figure 1, the HeLa and CasKi lines contained Smac mRNA, but very low levels were observed in SiHa and CaLo cells.

Next, we measured Smac mRNA levels using the same approach in 41 cervical tumor and 6 normal cervical samples. To ensure accurate determinations and to verify equal RNA input, GAPDH mRNA was amplified simultaneously. Figure 2 shows a representative panel of results, which are given in Tables 1 and 2. Unexpectedly, Smac mRNA was below the detection limit in normal cervical samples. In contrast, as expected from the cell line data, 13 (31.7%) of the 41 cervical cancer biopsies contained detectable levels of this transcript. The samples expressing Smac were distributed equally among the stages (5 in stage I, 4 in stage II and 4 in stage III). We found no significant correlation between Smac mRNA level and histology, menopause, clinical stage or disease status (Table 2). When the Smac expression levels in the tumor samples were analyzed, there were no significant differences between clinical stages (Figure 3), menopause status (Figure 4) or disease status (Figure 5). Similarly, a survival analysis of the patients showed no statistical differences.
between patients expressing or not expressing Smac mRNA (Figure 6).

**Discussion**

Tumors proliferate beyond the constraints that limit growth in normal tissue. Therefore, the resistance of tumor cells to apoptosis is an essential feature of carcinogenesis. This has been confirmed by the finding that deregulated proliferation alone is not sufficient for tumor formation because there is concomitant induction of cell death [25]. Overexpression of growth-promoting oncogenes such as c-Myc sensitize cells to apoptosis [26]. Thus, tumor progression requires the expression of anti-apoptotic proteins or the inactivation of essential pro-apoptotic proteins [27,28]. Indeed, it has been shown that survivin, a member of the Inhibitor of Apoptosis Protein (IAP) family, is upregulated in some tumors [29], correlating with prognosis [30,31].

Smac is a recently identified proapoptotic protein that interacts with and inhibits several IAPs, including survivin [6,11]. It has been shown that Smac mRNA levels in tumor tissues are significantly lower than in normal tissues [23]. Patients with lower Smac mRNA levels have worse prognoses. These results indicate that Smac expression may play a role in the progression of primary lung
cancer, as expected by the known role of this protein in cell death induced by chemotherapeutic drugs. Unexpectedly, we found that during cancer progression, some cervical tumors express this protein de novo.

Unfortunately, we found no correlation between Smac expression and any clinical variable. This could be attributed to differences in tissue expression of IAPs, which are reported to have different binding affinities for Smac. On the other hand, alternative IAPs such as the recently identified Omi/Htra2 [32] might play an important tissue- or tumor-specific role. This is supported by the recent report of a null phenotype in Smac-deficient mice, in which a role for other mitochondrial molecules such as Omi might substitute for its known proapoptotic function. There was no correlation between Smac expression and any clinical variable.

Cancer treatment by chemotherapy and $\gamma$-irradiation kills cells primarily by the induction of apoptosis. However, few tumors are wholly sensitive to these therapies, and the development of resistance to therapy is an important clinical problem. Failure to activate the apoptotic programme represents an important mode of drug resistance in tumor cells [34]. Modulation of the key elements in apoptotic signaling should directly influence therapy-induced tumor-cell death. Indeed, it has recently been suggested that peptides mimicking the Smac amino-terminus could be a novel therapeutic weapon [19]. Tumors with low or null Smac expression, such as the ones reported in this study, could be more susceptible to this approach.

Conclusions
During cervical cancer progression, a subset of tumors express the apoptotic protein Smac de novo. This finding contrasts with a previous report for lung cancer [23], underlining the notion that downregulation or even expression of Smac could be dispensable for tumor progression, at least in cervical cancer. This could be because other mitochondrial molecules such as Omi might substitute for its known proapoptotic function. There was no correlation between Smac expression and any clinical variable.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
JMZ: Conceived and coordinated the study.
VAML: Conceived and coordinated the study. Statistical Analysis
MEC: Performed RT-PCR assays
DCL: Provided the clinical samples and coordinated patient study
CMLG: Coordinated patient assessment, ethical guidelines.
JGGS: Provided clinical assessment

Acknowledgements
This work was supported by grants CONACYT-2002-C01-42040/A-1 and SALUD-2002-C01-6579 from Consejo Nacional de Ciencia y Tecnología, México.

References
1. Uren AG, Coulson EJ, Vaux DL: Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. Trends Biochem Sci 1998, 23:159-162.
2. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X: Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999, 15:269-290.
3. Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 2002, 9:469-470.
4. Deveraux QL, Reed JC: IAP family proteins--suppressors of apoptosis. Genes Dev 1999, 13:239-252.
5. Shi Y: A conserved tetrapeptide motif: potentiating apoptosis through IAP-binding. Cell Death Differ 2002, 9:93-95.
6. Du C, Fang M, Li Y, Li L, Wang X: Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000, 102:33-42.
7. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL: Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000, 102:43-53.
8. Srinivasula SM, Datta P, Fan XJ, Fernandes-Alnemri T, Huang Z, Alnemri ES: Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. J Biol Chem 2000, 275:36152-36157.
9. Tikoo A, O'Reilly L, Day CL, Verhagen AM, Pakusch M, Vaux DL: Tissue distribution of Diabolo/Smac revealed by monoclonal antibodies. Cell Death Differ 2002, 9:710-716.

10. Chai J, Du C, Wu JW, Kyrin S, Wang X, Shi Y: Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature 2000, 406:855-862.

11. Song Z, Yao X, Wu M: Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis. J Biol Chem 2003, 278:23130-23140.

12. Vucic D, Deshayes K, Ackerly H, Pisabarro MT, Kadkhodayan S, Fairbrother WJ, Dixit VM: SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). J Biol Chem 2002, 277:12275-12279.

13. Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X, Shi Y: Structural basis of IAP recognition by Smac/DIABLO. Nature 2000, 408:1008-1012.

14. Liu Z, Sun C, Olejniczak ET, Meadows RP, Betso SF, Oost T, Herrmann J, Wu JC, Fesik SW: Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature 2000, 408:1004-1008.

15. Yang L, Cao Z, Yan H, Wood WC: Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. Cancer Res 2003, 63:6815-6824.

16. Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N: Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res 2000, 6:127-134.

17. Ferreira CG, van der Valk P, Span SW, Jonker JM, Postmus PE, Kruyt FB, Zetter BR, Arap W, Reed JC, Pasqualini R: Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature 2000, 277:44236-44243.

18. Guo F, Nimmanapalli R, Paranawithana S, Wittman S, Griffin D, Bali P, Ou J, Yang L, Cao Z, Elia A, Duncan GS, Wakeham A, Itie A, Lowe SW, Wang X, Mak TW: Generation and characterization of Smac/DIABLO-deficient mice. Mol Cell Biol 2002, 22:3509-3517.

19. Kim R, Tanabe K, Uchida Y, Eni M, Inoue H, Toge T: Current status of the molecular mechanisms of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy. Cancer Chemother Pharmacol 2002, 50:343-352.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2407/4/84/prepub

Publish with BioMed Central and every scientist can read your work free of charge
"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime." Sir Paul Nurse, Cancer Research UK
Your research papers will be:
• available free of charge to the entire biomedical community
• peer reviewed and published immediately upon acceptance
• cited in PubMed and archived on PubMed Central
• yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp