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Anti-tumor Effect of SLPI on Mammary But Not Colon Tumor Growth

NICOLÁS O. AMIANO,[1] MARÍA J. COSTA,[1] R. MACARENA REITERI,[1] CRISTIAN PAYÉS,[1] DIEGO GUERRIERI,[1] NANCY L. TATEOSIAN,[1] MERCEDES L. SÁNCHEZ,[1] PAULO C. MAFFIA,[1] MIRIAM DIAMENT,[2] ROMINA KARAS,[2] ANDRÉS ORQUEDA,[3] MIGUEL RIZZO,[4] LAURA ALANIZ,[4] GUILLERMO MAZZOLINI,[4] SLOBODANKA KLEIN,[2] JEAN-MICHEL SALLENAVE,[5,6,7] AND H. EDUARDO CHULUYAN[1]*

[1]*Department of Pharmacology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina*

[2]*Animal Care and Experimental Cancer Research, Oncology Institute A.H. Roffo, Buenos Aires, Argentina*

[3]*Department of Biological Chemistry, FCEN, IFIBYNE – UBA – CONICET, Buenos Aires, Argentina*

[4]*Gene Therapy Laboratory, School of Biomedical Science, Austral University, Pilar, Prov. Buenos Aires, Argentina*

[5]*Unité de Défense Innée et Inflammation, Institut Pasteur, Paris, France*

[6]*Institut National de la Santé et de la Recherche Médicale (INSERM) U874, Paris, France*

[7]*Université Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, rue du Dr Roux, Paris, France*

Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor that was related to cancer development and metastasis dissemination on several types of tumors. However, it is not known the effect of SLPI on mammary and colon tumors. The aim of this study was to examine the effect of SLPI on mammary and colon tumor growth. The effect of SLPI was tested on in vitro cell apoptosis and in vivo tumor growth experiments. SLPI over-expressing human and murine mammary and colon tumor cells were generated by gene transfection. The administration of murine mammary tumor cells over-expressing high levels of SLPI did not develop tumors in mice. On the contrary, the administration of murine colon tumor cells over-expressing SLPI, developed faster tumors than control cells. Intratumoral, but not intraperitoneal administration of SLPI, delayed the growth of tumors and increased the survival of mammary but not colon tumor bearing mice. In vitro culture of mammary tumor cell lines treated with SLPI, and SLPI producer clones were more prone to apoptosis than control cells, mainly under serum deprivation culture conditions. Herein we demonstrated that SLPI induces the apoptosis of mammary tumor cells in vitro and decreases the mammary but not colon tumor growth in vivo. Therefore, SLPI may be a new potential therapeutic tool for certain tumors, such as mammary tumors.

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In recent years, cancer-related genes that code for proteins with whey acidic protein motifs have been identified (Bouchard et al., 2006). Among them secretory leukocyte protease inhibitor (SLPI) has been very well characterized as an anti-inflammatory agent (Williams et al., 2006). SLPI is mainly synthesized and secreted by inflammatory and epithelial cells of the respiratory, digestive, and genital mucosa. SLPI is an anti-protease active against serine proteases such as neutrophilic elastase, cathepsin G, trypsin, and chymotrypsin (Hiemstra, 2002). Other functions described for SLPI, such as bactericidal and anti-viral activity, are not related to their anti-protease activity (Williams et al., 2006; Sallenave, 2010). Furthermore, we have shown in our laboratory that SLPI is a secreted pattern recognition receptor for mycobacteria (Gomez et al., 2009).

The expression of SLPI has been described in several tumors. For instance, a significant increased expression was found in gastric (Cheng et al., 2008), pancreatic (Iacobuzio-Donahue et al., 2003), thyroidal (Jarzab et al., 2005), uterus cervix (Rein et al., 2004), endometrial (Zhang et al., 2002), and ovarian cancers (Israeli et al., 2005), whereas the expression was more modest in nasopharyngeal carcinoma (Sriuranpong et al., 2004; Huang et al., 2012), bladder tumors (Liang et al., 2002), and some mammary carcinomas (Hu et al., 2004). In ovarian cancer, SLPI is over-expressed and is thought to have a carcinogenic function (Hough et al., 2001; Clauss et al., 2005; Israeli et al., 2005; Devoogdt et al., 2009) independent of its anti-protease activity (Simpkins et al., 2008). However, in Lewis lung cancer cells, the pro-tumoral activity was shown to be dependent on its protease inhibitory activity (Devoogdt et al., 2003). More recently, significantly decreased SLPI was detected in oral squamous cell carcinoma compared with normal oral

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*Correspondence to: H. Eduardo Chuluyan, 3ra Cátedra de Farmacología, Piso 9, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, PC: C1121ABG, Buenos Aires, Argentina. E-mail: echuluyan@gmail.com

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epithelium (Wen et al., 2011). Moreover, an inverse correlation was also reported between SLPI and histological parameters associated with tumor progression (Wen et al., 2011). Interestingly, SLPI was shown to reduce hepatic Lewis lung carcinoma metastasis (Wang et al., 2006).

In breast tumors, SLPI mRNA expression either increases or decreases depending on the case (Kluger et al., 2004; Stoff-Khalili et al., 2005). Using a mammary tumor cell line, Sugino et al. (2007) showed that SLPI suppresses cancer cell invasion but promotes blood-borne metastasis via an invasion-independent pathway. More recently, it was demonstrated that SLPI inhibits cell growth of ovarian cancer cells through an apoptotic pathway (Nakamura et al., 2008), but conversely, other authors, have described that over-expression of SLPI is capable of producing more aggressive ovarian cancer (Devoogdt et al., 2009). On the other hand, we have demonstrated previously that an immunotherapy with SLPI over-expressing mammary tumor cells which do not develop tumor in immunocompetent mice, partially restrain the tumor growth induced by control cells and increase the survival of the mice (Amiano et al., 2011).

Although the studies mentioned above suggest a participation of SLPI in cancer, whether this molecule is beneficial or detrimental in the different situations is currently difficult to assess. Given the current state of knowledge, the aim of our present report was to elucidate the effect of SLPI in breast and colon cancer by performing in vitro and in vivo experiments with mammary and colon tumor cells.

Our results show that SLPI has anti-tumor activity in a mammary tumor model but not in a colon tumor model. Furthermore, SLPI induces the apoptosis of mammary tumor cells.

Materials and Methods

Tumor cell lines and culture conditions

F3II is a murine mammary carcinoma hormone-independent cell line derived from BALB/c (Alonso et al., 1996). These cells were cultured in RPMI1640 (Gibco, Grand Island, NY), 10% FBS, 2 mM L-glutamine, and 40 μg/ml gentamycin (37°C, 5% CO_2). LP07 is a murine lung carcinoma cell line derived from BALB/c that has been also characterized previously (Urtreger et al., 2001). CT26 tumor cell line (murine colon carcinoma), MCF7 (human breast adenocarcinoma), HeLa (human cervix adenocarcinoma), A549 (human lung carcinoma), and HCT116 (human colorectal carcinoma) were purchased from American Type Culture Collection. The cell lines MCF7, HeLa, and HCT116 were grown under the same conditions of F3II cells, while CT26 and LP07 were cultured in D-MEM (Gibco) 10% FBS, 2 mM L-glutamine, 100 U/ml streptomycin, and 100 mg/ml penicillin. The A549 cell line was cultured in Ham's F12K medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, and 40 μg/ml gentamicin. For all the experiments, low-passage cell lines were used. Quality control tests were done in all cell lines used in our studies at the beginning of the studies and periodically. These tests have included the check of cellular morphology with optical microscope, the growth curve analysis and mycoplasma detection with Hoeschst 33258. After 1 year, the clone B3 did not passed the quality control and, even, it lost the ability to secrete SLPI. Therefore, it was not used for further experiments.

Production and purification of human recombinant SLPI

rhSLPI was obtained as it was previously described (Maffia et al., 2007). The enzymatic activity of rhSLPI was analyzed in a trypsin inhibition assay using the chromogenic substrate N-succinyl–Ala–Ala–Pro–Phe-p-nitroanilide (Sigma, St. Louis, MO). Before using rhSLPI in in vivo and in vitro experiments, potential lipopolysaccharide (LPS) contaminant was removed with a polymyxin B column (Sigma) and the presence of remaining LPS was evaluated using the Lymulus test (Cambrex, Walkersville, MD).

Plasmid construction, transfection, and clones selection

Human SLPI cDNA (including the sequence codifying for the signal peptide) (Thompson and Ohlsson, 1986) was isolated by RT-PCR on total mRNA from HeLa cells using the following primers: 5′-GGCAGGAATCAAGCTTTCACA-3′ and 5′-TCACCATGAA-GTCCAGCGGC-3′. The fragment was first cloned into pGemT easy vector (Promega, Madison, WI) to amplify the sequence, and then it was subcloned into the *Eco*RI site of the pcDNA3 vector (Invitrogen, Carlsbad, CA). After sequence verification, the plasmid containing hSLPI cDNA and the vector alone were transfected into semi-confluent F3II, CT26, and MCF7 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Selection of stable transfectants was performed in the presence of 400 μg/ml of G418 Sulfate (Gibco). Colonies resistant to G418 Sulfate were selected by limiting dilution. hSLPI expression in the selected clones was evaluated measuring hSLPI concentration in the culture supernatants of clones by ELISA sandwich assay. For this ELISA assay, we used a mAb anti-hSLPI (Clone 20409, R&D Systems, Minneapolis, MN), a rabbit polyclonal antibody (Hbt, Uden, The Netherlands), and a goat polyclonal anti-rabbit IgG conjugated with peroxidase (Chemicon, Temecula, CA).

Cell treatment and apoptosis measurement

Cells were treated with rhSLPI (4 μg/ml) or control buffer for 48 h. Then, cells were harvested with 0.05% trypsin–EDTA (Gibco) and apoptosis was measured by: (1) detection of annexin V and propidium iodide (BD Biosciences, San Jose, CA) staining using a flow cytometer FACSCalibur (BD) and (2) cell staining with ethidium bromide/acridine orange assay (Duke and Cohen, 1992), using an ultraviolet fluorescence microscope (Nikon Eclipse TS100, Tokio, Japan). Cells were scored into categories: C1 as non-apoptotic cells; C2 as apoptotic cells; and C3 as necrotic cells. At least, 200 cells/samples were counted and scored.

Caspase-3-like activity assay

Cells were lysed in 50 mM Tris–HCl buffer pH 7.4 containing 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF, 1.5 μM aprotinin, and 14.6 μM pepstatin (30 min, 37°C). Cells lysates were clarified by centrifugation at maximum speed and 150 μl of the supernatant (30 μg protein) were incubated with incubation buffer (100 mM HEPES pH 7.5, 20% glycerol, 0.5 mM EDTA, and 5 mM dithiothreitol) containing 100 μM substrate acetyl-Asp–Glu–Val–Asp-7-amino-4 p-nitroanilide (Ac-DEVDpNA, Calbiochem, Merck, Darmstadt, Germany) at 37°C for up to 24 h. Controls were run containing either the substrate or the cell lysate alone. Caspase-catalyzed release of the chromophore pNA from the substrate was measured at 415 nm with a spectrophotometer (Benchmark, Bio-Rad, Hercules, CA) and the cleavage activity was expressed as pNA absorbance units per milligram protein. Protein concentration was determined using the Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) with BSA as a standard.

In vitro cell proliferation assays

The cells 12B3, 9H6, 5E3, CT26, and MCF7 (3×10^4/well) were incubated in RPMI 1640 (Gibco), supplemented or not (as indicated) with 10% FBS, 2 mM L-glutamine, and 40 μg/ml gentamicin at 37°C in a 5% CO_2 atmosphere. After 24 h, cells were pulsed with 5 μCi/ml [methyl-^{3}H] thymidine (specific activity 20Ci/mmol; Perkin-Elmer, Boston, MA) for the last 18 h of culture. Finally, the cells were harvested and radioactivity was measured in a liquid scintillation β-counter (Wallac 1214 Rackbeta, Turku, Finland).

Small interfering RNA

The small interfering RNA (siRNA) duplex for SLPI was synthesized by Invitrogen Corporation (Invitrogen). BLAST searches of the human genome database were carried out to ensure the sequences would not target other gene transcripts. SLPI over-expressing cells (2C1) were plated at 2.5×10^5 cells/well (for 24 h) and then transfected with 50 pmol siRNA using Lipofectamine^{TM} 2000 (Invitrogen), following the procedures provided by manufactures. Gene silencing was checked by measuring SLPI protein using a sandwich ELISA assay 24 h after transfection. Control cells were treated with siRNA duplex targeting scramble (Invitrogen).

In vivo assays

Female BALB/c mice (8 weeks old) were maintained at the Animal Facility of the Microbiology Department (School of Medicine, Buenos Aires University). The following protocol was approved by the Animal Care and Use Committee of the School of Medicine, Buenos Aires University. Mice were inoculated with either non-transfected or transfected F3II cells (8×10^5) or CT26 (5×10^5) or with LP07 (3×10^5) cells, subcutaneously in the right flank in a total volume of 200 µl. The tumor length (L) and width (W) were measured with a caliper three times a week and the tumor volume (V) was calculated as $V = (L \times W^2)/2$. No animals died during the experiments. The endpoints were arbitrarily set for a tumor volume of 1,500, 500, or 2,000 mm^3 for F3II, LP07, or CT26, respectively, in the case of inoculation of the peptide. The interval between tumor cell inoculation and the endpoint was defined as the survival time. Animals with tumor reaching the volume target were sacrificed. rhSLPI (4 µg/ml) or the dilution buffer (as control) were administered intraperitoneal (i.p. 40 µg/ml) or in the inoculation site of the tumor cells (intratumoral, i.t. – 4 µg/ml) three times a week. In another experimental setup, F3II (8×10^5) and 2C1 (3×10^6) cells were pre-mixed and co-administered in the same flank.

Statistical analysis

Student's *t*-test or ANOVA with the Dunnett post test was used. Kaplan–Meier plots were used for survival analysis and the comparison among curves was carried out with the Log-rank test.

Results

Effect of hSLPI expression on tumor growth

Mammary (F3II) and colon (CT26) murine carcinoma tumor cell lines were transfected with the pcDNA3 plasmid containing the hSLPI gene as described in the Materials and Methods Section. Cell clones 2C1 and B3 were obtained from F3II cells and produced over a 96-h cell culture period, 96 ± 8 and 52 ± 5 ng/ml of hSLPI, respectively. On the other hand, the transfection of CT26 cells generated only one clone (12B3) that produced significant levels (22 ± 6 ng/ml) of hSLPI. We have previously described that the administration of 5×10^6 F3II cells, but not 2C1 cells, developed tumors in BALB/c mice (Amiano et al., 2011). In the present study, the same result was obtained by the inoculation of 8×10^5 F3II and 2C1 cells. Accordingly, Figure 1A shows that the administration of 8×10^5 F3II control cells to BALB/c mice developed tumors. However, none of the animals developed tumors along the 90 days of our protocol when 2C1 cells were inoculated (Fig. 1A). On the other hand, all the animals inoculated with B3 cells developed smaller tumors and with a longer latency period (20 days for control cells vs. 32 days for B3 clone) than animals that received control cells (Fig. 1A). Furthermore, only one of the animals receiving the B3 clone had to be euthanized for having reached the experiment endpoint on Day 72 (Fig. 1B). Moreover, the control animals had multiple lung metastatic foci, while animals treated with the 2C1 (Supplementary Fig. 1) or B3 (data not shown) clones were completely free of metastasis.

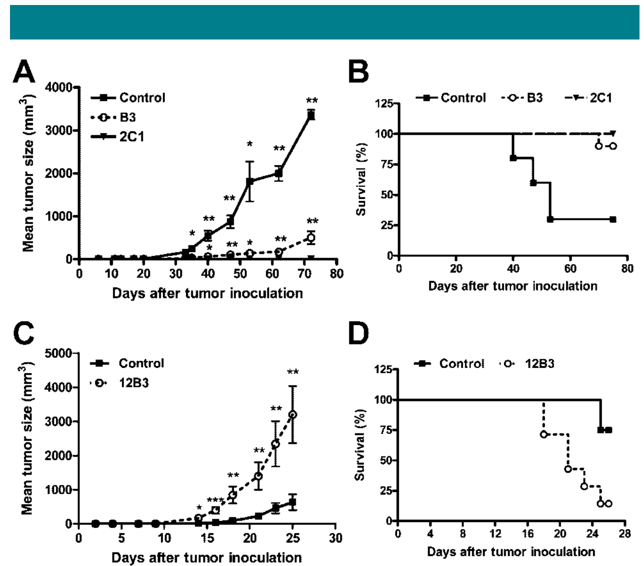

Fig. 1. Effect of hSLPI expression on tumor growth. BALB/c mice were inoculated s.c. with 8×10^5 F3II transfected cells: 2C1, B3, or control cells (A) or with 3×10^5 CT26 transfected cells: 12B3 or control (C) and the tumor growth was evaluated. It is shown the mean ± SD of one representative experiment of five for (A) and three for (C). Each group contained 10 animals. Tumor growth was monitored for 72 days (*$P < 0.05$ and **$P < 0.01$ B3 and 2C1 vs. control, ANOVA with Dunnett's multiple comparison post test) for (A) and 25 days (*$P < 0.05$ and **$P < 0.01$, unpaired Student's *t*-test) for (C). B and D: Survival curves of mice from the experiments showed in (A) and (C), respectively. Data are expressed as Kaplan–Meier survival curves. (*$P < 0.05$ 2C1 and B3 vs. control s.c. for (A), Log-rank test).

Next, we analyzed the growth of tumors induced by the administration of CT26 control cells and 12B3 clone. Figure 1C shows that the administration of CT26 control colon cells to BALB/c mice produced tumors faster than F3II cells (Fig. 1A). Furthermore, Figure 1C also shows that the subcutaneous (s.c.) administration of 5×10^5 12B3 cells generated tumors significantly larger than CT26 transfected with empty plasmid. Moreover, the animals inoculated with 12B3 survived less than control mice (Fig. 1D). These results suggested that the effect of SLPI expression on tumor growth could be different depending on the tumor cells; that is inhibitory on mammary and stimulatory on colon tumor cells.

Effect of SLPI administration on tumor growth

The effect of exogenous administration of SLPI was next examined on mammary, lung, and colon tumor growth. Different tumor cell lines (F3II, LP07, or CT26) were inoculated to BALB/c mice and rhSLPI was administered three times a week intraperitoneal (40 µg/ml) or in the inoculation site of the tumor cells (4 µg/ml). Figure 2A shows that i.p. rhSLPI administration did not modify the rate of tumor growth generated by mammary F3II tumor cells. In contrast, inoculation at the tumor site of rhSLPI (Fig. 2A) or denatured rhSLPI (Fig. 2C) led to significantly smaller tumors, with lower growth rate than control-treated animals. Furthermore, none of the rhSLPI-treated animals reached the end culling point (tumor size $< 1{,}300$ mm^3) throughout the experiment (day 43) (Fig. 2B). Also, survival in the denature rhSLPI group was significantly prolonged compared with the control group (Fig. 2D). These results indicate that the rhSLPI anti-tumoral activity was not dependent of its anti-protease activity. However, the administration of rhSLPI at the tumor site, did not reduce the volume of tumors induced by the administration of CT26

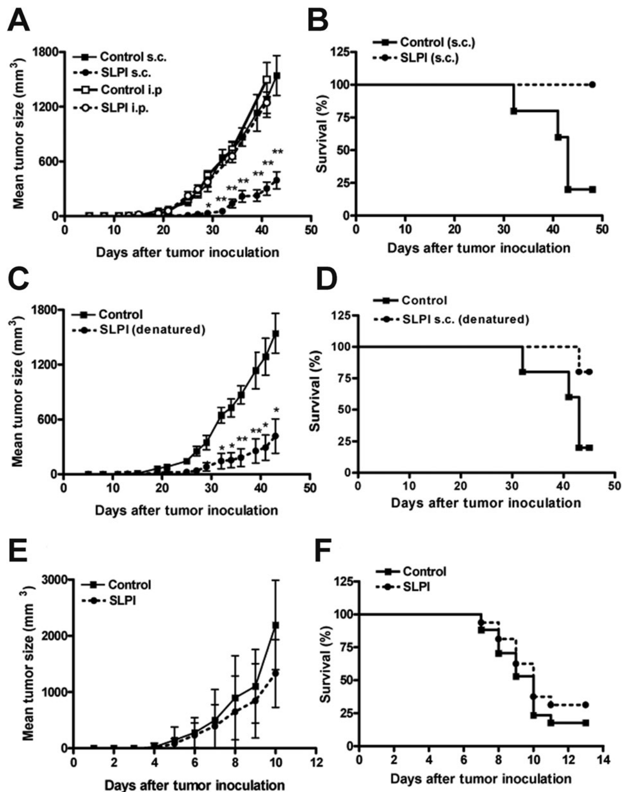

Fig. 2. In vivo effect of rhSLPI administration on tumors. (A) BALB/c mice were inoculated s.c. with 8×10^5 F3II cells. Animals were treated with rhSLPI i.p. (40 µg/ml), rhSLPI s.c. (4 µg/ml), or denature rhSLPI s.c. (4 µg/ml) (C) as described in the Materials and Methods Section. Tumor growth was monitored for 44 days and the protein was administered three times a week during the experiments. For each treatment, mean ± SD is shown (one representative experiment of four). Each group contained 10 animals ($^*P < 0.05$ and $^{**}P < 0.01$ SLPI s.c. vs. control s.c., ANOVA with Dunnett's multiple comparison post test for [A] and $^*P < 0.05$ and $^{**}P < 0.01$, unpaired Student's *t*-test for [C]). E: BALB/c mice were inoculated s.c. with 5×10^5 CT26 and treated s.c., at the tumor site, with either rhSLPI (4 µg/ml) or dilution buffer as a control, three times a week. Mean ± SD of one representative experiment of three is shown. B, D, and F: Survival curves of mice from the experiments showed in (A), (C), and (E), respectively. Data are expressed as Kaplan–Meier survival curves ($^*P < 0.05$ SLPI s.c. vs. control s.c. for (A), and $^*P < 0.05$ SLPI denatured vs. control for (D), Log-rank test).

(Fig. 2E) or LP07 (lung tumor cells, Supplemental Fig. 2A). Furthermore, the administration of rhSLPI did not affect the survival of mice bearing colon (Fig. 2F) or lung (Supplemental Fig. 2B) tumor cells. These results suggest that the anti-tumor effect of rhSLPI is specific, at least, for certain mammary tumors. Also suggest that SLPI needs to be administered locally at the tumor site to decrease mammary tumor growth. In fact, BALB/c mice inoculated with F3II and 2C1 cells at the same site (Supplementary Fig. 3A,B), but not in different flanks (Amiano et al., 2011), developed smaller tumors and survived longer. Together, these results reinforce the idea that the anti-tumoral activity of exogenous and 2C1-secreted SLPI is mediated locally.

Effect of SLPI on apoptosis

Although previous results suggest that an immune response may be developed after repeated administration of 2C1 cells protecting mice from F3II-induced tumors (Amiano et al., 2011), the current in vivo experiments suggested a direct effect of SLPI on mammary tumor cells. Furthermore, we also showed previously that 2C1 cells were more prone to apoptosis than F3II control cells (Amiano et al., 2011). Therefore, we next studied further the putative mechanism of action of SLPI on tumor cells by analyzing the induction of apoptosis in vitro under different conditions. For this, apoptosis of 2C1 cells was measured under starvation (cultured without FBS) and normal conditions (with FBS), by measuring Annexin V-FITC Apoptosis (BD Biosciences) and ethidium bromide/acridine orange staining. Figure 3A and 3B show that the apoptosis of 2C1 cells was significantly higher than that observed in F3II cells, regardless of the presence of FBS in the culture. However, this difference was more significant under serum deprivation conditions. Similar results were observed when apoptosis was measured by ethidium bromide/acridine orange staining under serum-containing (Amiano et al., 2011) and serum-deprived conditions (Fig. 3C,D). Moreover, 2C1 cells cultured in absence of FBS, but not in the presence of FBS, showed an 18-fold increase in caspase-3 activity compared with F3II control cells (Fig. 3E). By contrast, we did not observe differences on cell apoptosis in 12B3 cells compared with CT26 under any of the culture conditions tested by using either, the Annexin V-FITC assay (Fig. 3F) or by measuring ethidium bromide/acridine orange staining (data not shown). Furthermore, the proliferation of CT26 control cells decreased significantly in the absence of FBS. However, the cell proliferation of 12B3 was not affected when these cells were cultured under serum deprivation condition (Fig. 3G).

Then, SLPI knockdown experiments were performed in order to determine the role of this protein on 2C1 cell apoptosis. As shown in Figure 4A, SLPI siRNA treatment significantly reduced the SLPI levels in supernatant of 2C1 cells measured 24 h after transfection. Interestingly, 2C1 siRNA transfected cells showed a slight, but statistically significant, decrease in early apoptosis compared with control cells (Fig. 4B). In order to determine whether secreted SLPI was the inducer of 2C1 cell apoptosis, SLPI antibody-blocking experiments were also performed. Figure 4C shows that the 2C1 cells treated with blocking antibody (AB-260-NA; R&D Systems) but not with isotype control antibody (AB-108-C; R&D Systems) also decreased the early apoptosis. To confirm that SLPI was able to induce the cell apoptosis in vitro, F3II cells were cultured in the presence of rhSLPI (4 µg/ml, 48 h) and apoptosis was measured by flow cytometry (Fig. 4D) and ethidium bromide/acridine orange staining (Supplementary Fig. 4). Figure 4D shows that SLPI increased the F3II cells apoptosis in serum or serum-free conditions. However, like for 2C1 cells (Fig. 3B) the difference was more significant under serum-free conditions. Furthermore, a 3.5-fold increase in caspase-3 activity was observed on F3II cells treated with SLPI in serum-free culture condition (Fig. 4E). It is important to point out that, under the same culture conditions described for F3II cells, SLPI did not induce apoptosis (Supplementary Fig. 5) nor increased caspase-3 activity (data not shown) in SLPI-treated CT26 cells.

The relevance of these findings was further tested by analyzing the effect of SLPI on apoptosis in different human tumor cell lines, such as A549 (lung carcinoma), HeLa (cervix adenocarcinoma), HCT116 (colorectal carcinoma), and MCF7 (breast adenocarcinoma). Figure 5A shows that SLPI did not modify the apoptosis of HCT116; however, it slightly decreased the apoptosis of A549 cultured without FBS (Fig. 5B). By contrast (Fig. 5C), SLPI increased the apoptosis of HeLa (also under starving culture conditions). Remarkably, we observed a significant increase of apoptosis (Fig. 5D) and a decrease in cell proliferation (Fig. 5E) in MCF7 cells cultured in the presence of rhSLPI with or without FBS. However, the SLPI effect on

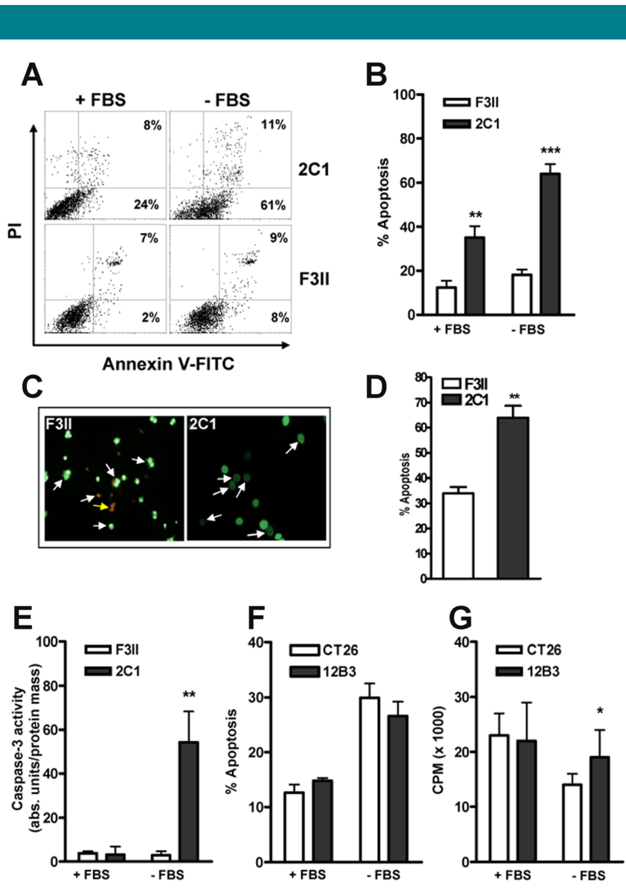

Fig. 3. Effect of hSLPI expression on cell apoptosis. F3II and 2C1 cells were cultured in complete medium (+FBS) or medium without serum (–FBS) for 48 h. After, cells were harvested and apoptosis was measured by flow cytometry (A) and (B) and ethidium bromide/ acridine orange staining (C) and (D). A: Flow cytometric analysis of a representative experiment of five. B: Percentage of total apoptosis (calculated as the sum of the percentages of lower right and upper right quadrants of the cytometric analysis) is shown as mean ± SEM of five independent experiments ($P < 0.01$ 2C1 vs. F3II [+FBS]; ***$P < 0.001$ 2C1 vs. F3II [–FBS], paired Student's t-test). C: Ethidium bromide/acridine orange staining of F3II and 2C1 cells cultured without FBS. Cells with large, bright green, non-condensed nuclei are non-apoptotic cells; cells with dimmed green nuclei that show signs of nuclear condensation (or with membrane blebbing) or cells with red/ orange nuclei were analyzed as apoptotic cells (white arrows shown as example). Cells with large red nuclei are necrotic cells (yellow arrows). D: Percentage of apoptotic cells cultured without FBS measured by ethidium bromide/acridine orange staining shown as mean ± SEM of five independent experiments (**$P < 0.01$ 2C1 vs. F3II, paired Student's t-test). E: Measurement of caspase 3 activity on F3II and 2C1 cells cultured with or without FBS (**$P < 0.01$ 2C1 vs. F3II [–FBS]; unpaired Student's *t*-test). F: Percentage of total apoptosis of 12B3 clone and CT26 control cells cultured in the same conditions than 2C1 and F3II cells in (B). It is shown the mean ± SEM of three independent experiments. G: Proliferation rate of 12B3 clone and CT26 cells (*$P < 0.05$ 12B3 [–FBS] vs. control [–FBS]; unpaired Student's *t*-test).**

both parameters was more significant under serum deprivation conditions.

In order to confirm the pro-apoptotic role of SLPI on human mammary tumors cells, MCF-7 were transfected with the pcDNA3 plasmid containing the hSLPI gene as described in the Materials and Methods Section. Two cell clones' producers of similar levels of hSLPI were obtained (5E3 and 9H6 produced 41 ± 15 and 25 ± 3 ng/ml, of hSLPI respectively). The MCF-7 clones had a lower proliferation rate under serum deprivation condition (Supplementary Fig. 6) compared with control MCF-7 cells. Furthermore, Figure 5F shows that, similarly to that observed with 2C1 cells, the total apoptosis of 5E3 and 9H6 cells was significantly higher than that observed in MCF-7 control cells, regardless of the presence of FBS in the culture. These results show that, in vitro, SLPI has a similar effect on a human mammary tumor cell line to that observed on murine mammary tumor cells.

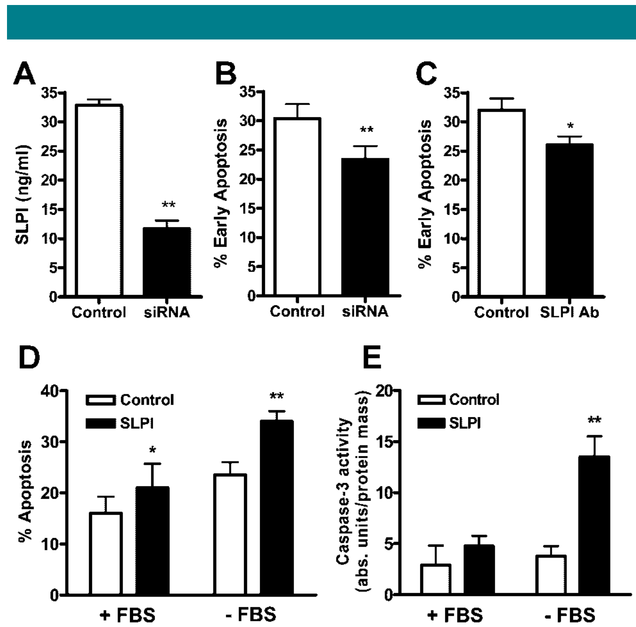

Fig. 4. Effect of blocking and SLPI treatment on cell apoptosis. A: SLPI expression by 2C1 siRNA transfected cells. SLPI levels were evaluated in culture supernatants collected 24 h after transfection ($P < 0.01$, unpaired Student's *t*-test). B: Measurement of apoptosis by flow cytometry of 2C1 cells transfected with siRNA-SLPI. Over-expressing SLPI cells (2C1) were cultured 24 h; then, cells were transfected with siRNA-SLPI or control siRNA. Cells were harvested and early apoptosis was measured by flow cytometry. Mean ± SEM of five independent experiments are shown (**$P < 0.01$, paired Student's *t*-test). C: Apoptosis measurement of 2C1 cells treated with a blocking SLPI antibody. 2C1 cells were cultured 24 h, after cells were treated with a blocking SLPI antibody or isotype control antibody. After 48 h, cells were harvested and apoptosis was measured as previously described. Mean ± SEM of three independent experiments are shown (*$P < 0.05$, paired Student's *t*-test). D: Percentage of total apoptosis of F3II cells cultured with or without FBS in presence of rhSLPI (4 μg/ml) or control buffer (*$P < 0.05$ and **$P < 0.01$ SLPI vs. control buffer, paired Student's *t*-test). E: Measurement of caspase 3 activity on treated F3II cells (**$P < 0.01$ SLPI vs. control buffer, unpaired Student's *t*-test).**

Discussion

Some reports associate SLPI with malignancy of tumors (Zhang et al., 2002; Devoogdt et al., 2003; Kluger et al., 2004; Tsukishiro et al., 2005; Devoogdt et al., 2009), while others show, conversely, that SLPI inhibits the aggressiveness of tumors, for example through inhibition of tumor-derived proteases (Alkemade et al., 1994; Westin et al., 2002). Our results demonstrate an anti-tumor activity of SLPI in mammary but not in colon tumors, in two different models: (1) in SLPI over-expressing tumor cells and (2) using exogenously intra-tumorally administered SLPI. In both models, we showed that the anti-tumor mechanism involves apoptosis. For example, over-expressing SLPI cells (2C1) but not control cells, showed to be apoptotic when either cultured with or without serum. However, the apoptosis of 2C1 cells is seen mainly under serum

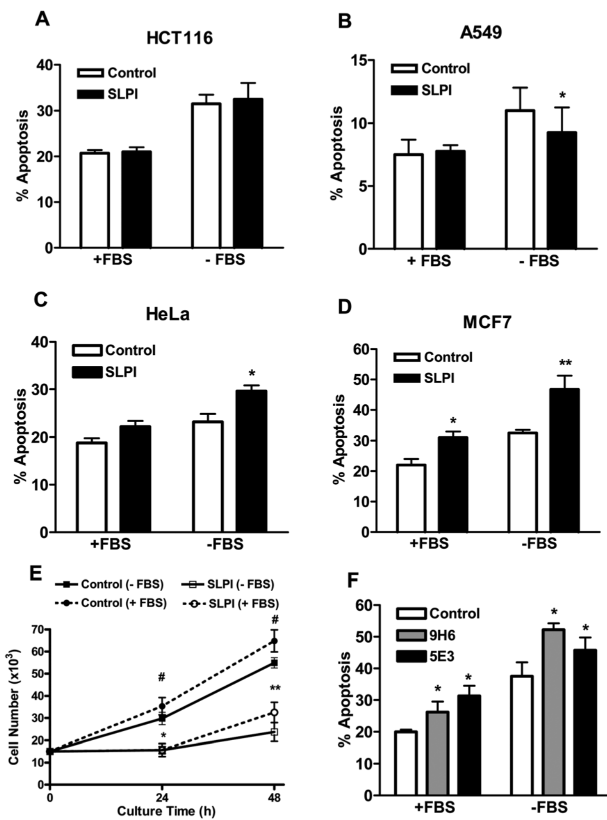

Fig. 5. Effect of SLPI on human tumor epithelial cell lines. A: HCT116 (colorectal carcinoma), B: A549 (lung carcinoma), C: HeLa (cervix adenocarcinoma), and D: MCF7 cells (breast adenocarcinoma) were cultured in complete medium (+FBS) or medium without serum (–FBS) and treated with rhSLPI (4 μg/ml) or control buffer for 48 h. After, cells were harvested and apoptosis was measured by flow cytometry. Percentage of total apoptosis is shown as mean ± SEM of three independent experiments (*P < 0.05 and **P < 0.01 SLPI vs. control, paired Student's *t*-test). E: In vitro growth of MCF7 cells incubated with or without FBS and treated with rhSLPI (4 μg/ml) or control buffer as described above. At the indicated time points, triplicate cultures were harvested and counted in Neubauer counting chambers (*P < 0.05 and **P < 0.01 control vs. SLPI under starving culture conditions; and #P < 0.05 control vs. SLPI cultured with FBS, unpaired Student's *t*-test). F: Apoptosis of MCF7 mock transfected, 9H6 and 5E3 cells cultured with or without FBS. Cells were cultured in complete medium (+FBS) or medium without serum (–FBS) for 48 h. After, cells were harvested and apoptosis was measured by flow cytometry. Percentage of total apoptosis is shown as mean ± SEM of four independent experiments (*P < 0.05 control vs. 9H6 and 5E3, paired Student's *t*-test).

starvation conditions. In fact, when apoptosis was assessed by measuring the caspase-3 activity, an increase in caspase-3 activity is observed only in serum-free cell cultures. The differences, observed among the assays used to evaluate apoptosis, are probably due to their sensitivity. The same differences in sensitivity of the apoptosis assays were seen when F3II cells were treated in vitro with SLPI. In these experiments we observed that SLPI induces F3II cells apoptosis when cells were culture with or without FBS. However, the increase in capase-3 activity is observed only under serum-free cell culture conditions. Remarkably, SLPI over-expressing 2C1 cells did not grow when they were injected into mice, suggesting that SLPI is a key factor responsible for anti-tumor activity. Mechanistically, this was supported in vitro by experiments blocking SLPI expression and by showing that recombinant SLPI, added to F3II cells, recapitulated the effect observed with 2C1 cells.

Overall, these results clearly show that SLPI can mediate the anti-tumoral effect on tumor mammary cells by inducing cell apoptosis. However, other mechanisms can also be involved in the differential growth of 2C1 cells, since we have described that these cells grow slowly in nude and do not grow in BALB/c mice (Amiano et al., 2011) and, also, the repeated administration of 2C1 lead to a reduction in tumor growth (Amiano et al., 2011). Therefore, an immune response is likely induced in mice inoculated with 2C1 cells. This immune response may be secondary to the apoptosis of these cells and it is probable that apoptosis of SLPI over-expressing cells induces a protective immune response leading to a reduced growth rate. Recently, we have described that SLPI inhibited CD4 lymphocyte proliferation and T helper type 1 cytokine secretion in vitro (Guerrieri et al., 2011). We do not know whether exogenous SLPI or SLPI over-expressing cells inhibits the Th1 response in our in vivo tumor model and further studies are required in order to address the immune response elicited by SLPI or 2C1 cells in in vivo tumor models.

Notably, we have used human SLPI in a murine model. This protein has 76% degree of homology with the mouse protein (Wright et al., 1999) and it has been shown to be active in rat and murine models in chronic inflammation (Lentsch et al., 1999; Mulligan et al., 2000; Cavarra et al., 2001). Therefore, it is unlikely that the anti-tumor effect of SLPI observed in our model was due to the immunogenicity of the molecule. The lack of SLPI anti-tumoral effect on LP07 cells (Fig. 2C) and the faster growth of 12B3 cells in vivo (Fig. 1C) support this view.

Another very important result in the present study is the demonstration that the anti-tumor effect of exogenous SLPI does not depend upon its anti-protease activity, as shown with denatured and inactive SLPI. This is very significant since tumor microenvironments are often acidic and they could have the capacity to inactivate SLPI in our experiments.

Importantly, we have also demonstrated that the pro-apoptotic effect of SLPI is also observed in human tumor cells. Among the cell lines studied, remarkably the main pro-apoptotic activity of SLPI is observed on a human breast tumor cell line. This result is very important considering that SLPI could become a new therapeutic tool in the treatment of, at least, certain mammary tumors. Here again, the inhibition of apoptosis of A549 cells and lack of effect on HCT116 cells, plus the increased apoptosis of MCF-7 and HeLa, suggest that the effect of SLPI is specific of some tumor cell lines.

Importantly, the route of administration was shown to be crucial for the anti-tumor effect, since intratumoral administration of the peptide exhibited a clear and significant inhibition of tumor growth whereas i.p. administration of 10 times more SLPI was inefficient. Furthermore, 2C1 SLPI over-expressing cells had to be inoculated in the same flank that F3II cells in order for SLPI to be active, suggesting that a high local concentration of SLPI is required for the anti-tumor activity. In line with the latter finding, the B3 clone (which produces approximately half the amount of SLPI, compared with the 2C1 clone) was able to grow. Remarkably, neither 2C1 nor B3 clones induced lung metastasis. Together, these results suggest a threshold levels for efficacy of SLPI on tumor growth inhibition and metastasis. Although the clones produce less amount of SLPI than that administered subcutaneously, we believe that the local concentration of the peptide could be comparable in both cases due to the diffusion of the protein in the tumor microenvironment and due to the constant production of SLPI by over-expressing tumor cells. However, the threshold level for the anti-tumoral effect of SLPI could be different depending on the tumor type. In fact, it seems clear that not only a threshold is important for SLPI to exert the anti-tumor activity but also the kind of tumor. This was clearly

shown by using the 12B3 clone cells. This clone was derived from CT26 and produces less SLPI than 2C1 but similar levels than B3. Unlike B3 (cells that grew slower than F3II control cells), 12B3 cells grew faster than empty plasmid transfected CT26 cells.

Overall, our results clearly show an anti-tumor effect of SLPI against mammary carcinoma and suggest that the discrepancies described in the field relating to SLPI activity could be explained by tumor types, the route of administration and the achieved local concentration of SLPI. Herein we described a clear pro-apoptotic pharmacological effect of SLPI on some tumor cells. However, under physiological conditions and based on the fact that SLPI is secreted in some tumors microenvironment, it is possible to speculate that SLPI might play a role in the immune evasion strategies by killing only the SLPI-sensitive cells and leaving the SLPI-resistant cells, and/or influencing the host immune response. Therefore, further studies are necessary to unravel the anti-tumor activity of SLPI before its therapeutic use as a pro-apoptotic agent in certain tumors.

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