Surfactant protein D in immune surveillance against cancer

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Summary

Surfactant protein D (SP-D) is an innate immune molecule that was originally discovered in the lungs as a part of pulmonary surfactant system. Later on, it became evident that SP-D has extensive tissue distribution that lent its credentials as a versatile innate immune molecule of mucosal system. In addition to its ability to protect against pathogens and allergens, and to modulate inflammatory reactions, SP-D has emerged as an immune surveillance molecule against cancer. SP-D can induce apoptosis in a variety of cancer cell lines and primary cancer cells derived from patients, including lung, pancreatic, prostate, ovarian and breast cancers. The apoptotic mechanisms including pathways, signaling and key mediators involved, have been delineated. The most striking feature of recent studies is the demonstration that a recombinant form of human SP-D (rfhSP-D) composed of homotrimeric C-type lectin domains can bring about these anti-tumor effects, raising the possibility of a therapeutic development. In addition to apoptosis induction, rfhSP-D can also interfere with epithelial-to-mesenchymal (EMT) transition in pancreatic cancer. In view of the above-mentioned *in vitro* studies, a recent bioinformatics analysis has examined if SP-D can serve as a potential prognostic marker for human lung cancer. It appears that compared to their normal tissue counterparts, there is a lower expression of SP-D in lung, gastric, and breast cancers, as opposed to ovarian cancer. In the lung cancer, the existence of SP-D is likely to be associated with a favourable prognosis.

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1. Introduction

Pulmonary surfactant protein D (SP-D) is a multi-functional, pattern recognition molecule involved in resistance to pathogen challenge and pulmonary inflammation including allergy (Madan et al., 1997, Borron et al., 2002, Brinker et al., 2001, Madan et al., 1997, Takeda et al., 2003, Pandit et al., 2016). The immunomodulatory properties of SP-D are reliant on its the domains: the C-terminal globular region, also called as carbohydrate recognition domains (CRDs), and the collagen-like region (CLR) connected through an α-helical neck region (Kishore et al, 2005; Waters et al., 2009). SP-D directly interacts with monocytes/ macrophages and enhance or suppress inflammatory mediator production depending on the binding of either of these two domains (Gardai et al., 2003, Ledford et al., 2014). Experiments using murine models of allergy of pulmonary hypersensitivity showed that SP-D (or a recombinant fragment of human SP-D containing trimeric neck and CRD region; rfhSP-D) treatment reversed hypersensitivity response by lowering blood and pulmonary eosinophilia specific IgE levels and a shift in cytokine profile from Th2 to Th1 type in the spleen cell culture (Madan et al., 2001, Singh et al., 2003). This was further confirmed by the negative regulation of allergic eosinophilic pulmonary inflammation and airway function with intratracheal administration of SP-D in the ovalbumin-induced murine models of lung allergy (Takeda et al. 2003). The eosinophilic inflammation of the airways has been directly correlated with the severity of asthma (Winqvist et al., 1982, Duncan et al., 2003, Bousqeut et al., 1990). SP-D knock-out (SP-D $^{-/-}$) mice showed elevated peripheral and pulmonary eosinophilia. Reconstituting the levels of SP-D by intranasal administration in the SP-D$^{-/-}$ mice reduced eosinophilia considerably. The susceptibility of SP-D $^{-/-}$ mice to allergen sensitization was consistent with severe pulmonary eosinophilia (Madan et al., 2005). These observations implicated a direct interaction of SP-D with the eosinophils.

2. Eosinophil leukemic cells

2.1. Interaction of SP-D with human eosinophilic leukemic cell line

Direct interaction of SP-D with human eosinophils inhibited eotaxin triggered chemotaxis and eosinophil cationic protein (ECP) degranulation stimulated by Ca^{2+} ionophore in the eosinophils derived from healthy donors (von Bredow et al., 2006). Our group also showed a CRD and dose dependent binding of SP-D (and rfhSP-D) to human eosinophils (Mahajan et al., 2008). SP-D resulted in a significant increase in oxidative burst and CD69 expression in

eosinophils derived from symptomatic allergic asthmatics; in addition, SP-D treatment induced apoptosis in these activated eosinophils *in vitro*. The viability of eosinophils from healthy donors was not affected following SP-D or rfhSP-D treatment. However, eosinophils from healthy donors, following priming with IL-5, showed apoptosis with rfhSP-D treatment (Mahajan et al., 2008). AML14.3D10 cell line, an advanced differentiated eosinophilic leukemic cell line, exhibits autocrine activation of the intracellular IL-3/GM-CSF/IL-5 signaling pathways (Baumann et al., 1998, Paul et al., 1997). The interaction of SP-D with AML14.3D10 cells was examined in view of the ability of SP-D to selectively induce apoptosis in the sensitized eosinophils. Native SP-D, purified from the lung lavage obtained from alveolar proteinosis patients, as well as rfhSP-D both showed dose and calcium dependent binding to AML14.3D10 cells. This binding was inhibited in the presence of cellular debris (known to interact with CRD of SP-D) suggesting the involvement of CRD region of SP-D in binding to AML14.3D10 cells (Mahajan et al., 2013).

2.2. Cell cycle arrest of leukemic cells

The hypotonic propidium iodide (PI) assay was used for cell cycle analysis by flow cytometry. The assay is based on measurement of DNA content by staining with PI. The rfhSP-D induced nuclear changes in the AML14.3D10 cells and led to accumulation of cells in the G2 phase. There was more than 20-fold increase in the G2 population of cells suggesting G2/M cell cycle arrest (Mahajan et al. 2013). The treatment also increased the sub G1 peak, i.e. the presence of fragmented DNA, suggestive of the cell apoptosis. The constant presence of rfhSP-D was required to induce cell apoptosis and the sustained downstream events leading to the cell death (Mahajan et al. 2013).

2.3. Induction of apoptosis

rfhSP-D treatment for 24 h caused significant G2/M cell cycle arrest in AML14.3D10 cells; however the cell viability was not significantly affected at this time point. Thus, Annexin V-FITC assay (Zhang et al., 1997) was used to allow the direct evaluation of Phosphatidylserine (PS) externalization, a very early stage marker of apoptosis; a significant increase in the annexin-V positive cells was observed following treatment of AML14.3D10 cells with rfhSP-D at 48h (Mahajan et al. 2013). Other assays including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as well as Trypan blue dye exclusion also showed a dose- and time-dependent decrease in the viability of rfhSP-D treated AML14.3D10 cells. NAD(P)H-dependent enzymatic reduction of MTT to MTT-formazan by Mitochondrial

succinate dehydrogenase indicates well-being of the cells with respect to respiration and mitochondrial level (Mosmann, 1983). The mitochondrial respiration efficiency of AML14.3D10 cells started to decline significantly by 48 h. At 72 h, nearly half of rfhSP-D-treated AML14.3D10 cells showed decrease in viability by MTT assay. At this time point, the rfhSP-D treated cells started to show DNA fragmentation, suggesting the progression of cells towards apoptosis (Riccardi et al, 2006). The trypan blue staining demonstrated that ~ 20% cells had lost the membrane integrity by 72 h. Thus, analysis of rfhSP-D treated cells by different assays at various time points such as release of intracellular oxidative burst, G2/M cell cycle arrest, increased levels of activated p53, cleavage of caspase-9 and PARP, and PS externalization suggest the sequential induction of events observed during rfhSP-D induced apoptosis in AML14.3D10 cell line (Mahajan et al. 2013) (Table 1).

2.4. Deciphering the proteome and phosphoproteome of SP-D treated eosinophilic leukemic cells

SP-D has immunomodulatory effects on a range of immune cells, including B- and T-lymphocytes, macrophages, dendritic cells and eosinophils (Singh et al., 2017, Lin et al., 2010, Brinker et al 2001, von Bredow et al., 2006, Mahajan et al., 2008). The large-scale molecular changes initiated by SP-D in a human cell were thus studied, for the first time, using proteomics approach. Comparative analysis of rfhSP-D (10 μg/ml for 48 h) treated AML14.3D10 cells showed a total of 134 proteins with a three-fold or more change in the expression (Mahajan et al., 2014). The important observations on proteomic profile of rfhSP-D treated AML14.3D10 cells included increased expression of oxidoreductases and stress-related molecules, and decreased expression of survival related proteins such as high-mobility group A1 (HMGA1) (Mahajan et al., 2014). In addition, the mitochondrial anti-oxidant defense system was found compromised in the rfhSP-D treated cells. There was a decreased expression of Ubiquinol-cytochrome c reductase (complex III of ETC), Peroxiredoxin 3 isoform b and Mitochondrial matrix superoxide dismutase. This is likely to cause mitochondrial dysfunction, and hence, triggering of the intrinsic pathway of apoptosis (Turrens et al., 2003).

2.5. Elucidation of apoptotic mechanisms

An important mechanism by which SP-D contributed to anti-leukemic activity was the reduced expression of survival related proteins, HMGA1, an oncogenic transcription factor that is over-expressed in various high-grade malignancies (Chiefari et al., 2013, Pierantoni et

al., 2007, Hillion et al., 2008, Fedele et al., 2005). Inhibition of HMGA1 expression has been shown to block phenotype transformation in many cancer cells (Scala et al., 2000). HMGA1 is known to inhibit the function of p53 family members, i.e. oncosuppressors, in cancer cells (Pierantoni et al., 2007). p53 is a tumor suppressor protein and plays a major role in cellular response to DNA damage or other genomic aberrations. Activation of p53 may lead to either cell cycle arrest and DNA repair, or apoptosis (Chen, 2016, Wang and El-Deiry, 2007). The decreased expression of HMGA1 in the rfhSP-D treated cells correlated with an increased level of p53 activation, i.e. phosphorylation of p53 at Ser15 (Mahajan et al., 2013). Another protein, heterogeneous nuclear ribonucleoprotein K (hnRNP K), known to be induced by stress, was found to be upregulated. hnRNP K is required for the induction of p53 target genes (Moumen et al., 2005, Mahajan et al., 2014). An increased level of p21 was also observed in rfhSP-D treated AML14.3D10 cells. The increased levels of activated p53 and p21 expression are known to inactivate cyclin B-cdc2 complex that regulates G2/M transition and leads to either DNA repair or apoptosis (Stewart et al., 1995, Bunz et al., 1998). The rfhSP-D treated cells also showed an increased Tyr15 phosphorylation of cdc2, suggesting activation block of the cdc2 (Li et al., 2011).

Treatment of AML14.3D10 cells with rfhSP-D resulted in the activation of caspase-9, a hallmark of the intrinsic pathway of apoptosis (Elmore et al., 2007). Once initiated, caspase-9 goes on to cleave procaspase-3 or procaspase-7, which in turn, cleaves several cellular targets, including Poly (ADP-ribose) polymerase (PARP), a well-known marker of apoptosis (Walsh et al., 2008). Although, PARP was found to be cleaved in the rfhSP-D treated cells, cleavage of caspase-7 was not observed (Figure 1). This indirectly suggests that an intermediate activation of caspase-3 may be leading to the cleavage of PARP.

2.6. Validation of anti-cancer activity of SP-D in other cancer cells

HMGA1 is over-expressed in various high-grade malignancies, including AML (acute myeloid leukemia), ALL (acute lymphoid leukemia) and Burkitt's lymphoma (Fusco et al., 2007). We found that the cell lines with reported increased HMGA1 expression, AML (AML14.3D10 cell and THP-1 cell line), ALL (Jurkat and Raji) and human breast epithelial cell line (MCF-7) demonstrated a significant decrease in the viability of cells on treatment with rfhSP-D, although, rfhSP-D treatment did not affect the viability of PBMCs isolated from healthy donors. The study indicated that rfhSP-D specifically exerted its apoptotic effect on the cancer cell lines.

3.Pro-apoptotic effects of rfhSP-D on pancreatic cancer cell lines

In view of the above-mentioned observations, rfhSP-D has been examined for its importance in pancreatic cancer. The fluorescence analysis revealed that rfhSP-D binds evenly in clusters on the cell membrane of three pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and Capan-2. No CY5 fluorescence was detected in the untreated controls suggesting the rfhSP-D binding observed in the treated cell lines was protein-specific. To determine whether rfhSP-D influences the cellular morphology and epithelial-to-mesenchymal transition (EMT) phenotypic expression, we treated a highly invasive pancreatic cancer cell line (e.g. Panc-1) with exogenous rfhSP-D (Kaur et al 2018a).

3.1. rfhSP-D induces morphological alterations in the pancreatic cell line Panc-1

The optimal dose was determined by observing the effects of rfhSP-D on cell morphology and cell division of Panc-1 cells incubated with 0, 5, 10, and 20 μg/ml. Images of colonies of 10-15 cells were taken at 0 h, 6 h and 24 h. Panc-1 cells, treated with rfhSP-D (5 μg/ml) and untreated cells, acquired spindle type cell morphology, reduced cell-cell contact, and continued to divide in a time dependent manner. No such alterations were seen in Panc-1 cells treated with rfhSP-D (10 and 20 μg/ml) and they appeared to be static. However, cell morphology at 10 μg/ml overcome these static effects by 24 h but not at 20 μg/ml dose. Interestingly, some dead cells were also seen at 20 μg/ml as compared to the other dose conditions. Subsequently, possible effects of rfhSP-D on EMT induction in Panc-1, MiaPaCa-2 and Capan-2 cells were investigated (Kaur et al, 2018a).

3.2. rfhSP-D suppresses the invasion ability/capacity in pancreatic cancer cell lines

The effects of rfhSP-D (20 μg/ml) on the invasion were analysed by incubating the pancreatic cancer cells in the upper surface of the matrigel chamber pre-coated with extracellular matrix proteins and serum containing media as a chemo-attractant in the bottom surface for 22 h. Both high grade Panc-1 (50%) and MiaPaCa-2 (65%) cell lines, treated with rfhSP-D (20 μg/ml), showed significantly reduced invasion in the matrigel; however, almost no invasion occurred in low-grade Capan-2 (Kaur et al 2018a) since Capan-2 is a non-invasive cell line.

3.3. rfhSP-D reduces the expression of EMT markers

EMT induction is characterized by morphological alterations, enhanced motility, reduced cell-cell contact (Ellenrieder et al., 2001), and upregulation of mesenchymal markers, such as Vimentin (Maier et al., 2010), Snail (Peinado et al., 2003), and Zeb1 (Wellner et al., 2009). Most pancreatic cancer cells overexpress TGF-β, which suppresses immune surveillance and facilitates the escape, migration and increased resistance to anti-tumor immune responses (Sun et al., 1994; Beauchamp et al., 1990; Reiss, 1999). rfhSP-D (20 μg/ml) treatment significantly downregulated the gene expression of TGF- β in the treated Panc-1 and MiaPaCa-2 at 12 h whereas no difference was seen in Capan-2 as analysed by qPCR and western blot. Fluorescence microscopy analysis revealed that TGF-β expression at 24 h diminished considerably within the cytoplasm of the treated Panc-1 and MiaPaCa-2 cell lines. During TGF-β induced EMT pathway, Smad2/3 are phosphorylated in the cytoplasm, followed by translocation into nucleus; however, Smad2/3 staining appeared very weak in the cytoplasm of the rfhSP-D treated Panc-1 and MiaPaCa-2 cell lines. Furthermore, gene expression of key markers of EMT, regulated by TGF-β such as Vimentin, Zeb1 and Snail, was also downregulated in all cell lines treated with rfhSP-D at various times ranging between 1h and 12h. Fluorescence microscopy and flow cytometry analysis also confirmed a significant decrease (~50%) in the cytoplasmic presence of these proteins in the treated as compared to untreated cells. Interestingly, blocking TGF-β via neutralizing antibody reduces the expression of EMT markers in a similar fashion as rfhSP-D. Interestingly, the effect was even more prominent when rfhSP-D and rabbit anti- human TGF- β were added together (Kaur et al, 2018a). Similarly, visual assessment of cell proliferation and migratory capacity of ovarian cancer cells, SKOV3, following treatment with rfhSP-D (10 μg/ml) for 24h also revealed inhibition of growth as compared to the untreated cells (Kumar et al, 2019). These observations suggested that rfhSP-D interfered with EMT, therefore, we investigated the static affects occurred due to cell cycle arrest.

3.4. rfhSP-D induces cell cycle arrest in G1 phase

Panc-1, MiaPaCa-2, Capan-2 cell lines treated with rfhSP-D (20 μg/ml) for 24 h were subjected to DNA quantitation using DNA binding dye, PI, to determine whether the cytostatic effect seen was due to growth arrest. rfhSP-D treatment inhibited the DNA synthesis during G1 phase in treated Panc-1 (68%) and MiaPaCa-2 (50%) as compared to untreated Panc-1 (3%) and MiaPaCa-2 (2%) cells, respectively. The untreated cells for all cell lines as well as treated Capan-2 remained unaffected as cell cycle into next S and G2

phase continued (Kaur et al 2018b).

3.5. rfhSP-D induces apoptosis in pancreatic and ovarian cancer cells

The fluorescence microscopy revealed that the cell membrane was no longer intact and the propidium iodide bound to DNA in the rfhSP-D (20 μg/ml) treated pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and Capan-2 as compared to untreated cells, where no fluorescence was detected, indicating that cells were undergoing apoptosis at 48 h. The quantitative flow cytometry analysis was carried out by measuring the Annexin V/FITC binding to phosphatidylserine (PS), a cell membrane phospholipid, which is externalized during early apoptotic stage and PI, a DNA stain, passes through the porous cell membrane into the nucleus. It showed that rfhSP-D induced apoptosis in ~70% of Panc-1 and MiaPaCa-2 cells and ~43% in Capan-2 as compared to ~80% unstained, viable cells in the untreated samples at 48 h. A significant percentage of Panc-1 cells (~43%) and some MiaPaCa-2 and Capan-2 (~12%) were positive for PI alone, suggesting that these cells were either dead or in late apoptotic stage (Kaur et al 2018b). The quantitative and qualitative analysis of ovarian cancer cells, SKOV3 treated by rfhSP-D (20 μg/ml) for apoptosis induction revealed similar trends as seen in pancreatic cancer cells. Approximately, 68% cells underwent apoptosis at 48 h of treatment (Kumar et al, 2019) Then, activation of apoptosis pathway was determined by assessing key markers of both intrinsic and extrinsic apoptosis pathway.

3.6. rfhSP-D activates apoptosis via extrinsic pathway in pancreatic and ovarian cancer cells

The treatment with rfhSP-D (20 μg/ml) activated cleavage of caspase 8 and 3, in addition to upregulation of pro-apoptotic gene, Fas at 12 and 24 h in all the cell lines. Moreover, both TNF-α and NF-κB mRNA expression levels showed a significant up-regulation in all the rfhSP-D treated cell lines at 12 and 24 h. Fluorescence microscopy of Panc-1, MiaPaCa-2 and Capan-2 cell lines showed that NF-κB was translocated to the nucleus at 24 h, which was not seen in the untreated cells (Kaur et al, 2018b). TNF-α and NF-κB are crucial factors in the apoptotic pathway and they can regulate Fas expression (Fulda and Debatin, 2006). Therefore, it appeared that apoptosis occurred via extrinsic pathway as upregulated TNF-α binds to TNF type I receptor (TNFR1), which is internalized and forms a complex with

TNFR1-associated DEATH domain (TRADD) (complex I), stimulating the upregulation of NF-κB. Then, a complex II is formed upon binding of complex I to Fas-Associated protein with Death Domain (FADD), which is formed when Fas is activated. NF-κB upregulation promotes Fas upregulation as it acts as a transcription factor for Fas Subsequently, Complex II activates downstream caspase cascade, which causes the cleavage of caspase 8 followed by effector caspase 3 cleavage, which brings about apoptosis. These findings indicated that cell death is likely to occur via TNF-α/Fas-mediated apoptosis pathway (Kaur et al., 2016; Liu et al., 2012; Ashkenazi et al., 1998). Intrinsic markers such as caspase 9 and pro-apoptotic gene, Bax, remained unaffected. Moreover, the survival pathway such as mTOR is often deregulated in pancreatic cancer (Semba et al., 2003) and its activation is associated with poor prognosis (Kennedy et al., 2011). Upon treatment with rfhSP-D (20 μg/ml), mRNA expression of mTOR was downregulated in Panc-1 and MiaPaCa-2 cell line at 12 h, however, no difference was seen in Capan-2. In addition, fluorescence analysis revealed significant decrease in the cytoplasmic levels and an increased accumulation of mTOR in the nucleus of the treated cells in comparison to the untreated cells, where it has been shown to be present in its inactive form in previous studies (Betz et al., 2013). Interestingly, ovarian cancer cells, SKOV3, also appeared to undergo apoptosis via extrinsic pathway as pro-apoptotic gene Fas and TNF-α were upregulated and survival pathway mTOR was downregulated (Kumar et al. 2019).

4. Anti-prostate tumor effects of SP-D

4.1 SP-D expression in Prostate and correlation with Gleason score

Although the lung remains the major site of SP-D synthesis, its presence has been reported in non-pulmonary human tissues, including trachea, brain, testis, salivary gland, heart, prostate gland, kidney, and pancreas (Madsen et al., 2000). Elevated levels of SP-D were observed at inflamed sites in the prostate, manifesting protection against bacterial infection (Oberley et al., 2005). Testosterone withdrawal showed upregulation of TLR4 pathway and improved SP-D-mediated bacterial clearance in rat prostate cells (Quintar et al., 2012; Oberley et al., 2007). Differential SP-D protein expression in the glandular structures of inflamed malignant and non-malignant human prostate tissues has also been reported. A significant correlation between lower expression of SP-D and increased Gleason score and prostate tumor volume has been noted earlier (Kankavi et al., 2014). A low level of SP-D, a known anti-

inflammatory molecule, may contribute to the development and/or progression of the human prostate cancer (Kankavi et al., 2014).

SP-D expression in LNCaP (androgen dependent) tumor cells was significantly lower in comparison to DU145 and PC3 (androgen-independent) tumor cells and primary prostate epithelial cells. Furthermore, treatment with Dihydrotestosterone (DHT) upregulated levels of SP-D transcripts in Primary epithelial cells, LNCaP, but not in PC3 tumor cells, suggesting that SP-D expression was regulated by androgens (DHT) in an androgen-dependent cancer (Thakur et al., 2019). Similarly, SP-D expression was weaker in seminoma compared to normal testicular tissue that may contribute to reduced immunomodulatory and rheology processes in germ cell tumor (Beileke et al., 2015).

4.2 SP-D binds prostate tumor cells and induces apoptosis via intrinsic pathway

A significantly higher calcium dependent binding of rfhSP-D was observed with the androgen independent prostate cancer cells (DU145 and PC3) than the androgen-dependent prostate cancer cells (LNCaP). Primary prostate epithelial cells showed comparatively less binding to rfhSP-D than any of the prostate cancer cells, suggesting an involvement of certain interacting cell membrane proteins that are upregulated or differentially expressed on prostate cancer cells (Thakur et al., 2019). rfhSP- D caused a dose- and time-dependent reduction in the viability of prostate cancer cells (LNCaP, DU145 and PC3) irrespective of their androgen sensitivity. This effect was also observed in primary prostate cancer epithelial cells isolated from the metastatic PCa patients. Various attributes of apoptosis such as PS externalization, mitochondrial dysfunction and DNA fragmentation were evident in the rfhSP-D treated prostate cancer cells (Thakur et al., 2019). Anti-prostate cancer activity of rfhSP-D via induction of apoptosis in tissue explants from metastatic prostate cancer patients has also been demonstrated.

4.3 Mechanisms involved in anti-prostate cancer activity of rfhSP-D

p53 pathway plays a crucial role in the transmission of pro-apoptotic signals (Gottlieb et al., 2002). rfhSP-D treated LNCaP (p53 +/+, androgen dependent) cells showed significant upregulation in phosphorylated p53 (Thakur et al., 2019). rfhSP-D treatment led to a decreased level of Bcl2, with a concomitant increase in Bax, cytochrome c and cleavage of caspase 7, confirming induction of intrinsic apoptosis pathway (Thakur et al., 2019).

PC3 cells, a p53 null and highly metastatic prostate cancer cell line, also showed significant apoptosis following treatment with rfhSP-D, which suggested involvement of a p53 independent mechanism of apoptosis. Among 25% prostate cancer cases, diallelic deletion of the Phosphatase and tensin homolog (PTEN) gene and the associated increase in Akt phosphorylation correlates with hormone refractory prostate cancer (Sircar et al., 2009). A significant downregulation of phosphorylated Akt was observed in both rfhSP-D treated PC3 (p53 -/-, androgen independent) and LNCaP cells (Thakur et al., 2019). Decreased levels of activated Akt may lead to decreased levels of phosphorylated Bad (Bcl-2 associated death promoter). Dephosphorylated Bad interferes with interaction of activated Bcl2 with Bax. Thus, an increased release of Bax triggers apoptosis (Ruvolo et al., 2001; Oltvai et al., 1993). Our studies suggested that besides activation of p53 pathway, rfhSP-D also inhibited Akt-PI3K pathway leading to Bax mediated apoptosis. Thus, this study unraveled PI3K/Akt, an anti-apoptotic pathway, as a novel target of rfhSP-D mediated anti-prostate cancer activity (Thakur et al., 2019) (Figure 2).

5. Conclusions and Perspectives

A great advantage associated with the anti-cancer activity of SP-D (especially a recombinant fragment of human SP-D i.e. rfhSP-D) is induction of apoptosis by simultaneous targeting of multiple cellular signaling pathways including transcription factors, tumor cell survival factors, protein kinases, resulting in the efficient and selective killing of cancer cells (Table-2). SP-D has been shown to inhibit the proliferation, migration and invasion of A549 human lung adenocarcinoma cells by binding to N-glycans of epidermal growth factor receptor (EGFR) via its CRD region, and thus, interfering with EGF signalling (Hasegawa et al., 2015). In UV treated apoptotic Jurkat T cells, SP-D enhanced membrane and nuclear blebbing, suggesting involvement of SP-D in induction of apoptosis (Djiadeu et al, 2017). rfhSP-D induced apoptosis in pancreatic adenocarcinoma cells via Fas-mediated pathway in a p53-independent manner (Kaur et al., 2016). Exogenous treatment of SKOV3 cells (an ovarian cancer cell line) with rfhSP-D led to increased caspase 3 cleavage and induction of pro-apoptotic genes, Fas and TNF-α (Kumar et al., 2019). Recently, Kaur et al. reported that rfhSP-D can suppress the invasive-mesenchymal properties of highly aggressive pancreatic cancer cells by inhibiting TGF-β expression in a range of pancreatic cancer cell lines via Smad2/3 signaling (kaur et al, 2018a).

Bioinformatics analysis of SP-D presence/levels in normal and cancer tissues was performed to assess if SP-D can serve as a potential prognostic marker for human lung, gastric, breast, and ovarian cancers. Cancer tissues with significantly higher levels of SP-D compared to their normal tissue counterparts are more susceptible to SP-D-mediated immune surveillance mechanisms via infiltrating immune cells (Mangogna et al., 2018). In view of these poignant evidences, SP-D is likely to act as an integral component of the human innate immune surveillance against cancer cells.

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Table: 1

Sequential events on rhSP-D (10 μg/ml) treatment of eosinophilic leukemic cells (AML14.3D10 cell line)

Increased Oxidative burst	Decrease in HMGA1 level	Increased p53 phosphorylation	Subsequent Observations / Outcomes →	G2/M cell cycle arrest	MTT assay	Annexin-V FITC assay	Sub-G1 peak (DNA fragmentation)	Trypan blue staining
Induced at 24 h on treatment of AML14.3D10 cells with SP-D			Time points ↓					
+	++	++	24 h	+	+	---	-	-
---	++	---	48 h	++	++	+	+	+/-
+	---	---	72 h	+++	+++	---	+++	++

Key:

+ : Results Positive

++/+++ : Further Increase in Results

- : Results Negative

+/- : Low yet significant levels

--- : Results not observed

Table 2. Multiple signaling pathways targeted by rfhSP-D in various cancer cells

Cancer	Source of cancer cells	Targeted Pathways	Reference
Eosinophilic leukemia	AML14.3D10	Intrinsic apoptosis, p53 upregulated	Mahajan et al, 2008
Lung	A549 cells	Reduced viability, EGF signalling inhibited by binding to EGFR	Hasegawa et al, 2015
Pancreatic	Panc-1, MiaPaCa-2, and Capan-2	Extrinsic apoptosis, induction of Fas	Kaur et al, 2016
Pancreatic	Panc-1, MiaPaCa-2, and Capan-2	Inhibited invasion, reduced TGF-β	Kaur et al, 2018
Ovarian	SKOV3 cells	Extrinsic apoptosis, induction of Fas and TNF-α	Kumar et al, 2019
Prostate	LNCaP, PC3, DU145, Primary tumor explants/ cells isolated from biopsies of metastatic prostate cancer patients	Intrinsic apoptosis, p53 and pAkt pathways	Thakur et al, 2019

Figure legends:

Figure 1: Predicted steps of SP-D induced apoptosis of AML14.3D10 cells. Treatment with rfhSP-D leads to oxidative stress and reduction in the levels of HMGA1. Consequently, there is increased phosphorylation of p53 (Ser15) and p21 levels. Cdc2 phosphorylation contributes to inhibition of Cdc2 leading to arrest of cells in G2/M phase followed by apoptosis and cell death.

Figure 2. Proposed mechanisms for rfhSP-D mediated apoptosis in androgen dependent (LNCaP) and independent (PC3) prostate cancer cells. rfhSP-D (20µg/ml) treatment for 24h upregulates p53 and downregulates pAkt, resulting in upregulation of Bad, Bax and release of cytochrome c leading to cleavage of caspase 7 in prostate cancer cells. SP-D interaction with some key molecules like HMGA1, CD14, SIRPα and EGFR has been reported previously and may be relevant as part of the proposed mechanisms of p53 and Akt.