IL-35 promotes pancreas cancer growth through enhancement of proliferation and inhibition of apoptosis: Evidence for a role as an autocrine growth factor

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ABSTRACT

Interleukin-35 (IL-35), an IL-12 cytokine family member, mediates the immune inhibitory function of regulatory T cells (Treg). We assayed the presence of IL-35 in paraffin-embedded human pancreas cancer (PCAN) and unexpectedly found IL-35 was expressed mainly by epithelial derived PCAN cells, but not by Treg. We further examined the expression and effect of exogenous IL-35 in human PCAN cell lines and found IL-35 promoted growth and inhibited apoptosis in PCAN cell lines. IL-35 induced proliferation correlated with an increase in cyclin B, cyclin D, cdk2, and cdk4 and a decrease in p27 expression, while inhibition of apoptosis was associated with an increase in Bcl-2 and a decrease in TRAILR1. We conclude IL-35 is produced by PCAN in vivo and promotes PCAN cell line growth in vitro. These results might indicate an important new role for IL-35 as an autocrine growth factor in PCAN growth.

1. Introduction

Regulatory T cells (Treg) govern the immune response to cancer and may be a factor in inhibition of the antitumor immune response in human pancreas adenocarcinoma (PCAN) [1,2]. Depletion of Treg in murine PCAN promotes tumor elimination and enhances animal survival [3]. Detection of increased numbers of Treg in the peripheral blood of PCAN patients and in the draining lymph nodes of human PCAN specimens implicates Treg in the human disease process [4].

The mechanism through which Treg exert their effects is complex and interleukin-35 (IL-35) has recently been proposed as a key effector molecule of Treg function [5]. IL-35 is an IL-12 cytokine family member primarily produced by inducible Treg [6]. This cytokine functions to promote Treg proliferation and inhibits CD8 T cell function [7,8]. Through these effects, IL-35 plays a role in immune regulation [9]. IL-35 contributes to the development and prevention of infectious and autoimmune diseases, but the role of IL-35 in malignant disease has yet to be established [10–15].

There is a paucity of data regarding the role for IL-35 in PCAN, but the previously demonstrated importance of Treg in this disease process implies a possible role for IL-35 in PCAN growth and/or progression [16]. We surmised that IL-35 is detectable in human PCAN and this expression might be associated with Treg. Surprisingly, we found IL-35 was mainly expressed by tumor cells. In this report, we further elucidate the role it may have in tumor progression.

2. Materials and methods

2.1. Statement of compliance

Approval was obtained from the University of Missouri IRB for use of human cell lines and paraffin embedded specimens.
2.2. IHC

Paraffin embedded samples from patients having undergone surgery for pancreas adenocarcinoma were obtained from the Department of Pathology, University of Missouri, Columbia. Six paraffin slides from randomly selected primary tumor specimens from patients diagnosed and treated for PCAN were used for detection of IL-35 and its receptor. Immunohistochemistry (IHC) staining (including fluorescence IHC) for IL-35, gp130, IL-12Rb2 were described previously [17–19]. IHC staining for PCNA, cdk4, TRAILR1 and Bcl-2 of pancreatic cell lines was also described previously [17–19]. Mouse anti-IL-35 monoclonal antibody was generously provided by Dr. Dario Vignali (Clone V1.4F5 IgG2b, St. Jude Children’s Research Hospital, Memphis, TN). All other antibodies were purchased from Santa Cruz Biotechnology. To quantify the number of PCNA + cells, all cells in 3–5 randomly selected high power fields were manually counted using image analysis software MetaMorph version 6.3r6 (Molecular Devices Analytical Technologies). Average staining intensity for proteins within the area covered by cells was measured using MetaMorph image analysis software. Results are expressed as the average integrated immunostaining intensity of 3 slides ± SEM relative to that in control cells.

2.3. Tumor cell lines

Human MiaPaCa-2, Pan-28 and Pan-48 pancreatic cell lines were provided by Dr. Citrin (Radiation Oncology Branch, NIH) and Dr. Chiao (M.D. Anderson Cancer Center, University of Texas). Cells were maintained in DMEM (Invitrogen), supplemented with 7% heat-inactivated FBS and 1% penicillin–streptomycin (Invitrogen). Cultures were maintained under conditions as previously described [20,21]. Cells were grown until they reached 70% confluence, at which time they were subjected to the designed experimental treatment regimens.

2.4. ELISA assay

After growth in medium for 6 days, supernatants of pancreatic cancer cell lines were collected and frozen at −20 °C until the assay was performed. IL-35 concentrations in supernatants and medium alone were determined using Legend Max human heterodimer IL-35 ELISA kit (Biolegend) per the manufacturer’s instructions. The OD450 was measured on a SpectraMax 340 counter using SoftMAX PRO 1.2.0 software (Molecular Devices). The reported concentration IL-35 was determined by subtracting the concentration of IL-35 in medium alone from the concentration measured in supernatants to yield the reported concentration. All assays were performed in triplicate.

2.5. Treatment with IL-35 of pancreatic cancer cell lines

70% confluent pancreatic cancer cells were treated for 3 d with escalating concentrations of IL-35 (2–50 ng/ml, Enzo life sciences) or medium alone. IL-35 concentrations and the duration of cytokine treatment were based on our pilot experiments and our previous cytokine studies [22,23].

2.6. Clonogenic survival assay

Three days after IL-35 treatment, cells were detached and counted in a hemocytometer. Clonogenic survival assay was performed as described previously [20,21]. The number of colonies was counted and expressed as a percentage of total colonies in controls.

2.7. RT-PCR

Cells were washed with PBS, centrifuged and homogenized in TRIzol (Invitrogen). RNA was extracted and its concentration was determined. 1 µg RNA was reverse transcribed as previously described [17,18]. GAPDH was used as a housekeeping gene to verify that the same amount of RNA was amplified. Primer sequences were described previously [20,21].

2.8. Determination of proliferation with the Quick Cell Proliferation Assay Kit

Cell proliferation was also determined using the Quick Cell Proliferation Assay Kit (BioVision) according to the manufacturer’s instructions. Expansion of the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases [20,21], leading to an increase in the amount of formazan dye, which can be detected by spectrometry.

2.9. Neutralization of IL-35 with anti-IL-35 in culture

Mouse Neutralizing anti-IL-35 (Clone V1.4F5 200 ng/ml) or isotype was used to block any possible effect of IL-35 on tumor cells. Anti-IL-35 was added before adding IL-35 (50 ng/ml).

2.10. TUNEL staining

Apoptosis was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay using an Apoptag kit (Chemicon) as previously described [20,21]. To quantify the number of apoptotic cells, all cells in 5–6 randomly selected high power fields (magnification: ×400) were manually counted using image analysis software MetaMorph. TUNEL+ cells were expressed as a percentage of total cells.

2.11. Measurement of caspase-3 activity

Cellular caspase-3 activity of PC-3 cells recognizing the sequence DEVD (Asp-Glu-Val-Asp) was measured using a caspase-3/CPP32 colorimetric assay kit (BioVision) as described before [20,21].

2.12. Statistics

All experiments were repeated at least two to three times. Statistical analysis of data was performed using an unpaired two-tailed Student’s t test. A p value <0.05 was considered significant.

3. Results

3.1. IL35 and its receptor are detectable in human pancreatic cancer

Paraffin-embedded (PE) sections of human PCAN were stained by IHC for the presence of IL-35. Staining confirmed the presence of IL-35 in all PE samples (6/6). Unexpectedly, IL-35 was found to be concentrated in the PCAN epithelium and not in the tumor microenvironment (Fig. 1A). Dual-color immunofluorescence microscopy was used to determine with which cells IL-35 localized. Pancytokeratin (PCK) was used as a marker for PCAN. The results shown in Fig. 1B support the IHC finding of PCAN epithelial cells as the main site for IL-35 (Fig. 1B, top panels, merged, yellow, overlay). Next, we investigated if the IL-35 receptor, a heterodimer of gp130 and IL12Rb2, was detectable in human PCAN. The results of dual-color immunofluorescence microscopy showed that both gp130 and IL12Rb2 (Fig. 1B, middle and bottom panels, red) were...
detectable in the PCAN PE samples and both subunits localized to PCK + cancer cells (Fig. 1B, middle and bottom panels, merged, yellow, overlay). These results confirm the presence of IL-35 and IL-35 receptor in human PCAN.

3.2. IL-35 and its receptor are detectable in human pancreatic cancer cell lines

To determine the suitability of human PCAN cell lines for further study of IL-35 in PCAN, we determined the expression of IL-35 and its receptor in the well-established human PCAN cell lines, MiaPaCa-2, Pan-28 and Pan-48. IL-35 was detected by ELISA in the supernatant of all three unstimulated PCAN cell lines (MiaPaCa-2 0.28 ± 0.01 ng/ml, Pan-28 0.35 ± 0.02 ng/ml, and Pan-48 0.12 ± 0.04 ng/ml). As shown in Fig. 2A, IL-35, as well as the IL-35 receptor subunits gp130 and IL12Rβ2, were detectable in these human PCAN cell lines. Colocalization of subunit gp130 (Fig. 2B, top panels, red) and subunit IL12Rβ2 (Fig. 2B, middle panels, green) were further confirmed by dual-color immunofluorescence microscopy (Fig. 2B, bottom panels, merged, yellow, overlay). Thus, we determined these cell lines were suitable for studying IL-35 in PCAN.

3.3. IL-35 promotes survival and proliferation of MiaPaCa-2 cells

We utilized MiaPaCa-2 cells to investigate the direct effect of exogenous IL-35 on PCAN. 70% confluent MiaPaCa-2 cells were treated with escalating doses of IL-35 (2 ng/ml, 10 ng/ml, or 50 ng/ml) or medium alone for 3 days and cell survival was evaluated by clonogenic survival assay (Fig. 3A). The percentage of colonies of MiaPaCa-2 cells treated with IL-35 at the concentration of 2 ng/ml was comparable with that in controls treated with medium alone, while the percentage of colonies of MiaPaCa-2 cells was higher at 10 ng/ml of IL-35 and the highest at the concentration of 50 ng/ml, demonstrating that exogenous IL-35 promotes increased MiaPaCa-2 cell survival in a dose-dependent manner. The proliferative effect of IL-35 (50 ng/ml) on MiaPaCa-2 cells was also evident by staining for PCNA (Fig. 3B) and evaluation of mRNA of PCNA, a marker for cell proliferation (Fig. 3C). Similar results were also obtained by using a Quick Cell Proliferation Assay Kit to analyze cell proliferation (Fig. 3D). Despite the fact that IL-35 shares a subunit of EBI3 with IL-27, our unpublished data indicates that IL-27 has an inhibitory effect on proliferation of pancreatic cancer cells. Thus, our results indicate that IL-35, but not IL-27, promotes survival and proliferation of pancreatic cancer cells.
3.4. IL-35 neutralizing antibody reverses the effect of IL-35 on proliferation of human PCAN cell lines

To investigate if the proliferation promoting effect of IL-35 on MiaPaCa-2 cells is IL-35 specific, a neutralizing anti-IL-35 was used to block the effect of IL-35 on MiaPaCa-2 cells. As shown in Fig. 3E, the percentage of colonies of MiaPaCa-2 cells was higher after treatment with IL-35 (50 ng/ml) and was significantly lower after treatment with anti-IL-35. Similar results were also found in Pan-28 and Pan-48 cell lines. These results indicate that the proliferation promoting effect is IL-35 specific and it is not cell line specific.

3.5. Effect of IL-35 on expression of pro- and anti-proliferative molecules

We examined MiaPaCA-2 cells for molecular changes in proliferation after treatment with IL-35. mRNA expression of major pro-proliferative molecules (cyclin B, cyclin D, cyclin E, cdk2, cdk4) and anti-proliferative molecules (p15, p18, p21, p27, p53) were determined by RT-PCR and IHC in MiaPaCa-2 cells in the presence or absence of IL-35 (50 ng/ml) (Fig. 4). mRNA expression of cyclin B, cyclin D, cdk2 and cdk4 was significantly higher in cells treated with IL-35 than in cells treated with medium alone (p < 0.05). The mRNA expression of anti-proliferative molecule p27 was
increased in cells treated with IL-35 compared to that in cells treated with medium alone; however, the expression of p15, p18, p21, and p35 were not significantly altered. Consistent with the mRNA expression of cdk4, the relative immunostaining intensity for cdk4 was stronger in cells treated with IL-35 than that in cells treated with medium alone (Fig. 5A and B, \( p < 0.05 \)). These results indicate that increased expression of pro-proliferative molecules cyclin B, cyclin D, cdk2 and cdk4 correlated with the effect of IL-35 on the proliferation of MiaPaCa-2 cells.

3.6. IL-35 decreases apoptosis of MiaPaCa-2 cells

In addition to enhanced proliferation, decrease of apoptosis could also result in IL-35 promotion of PCAN growth. To address this possible effect of IL-35, we examined apoptosis of MiaPaCa-2 cells treated with IL-35. 70% confluent MiaPaCa-2 cells were treated with IL-35 (50 ng/ml) or medium alone for 3 days and apoptosis was evaluated by TUNEL staining (Fig. 6A and B). 12 ± 3% of MiaPaCa-2 cells treated with medium alone were TUNEL+, however, few of MiaPaCa-2 cells treated with IL-35 were TUNEL+ and their difference in TUNEL+ cell was significant (\( p < 0.05 \)). Similar results were obtained when caspase-3 activity was assayed.
These results suggest that IL-35 inhibits apoptosis of MiaPaCa-2 cells.

3.7. IL-35 alters the expression of pro- and anti-apoptotic molecules

mRNA expression of pro-apoptotic molecules (Fas, Fasl, TRAILR1, TRAIL, Bax) and anti-apoptotic molecules (FLIP, Bcl-2, survivin) in MiaPaCa-2 cells treated with IL-35 (50 ng/ml) was determined by RT-PCR (Fig. 7). mRNA expression of the pro-apoptotic molecule TRAILR1 was lower and the anti-apoptotic molecule Bcl-2 was higher in cells treated with IL-35 than that in cells treated with medium alone ($p < 0.05$). Consistent with the mRNA expression patterns of these two proteins, the relative immunostaining intensity for TRAILR1 was weaker and for Bcl-2 was stronger in cells treated with IL-35 than that in cells treated with medium alone (Fig. 5A, C and D, $p < 0.05$). These results indicate that downregulation of TRAILR1 and upregulation of Bcl-2 correlated with the decreased apoptosis of MiaPaCa-2 cells induced by IL-35.

4. Discussion

The IL-12 cytokine family members (IL-12, IL-23, IL-27, IL-35) are important regulators of the pro- and anti-inflammatory immune response [24–26]. Each member of the IL-12 cytokine family is a heterodimer which shares alpha and beta chains as well as receptor subunits with other members of the family [24–26]. IL-35 is composed of the p35 (shared with IL-12) and Ebi3 (shared with IL-27) subunits [24–27]. IL-35 signals through a heterodimer IL-35 receptor (IL35R) which is composed of IL12Rb2 (shared with IL-12) and gp130 (shared with IL-27) subunits [24–27]. As with other IL-12 cytokine signaling, IL-35 signaling is mediated through the JAK-STAT pathway (STAT1 and STAT4) [27]. IL-35 signaling through IL12Rb2 and gp130 homodimers has been described, but the physiologic significance is underdetermined [27].

Each IL-12 family cytokine member has a unique functional profile and, in a sense, the function of each IL-12 cytokine family member compliments the others. IL-12 augments the TH1 response and drive cellular differentiation in this pathway, while IL-23 drives IL-17 production in the TH17 pathway [28–30]. These cytokines are produced by dendritic cells and activated macrophages as part of the pro-inflammatory response. IL-27 is also produced by antigen presenting cells. IL-27 has mainly anti-inflammatory effects by inducing IL-10 production while diminishing the function of helper T cells [31]. IL-35 is the most recently described IL-12 family member. Like IL-27, its role is anti-inflammatory; however unlike other IL-12 family members, IL-35 is produced primarily by regulatory T cells (Treg). The mechanism by which cytokines govern Treg function is complex and IL-35 has recently been proposed as a key effector molecule of Treg function [5]. IL-35 functions as an autocrine growth factor for Treg wherein production of IL-35 by Treg enhances the secretion of IL-35 by Treg. Furthermore, IL-35 secreting Treg may be an inducible Treg (iTreg) subset with a different role from TGF-β and IL-10 secreting Treg which purportedly play a housekeeping role in immunomodulation [32]. Despite recent discoveries about the role IL-35 in immune function and disease states, many of its major functions are still being elucidated.

While our hypothesis regarding the detection of IL-35 in human PCAN was correct, we thought we would find IL-35 primarily...
associated with Treg. Instead, we found IL-35 expression in PCAN was principally associated with the epithelial derived cancer cells themselves. Our finding of PCAN associated IL-35 expression is significant. We have demonstrated IL-35 expression in epithelial derived tumor cells both in vivo by use of paraffin embedded human PCAN samples, and in vitro by use of PCAN cell lines. Although IL-35 expression has recently been demonstrated in a cross section of cancer cell lines, we are the first to show in vivo expression by using human PCAN tissue samples [16]. IHC and fluorescence microscopy confirm IL-35 expression in human PCAN specimens.

Our data suggest IL-35 has a functional role to support the growth of PCAN cell lines by promoting proliferation and inhibiting apoptosis. We have determined which changes in pro-proliferative and anti-apoptotic molecules correlated with treatment of the PCAN cell line with IL-35. Recent data confirms the expression of IL-35 in many cancer cell lines, thereby implicating IL-35 broadly in tumorigenesis [16]. In their study, transfection of a hepatocellular cancer cell line with IL-35 had an anti-proliferative and pro-apoptotic effect in vitro in contradistinction to our results achieved when IL-35 was exogenously administered to PCAN cell lines. Decreases in cyclin D and Bcl-2 expression in the transfected cell lines were described, in opposition to the increases we found in these molecules when PCAN cells were treated with IL-35. Additionally, we demonstrated IL-35 blockade inhibited proliferation. When these studies are considered together, the results may indicate a technical problem or a lineage and/or context dependent role for IL-35, which has not been suggested previously. There is precedent for contextual behavior as demonstrated in studies of sirtuin 1 in cancer [33]. Further investigation is necessary to understand the divergent results.

Analogous to previous studies of PCAN cytokine secretion, our data supports IL-35 as an autocrine growth factor for PCAN. Autocrine secretion of cytokines IL-1β, IL-4, IL-6, IL-8, and IL-13 has been previously been demonstrated in human PCAN cell lines [34–38]. IL-1β has been shown to have an autocrine activity in some, but not all PCAN cell lines, wherein IL-1β increases chemoresistance [34]. IL-4 is produced by the majority of investigated PCAN cell lines and its expression promotes cell proliferation [35]. IL-6 expression supports PCAN cell line survival and its upregulated expression correlates with mesothelin expression [36]. IL-8 promotes proliferation of PCAN [39]. IL-13 has been shown to increase PCAN proliferation and likelihood of metastasis [38]. It is unknown at this time whether IL-35 expression in PCAN is related to the expression of other autocrine cytokines in PCAN, however is an exciting possible line of investigation.

Our results do not exclude the possibility of IL-35 expression by Treg in PCAN nor diminish the previously demonstrated importance of Treg in PCAN. Reports derived from human specimens and mouse models reveal Treg are present in the immune infiltrate in PCAN. Dominant IL-35 expression by the PCAN epithelium could suggest that iTreg may not be present or may not play a role in PCAN. Alternatively, it may be that Treg are infiltrating the tumor but cannot be differentiated from the background or that Treg secrete IL-35 but at levels below the limits of detection of our IL-35 assay. Treg may exert their effects in PCAN through other modes of action such as IL-10 or TGF-β expression without Treg IL-35 playing much of a role in PCAN [40,41]. Support for this hypothesis comes from murine studies of PCAN, in which PCAN secretion of TGF-β into the tumor microenvironment induces Treg [42]. Elimination of these Treg promotes animal survival. To date, IL-35 has not been explored in murine PCAN.

There is little data regarding the role of IL-35 in cancer genesis, proliferation, or cancer treatment [14,15]. We have shown the presence of IL-35 in human PCAN specimens and the production of IL-35 by human PCAN cell lines. The findings presented in this report add to the burgeoning understanding of IL-35 in malignancy and the tumor immunology of PCAN. The role of IL-35 in PCAN progression in vivo, on the microenvironment, and interaction with Treg has yet to be explored. We anticipate this report will open a new line of research and potential arena for the development of anticancer therapies.

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