**The alarm anti-protease, secretory leukocyte protease inhibitor, is a proliferation and survival factor for ovarian cancer cells**

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**Alarm anti-proteases are secreted locally in response to inflammation and have been shown to be elevated in cancers. Secretory leukocyte protease inhibitor (SLPI), an alarm anti-protease, is amplified in ovarian carcinoma and is induced and binds to and protects progranulin (prgn) in inflammation. We reported prgn is a survival protein in ovarian cancer and now hypothesize that SLPI/prgn would promote proliferation and survival. Neutralizing anti-SLPI antibody treatment of HEY-A8 and OVCAR3 ovarian cancer cells decreased cell number (P < 0.001), induced apoptosis and reduced prgn quantity. This was confirmed using SLPI small interfering RNA. Prgn and SLPI were co-immunoprecipitated and co-localized by confocal microscopy. Prgn is a substrate of the serine protease elastase and SLPI is an inhibitor of elastase. Elastase reduced prgn expression, inhibited proliferation in a dose-dependent manner (P ≤ 0.01) and was pro-apoptotic. SLPI protected prgn from elastase-mediated degradation and restored prgn survival and proliferative function (P ≤ 0.04). SLPI also reversed elastase’s pro-apoptotic effects (P ≤ 0.03), yielding recovery of S-phase fraction (P ≤ 0.001) and increased cyclin D1. Treatment with a general serine protease inhibitor increased prgn, but did not reverse elastase-mediated prgn loss or apoptosis. These data demonstrate that inappropriate over-expression of the alarm anti-protease, SLPI, creates a pro-survival milieu for ovarian cancer.

**Ovarian cancer is the fifth most common cause of death from cancer in women (7). SLPI has been described as amplified and over-expressed in ovarian carcinoma (1.8–11). Its messenger RNA and protein have been reported up-regulated in ovarian tumors compared with normal surface epithelium (12). SLPI and other whey acidic protein alarm anti-proteases, such as elafin and HE-4, have been proposed as putative serum biomarkers for malignant ovarian masses (13–16). HE-4 has been shown to be expressed in ovarian inclusion cysts lined by metaplastic epithelium and to be expressed highly in serous and endometrioid ovarian cancers (13). Together these data suggest that SLPI, as an alarm anti-protease, may have context-dependent function. We hypothesize that SLPI has a pro-cancer function in ovarian cancer, independent of its anti-protease activity.

We identified progranulin (prgn), a 68 kDa protein with multiple polyglycosylated higher molecular weight isoforms, to be up-regulated in invasive serous ovarian carcinoma (17–19). It was present selectively in stage III epithelial ovarian cancer compared with serous borderline tumor. Blocking prgn expression by transfection of OVCAR3 human ovarian cancer cells with anti-sense prgn reduced monolayer and density-independent cell growth (19) and caused apoptosis (20). Prgn has also been shown to be induced in fibroblasts and endothelial cells after injury to promote proliferation, migration and formation of capillary-like structures, indicating a role for prgn in angiogenesis (21). It was also shown to be present in activated blood vessels in ovarian cancers, co-localizing with perlecan (22). The results indicate a role for prgn in growth and survival of ovarian cancer. Zhu et al. (3) describes a balanced interaction between prgn and SLPI necessary for wound healing, where SLPI, prgn and elastase elaborted by activated neutrophils come together to regulate the pro-inflammatory microenvironment. That both SLPI and prgn are over-expressed in ovarian cancer led us to propose that these two proteins may function to support the tumorigenic and malignant activity of this cancer. We now report SLPI promotes proliferation and survival of ovarian cancer cells. It furthers their survival through partnering with and protection of prgn.

**Materials and methods**

**Reagents**

Recombinant human SLPI (rhSLPI) and goat anti-rhSLPI antibody were purchased from R&D Systems (Minneapolis, MN). Human neutrophil elastase was acquired from Calbiochem (La Jolla, CA). ELISA kits measuring SLPI and elastase were from Cell Sciences (Canton, MA). Precast PAGE gels were from Invitrogen (San Diego, CA). XTT (3-[4,5-phenylaminocarbonyl]-3-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling agent was obtained from Roche Applied Science (Indianapolis, IN). DAPI vectashield mounting medium was purchased from Vector Laboratories (Burlingame, CA) and propidium iodine, donkey anti-goat alexafluor 488 and donkey anti-rabbit alexafluor 548 were obtained from Molecular Probes (Eugene, OR). Small interfering RNA (siRNA) against SLPI, control siRNA and transfection reagents were obtained from Dharmacol (Lafayette, CO). Antibody to glicereraldehyde dehydrogenase (GAPDH) was from Abcam (Cambridge, MA). All other reagents were of molecular or imaging grade. A peptide-specific anti-SLPI antibody was generated against the peptide DTPNPTRRKPQGC in the A2 segment of SLPI (19,23). This antibody was peptide-purified, titered for optimal immunoprecipitation and immunoblot and peptide competed for validation prior to use. The BAG3 antibody has been reported (24–26).

**Cell culture**

Ovarian cancer cells expressing a range of SLPI were selected for study. The OVCAR3 human ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). HEY-A8 and SKOV3 lines were a generous gift of Dr G.Mills (M.D. Anderson Cancer Center, Houston, TX). Human ovarian surface epithelium cultures were a generous gift of Dr B.Karlan (Cedars Sinai Cancer Center, Los Angeles, CA). All ovarian cancer cell lines were cultured in RPMI 1640 with 10% fetal bovine serum unless otherwise
SLPI is a survival factor in ovarian cancer

Addition of SLPI protected prgn from elastase shown both by western blots and of GAPDH (in part as a loading control; Figure 2B). Neither SLPI nor prgn are shown to have initiated DNA fragmentation, nuclear blebbing and apoptotic changes by DAPI stain. The general co-localization of SLPI and prgn also is reduced in late apoptotic cells (arrow heads). These cells are shown to have initiated DNA fragmentation, nuclear blebbing and chromatin condensation. This confirms SLPI-mediated protection of prgn and cell survival. The specificity of silencing of SLPI to SLPI and prgn was tested by examination of expression of BAG3, a stress co-chaperone protein under investigation in our laboratory (24–26) and of GAPDH (in part as a loading control; Figure 2B). Neither BAG3 nor GAPDH were reduced under conditions in which both SLPI and prgn were diminished.

SLPI and prgn are binding partners

We next asked if both SLPI and prgn interact in ovarian cancer cells and their local microenvironment. A SLPI/prgn complex was shown by co-immunoprecipitation of both lysates and CM from OVCAR3 and SKOV3 cells, those containing the greatest quantity of SLPI (Figure 3A). More SLPI/prgn complex was present in CM consistent with the secretion of both proteins. Binding was confirmed by reverse order immunoprecipitation (data not shown). Co-localization was further demonstrated using fluorescence confocal microscopy (Figure 3B). Prgn and SLPI are both cytosolic, found in the region of the golgi, typical of secreted proteins.

SLPI reverses both anti-proliferation and pro-apoptotic effects of elastase

Prgn has been reported to be susceptible to cleavage by serine proteases such as elastase, which was shown to be necessary for the balance of prgn and SLPI in a wound-healing model (3). The ovarian cancer cell lines produce no detectable elastase measurable by ELISA (data not shown). Therefore, purified elastase was introduced to cultures for 24 h causing a marked and dose-dependent decrease in prgn protein in both HEY-A8 and OVCAR3 cells (Figure 4A, left panel). Addition of SLPI protected prgn from elastase shown both by

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The provided text contains biological and experimental details regarding the role of SLPI in ovarian cancer cell survival and proliferation. It describes methods for protein and RNA analysis, immunoprecipitation, and apoptosis induction. The text also discusses the effects of SLPI on cell survival and the interactions between SLPI and prgn in ovarian cancer cells.
immunoblot (Figure 4A, right panel) and confocal imaging (Figure 4B). These data demonstrate the ability of SLPI to protect prgn from serine protease-mediated degradation. The functional consequence of SLPI protection of prgn was investigated next. Figure 4B shows loss of apoptosis in cells exposed to elastase concomitantly with SLPI. This was further measured in a dose-dependent fashion in all three cell lines using an XTT assay ($P \leq 0.01$; Figure 4C). Cell cycle analysis was done to determine whether this was due to the anti-proliferative and/or pro-apoptotic effects of elastase. Treatment with elastase significantly decreased the S-phase fraction in HEY-A8 and OVCA3 cells (Table I). Addition of SLPI to elastase partially reversed the loss of DNA synthesis, with little proliferative activity of its own.

Fig. 1. Neutralizing anti-SLPI antibody decreases ovarian cancer cell proliferation and prgn production and induces apoptosis. (a) SLPI is present in ovarian cancer cell lines. Primary culture HOSE cells do not produce SLPI, while it is detectable in lysate of malignant cells. Lanes 1: recombinant huSLPI; 2: HOSE; 3: HEY-A8; 4: OVCAR3; 5: SKOV3. (b) Neutralizing polyclonal anti-SLPI antibody inhibits proliferation of Hey-A8 and OVCAR3. Cell viability was measured using the XTT assay for cells starved for 24 h then exposed to control IgG or anti-SLPI antibody for 48 h. Data shown represents the mean ± SEM of at least three independent experiments; $P$ value is comparison to control. (c) Apoptosis is demonstrated by DAPI immunofluorescence. Reduced cell number, nuclear degradation and apoptotic bodies are present when cells were exposed to neutralizing anti-SLPI antibody for 16 h. A shorter time was selected due to cell loss. Inset: apoptotic bodies. (d) Prgn is protected by SLPI. The left panel shows loss of prgn in HEY-A8 cells in the presence of anti-prgn antibody and recovery when SLPI is added. Prgn is lost when the anti-SLPI antibody is used in HEY-A8 cells (right panel). The 50 kDa prgn band is a previously documented prgn fragment (20).

Fig. 2. SLPI siRNA selectively reduces SLPI and prgn protein and ovarian cancer cell survival. (a) siSLPI reduces expression of both SLPI (red) and prgn (green). Control or SLPI siRNA were introduced and cells incubated for 96 h prior to fixation and staining. The right most panel shows the confocal overlay with yellow indicating presence of both proteins. Apoptosis is shown by loss of cells and nuclear changes (DAPI, blue). There is a dissociation of SLPI from prgn seen in apoptotic cells shown by arrow heads. Two independent fields of siRNA-treated cells are shown (rows 2 and 3), representative of at least three replicate transfection experiments. (b) Silencing of SLPI does not reduce BAG3 quantity. Cells were exposed to siSLPI as in A, lysed and subjected to immunoblot for SLPI, prgn and BAG3 as indicated. No loss of BAG3 or GAPDH (as loading control) is seen under conditions where SLPI and prgn are markedly reduced.
This effect was further evaluated by assessing cyclin D1 (Figure 4D). In parallel with the other findings, there is a reduction in cyclin D1 expression in elastase-exposed cells that is restored when SLPI is added with elastase. Thus, SLPI reversed both the negative effects of elastase on proliferation and its pro-apoptotic activity.

**SLPI reverses elastase effects through prgn interaction and not by protease inhibition**

SLPI has been shown to bind both prgn and elastase, providing two opposing mechanisms for its protection of prgn (3). TAME, a general serine protease inhibitor, was used to address the protease inhibition function. Exposure of HEY-A8 and OVCAR3 cells to increasing concentrations of TAME under the same experimental conditions in which the elastase or SLPI were included caused small but reproducible increases in prgn in a dose-dependent manner, suggesting the involvement of a cellular serine protease in local prgn regulation (Figure 5A). However, TAME treatment did not protect prgn from elastase-mediated degradation (Figure 5B), strongly suggesting that SLPI protects prgn through a mechanism unrelated to its protease inhibitory activity. TAME also did not protect cells from elastase-mediated inhibition of proliferation although SLPI significantly overcame elastase-mediated injury (Figure 5C). This demonstrates that the

**Fig. 3.** SLPI and prgn are binding partners. (a) SLPI and prgn co-immunoprecipitate. Both cell lysates and CM were subjected to immunoprecipitation with anti-prgn and probed for SLPI. Similar results were seen with anti-SLPI immunoprecipitation followed by immunoblot with anti-prgn (not shown). (b) Confocal imaging demonstrates cytosolic co-localization of prgn and SLPI.

**Fig. 4.** SLPI protects cells from elastase-mediated apoptosis. (a) Prgn is markedly reduced when cells are exposed to elastase in a dose-dependent fashion. This loss is reversed upon concomitant exposure to SLPI (right panel). (b) Elastase-mediated injury is reversed by SLPI. The left panel confirms the loss and recovery of prgn upon elastase treatment in the absence and presence of SLPI. Elastase apoptotic injury and SLPI-associated protection from injury is seen under the same conditions. (c) SLPI reverses elastase injury in a dose-dependent fashion in HEY-A8 cells. XTT assay is used to quantitate cell injury with elastase and recovery with concomitant SLPI (mean and SEM, n = 3; similar results are shown for OVCAR3 and SKOV3). (d) Cyclin D1 expression is reduced when cells are exposed to elastase and restored with co-treatment with SLPI.
protective role of SLPI on prgn and on cell proliferation is independent of its protease inhibitory activity. Thus, both SLPI and prgn have key roles in ovarian cancer but may synergize to make a locally permissive microenvironment.

Discussion

Genomic analysis of ovarian cancer identified amplification of SLPI and the whey acidic protein locus in a large proportion of epithelial ovarian cancers (28,29). Multiple functions of SLPI have been reported previously, including function as a protease inhibitor and as a regulator of local inflammatory responses downstream of chemokines and tumor necrosis factor-α (TNF-α) (5,30). One mechanism of its anti-inflammatory activity is through inhibition of cleavage of prgn into component pro-inflammatory granulins (3). We identified overexpression of prgn in epithelial ovarian cancer and demonstrated that it is both a proliferation and survival factor for ovarian cancer cell lines (19,20). The findings linking prgn and SLPI in inflammation led to the hypothesis that they would interact in malignancy to promote stabilization of the pro-survival prgn form. We now show that SLPI itself has pro-survival function, that by direct interaction it stabilizes prgn, and that SLPI protects prgn from serine protease-mediated degradation. This interaction makes a more potent anti-apoptotic and pro-growth environment for the ovarian cancer cells. Further, SLPI is an independent pro-survival factor for ovarian cancer, as shown by the stimulation of apoptosis in response to its neutralization and silencing. Thus, these results identify prgn and SLPI as putative molecular therapeutic targets in ovarian cancer.

Table I. Elastase alters proliferation and apoptosis in a SLPI-sensitive fashion

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<td>2.5</td>
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*a*Average of three independent experiments.

*b*Control versus elastase.

*c*Elastase versus SLPI + elastase.

Fig. 5. The protective effects of SLPI are not caused by its protease inhibitory activity. (a) The general serine protease inhibitor, TAME, increases prgn quantity in a dose-dependent manner in both HEY-A8 and OVCAR3 cells. (b) Immunofluorescence demonstrates protection of prgn only when SLPI is added to elastase. (c) SLPI but not TAME protects cells from elastase-mediated inhibition of proliferation. Elastase versus SLPI + elastase: *P* = 0.01.
with elastase and immunoneutralization of prgn (20). One mechanism of this may be the small but dose-dependent increase in prgn observed when cells are incubated with SLPI. It may be due to the protective role of SLPI on prgn, such that loss of SLPI may leave prgn open to cleavage by elastase or other, as yet undefined serine proteases. The findings with recombinant SLPI also may be limited by the nature of the bacterial recombinant protein (11 kDa) that may not be recognized as physiologically as the endogenously produced glycosylated form (14 kDa). It is possible that there may be differential activity of the modified forms of SLPI; we found more expression of the higher molecular weight SLPI in the HEY-A8 cells which also seemed more susceptible to neutralization of SLPI than would be expected by their relatively lower secreted concentrations. These findings are supported by the increase in prgn quantity in the presence of TAME. SLPI appears to have its pro-malignant effects more through inhibition of cell death than promotion of cell proliferation in ovarian cancer, contrary to reports assessing its role in other cancers (6,8,30,34,35). Preliminary data indicates that SLPI has a limited direct proliferative drive on these ovarian cancer cells in the absence of elastase or neutralizing antibody, despite the marked protective effects observed when the injuring agent is present.

Findings in the literature indicate that the anti-protease-independent role for SLPI in cancer may be either context or cancer dependent. SLPI has been implicated in HIV disease in an anti-protease-independent fashion (36–38). Induction of SLPI in response to inflammatory cytokines has been shown in several systems (39,40). TNF-α, produced by many types of cancer stimulates SLPI production (41). Devoogdt et al. (5) showed previously that TNF-α producing macrophages enhanced resistance to TNF-α mediated lysis and promoted malignancy of 3LL cells associated with up-regulation of SLPI. Further studies have revealed that although induction of SLPI occurred during TNF-α treatment, the promotion of tumor progression by SLPI abrogated tumor advance in a TNF-α-independent fashion (5,30,41,42). Thus, induction of SLPI in the microenvironment whether from tumor or local inflammatory cells can shift the local balance to favor tumor progression.

SLPI and prgn have both been shown to be over-expressed in ovarian cancer. This over-expression is now reduced to function showing that these proteins have proliferative and anti-apoptotic activity independently, but that they work in concert to yield a stronger effect as a partnered complex to promote survival and proliferation of the malignant cells. Interruption of this complex is a logical molecular therapeutic target and one that may also be monitored as a biomarker due to the secreted nature of both proteins.

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References

32. Xu, L. et al. (2000) Inhibition of malignant ascites and growth of human ovarian carcinoma by oral administration of a potent inhibitor of the SLPI is a survival factor in ovarian cancer

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