POTENTIALS OF TYPE I INTERFERON THERAPY IN THE TREATMENT OF PANCREATIC CANCER

Stephanie Booy

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POTENTIALS OF TYPE I INTERFERON THERAPY IN THE TREATMENT OF PANCREATIC CANCER

MOGELIJKHEDEN VAN TYPE I INTERFERON THERAPIE IN DE BEHANDELING VAN ALVLEESKLIERKANKER

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1 General introduction and aims of this thesis

Partly based on:
Potentials of interferon therapy in the treatment of pancreatic cancer

Stephanie Booy, Leo J. Hofland and Casper H.J. van Eijck.

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1. PANCREATIC CANCER

Pancreatic cancer, with 165.100 estimated new cases and 161.800 estimated deaths, is the fourth leading cause of cancer related death in the western world[1]. Surgery is the only curative therapy, but due to early metastasis and/or locally advanced disease only 15-20% of the patients are eligible for resection at time of presentation. Nevertheless, even after successful surgery prognosis remains poor, resulting in a total overall 5-year survival rate of less than 6% [2].

In addition to tumors of pancreatic ductal origin, in the same anatomical area other tumors may arise as well. These cancers are known as periampullary cancers consisting of distal bile duct and ampullary carcinoma's and present with the same clinical symptoms as pancreatic cancer. However, after correction for tumor size, positive lymph nodes and stage, the survival of periampullary cancer patients is significant favorable compared to pancreatic cancer patients (45 months *versus* 15 months, respectively), which may indicate that periampullary cancers represent a different family of tumors with a different biological behavior. Nevertheless, patients with periampullary cancers are subjected to the same surgical procedure as patients with pancreatic carcinomas[3,4]. Additionally, it has recently been demonstrated that periampullary cancer patients have a small survival benefit of adjuvant chemotherapy as well[5].

Current chemotherapeutic agents used in pancreatic cancer, mainly, consist of gemcitabine and 5-FU, which may be complemented with platinum analogues (cisplatin, oxaliplatin), capecitabine, taxanes and irinotecan. The use of adjuvant radiotherapy in the treatment of pancreatic cancer is still controversial and depends on the country in which the patient is being treated (chemo-radiotherapy in North America *versus* chemotherapy alone as standard in Europe)[6]. Pancreatic cancers are very heterogeneous tumors with a restricted vasculature and appear to be very resistant to chemo- and radiotherapy[7]. Recently, the importance of the tumor-stroma component in pancreatic cancer is becoming clearer. The desmoplastic reaction of the stroma to the cancer is an active player in the carcinogenesis of pancreatic cancer and can explain the aggressive behavior of this tumor. In addition, it has been described that increased levels of stroma correlate with poorer prognosis, although in pre-clinical and clinical trials, targeting the stromal component has been associated with a better prognosis for patients with pancreatic cancer[7,8]. Nevertheless, despite the multiple different treatment strategies that have been attempted over the last 30 years, pancreatic cancer survival has barely improved. Nowadays median survival of patients with pancreatic cancer varies from 24.1 months for patients having stage IA disease, to 4.5 months for patients with metastatic, stage V disease[9]. Therefore, in order to improve survival of patients with pancreatic cancer new treatment strategies are warranted.

A few years ago several clinical studies showed some evidence that there might be a role for interferon-α in the adjuvant treatment of pancreatic cancer[10-14]. Although the only randomized clinical trial did not show a significant increase in overall survival, the difference in median survival of 3.6 months implies that some patients probably benefited from the experimental treatment [14]. Furthermore, median survival in these clinical trials was notably high and therefore this treatment strategy seems promising.

More recently, several *in vitro* studies showed promising results regarding IFN-β therapy and demonstrated that in human pancreatic cancer cells IFN-β induces, already at low concentrations, a much more potent anti-cancer effect compared to IFN-α. Additionally, IFN-β is capable to induce strong chemo- and radiosensitizing effects even in cell lines non-responsive to IFN-α. However, the use of interferons (IFNs), mainly IFN-α, in the treatment of pancreatic cancer remains controversial[15-17].

2. INTERFERONS

Interferons are cytokines that have been discovered over 50 years ago, and are known to have antiproliferative, antiviral and immunoregulatory activities[18-22]. They can be divided into two major categories, type I and type II IFNs. Type I IFNs are produced in direct response to viral infection and include IFN-α,-β, -ω,-δ and –τ. Type II IFNs consists only of IFN-γ[23]. Of all the various subtypes, IFN-α and IFN-β are the most frequently used subtypes in clinical practice and subsequent information in this review refers to these subtypes. In humans, IFN-α is predominately synthesized by leukocytes. IFN-β is particularly synthesized by fibroblasts[21,24]. There are 13 human IFN-α genes located on the short arm of chromosome 9 encoding for 12 different functional human IFN-α proteins since there are two genes (α1 and α13) that encode for identical proteins. The human IFN-β gene is present as a single copy on chromosome 9. IFN-α and –β share around 30% of their amino acid sequence identity[25].

2.1 Type I interferon receptor

Both IFN-α as IFN-β act via the type I IFN receptor complex (IFNAR), which is composed of two subunits; IFNAR-1 and IFNAR-2. Of the IFNAR-2 subunit there are three isoforms known that are differentially spliced from a common gene. IFNAR-2a is the soluble form and can act as a dominant negative regulator of free IFNs. IFNAR-2b is the shorter form, lacking regions of the cytoplasmic domain, is unable to couple to signal transduction but may act as a negative regulator of IFN responses. The latter isoform, the IFNAR-2c, contains the entire cytoplasmic domain and along with the IFNAR-1 makes up the functional IFN receptor complex[21,26,27].

Binding of IFNs to the receptor complex leads to the activation and phosphorylation of the receptor associated Janus kinase JAK1 and tyrosine kinase TYK2 and thereby to the phosphorylation of signal transducer and activator of transcription (STAT) proteins. After phosphorylation, STAT-1 and STAT-2 forms a complex named IFN-stimulated gene factor-3 (ISGF3) with the DNA binding protein p48. The ISGF3 complex moves into the nucleus where it binds to IFN-stimulated response elements (ISRE) resulting in transcription of interferon stimulated genes (ISGs) encoding for multiple different proteins that effectuate the different activities of IFNs[19,20,23,28](Figure 1).

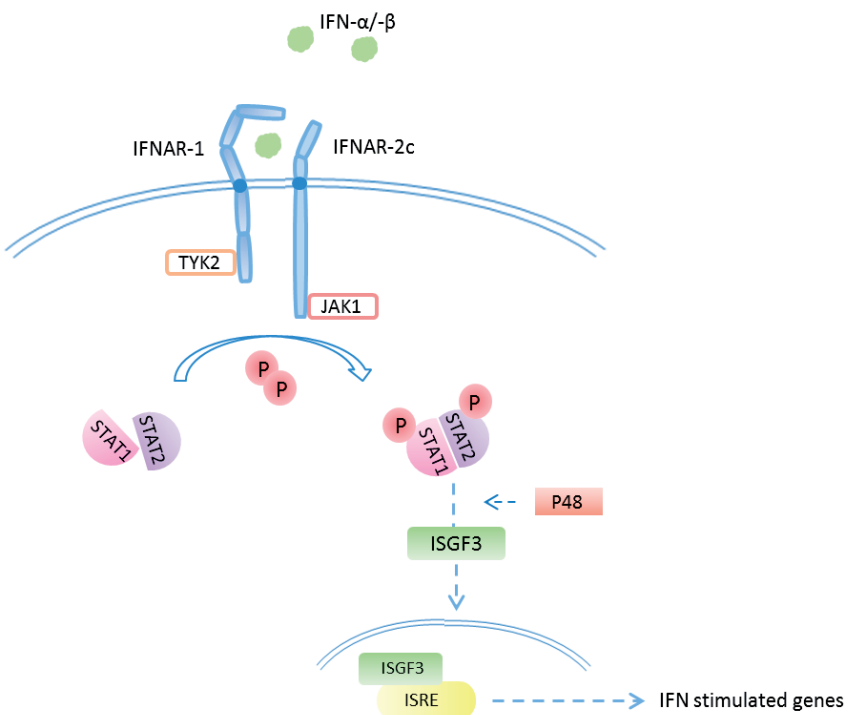

Figure 1: Signaling pathway activated by IFN-α/-β via the type I IFN receptor complex (IFNAR-1 and IFNAR-2c). Binding of IFN-α/-β to the receptor complex leads to the phosphorylation of the receptor-associated tyrosine kinases which in their turn phosphorylate STAT-1 and STAT-2. Together with the DNA binding protein p48 the ISGF3 complex is formed which enter the nucleus and binds to interferon stimulated response element (ISRE) resulting in the transcription of interferon stimulated genes (ISGs).

2.2 Antitumor defense processes of type I IFNs

In addition to the antiproliferative, antiviral and immunoregulatory activities IFN-α and-β, both cytokines are also involved in cell differentiation and antitumor defense processes (figure 2). There are several mechanisms for the anti-cancer effects of IFN-α and-β. The direct effects include the induction of apoptosis and blocking of the cell cycle. The initiation of apoptosis can act through an extrinsic pathway by the activation of the death receptors, via an intrinsic and/or mitochondrial pathway or by the stress kinase cascade[29-32]. IFNs can affect different phases of the mitotic cycle, mostly IFN therapy results in a cell cycle arrest at the G1 phase or in a prolongation and accumulation of cells in the S-phase due to the disability to complete DNA replication by the down regulation and impaired activity of cyclin and cyclin dependent kinases.[30,33,34]. As a result, tumor cells are more vulnerable to chemo- and radiotherapy.

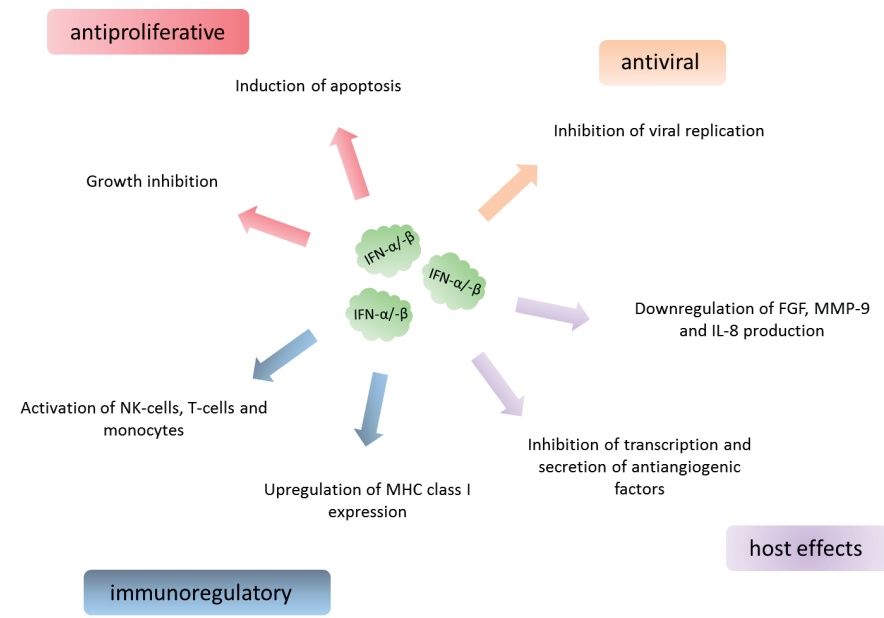

Figure 2: Overview of the activities of IFN-α and –β in humans. Besides the direct effects, several indirect anti-cancer effects of type I IFNs have been described. These effects include the induction of immunomodulatory functions, like the activation of cytotoxic t-lymphocytes, natural killer (NK)-cells and monocytes[35] and the induction of increased cell surface expression of class I major histocompatibility complex antigens[36,37]. Furthermore, several non-immunomodulatory host effects, like the inhibition of transcription and secretion of anti-angiogenic factors[38-40] and the interaction between tumor and surrounding stroma[41] have been demonstrated.

Besides the direct effects, several indirect anti-cancer effects of type I IFNs have been described. These effects include the induction of immunomodulatory functions, like the activation of cytotoxic t-lymphocytes, natural killer (NK)-cells and monocytes[35] and the induction of increased cell surface expression of class I major histocompatibility complex antigens[36,37]. Furthermore, several non-immunomodulatory host effects, like the inhibition of transcription and secretion of anti-angiogenic factors[38-40] and the interaction between tumor and surrounding stroma[41] have been demonstrated.

3. INTERFERONS IN CANCER TREATMENT

The wide range of potential anti-cancer effects of IFNs led to exploration of IFN-α and -β, in the treatment of multiple malignant and viral diseases. Although IFN-α and –β act via the same receptor complex, they display significant functional differences and do not share the same clinical indications (Table 1).

3.1 Interferon-α

In 1985 IFN-α was approved for the treatment of hairy cell leukemia[42] hereafter it was found that IFN-α was therapeutically effective in the treatment of other malignancies like chronic myelogenous leukemia[43], multiple myeloma[44], malignant cutaneous melanoma[45], Kaposi's sarcoma[46], gastroenteropancreatic neuroendocrine tumors[47] and renal cell carcinoma[48] as well. However, IFN-α therapy can give considerable side effects, which may be acute (fever, chills, headache and myalgia) or delayed (nausea, vomiting, fatigue, anorexia, leukopenia and less frequently hyper- or hypothyroidism, neutropenia and neurotoxicity) and are usually dependent on the amount of the dosage. Some other side effects that were reported are depression and confusion (0.1-1% of the patients) and rarely, though notable, (attempted) suicide (0.01-0.1% of the patients). Nowadays, due to the increased understanding of molecular mechanisms of various types of cancer, IFN-α is only commonly employed as a first line, salvage, therapy in the treatment of metastatic renal cell carcinoma and malignant melanoma.

3.2 Interferon-β

To date, the use of IFN-β is only approved in the treatment of relapsing remitting multiple sclerosis(RRMS). Although multiple clinical studies have been conducted regarding the use of IFN-β in RRMS, the mechanism underlying the therapeutic effects is not fully understood.

Table 1. Clinical indications of type I interferons

Interferon-type	*Name*	*Indications*
Interferon-α2a	Roferon-A	Hairy cell leukemia AIDS related Kaposi sarcoma Chronic hepatitis B Chronic hepatitis C Cutaneous T-cell lymphoma Chronic myeloid leukemia Follicular non-hodgkin lymphoma Advanced renal cell carcinoma Malignant melanoma
Interferon-α2b	Intron-A	Hairy cell leukemia Juvenile laryngeal pappilomatosis AIDS related Kaposi sarcoma Chronic hepatitis B Chronic hepatitis C Multiple myeloma Chronic myeloid leukemia Non-Hodgkin lymphoma Carcinoid tumor Malignant melanoma
Peg-Interferon-α2a	Pegasys	Chronic hepatitis B Chronic hepatitis C
Peg-Interferon-α2b	Pegintron	Chronic hepatitis C
Interferon-β1a	Avonex Rebif	Relapsing remitting multiple sclerose
Interferon-β1b	Betaferon Betaseron	Relapsing remitting multiple sclerose

Adapted from: Bracarda et al.

Nevertheless, IFN-β therapy reduces relapse rates in approximately one third of the patients and reduces the appearance of new MRI lesions in around two third of the patients[49-51].

In cancer treatment, simultaneous administration of IFN-β with adjuvant treatment has been clinically tested in phase II and III trials in patients with glioblastoma[52], metastatic breast cancer[53,54], advanced pancreatic cancer[55] and in patients with locally advanced non-small-cell lung cancer[56]. In patients with locally advanced non-small-cell lung cancer the concomitant use of IFN-β did not lead to an improvement in the 1-year survival. In one of the two studies regarding the additional use of IFN-β in metastatic breast cancer and in the phase II trials in patients with glioblastoma and advanced pancreatic carcinoma a trend towards a prolonged progression free survival was observed[52,53,55].

4. INTERFERONS IN THE TREATMENT OF PANCREATIC CANCER - *IN VITRO*

One of the first reported *in vitro* studies regarding the anti-cancer effect of type I IFNs in human pancreatic cancer cell lines was in 1991 by Matsubara et al[57], in which the antiproliferative effects of natural human tumor necrosis factor-α (TNF-α), IFN-α and interferon-γ (IFN-γ) alone and in combination, in five human pancreatic cancer cell lines (HuP-T1, HuP-T3, HuP-T4, BxPC-3 and MIA-PaCa-2) were described. After 96 hours of incubation with IFN-α, the authors showed a dose-dependent growth inhibition, ranging from 35 to 70%, in four of the five cell lines[57]. In 1996, Rosewicz et al[58] described that protein kinase C (PKC) expression might determine the sensitivity of pancreatic cancer cells to the antiproliferative effects of IFN-α, although this mechanism is not completely elucidated.[58].

Despite the paucity of available *in vitro* data, multiple clinical studies were conducted in the following years, owing to the poor perspectives and limited treatment options of pancreatic cancer patients. Nearly 10 years later several researchers aimed to expand the knowledge on the anti-cancer effects of IFNs in the treatment of pancreatic cancer. In 2005, the group of Angela Marten from the university of Heidelberg determined whether IFN-α acts in a synergistic manner when combined with chemotherapy (5-FU and cisplatin) and/or radiotherapy in 8 human pancreatic cancer cell lines. The results of this study showed that IFN-α has a limited chemosensitizing effect, but a direct inhibitory effects (cell survival, apoptosis and proliferation) and potent radiosensitizing effects. In addition, the authors showed that three cell lines secreted VEGF and that INF-α was able to inhibit this enhanced VEGF secretion of 5-FU and cisplatin resistant cells. Besides that, IFN-α was able to diminish the decrease of MHC class I expression occurring after 5-FU treatment[59].

Subsequently, the same research group demonstrated that IFN-α is capable to activate NK-cells against pancreatic carcinoma cells and that treatment with 5-FU makes pancreatic cancer cells more susceptible against these immunological attacks[60].

Other research groups focused more on the antiproliferative effects of IFNs and the importance of the IFN receptor expression. Saidi et al.[61] showed that pancreatic cancer cell lines differently express the IFNAR-2 receptor and that cell lines with a higher IFNAR-2 receptor expression are more prone to the direct effects (cell growth and apoptosis) of IFNs. Moreover, Vitale et al.[30] evaluated the role of the type I IFN receptor in the responsiveness to IFN-α and –β as well. The growth inhibitory effects of IFN-α and –β were determined in three human pancreatic cancer cell lines as well as the mechanisms that are involved in this growth inhibition (apoptosis and cell cycle arrest). Furthermore, the presence of all type I IFN receptor subunit transcripts (e.g. IFNAR-1, IFNAR-2 total, IFNAR-2a, -2b and -2c) were

demonstrated. The study of Vitale et al. showed, for the first time, that the antitumor activity of IFN-β in human pancreatic cancer cell lines is significantly more effective compared to the effects of IFN-α. IFN-β induces a more potent and early apoptosis and cell cycle arrest compared to IFN-α. In addition, this effect was even more pronounced in cell lines with a higher receptor expression, suggesting an association between type I IFN receptor expression and the response to IFN-α and –β.

The promising effects of IFN-β on human pancreatic cancer cell lines encouraged other research groups to determine the chemo and radiosensitizing properties of IFN-β as well. The study of Jost et al.[62] showed a heterogeneous response to IFN-α and –β to ionizing radiation in 10 human pancreatic cancer cell lines. IFN-β induced stronger cytotoxic and radiation enhancing effects compared to IFN-α, which seems to be due to the increase of radiation induced apoptosis by IFN pre-treatment. The potent radiosensitizing properties of IFN-β were confirmed by the study of Morak et al[63]. Moreover, it is demonstrated that IFN-β is able to act synergistically with gemcitabine to reduce the cell growth of pancreatic cancer cells, even in cell lines that are non-responsive to IFN-α and have a low IFNAR-2 expression.[64] However, the correlation between type I interferon receptor (IFNAR-1 and IFNAR-2) expression and response of pancreatic cancer cells to type I interferons (IFN-α and IFN-β) has not been demonstrated to date.

5. INTERFERONS IN THE TREATMENT OF PANCREATIC CANCER – *IN VIVO* AND *EX VIVO*

Following *in vitro* research, the next step is to demonstrate the effects of IFNs *in vivo*. Despite the fact that the potent anti-tumor effects of IFN-β were demonstrated *in vitro*, all the *in vivo* research that has been done is solely focused on the effects of IFN-α.

5.1 Interferon-α therapy *in vivo* in experimental models

In the first *in vivo* study researchers aimed to determine the most optimal and effective IFN-α treatment schedule in a orthotopic pancreatic cancer mouse model. Mice were randomized in different treatment groups receiving divergent doses of IFN-α at different time points with or without gemcitabine. After 35 days of treatment with daily subcutaneous injections of 10.000 IU IFN-α and twice a week intraperitoneal injections of 125 mg/kg gemcitabine pancreatic tumor growth and the number of metastasis was significantly inhibited in nude mice. Treatment alone with IFN-α was effective as well, though combination therapy further decreased tumor growth and significantly prolonged survival. Further-

more, tumors of treated mice showed a significant increase of apoptotic cells, a decrease in mean vessel density and a down regulation of bFGF, MMP-9, IL-8 and VEGF which, in turn, can be responsible for the reduced tumor growth[65]. Only two other studies used the cytotoxic, and most active, agent gemcitabine as combination therapy as well. The study of Saidi et al.[66] mainly focused on the relationship between the IFN receptor status of the pancreatic cancer cell lines that were injected orthotopically in nude mice and the effect of IFN-α combined with gemcitabine. The authors showed that IFN-α treatment enhanced the effects of gemcitabine therapy. In addition, treatment with IFN-α appeared to be only effective in the IFNAR-2 expressing cell line[66]. The other study that explored the effects of IFN-α and gemcitabine demonstrated that IFN-α is able to increase the chemosensitivity for gemcitabine of pancreatic cancer cells with cancer stem cell properties in a subcutaneous xenograft mice model[67].

Another study demonstrated the effect of IFN-α in addition to S-1, an oral fluoropyrimidine derivative. In a subcutaneous heterotopic xenograft model a significant effect of the combination therapy on the reduction of tumor growth was observed. Furthermore, an inhibition of angiogenic and survival factors (VEGF, bFGF, MMP-2, MMP-7, MMP-9) was demonstrated, as well as a downregulation of 6 genes that are linked to cell invasion, proliferation and metastasis[68].

Three studies, all from the group of Heidelberg, investigated the effects of IFN-α combined with mainly 5-FU and focused on the different anti-cancer features of IFN-α[69-71]. First, the effect of IFN-α combined with 5-FU on tumor growth and immune response was determined. The addition of IFN-α to 5-FU, and cisplatin treatment significantly improved the outcome. In contrast to the *in vitro* results, the effect of IFN-α combined with radiotherapy were less clear due to adverse reactions in the mice. Furthermore, by the addition of IFN-α an improved adherence of leukocytes to the endothelium was observed which is of importance for the infiltration of immune cells into the tumor[70].

In the second study the authors showed, by intravital microscopy, in an orthotopic murine pancreatic cancer model, that the addition of IFN-α to 5-FU has strong anti-angiogenic properties. Tumor regression, a decrease in serum VEGF and VEGF receptor expression and a reduced, and less chaotic in structure, vessel density was demonstrated. Furthermore, treatment with IFN-α monotherapy led to the normalization of vessel structure assuming that this results in a better distribution of chemotherapy[69]. Lastly, the effect of IFN-α on multidrug resistance was demonstrated in the same murine orthotopic pancreatic cancer model. After combined treatment with IFN-α and chemotherapy (5-FU, cisplatin and

gemcitabine) a decrease (though not in all treatment modalities significant) of multidrug resistance-associated proteins (MRPs) and P-glycoprotein was observed as well as a significantly reduced tumor size, peritoneal carcinomatosis, hepatic seeding and pulmonary metastasis[71].

In conclusion, the *in vivo* effect of adjuvant IFN-α therapy is at multiple levels, with different combination therapies broadly demonstrated. Nevertheless, the effects of IFN-β treatment *in vivo* remain unexposed.

5.2 *ex vivo* interferon receptor expression

Type I IFNs exert their effect via the type I IFN receptor. The importance of the receptor for the direct anti-cancer effect has been described for melanoma, breast cancer, fibrosarcoma and hepatocellular cancer cells. Surprisingly, in none of the pancreatic cancer patients receiving adjuvant IFN-α therapy IFN receptor status known.

Two, nearly identical, studies of Saidi et al.[72,73] demonstrated the IFNAR-α/β (IFNAR-2, unknown isoform) expression in 46 pancreatic cancer patients. The majority of the pancreatic tumors (35/46) showed no or faint expression of the IFNAR-α/β, 9 tumors showed a moderate expression and only 2 of the pancreatic tumors showed a high expression. The expression of the IFNAR-α/β receptor did not correlate with clinical and/or pathological parameters. Patients with tumors expressing a high-moderate IFN-α/β receptor had a significant better survival compared to patients with tumors with no detectable IFN-α/β expression (22 months *versus* 13 months; log rank p=0.012). In addition, patients with tumors with a positive receptor expression and whom received adjuvant chemotherapy (n=7) had a significant better survival compared to the patients that received adjuvant therapy but whose tumors showed no receptor expression (n=25) (24 months *versus* 14.7 months; log rank p=0.012). However, in these studies no distinction was made regarding cancer origin. Although periampullary cancers, like ampullary carcinomas and distal bile duct cancers, arise in the same anatomical area and are subjected to the same surgical procedure as pancreatic cancers, they have different prognosis and distinction between these two cancers is of importance[3,74].

6. INTERFERONS IN THE TREATMENT OF PANCREATIC CANCER – CLINICAL STUDIES

Similar to most other clinical studies, in the clinical trials with respect to pancreatic cancer, IFN-α is the most evaluated type I IFN. The use of IFN-α in the treatment of pancreatic cancer

was at first evaluated in the palliative setting, later on, as adjuvant treatment. Many different treatment strategies combining a variety of chemotherapeutic agents with or without radiation therapy and different dosages of IFNs have been tested and will be discussed below.

6.1 Interferon therapy in the palliative treatment setting

From 1992 until 2000 11 phase II single treatment arm studies were performed using INF-α in the treatment of advanced pancreatic cancer[16,17,75-83] (Table 2). There was only 1 phase II randomized clinical trial[15] and only 1 study that explored the effect of IFN-β[55](Table 3). In the majority of the studies the outcome of interest was the feasibility of IFN therapy, the response rate and the related toxicities. Overall, the number of patients included in the studies were small, ranging from 6 patients in the study of Moore et al.[80] to 57 patients in the study of Bernhard et al.[75]. The chosen treatment regimen included in the majority of the studies included IFN-α and 5-fluoracil (5-FU) combined with inter alia cisplatin (CDDP), leucovorin (LV) and/or 13-cis retinoid acid. One study[79] also included etoposide, a topoisomerase inhibitor, another study added folinic acid to the treatment regimen[75]. The treatment dose of IFN-α ranged from 9 million international units (IU) a week up to 6 million IU daily. In the palliative setting the median survival ranged from 4.6 to 11 months. Treatment toxicities were in most of the studies of gastrointestinal and/or hematological origin. Only a few studies showed the proportion of patients experiencing treatment toxicities[75,76,79-81,83]. Solely the study of MacDonald et al[79] reported a case of complete response, implying the disappearance of tumor mass, on IFN-α therapy. A partial response, in most of the studies defined as a reduction of tumor mass greater than 50%, was variably, though not extensively, observed in the majority of the studies. Despite the fact that in all studies most of the patients did not show a reduction in tumor mass, in some patients the disease did remained stable for a, unfortunately not specified, period of time on IFN-α therapy.

The randomized clinical trial of Wagener et al[15] aimed to demonstrated an effect of IFN-α on rate and the duration of response. In total, 33 patients were randomized to either the CDDP+5-FU arm (18 patients) or the CDDP+5-FU+INF-α arm (15 patients). Median survival was not different between the two groups (6.5 *versus* 5 months) and treatment related toxicity were in both arms considerable. In the CDDP/5-FU arm there were no partial responses, 50% of the patients had stable disease with a median time to progression of 5 months. Although there were two partial responses achieved in the CDDP/5-FU/INF-α

Table 2. Interferon based therapy in the palliative setting

Study	Year	Total no. patients	Treatment	IFN dosage	partial response(%)[b]	Grade III/IV toxicity (%)	Stable disease(%)	Median survival (months)
Padzur et al.[10]	1992	49	5-FU+IFN-α2a	3* 9 MIU/ week	4.0	NR	28.5	5.5
Scheithauer et al.[83]	1992	32	5-FU+LV+IFN-α2b	3* 10 MIU/ week	12.5	9.3[b]	40.5	5.5
Moore et al.[81]	1993	22	5-FU+LV+IFN-α2a	3* 3 MIU/ week	14.0	73[b]	18.0	NR
Bernhard et al.[75]	1995	57	5-FU+FA+IFN-α2a	1* 6 MIU/ week	14.0	28[b]	49.0	10.0
Moore et al.[80]	1995	6	13-cis RA+IFN-α2a	1* 6 MIU/ daily	0.0	16[b]	NR	NR
Sparano et al.[9]	1996	26	5-FU+IFN-α2b	3* 5 MIU/ m^2/week	8.0	NR	NR	4.6
Sporn et al.[82]	1997	18	5-FU+LV+CDDP +IFN-α2b	1* 5 MIU/ m^2/daily	37.5	NR	NR	5.0
John et al.[78]	1998	13	5-FU+IFN-α	3* 3 MIU/ week	1.0	NR	NR	8.0
Brembreck et al.[76]	1998	26	13-cis RA+IFN-α2a	1* 6 MIU/ week	4.5	13.6[c]	63.6	7.7
Recchia et al.[55]	1998	23	Epirubicin+ Mitomycin C+ racemic FA+ 5-FU+IFN-β+ retinol palmitate	3* 1 MIU/ week	26.0	NR	35.0	11.0
MacDonald et al.[79]	2000	55	Etoposide + 5 -FU+LV+IFN-α2b	3* 3 MIU/ week	9.0	32.7[d]	27.2	5.0
David et al.[77]	2000	23	5-FU+LV+IFN-α2a	5* 5 MIU/ week	0.0	NR	17.0	NR
Wagener et al.[8]	2002	15[a]	CDDP+5-FU+IFN-α2b	5* 3 MIU/ week	2.0	46.6	46.6	5.0

IFN, interferon; LV, leucovorin; FA, folinic acid; 13-cis RA, 13-cis retinoid acid; CDDP, cisplatin; NR, not reported;
[a] A total of 33 patients were randomly assigned to IFN-α based therapy or 5-FU+ cisplatin based chemotherapy. OS of patients in the 5-FU+cisplatin treatment arm (6.5 months) was not significantly different from patients in the IFN-α based treatment arm.
[b] Partial response was overall defined as 50% or greater reduction in the bidimensional product of the tumor mass for at least 4 weeks or one interval with no appearance of new disease.
[c] All grade III events

Table 3. Interferon based therapy in the adjuvant treatment setting

Study	Year	Total no. patients	Treatment	IFN dosage	Median survival (months)	2-year survival (%)	DFS (months)	grade III/IV toxicity (%)
Nukui et al.[4]	2000	17	EBRT +5-FU+CDDP+ IFN-α	3* 3 MIU/ every other day	>24.0[b]	84.0	NR	53.0
Picozzi et al.[6]	2003	43	EBRT +5-FU+CDDP+ IFN-α	3* 3 MIU/ every other day	44.0	64.0	16.0	70.0
Linehan et al.[85]	2008	22	EBRT +5-FU+CDDP+ IFN-α	3* 3 MIU/ week	25.0	56.0	25.0	68.0
Nitsche et al.[84]	2008	11	EBRT +5-FU+CDDP+ IFN-α	3* 3 MIU/ week	NR	55.0	NR	73.0[c]
Picozzi et al.[5]	2010	80	EBRT +5-FU+CDDP+ IFN-α2b	3* 3 MIU/ week	36.0	59.0	14.1	95.0
Katz et al.[3]	2011	28	EBRT +5-FU+CDDP+ IFN-α2b	3* 3 MIU/ week	42.0	79.0	28.9	89.0[c]
Schmidt et al.[7]	2012	53[a]	EBRT +5-FU+CDDP+ IFN-α2b	3* 3 MIU/ week	32.1	62.0	15.2	85.0

DFS, disease free survival; EBRT, external-beam radiotherapy; CDDP, cisplatin; IFN, interferon; FA, folinic acid; MIU, million international units; NR, not reported;

[a] A total of 110 patients were randomly assigned to IFN-α based chemoradiation or systemic 5-FU+FA based chemotherapy. OS of patients in the 5-FU treatment arm (28.5 months) was not significantly different from patients in the IFN-α based treatment arm.

[b] Median survival not calculated as 67% of the patients were still alive at time of report.

[c] All grade III events, no grade IV events observed

arm, only 46.6% of the patients had stable disease with a median time to progression of 3 months.

Only one phase II non-randomized clinical study explored the effect of IFN-β in the treatment of advanced pancreatic carcinoma[55]. In this study 23 patients received a treatment regimen of epirubicin, mitomycin C, racemic folinic acid, 5-FU, retinol palmitate and IFN-β. The median survival was 11 months and the progression free survival was 7.5 months. Treatment toxicity was mainly hematological (leukopenia, thrombocytopenia and anemia).

Complete response was achieved in 9% of the patients; a partial response was achieved in 26% of the patients and 35% of the patients showed stable disease on the treatment.

Unfortunately, only a few conclusions can be drawn from the above-discussed studies. The majority of the studies are single treatment arm studies in which only 2 studies showed a case of complete response on IFN therapy. In addition, in all studies treatment toxicities were considerable. In the only randomized clinical study partial responses were observed, and treatment toxicities were similar among the two treatment arms, unfortunately this did not translate into a (progression free) survival benefit for patients in the experimental arm of the study. Based on these findings, until more advanced and better-designed studies are developed, IFN-α therapy in the palliative setting is not preferred.

6.2 Adjuvant interferon-α therapy

In the adjuvant treatment setting of pancreatic cancer, from 2000 until 2012, 6 phase II IFN-α based clinical studies[10-13,84,85] and one phase III randomized clinical trial[14], have been conducted (Table 3). In all of the studies the added value of concomitant IFN-α to external beam radiation therapy (EBRT), 5-FU and cisplatin was explored. In total, the outcome of interest was overall survival, toxicity and in some studies quality of life. The study of Katz et al[10]. reported the highest median survival of 42 (32.1-64.5) months with a 2-year and 5-year survival of 79% and 35%, respectively. Picozzi et al[13] reported a 5-year survival of 55% of patients receiving adjuvant IFN-α therapy. Additionally, high overall survival rates were described by the other studies as well. Nevertheless, grade III and IV treatment toxicities, mostly from gastrointestinal and hematological origin, were in all studies considerable and ranged from 53% in the study of Nukui et al[11] up to 95% in the study of Picozzi et al[12]. In the study of Nitsche et al[85]. and Katz et al[10]. no grade IV toxicity was observed.

The only randomized clinical trial was performed by Schmidt et al[14]. at the university of Heidelberg in which a total of 110 patients were randomized to either the experimental arm of the study (53 patients) receiving 5-FU, cisplatin, IFN-α and radiotherapy, or to the 5-FU monotherapy arm (57 patients). The total follow up duration was 45.9 months. The median survival for patients randomized to the experimental IFN-α arm was 32.1 months (95% CI 22.8 to 42.2 months) and for patients in the monotherapy arm 28.5 months (95% CI, 19.5 to 38.6 months) which was not statistically significant different. Although the survival of patients receiving IFN-α did not improve significantly compared with 5-FU monotherapy, the difference in median survival of 3.6 months implies that some patients probably benefited from the experimental treatment. Furthermore, grade III and IV toxicity was experienced in

85% and 16% of the patients in the experimental arm and in the monotherapy arm, respectively, although the difference cannot only be attributed to the addition of IFN-α.

Despite the strong evidence that IFN-β has more potent anticancer features compared to IFN-α, no clinical studies have been conducted regarding adjuvant IFN-β therapy in pancreatic cancer.

7. AIMS AND OUTLINES OF THIS THESIS

As indicated in the previous paragraphs, in addition to the antiviral and immunoregulatory activities, type I interferons are also able to inhibit cell proliferation, induce apoptosis, block cell cycle and sensitize tumor cells for chemo- and radiotherapy. In pancreatic cancer, several studies, including some clinical studies, demonstrated promising, although variable, results of (adjuvant) interferon (predominantly IFN-α) therapy. However, several important aspects of type I interferon therapy, like the importance of receptor expression, the influence of growth factors and the use of IFN-β, remain underexposed. Therefore, the main aim of this thesis is to further elucidate the role of type I interferon therapy in the treatment of pancreatic cancer.

The first aim was to evaluate the relationship between type I interferon receptor expression (IFNAR-1 and IFNAR-2c) and the antitumor effect of IFN-α and –β *in vitro* **(chapter 2)**. The second aim of this thesis was to determine the influence of insulin and insulin-like growth factors on the proliferation and migration of human pancreatic cancer cells and to investigate to what extent IFN-β is capable of inhibiting the basal- and growth factor stimulated cell proliferation and migration **(chapter 3)**. The third aim of this thesis was to investigate the role of IFN-β therapy alone and in combination with gemcitabine *in vivo* **(chapter 4).** The fourth aim of this thesis was to determine the expression of the IFNAR-1 and IFNAR-2c receptor in human pancreatic cancer specimens and to investigate whether interferon receptor expression is associated with clinicopathological factors and survival **(chapter 5)**. Finally, the experimental findings of this thesis and some future perspectives will be discussed **(chapter 6)**.

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2

Influence of type I Interferon receptor expression level on the response to type I Interferons in human pancreatic cancer cells

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ABSTRACT

Background: Pancreatic cancer is a highly aggressive malignancy with limited treatment options. Type I Interferons (e.g. IFN-α/-β) have several anti-tumor activities. Over the past few years clinical studies evaluating the effect of adjuvant IFN-α therapy in pancreatic cancer yielded equivocal results. Although IFN-α and -β act via the type I IFN receptor, the role of the number of receptors present on tumor cells is still unknown. Therefore, this study associated, for the first time, in a large panel of pancreatic cancer cell lines the effects of IFN-α/-β with the expression of type I IFN receptors.

Methods: The anti-tumor effects of IFN-α or IFN-β on cell proliferation and apoptosis were evaluated in 11 human pancreatic cell lines. Type I IFN receptor expression was determined on both the mRNA and protein level.

Results: After 7 days of incubation, IFN-α significantly reduced cell growth in 8 cell lines by 5-67%. IFN-β inhibited cell growth statistically significant in all cell lines by 43-100%. After 3 days of treatment, IFN-β induced significantly more apoptosis than IFN-α. The cell lines variably expressed the type I IFN receptor. The maximal inhibitory effect of IFN-α was positively correlated with the IFNAR-1 mRNA ($p<0.05$, $r=0.63$), IFNAR-2c mRNA ($p<0.05$, $r=0.69$) and protein expression ($p<0.05$, $r=0.65$) expression.

Conclusion: Human pancreatic cancer cell lines variably respond to IFN-α and -β. The expression level of the type I IFN receptor is of predictive value for the direct anti-tumor effects of IFN-α treatment. More importantly, IFN-β induces anti-tumor effects already at much lower concentrations, is less dependent on interferon receptor expression and seems therefore more promising than IFN-α.

1. INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in the western world[1]. Surgery is the only curative therapy but due to early metastases and local invasion only 15-20% of the patients are eligible for resection at time of presentation. After resection prognosis remains poor resulting in an overall 5-year survival for patients diagnosed with pancreatic cancer of less than 5%. Several larger clinical studies have suggested the benefit of adjuvant therapy; however no definite consensus about the optimal treatment regime has been established [2-5]. To further improve survival, research has focussed on new and other medical treatment options, like adding biological modulators as interferon[6-8].

Interferons (IFNs) are known to have antiproliferative, antiviral and immunoregulatory activities. Type I IFNs (e.g. IFN-α, IFN-β and IFN-ω) are also involved in cell differentiation and anti-tumor defence processes, and besides that they are also able to sensitize tumor cells for chemo- and radiotherapy [9-11]. Type-I IFNs act via the type-I IFN receptor complex which is composed by two subunits; IFNAR-1 and IFNAR-2, of which there are three isoforms that are differently spliced from a common gene. IFNAR-2a is the soluble form and can act as a dominant negative regulator of free IFNs, IFNAR-2b is a shorter form lacking regions of the cytoplasmic domain and unable to activate JAK-STAT signaling once the receptor binds IFNs. IFNAR-2c contains the entire cytoplasmic domain and along with IFNAR-1 makes up the functional IFN receptor complex, capable of binding IFNs and inducing JAK-STAT signaling [12,13]. Currently, IFN-α is used in the treatment of several malignancies like chronic myeloid leukaemia, metastatic melanoma, renal cell carcinoma and Kaposi sarcoma [14,15]. IFN-β is only used in the treatment of multiple sclerosis [16].

In experimental models *in vitro* and *in vivo*, the anti-tumor effect of IFN-α has been demonstrated [11,17-21] and in the past years a number of clinical studies have been conducted regarding adjuvant IFN-α therapy. The study of Picozzi *et al.* [7] reported an actuarial 5-year survival of 55%, but regrettably none of the other studies achieved an overall survival that high and also treatment toxicities were very high [6,8].

Surprisingly, very few studies investigated the effect of IFN-β, even though some *in vitro* studies showed that IFN-β binds the receptor complex with a higher affinity and has greater anti-tumor effects than IFN-α [11,17,18,22]. Despite the fact that the approximate amount of receptors may determine the effect [10], the relationship of type I IFNs receptor expression with the anti-tumor effect of IFN-α/β in pancreatic cancer cell lines is not established. Therefore, in the present study we evaluated the anti-tumor activity of IFN-α and IFN-β in

11 human pancreatic adenocarcinoma cell lines and assessed the correlation between the responsiveness to type I IFNs and the expression of IFNAR-1 and IFNAR-2c receptors.

2. MATERIALS AND METHODS

2.1 Cell lines and culture conditions

The human pancreatic cell lines AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAF-II, Hs 700T, Hs 766T, MIA PaCa 2, PANC-1 and SU.86.86 were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were allelotyped and the DNA (STR) profile corresponded with the profile of the ATCC. The cells were cultured in a humidified incubator at 5% CO_2 and 37°C. The culture medium consisted of RPMI 1640 supplemented with 5% FCS, penicillin ($1x10^5$ U/L) and L-glutamine (2 mmol/l). Capan-1, Capan-2 and SU.86.86 were cultured in medium consisting of RPMI 1640, supplemented with 10% FCS, penicillin ($1x10^5$ U/L) and L-glutamine (2 mmol/l). Periodically, cells were confirmed as Mycoplasm-free. Cells were harvested with trypsine (0.05%) EDTA (0.53 mM), counted microscopically using a standard haemocytometer, resuspended in medium and plated in 24-well multiwell plates. Trypan blue staining was used to asses cell viability. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

2.2 Drugs and Reagents

Human recombinant IFN- α-2b (Intron-A) was obtained from Schering-Plough Corporation (Utrecht, The Netherlands), while human recombinant IFN- β-1a was acquired from Serono Inc. (Rebif, Rockland, MA). All compounds were stored at -20°C and stock solutions were constituted in distilled water according to the manufacturer's instruction.

2.3 Cell proliferation assay

For each cell line the optimal cell number plating density was determined (data not shown). After trypsinization, the cells were plated in 1 ml of medium in 24 well plates at the correct cell density. The plates were placed in a 37°C, 5% CO_2 incubator and cells were allowed to attach overnight. The next day increasing concentrations (0-1000 IU/ml) of IFN-α or IFN-β were added. Each treatment was performed in quadruplicate. After 3 and 7 days of treatment, the cells were harvested for DNA measurement. For the 7-day experiments, the medium was refreshed after 3 days and compounds were added again. Measurement of

total DNA contents was performed using the bisbenzimide fluorescent dye (Hoechst 33258) (Boehring Diagnostics, La Jolla, CA) as previously described [23].

2.4 Quantitative RT-PCR

For the detection of interferon receptors (IFNAR-1, IFNAR-2 total, IFNAR-2b and IFNAR-2c) and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT), mRNA expression was evaluated by quantitative RT-PCR in all 11 pancreatic adenocarcinoma cell lines.

The isolation of total RNA (tRNA), complementary DNA (cDNA) synthesis and the primer and probe sequences that were used for the detection of IFNAR-1, IFNAR-2 total, IFNAR-2b, IFNAR-2c and HPRT have been described previously. The soluble form of IFNAR-2a subunit was calculated indirectly by subtracting IFNAR-2b and IFNAR-2c from IFNAR-2 total. [24]. All the primer and probe sequences were purchased from Biosource (Nivelles, Belgium). The primer set that was used to detect an interferon response (interferon stimulated gene 56; ISG56) was purchased from Applied Biosystems (Hs00356631).

Dilution curves were constructed for calculating the PCR efficiency for every primer set and have been described by Koetsveld *et al.* [24]. After efficiency correction of target and reference gene transcripts (HPRT), the comparative threshold method, $2^{-\Delta Ct}$ was used to calculate the relative expression of genes [25]. Interferon treatment did not change HPRT mRNA expression. As a positive control for the PCR reactions of HPRT and type I IFN receptors human cDNA of human carcinoid tumor cells was amplified in parallel with the cDNA samples[26].

2.5 Protein extraction

After trypsinization the cells were plated in 2 ml of medium in 6 well plates and placed in a 37ºC, 5% CO_2 incubator and allow to grow until a confluence of 90% was observed.

Cells were washed with ice-cold PBS. Whole-cell lysates were prepared by adding 200 µl ice-cold RIPA lysis buffer (Pierce Biotechnology, Inc., Rockford, IL, USA.) with the addition of 1% Halt Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Inc.) to each well and incubated for 1 minute on ice. Cell lysates were transferred to labeled tubes and incubated for 15 minutes on ice (mixing every 5 minutes) and spun down at 18 000x*g* at 4°C. Supernatants were stored at -80°C.

With the dye-binding assay (Bio-Rad Protein Assay) the total amount of protein was calculated. BSA was used as standard curve and a spectrophotometer set to 595 nm as reader.

2.6 Western blotting

Total protein solution (50µg) diluted in a water solution containing 25% SDS sample buffer was denatured (2-3 minutes in a bath at 95°C) and separated by electrophoresis on 10% SDS-page gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membranes were blocked in 0.1% Tween 20-PBS/ 3% non-fat dry milk for 1h and incubated overnight at 4°C with the primary antibody (human IFNAR1 (mouse monoclonal antibody; Sigma-Aldrich; concentration 1:1000) and human IFNAR2c (monoclonal antibody 27D11, kindly provided by Dr. E. Croze, International Review of Investigational Science, San Francisco Bay Area, USA; concentration 1:700)). After 3 times 5 minutes washing in 0.1% Tween 20-PBS the membranes were incubated, for 1h at room temperature, with the secondary antibody (Alexa Fluor® goat anti-mouse IgG, Invitrogen; concentration 1:15000). Starting from the incubation with the secondary antibody membranes were kept in dark condition. After the incubation with the secondary antibody, membranes were washed twice 5 minutes with 0.1% Tween 20-PBS and finally once 5 minutes with only PBS.

Using the odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK) immunodetection was performed. The optical density of the sized bands was measured using the Odyssey molecular imaging software (LI-COR Biosciences). Relative expression of total IFNAR-1 or IFNAR-2c was calculated as a ratio to the expression of *beta*-actin.

2.7 DNA fragmentation (Apoptosis)

Cells were plated, according to the optimal plating density of each cell line, in 1ml of medium in a 24-well plate and allowed to attach overnight. The next day, medium was replaced with 1ml fresh medium containing 1000 IU/ml IFN-α or IFN-β. Each treatment was performed in quadruplicate. After 3 days of treatment, apoptosis was measured using a commercially available ELISA kit (Cell death detection ELISA^{plus}, Roche Diagnostics GmbH, Penzburg, Germany) according to manufacturer's instructions. Apoptosis was expressed as percentage of control (untreated) cells, and data was corrected for the total DNA content in each well.

2.8 Staining of apoptotic cells

Cells were plated and treated in the same manner and order as in the DNA fragmentation section, described above. After 3 days of treatment cells were washed twice with PBS and fixed for 10 minutes with methanol/acetic acid (3:1). After fixation, cells were washed twice with distilled water and incubated for 8 minutes with 5µg/ml Hoechst 33258 (Sigma-

Aldrich, Zwijndrecht, the Netherlands). After the incubation cells are washed again twice with distilled water and apoptotic cells were evaluated under fluorescence microscope (Axiovert 200M, HXP 120 external lamp). Cells with condensed or fragmented chromatin were considered apoptotic [27].

2.9 Statistical analysis

All experiments were carried out at least twice, with exception of the western blot, and gave comparable results. For statistical analysis GraphPad Prism 5.0 (GraphPad software, San Diego, CA) was used. Concentrations that induced 50% growth inhibition (IC_{50}) and maximal inhibitory effects were calculated using non-linear regression curve fitting program. The comparative statistical evaluation among groups was performed by a one-way ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The unpaired student t-test was used to analyze differences in concentration-effect curves (IC_{50} and maximal inhibitory effect (Emax)). After log-log transformation the results were normally distributed and correlation analyses were performed using Pearson's coefficients. In all analyses, values of $P<0.05$ were considered statistically significant. Data are reported as mean ±SEM.

3. RESULTS

3.1 Antiproliferative effect of type I IFNs

After 7 days of incubation, IFN-α significantly suppressed the growth in 8 of the 11 cell lines, IFN-β significantly suppressed the growth of all the cell lines (table 1). The effects of both type I IFNs were time- and dose- dependent. The maximal inhibition of cell proliferation induced by both compounds was significantly higher after 7 days, compared to 3 days of incubation (data not shown). Furthermore, the overall growth inhibitory effect of IFN-β after 7 days was significantly more potent than the growth inhibitory effect of IFN-α ($P<0.0001$). Figure 1 illustrates 3 cell lines, AsPC-1, Capan-1 and PANC-1, which represent the spectrum of effects of IFN-α/-β treatment in the 11 cell lines. In addition, the maximal inhibitory effect of IFN-α is correlated with the maximal inhibitory effect of IFN-β ($p<0.05$ $r=0.61$).

3.2 Effect on apoptosis of type I IFNs

To evaluate the effect of type I IFNs on apoptosis, we first measured the percentage of DNA fragmentation after 2, 4, 8, 24, 48 and 72u of incubation with 1000 IU/ml IFN-α or 1000 IU/

Table 1. IC_{50} and maximal inhibitory effect (Emax) of 11 pancreatic adenocarcinoma cell lines after 7 days of incubation with IFN-α and IFN-β.

	Alpha		Beta	
Cell lines	IC_{50} (IU/ml)	Emax (%)	IC_{50} (IU/ml)	Emax (%)
AsPC-1	> 1000	38 ± 3.3 ***	272	68 ± 1.7 ***
BxPC-3	> 1000	20 ± 4.2 *	114	89 ± 2.3 ***
Capan-1	378	67 ± 3.6 ***	70	96 ± 1.2 ***
Capan-2	> 1000	10 ± 3.6	>1000	43 ± 2.9 ***
CFPAC-1	841	54 ± 2.6 ***	131	100 ± 1.5 ***
HPAF-II	> 1000	21 ± 4.3 ***	192	94 ± 2.0 ***
HS 700T	> 1000	5 ± 4.2	272	62 ± 1.8 ***
Hs 766T	> 1000	17 ± 3.2 **	169	76 ± 1.7 ***
MIA PaCa2	> 1000	14 ± 3.6 *	422	75 ± 1.4 ***
PANC-1	> 1000	11 ± 2.9	710	54 ± 5.3 ***
SU.86.86.	> 1000	17 ± 2.3 ***	>1000	44 ± 2.4 ***
	378 - >1000†	25 ± 3.4	70 - >1000†	73 ± 2.2

* p<0.05, ** p<0.01 and ***p<0.001 versus control.
The IC_{50} is expressed as the concentration needed for 50% of cell growth reduction; concentrations that exceeded 1000 IU/ml were mentioned as > 1000. Mean IC_{50} of IFN-α and –β is expressed as the range†.
The maximal inhibitory effect is expressed as the percentage inhibition compared with the untreated control ± SEM.

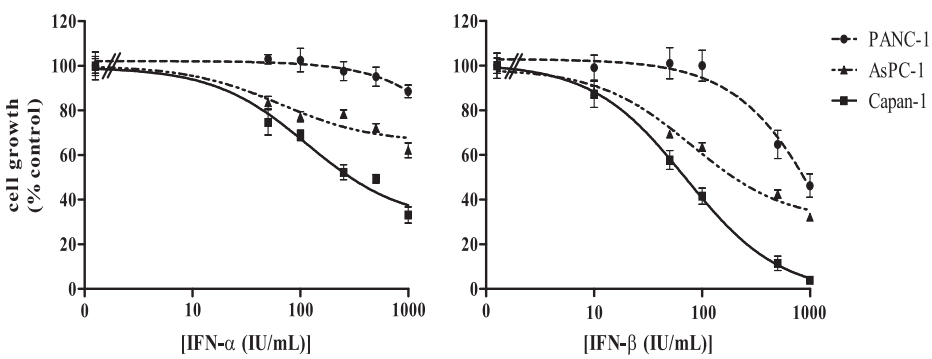

Figure 1. Treatment effects on cell proliferation after 7 days of incubation with increasing concentrations of IFN-α and IFN-β in 3 human pancreatic cancer cell lines. Values are expressed as the percentage of control and represent the mean ± SEM of at least 2 independent experiments in quadruplicate

ml IFN-β in the relatively sensitive cell line BxPC-3 (data not shown). After 8h of incubation the effect of IFN-β on apoptosis became apparent. Starting from 24h the effect of IFN-α and IFN-β on DNA fragmentation was already significant and continued to increase significantly up to 72h of interferon incubation ($p<0.05$). Since BxPC-3 is a relatively sensitive cell line and because the effect of IFN-α is less pronounced than the effect of IFN-β, we decided to measure the amount of induction of DNA fragmentation of the remaining 11 cell lines only after 72h (Figure 2a). In total, of the 11 cell lines, 4 cell lines showed, compared to the untreated control, a significant increase of DNA fragmentation after 1000 IU/ml of IFN-α treatment (CFPAC-1 and HS 700T $p<0.05$; BxPC-3 and HPAF-II $p<0.01$). After 3 days of IFN-β treatment (1000 IU/ml), 8 of the 11 cell lines showed a significant increase in DNA fragmentation ($p<0.001$; only HS 700T $p<0.01$). Only Capan-2, Panc-1 and SU.86.86 did not show a significant increase in DNA fragmentation after 3 days of IFN-α nor IFN-β treatment. In addition, the maximal effect of IFN-α on DNA fragmentation was significantly correlated with the maximal effect on DNA fragmentation of IFN-β ($p<0.001$, $r=0.87$). Moreover, the

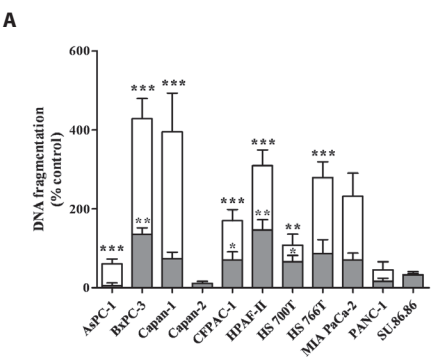

Figure 2A: Effects of IFN-α (greys bars) and IFN-β (white bars) treatment on apoptosis (DNA fragmentation) in 11 human pancreatic cancer cell lines. The cells were incubated for 3 days with 1000 IU/ml of IFN-α or IFN-β. Values are absorbance units and are expressed as the percentage of change in DNA fragmentation compared to control. Data are the mean ± SEM of at least 2 independent experiments in quadruplicate. * $p<0.05$, ** $p<0.01$ and ***$p<0.001$ versus control.

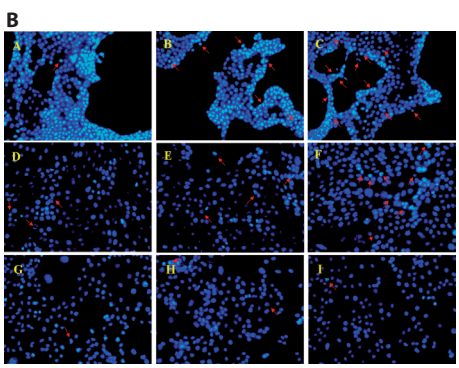

Figure 2B: Visualisation of apoptosis (nuclear condensation, fragmentation and forming of apoptotic bodies) with Hoechst 33258 after 3 days without treatment (A,D,G), IFN-α (B, E, H) or IFN-β (C, F, I) treatment in Capan-1 (A-C), CFPAC-1(D-F) and PANC-1 (G-I).The red arrows indicate condensated or fragmented nuclei or apoptotic bodies. Original magnification x200.

maximal inhibitory effect of IFN-β on proliferation was significantly correlated with the maximal effect of IFN-β on DNA fragmentation ($p<0.05$, $r=0.65$).

Besides the quantitative measurement of DNA fragmentation we also visualized, in all 11 cell lines, nuclear condensation, fragmentation and apoptotic bodies with the Hoechst 33258 staining. Figure 2b shows the Hoechst staining of Capan-1 a cell line with much DNA fragmentation, the PANC-1 a cell line with very little DNA fragmentation as well as CFPAC-1, a cell line with an intermediate increase of DNA fragmentation, after IFN-α/-β treatment. The Hoechst 33258 staining of the three cell lines that is shown was in accordance with the DNA fragmentation measurement, this also applied to the remaining 8 cell lines (data not shown).

3.3 Expression of type I IFN receptor mRNA

We analyzed the receptor mRNA expression of type I IFNAR by quantitative RT-PCR in the 11 pancreatic adenocarcinoma cell lines. As shown in Figure 3a, all cell lines expressed IFNAR-1 and IFNAR-2, but with great variability. IFNAR-1 expression was considerably higher (on the average 3.6-fold) than IFNAR-2 total (sum of IFNAR2a, IFNAR2b and IFNAR2c isoforms) expression. The expression of the IFNAR-2 total was significantly correlated with both the expression of the IFNAR-2b ($p<0.0001$, $r=0.92$) and the IFNAR-2c ($p<0.005$, $r=0.78$). Furthermore, the expression of the IFNAR-1 mRNA is significantly correlated with the expression of the IFNAR-2c ($p<0.05$, $r=0.66$). Doubling time varied between cell lines (between 28.81 and 48.26 hours). No significant correlations were found between doubling time and IFNAR-1 or IFNAR-2 expression (data not shown).

3.4 Western blotting

Since receptor mRNA expression does not necessarily correlate with receptor expression at the protein level, we also characterized the receptor expression at protein level by western blotting. The IFNAR-1 and IFNAR-2c are both expressed at an anticipated[28,29] band of ~100 kDA (Figure 3b right panel; arrowhead)although with great variability in the level of protein expression (Figure 3b, left panel). The expression of the IFNAR-1 was in all of the cell lines on average 17-fold higher than the expression of the IFNAR-2c. The mRNA expression of the IFNAR-1 did not correlate with the expression of the IFNAR-1 at the protein level. On the other hand, the receptor expression of the IFNAR-2c subunit at mRNA level showed a significant positive correlation with the receptor expression of the IFNAR-2c at protein level ($p<0.05$ $r= 0.69$).

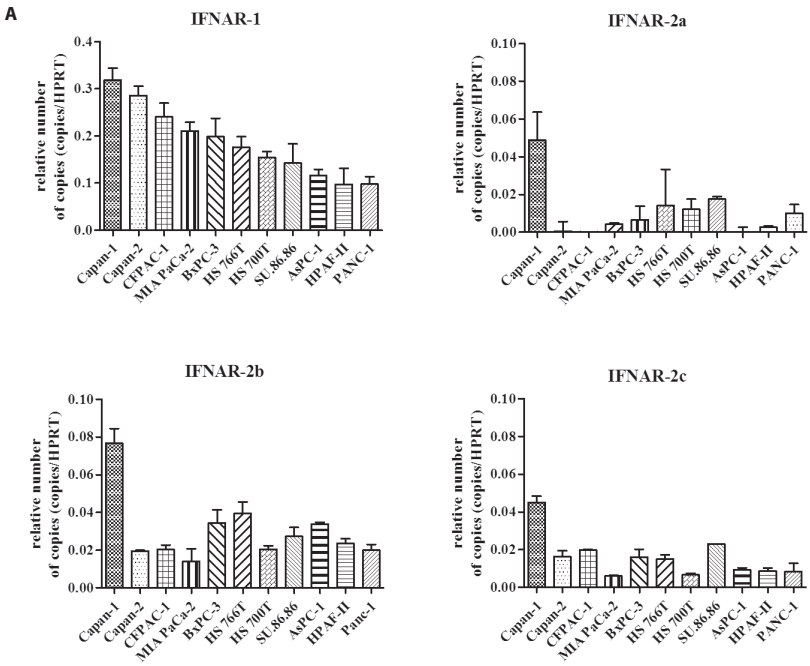

Figure 3A: Relative expression of IFNAR-1, IFNAR-2a, IFNAR-2b and IFNAR-2c mRNA in 11 human pancreatic adenocarcinoma cell lines, normalized to HPRT mRNA. Values represent mean ± SEM;

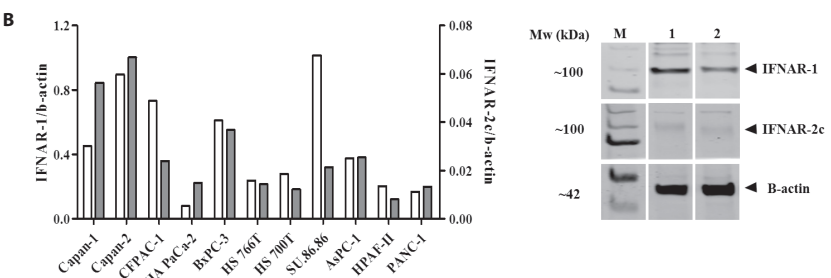

Figure 3B: Right panel: Western blot analysis of high and low IFNAR-1 and IFNAR-2c protein expression in 2 human pancreatic cancer cell lines Both IFNAR-1 and IFNAR-2c are expressed at approximately 100 kDa, previously reported to be the main Mw of both IFNAR receptors. b-actin is expressed at 42 kDa. (M=marker, 1.Capan-1, 2. MIA PaCa-2) Left Panel; IFNAR-1 (left axis, white bars) and IFNAR-2c (right axis, grey bars) protein band density relative to b-actin band density in 11 human pancreatic adenocarcinoma cell lines.

3.5 Correlation of the receptor expression with the anti-proliferative effect of type I IFNs

Amongst the panel of human pancreatic cancer cell lines we found a considerable variation in their sensitivity to type I IFNs. Considering the fact that there was also a lot of variation in the expression of the receptor complex of IFNAR-1 and IFNAR-2c, which is responsible for initiating signal transduction, we determined the relationship between the maximal inhibitory effects of IFN-α and IFN-β and the IFNAR-1 and IFNAR-2c mRNA expression levels. Overall, only a significant correlation was found between the maximal inhibitory effect of IFN-α and the expression of IFNAR-2c ($p<0.05$, $r=0.63$).

However, after evaluation of the individual cell lines, there was one cell line that responded completely different to type I IFNs compared to the other cell lines. For this cell line, e.g. Capan-2, it is remarkable that there is a poor response to IFN-α and IFN-β, despite the fact that this cell line does express a significant number of IFNAR-1 and IFNAR-2c receptors to initiate an effect. This could be due to a defect in post receptor signal transduction, which was illustrated by the fact that there was only a very low up-regulation of ISG56 after IFN-α (4.47 ± 0.45 fold compared to control) and IFN-β (7.00 ± 0.50 fold compared to control) treatment. This was not in line with the amount of up-regulation of ISG56 in the BxPC-3 cell line after IFN-α (41.24 ± 12.1 fold) or IFN-β treatment (99.47 ± 5.61 fold), a cell line with nearly the same amount of IFNAR expressed.

For this reason we excluded Capan-2 from further correlation analysis. Figure 4 and 5 show the correlations between the growth inhibitory effect of IFN-α and IFN-β and IFNAR mRNA and protein expression, respectively, in the remaining 10 cell lines. There was a significant correlation between the IFNAR-1 mRNA expression and the response to IFN-α ($p<0.05$, $r=0.63$) (Figure 4, left upper panel). This correlation was also found regarding IFNAR-2c mRNA expression and the response to IFN-α ($p<0.05$, $r=0.69$) (Figure 4, right upper panel).

Furthermore, there was a significant correlation between the IFNAR-2c mRNA expression and the IFNAR-2c protein expression ($p<0.01$, $r=0.77$). The IFNAR-2c protein expression also correlated significantly with the response to IFN-α ($p<0.05$, $r=0.65$; Figure 5, right upper panel). IFNAR-2a and IFNAR-2b expression did not correlate with the effects of IFN- α or IFN-β treatment.

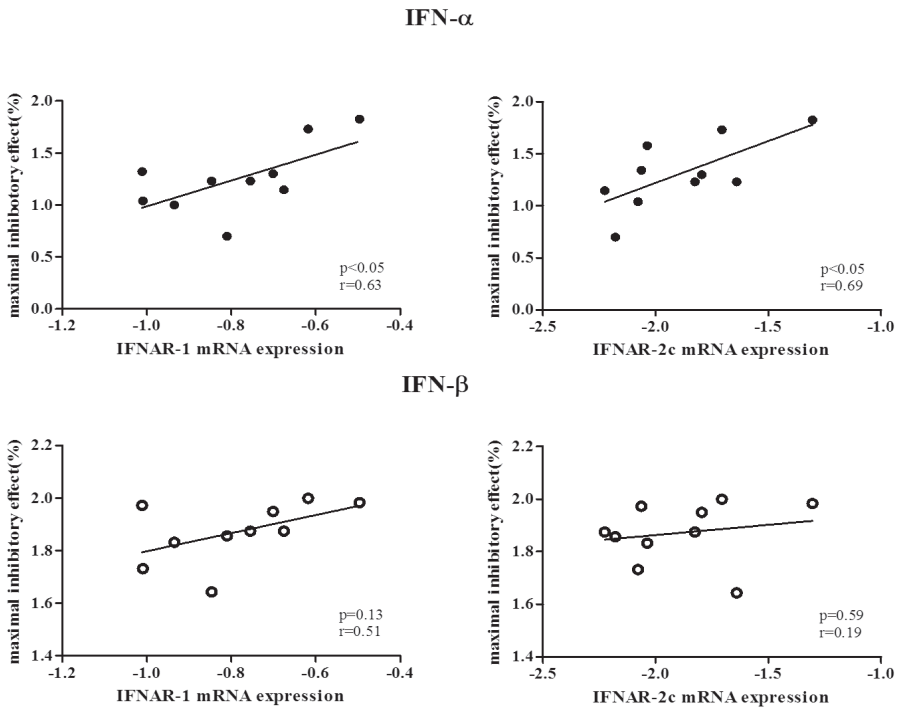

Figure 4: Correlation of the maximal inhibitory effect of IFN-α (1000 IU/mL) and IFN-β (1000 IU/mL) with the expression level of IFNAR-1 and IFNAR-2c mRNA in 10 human pancreatic adenocarcinoma cell lines (log-log transformed scale). The maximal inhibitory effect is expressed as percentage inhibition compared with untreated control. There was a significant correlation between the expression of IFNAR-1 mRNA and the maximal inhibitory effect of IFN-α ($p<0.05$, $r= 0.63$). A significant correlation was found as well between the expression of the IFNAR-2c and the maximal inhibitory effect of IFN-α ($p<0.05$, $r=0.69$). However there was no significant correlation regarding the maximal inhibitory effect of IFN-β and the expression of the IFNAR-1 or IFNAR-2c mRNA.

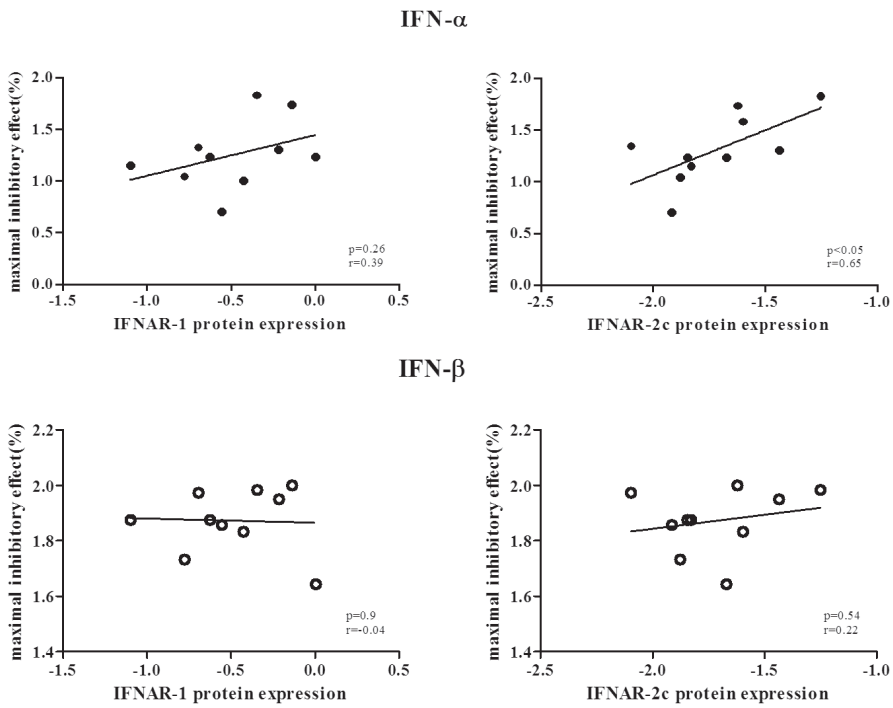

Figure 5: Correlation of the maximal inhibitory effect of IFN-α (1000 IU/ml) and IFN-β (1000 IU/ml) with the expression of IFNAR-1 and IFNAR-2c at protein level in 10 human pancreatic adenocarcinoma cell lines (log-log transformed scale). The maximal inhibitory effect is expressed as percentage inhibition compared with untreated control. There was a significant correlation between the expression of IFNAR-2c protein and the maximal inhibitory effect of IFN-α ($p<0.05$, $r= 0.65$), this correlation was not found for IFN-β. No correlation was found between the IFNAR-1 mRNA expression and the maximal inhibitory effect of IFN-α or IFN-β.

4. DISCUSSION

Pancreatic cancer is a highly aggressive malignancy, with very limited treatment outcome. The effect of adjuvant treatment modalities like chemo- and radiotherapy are still marginal. To improve survival in patients with pancreatic cancer additional treatment options are clearly required[2,3]. Several years ago a number of clinical studies have been conducted regarding adjuvant IFN-α therapy in the treatment of pancreatic cancer. Some studies reported a remarkable increase in the 2- and 5-year survival[6,7,30]. On the other hand, the only randomized clinical trial did not show a significant increase in overall survival, although the

increased median survival implicated that some patients in the experimental arm benefited from adjuvant IFN-α therapy[31]. Despite that some *in vivo* and *in vitro* studies have investigated the role of type I IFNs, in particular IFN-α in pancreatic cancer, the importance of the IFN receptor expression in relation with the effect of type I IFNs is not clarified. Previous research of Vitale *et al.* [17] and Saidi *et al*. [20,32,33] established a trend towards the importance of the IFN receptor in the response of IFN-α and -β, however, the number of cell lines used in these studies were very low. Furthermore, despite the fact that some studies did show promising results [11,17,18,22], the effect of IFN-β in the treatment of pancreatic cancer still remains underexposed. Therefore, in the present study we evaluated the type I IFN receptor expression in a large panel of available human pancreatic cancer cell lines and associated the receptor expression with the anti-tumor potencies of IFN-α and IFN-β.

In the panel of 11 human pancreatic adenocarcinoma cell lines there was a considerable variability in the response to the type I IFNs. Overall, IFN-β is a significantly more potent inhibitor of cell proliferation compared with IFN-α. IFN-β inhibited cell proliferation already at very low concentrations (10-50 IU/ml) in the majority of the cell lines, which is in agreement with the study of Vitale *et al.* [17]. and Jost *et al.* [18]. The maximal inhibitory effect of IFN-α significantly correlated with the maximal inhibitory effect of IFN-β. Consequently, cell lines that achieve a higher maximal inhibitory effect with IFN-α will also achieve a higher maximal inhibitory effect with IFN-β.

Type I IFNs are also known to induce apoptosis which can act via the intrinsic mitochondria mediated pathway or the extrinsic death receptor induced pathway, both resulting in nuclear condensation and fragmentation, followed by fragmentation of the cell into apoptotic bodies. Both type I IFNs were able to induce apoptosis, however, the increase of DNA fragmentation after IFN-β therapy was considerably more potent compared to the increase in DNA fragmentation after IFN-α treatment. The increase in DNA fragmentation was also visualised by Hoechst 33258 and was consistent with the quantitative DNA fragmentation measurements.

By quantitative RT-PCR and western blotting we demonstrated that all pancreatic cell lines expressed the IFNAR-1 and IFNAR-2c receptors, although receptor expression levels varied considerably. The maximal inhibitory effect of IFN-α was positively correlated with the expression of IFNAR-2c. Remarkably, one cell line, Capan-2, did express a significant amount of the type I IFN receptor but showed only a marginal response to IFN-α and-β. Type I IFNs act via several signalling pathways [34]. Like in many different types of cancers, there can be a defect in the interferon post receptor signalling pathway, resulting in the absence

of an up-regulation of interferon stimulated genes. For this reason, we measured the up-regulation of ISG56 after IFN-α and IFN-β treatment in the Capan-2 cell line. Compared to BxPC-3, Capan-2 showed only very low ISG56 up-regulation. Therefore, a defect in the post receptor signalling pathway in Capan-2 is likely. The development of pancreatic cancer often occurs through the accumulation of genetic mutations, including frequent mutations in the KRAS (Kirsten rat sarcoma viral oncogene homolog), p53, CDKN2A (p16) and SMAD4 (DPC4)[35]. The presence or absence of genetic mutations in these cell lines could determine the amount of interferon receptors expressed and/or influence the effect of IFN-α and-β. The cell lines included in our study displayed a wide variability in genetic alterations. There was no difference between wild type and mutated cell lines in their response to IFN-α and-β treatment, nor in the number of type I IFNARs. Nevertheless, it is notable thatCapan-2 is the only wild type p53 cell line in this panel of pancreatic cancer cell lines. Literature data shows that type I IFNs can up-regulate p53 which can initiate apoptotic pathways [36]. The fact that we did observe an up-regulation of apoptosis in the mutated p53 cell lines, while very little induction of apoptosis in the Capan-2 wild type p53 cell line strengthens the concept that the Capan-2 cell line has an impaired post receptor interferon signalling pathway. For this reason, the Capan-2 cell line was excluded from the further analysis. In the remaining 10 cell lines there was a significant positive correlation between the IFNAR-1 mRNA, but not protein, expression and the maximal inhibitory response to IFN-α. In addition, IFNAR-2c mRNA and protein expression showed a positive correlation with the maximal inhibitory effect of IFN-α. Our findings in human pancreatic cancer cells endogenously expressing the IFNAR-2c add to the observations by Wagner *et al.*[10], who showed in melanoma, breast cancer and lung fibrosarcoma cell models that transfection and overexpression of IFNAR-2c enhances their sensitivity for type I IFNs *in vitro* and *in vivo*. Based on these findings it is concluded that the number of IFNAR-2c receptors can be of predictive value in determining responsiveness of human pancreatic cancer cells to IFN-α therapy. However, one should realize that in selected pancreatic cancers, reflecting our observations in Capan-2 cells, defects in IFNAR signalling can occur, rendering such cancers insensitive to IFN treatment.

Although IFN-α and –β act via the same receptor complex, both cytokines display functional differences. In addition to the fact that IFN-α and –β share only 35% of their sequence identity and that IFN-β, unlike IFN-α is a glycosylated protein, a study by Johns *et al*. demonstrated that IFN-β has a 10-fold higher binding affinity with the receptor complex compared to IFN-α[37] Moreover, IFN-β induced an up-regulation of 338 genes in human fibrosarcoma cells, whereas IFN-α induces an up-regulation of only 130 genes[38]. A different interaction

with the type I IFN receptor complex between IFN-α and –β can be an explanation for the more potent activity of IFN-β as well. Recently, an elegant study by de Weerd *et al*. using IFNAR-1 and IFNAR-2 knockout mice, demonstrated that IFN-β can induce functional signal transduction, via the IFNAR-1, independently of the IFNAR-2c receptor[39]. In addition, it was found that 104 unique genes were induced by this IFNAR1-IFN-β signalling axis. These observations may form an explanation for the lack of correlation between the IFNAR-1 and/or IFNAR-2c receptors and the maximal inhibitory effect of IFN-β, as we found in our study, but also for the much higher potency of the antitumor effects of IFN-β, compared to IFN-α.

Although hypothetical, several potential pathways may be involved in the above indicated differential direct anti-tumor activities of IFN-α and IFN-ß. First, IFN-β may be more potent than IFN-α in stimulating the protein kinase dependent on double stranded RNA (PKR). PKR is involved in the regulation of protein synthesis and the action of transcription factors and is thereby able to control several cellular processes, including cell growth[40,41]. Secondly, IFN-β may be more effective in down-regulating cdk activity or up-regulating cdk-inhibitory proteins, as the compound was shown to be more potent than IFN-α in inducing cell cycle arrest in human pancreatic cancer cell lines[17]. Thirdly, fewer or less effective survival mechanisms may be induced (i.e. up-regulation of the EGFR, stimulation of STAT-3, induction of SOCS-1 and -3 or the stimulation of the MAPK cascade;[41]) after IFN-β treatment compared to IFN-α treatment. However, to the best of our knowledge there are no studies that made a direct comparison between the effects of IFN-α and IFN-ß on PKR, cdk's and survival pathways in human pancreatic cancer cells.

Regarding IFN-β, the concentration required to reduce cell growth with 50% ranged from 70 to over a 1000 IU/ml. These concentrations seem not easily reached in serum of human healthy volunteers after s.c. administration (4-10 IU/ml after four doses of 18 MIU IFN-β at 48-h intervals)[42]. However, the recently developed PEGylated form of IFN-β, which is currently being tested in phase III clinical trial (ADVANCE) in patients with multiple sclerosis seems very promising in this respect. In experimental models, after a single s.c. dose of PEG-IFN-β (3.0 MIU/kg in monkeys), serum concentrations of 100 IU/mL were reached after 20 hours[43,44]. Although this serum concentration is for some cell lines still not high enough, it does get into the sensitivity range to IFN-β of several well responding pancreatic cancer cell lines

Our study showed four cell lines in which IFN-α and -β had only a marginal anti-tumor effect, but over time an increasing trend in anti-tumor activity was observed. This advocates for a longer treatment period, combined with DNA damaging agents like 5-fluorouacil,

gemcitabine or radiotherapy. Tomimaru *et al*. confirms this hypothesis and showed that IFN-β combined with gemcitabine was able to induce synergistically anti-tumor effects even in the pancreatic cancer cells with low IFN receptor expression[22]. Taken this together, this seems promising and further *in vivo* research is definitely necessary. Furthermore, the interaction of type I IFNs with the cells representing the host immune response cannot be neglected as the relevance of cancer immunoediting is becoming clearer [45].

In conclusion, IFN-β is a significant more potent growth inhibitor in pancreatic cancer than IFN-α. Although there is a lot of heterogeneity amongst the panel of pancreatic cancer cell lines, there is a significant correlation between the expression level of the IFNAR-2c receptor and the maximal inhibitory effects of IFN-α. This study provides for the first time extensive and well substantiated evidence that the expression of these receptors in pancreatic cancer can be of predictive value in the responsiveness to the direct tumor growth inhibitory effects IFN-α therapy. More importantly, IFN-β induces a tumor growth inhibitory effect already at lower concentrations, is less dependent on receptor status, and seems therefore more promising than IFN-α.

ACKNOWLEDGEMENTS

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3

IFN-β is a potent inhibitor of insulin and insulin-like growth factor stimulated proliferation and migration in human pancreatic cancer cells

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ABSTRACT

Introduction*:* Pancreatic cancer is a highly aggressive malignancy with few treatment options. The overexpression of several growth factors, including insulin and insulin-like growth factors (IGFs), can underlie the aggressive nature of this disease. Previous research has demonstrated potent effects of interferon (IFN)-β on pancreatic cancer cell growth, however up till now it is unknown whether IFN-β is able to counteract IGF1, IGF2 and insulin-induced pancreatic cancer cell proliferation and migration.

Methods: Expression of IGF- and insulin receptors was determined and the stimulatory effects of IGF1, IGF2 and insulin on cell proliferation and migration, as well as the inhibitory effects of IFN-β were evaluated in 3 human pancreatic adenocarcinoma cell lines.

Results: Both the insulin- and the IGF1 receptor were variably expressed in the cell lines. IGF1, IGF2 and insulin were capable of stimulating cell proliferation in all three cell lines, however cell migration was significantly enhanced only in the BxPC-3 cell line. IFN-β significantly inhibited IGF1-, IGF2- and insulin-stimulated proliferation in all three cell lines in a dose and time dependent manner. Furthermore, in the BxPC-3 cell line IFN-β significantly inhibited both basal and IGF1-, IGF2- and insulin-stimulated cell migration.

Conclusion: Both IGF1, -2 and insulin were capable of stimulating proliferation and migration in human pancreatic cancer cells irrespective of the type of receptor expressed. This study demonstrates that insulin, in addition to IGF1 and IGF2, may play an important role in the progression of pancreatic cancer. Moreover, IFN-β strongly inhibits growth factor stimulated cell proliferation and migration. Our study supports previous findings which have suggested that IFN-β can be a potential promising anti-cancer agent in pancreatic cancer.

1. INTRODUCTION

Pancreatic cancer, with an overall 5-year survival of less than 6%, is the fourth leading cause of cancer related death in the western world[1]. At time of presentation over 80% of the patients are diagnosed with locally advanced or metastatic disease, indicating that pancreatic cancer can be considered as a systemic disease[2]. Several factors, including the overexpression of growth factors receptors, like insulin and insulin like growth factor (IGF) receptors, can underlie the highly aggressive nature of this disease[3,4]. Additionally, previous research reported in 38-64% of the investigated pancreatic cancer specimens an overexpression of the IGF1 receptor (IGF1R), which was associated with more proliferating and invasive tumors leading to a poorer survival[5].

The IGF system is a highly complex system consisting of two growth factors (IGF1 and IGF2), two receptors (IGF1R and IGF2R) and six binding proteins (IGFBP 1-6)[6]. IGFs are very similar in function and structure to insulin, produced by the liver and several other tissues in response to pituitary growth hormone, and implicated as regulators of cell differentiation and cell proliferation in number of cell systems. Both IGF1 and –2 can interact with the IGF1R. However, only IGF2 can bind to the IGF2R, which is a scavenger receptor. Insulin signals via the insulin receptors (IRs) of which there are 2 known isoforms, the IR-A and IR-B. The IR-B receptor mainly activates the metabolic signaling pathway, whereas signaling via the IR-A induces primarily mitogenic effects. Besides that, insulin and IGFs may interact with each other's receptors, although with different affinities[7,8]. Furthermore, high levels of insulin can increase the hepatic IGF1 production by the upregulation of the growth hormone receptor (GHR). In addition, insulin may (independent from GH) directly increase IGF1 expression in the liver [9].

In cancer tissue in general, IGF1R is frequently overexpressed and in this respect pancreatic cancer is not different from other cancers[6,10,11]. Binding of IGFs to the IGF1 receptor induces activation of several pathways including the MAPK and PI3K pathways, which are associated with growth, proliferation, migration and the prevention of apoptosis[8]. Besides that, several epidemiological studies have also shown associations between diabetes mellitus and hyperinsulinemia on the one hand and an increased risk of cancer on the other hand[12-14]. Although the underlying mechanism still needs to be clarified, insulin and/or insulin receptors may play an important role in the development of cancer[15].

Although IGFs and their receptors have shown to be of importance in different cancer cell features it is, to the best of our knowledge, unknown to what extent IGFs (i.e. IGF1 and IGF2) are able of inducing cell migration in human pancreatic cancer cells. Furthermore, in

many studies the role of insulin on tumor cell proliferation and migration is underexposed even though insulin is closely related to the IGF system.

Moreover, type I IFNs, particularly IFN-β, are able to modulate the IGF system in tumor cells by the suppression of endogenous production of IGF2 and by inhibiting the expression of the IGF1R[16]. Type I interferons (e.g. IFN-α and -β) are cytokines that are able to inhibit cell proliferation, induce apoptosis, block cell cycle and to sensitize tumor cells for chemo-and radiotherapy[17]. In a recent study, we have demonstrated these anti-tumor effects in human pancreatic cancer cells, in which the effects of IFN-β were significantly more potent compared to IFN-α[18]. However, it is unclear to what extent IFN-β is capable of inhibiting basal and growth factor stimulated cell proliferation and migration.

The aim of the present study is to evaluate the effect of IFN-β on the IGF1-, IGF2- and insulin-stimulated proliferation and migration of human pancreatic cancer cell lines.

2. MATERIALS & METHODS

2.1 Cell lines and culture conditions

The human pancreatic cell lines BxPC-3, Hs 766T and PANC-1 were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell lines were allelotyped and the DNA (STR) profile corresponded with the profile provided by the ATCC. The cells were cultured in a humidified incubator at 5% CO_2 and 37°C. The culture medium consisted of RPMI 1640 supplemented with 5% FCS, penicillin ($1x10^5$ U/L) and L-glutamine (2 mmol/l). Periodically, cells were confirmed as Mycoplasm-free. Cells were harvested with trypsine (0.05%)EDTA (0.53 mM) solution. Before plating, cells were counted microscopically using a standard haemocytometer. Trypan blue staining was used to determine cell viability. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

2.2 Drugs and reagents

IGF1 and IGF2 were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Human recombinant insulin was obtained from Novo Nordisk (Actrapid®, Bagsvaerd, Denmark). Human recombinant IFN- β-1a was acquired from Serono Inc. (Rebif, Rockland, MA). IGF1, IGF2 and IFN- β-1a were stored at -20ºC, insulin was stored at 4 ºC. Stock solutions of IGF1 and IGF2 were constituted in 0.01M of acetic acid, insulin and IFN-β were constituted in distilled water, all according to the manufacturer's instruction.

2.3 Quantitative RT-PCR

By quantitative RT-PCR mRNA expression of *IGF1, IGF2, IGF1R, IR-A, IR-B* and the housekeeping gene *hypoxanthine phosphoribosyltransferase (HPRT)*, was evaluated. The isolation of total RNA, complementary DNA (cDNA) synthesis and the primer and probe sequences (Sigma-Aldrich) that were used for the detection of *IGF1, IGF2, IGF1R, IR-A, IR-B* and *HPRT* have been described previously.[16,19-21] Dilution curves were constructed for calculating the PCR efficiency for every primer set and have been described by van Adrichem *et al*[21], Varewijck *et al.*[20] and Vitale *et al*.[16] After efficiency correction of target and reference gene transcripts (*HPRT*), the comparative threshold method, $2^{-\Delta Ct}$ was used to calculate the relative expression of genes.

2.4 Cell proliferation assay

For each cell line the optimal cell number plating density was determined (data not shown). After trypsinization, the cells were plated in 1 ml of medium in 24 well plates at the correct cell density. The plates were placed in a 37ºC, 5% CO_2 incubator and cells were allowed to attach overnight. The next day, after washing the plates three times, the culture medium was replaced with 1ml/well medium containing 0.5% FCS. Increasing concentrations ($1x10^{-10}$ – 10^{-8}) of IGF1, -2 and insulin were added. Each treatment was performed in quadruplicate. After 3 and 7 days of treatment, the cells were harvested for DNA measurement. For the 7-day experiments, the medium containing 0.5% FCS was refreshed after 3 days and compounds were added again. As previously described, the measurement of total DNA contents was performed using the bisbenzimide fluorescent dye (Hoechst 33258) (Boehring Diagnostics, La Jolla, CA)[18].

2.5 Cell migration assay

The *in vitro* cell migration was measured by the scratch assay method described by Liang et al[22], with some modifications. After trypsinization, cells were plated in 2 ml of medium in poly-L-lysine coated 12-wells plates and placed in a 37ºC, 5% CO_2 incubator. Cells were grown until a confluent monolayer was formed. With a 200µl pipet tip a scratch was made in the cell monolayer. The debris was removed by washing the cells once with 1ml of growth medium and twice with 1ml of medium containing 0.5% FCS. Hereafter, 2ml of medium containing 0.5% FCS and the different compounds of interest were added. The ability of cells to migrate into the scratch area was assessed after 2, 4, 8 and 24 hours by comparing the 0- and 2,4,8 and 24-hour photomicrographs (Zeiss, Axiovert 40c, x50 magnification) of 4 fixed points along the scratch area. The percentage of non-recovered scratch area was

calculated by dividing the non-recovered area after 2, 4, 8 and 24-hours by the initial scratch area (t=0) using the image software (http://rsb.info.nih.gov/ij/).

The scratch assay method was compared with a wound healing assay using the Cytoselect ™Wound Healing Assay Kit (Cell Biolabs, Inc., San Diego, USA). Briefly, after trypsinization cells were seeded in 1ml of growth medium into 24-wells tissue culture plates containing inserts and incubated until a monolayer of cells was formed. After removing the inserts from the wells, the cells were treated according to the scratch assay method as described above.

2.6 Statistical analysis

All experiments were performed at least twice, with the exception of the migration assay, which was performed at least three times. For statistical analysis GraphPad Prism 5.0 (GraphPad software, San Diego, CA) was used. The comparative statistical evaluation among groups was performed by a one-way ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. In all analyses, values of $P<0.05$ were considered statistically significant. Data are reported as mean ±SEM.

3. RESULTS

3.1 mRNA expression.

By quantitative RT-PCR we analyzed the receptor mRNA expression of the *IGF1R, IGF1, IGF2*, *IR-A* and *IR-B* (Figure 1). All 3 human pancreatic adenocarcinoma cell lines expressed the *IGF1R* (highest in PANC-1), *IR-A* (highest in Hs 766T) and *IR-B* (highest in BxPC-3). None of the cell lines expressed detectable levels of *IGF1* mRNA (data not shown). *IGF2* mRNA was only expressed in the PANC-1 and Hs 766T cell lines, but not in the BxPC-3 cell line.

3.2 Growth factor stimulated proliferation.

As shown in figure 2, in two of the three cell lines growth factor stimulated proliferation was time- and dose dependent. After 3 days of incubation, at a concentration of 10 nM, IGF1 and insulin significantly stimulated the cell proliferation in 2 of the 3 cell lines. IGF2 significantly increased cell growth after 3 days in all three cell lines (Figure 2, open bars). After 7 days of incubation IGF1 and insulin stimulated the cell growth significantly at a concentration of 10nM, in all three cell lines, whereas IGF2 increased cell proliferation in 2 of the 3 cell lines (Figure 2, black bars). Table 1 shows maximal stimulatory effect of 3 and 7 days incubation with IGF1, IGF2 and insulin (all at 10nM) in the 3 cell lines.

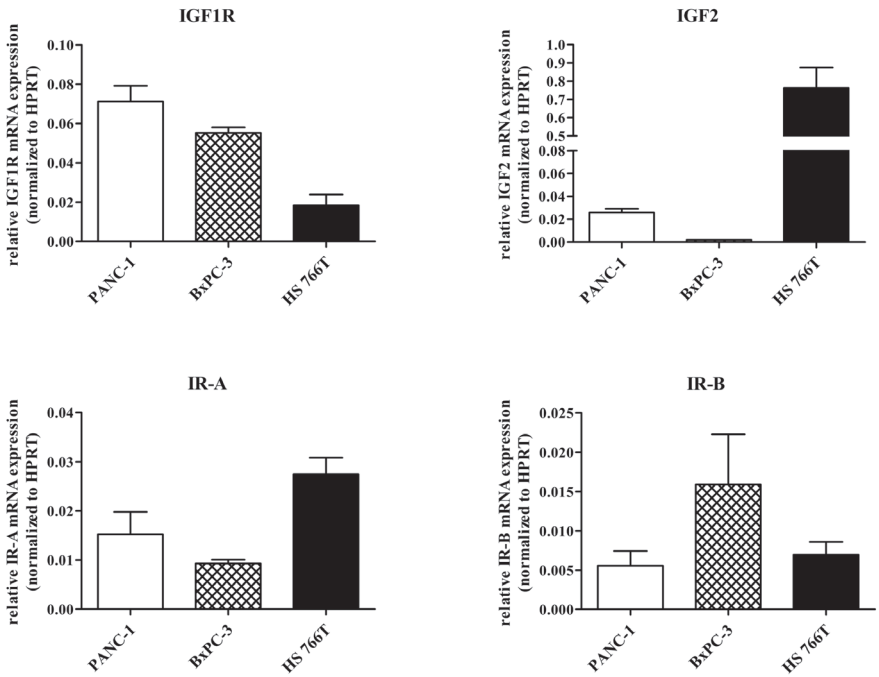


Figure 1: Relative expression of *IGF-1R, IGF2, IRA* and *IRB* mRNA in 3 human pancreatic adenocarcinoma cell lines, normalized to *HPRT* mRNA. Values represent mean ± SEM.

Table 1. Maximal stimulatory effect (Emax) of IGF1, IGF2 and insulin in three human pancreatic cancer cell lines.

	PANC-1		BxPC-3		HS 766T	
	Emax (%) 3d	Emax (%) 7d	Emax (%) 3d	Emax (%) 7d	Emax (%) 3d	Emax (%) 7d
IGF1 [10nM]	39.1 ± 5.6 ***	54.5 ± 8.6 ***	20.4 ± 4.8 ***	50.4 ± 6.8 ***	18.4 ± 7.58	27.4 ± 7.9 **
IGF2 [10nM]	19.7 ± 6.3 *	79.1 ± 17.8 ***	27.5 ± 4.9 ***	44.5 ± 10.5 ***	16.4 ± 5.8 *	1.6 ± 2.9
INS [10nM]	20.2 ± 5.2 *	24.0 ± 6.8 *	44.0 ± 4.6 ***	50.4 ± 8.5 ***	14.2 ± 4.4	23.3 ± 6.9 *

The maximal stimulatory effect is expressed as the percentage stimulation compared to the untreated control
± SEM. * p<0.05, ** p<0.01 and ***p<0.001 versus control.

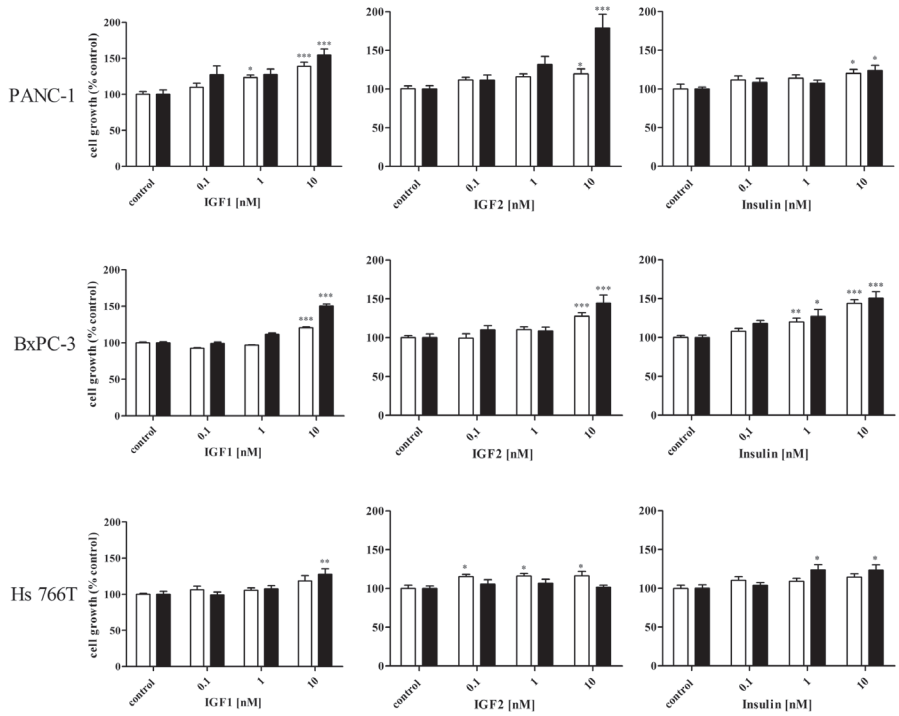


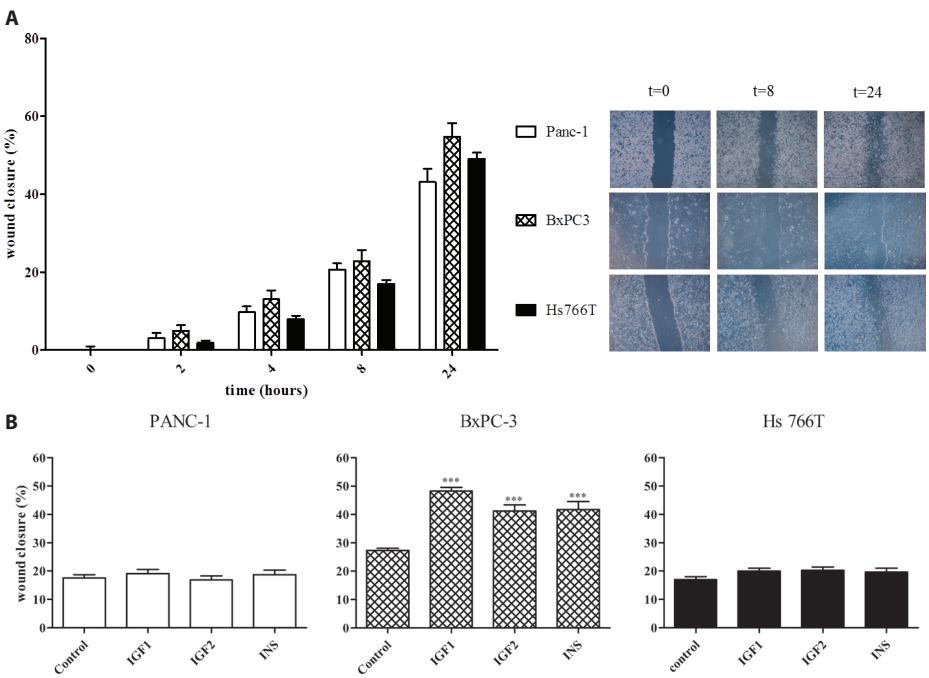
Figure 2: Treatment effects on cell proliferation after 3 days (open bars) and 7 days (black bars) of incubation with increasing concentrations of IGF1, IGF2 and insulin in 3 human pancreatic cancer cell lines. Values are expressed as the percentage of control and represent the mean ± SEM of at least 2 independent experiments in quadruplicate * p<0.05, ** p<0.01 and ***p<0.001 versus control.

3.3 Basal and growth factor stimulated migration

To assess cell migration the scratch assay method was used in the three cell lines. First, basal cell migration was evaluated after 2, 4, 8 and 24 hours (Figure 3a). Although differences were relatively small, overall, the BxPC-3 cells migrated the fastest. After 8 hours 22.9% of the scratch was closed and after 24 hours closure was 54.7%. In the PANC-1 and the HS 766T cell line values were 20.7% and 16.9% after 8 hours, and 43.1% and 49% after 24 hours, respectively.

Furthermore, we evaluated the growth factor stimulated migration (Figure 3b). Of each compound (IGF1, IGF2 and insulin) a concentration of 10 nM was used and scratch closure was assessed after 8 hours to avoid the possibility of involvement of cell growth. Only in

the BxPC-3 cell line a statistically significant increase in migration by all growth factors was observed ($p<0.001$). After stimulation with IGF1, 8 hours after scratch 48.2% of the wound was closed (76.4% increase compared to control). After stimulation with IGF2 and insulin scratch closure was 41.2% and 41.7%, respectively (50.8% and 52.7% increase compared to control, respectively).



Figuur 3

A: Left panel: Percentage cell migration in 3 human pancreatic cancer cell lines, after 2, 4, 8 and 24 hours after scratch. After scratch, medium was removed and cells were placed in medium containing only 0.5% FCS. The percentage of non-recovered wound area was calculated by dividing the non-recovered area after 2,3,8 and 24 hours by the initial wound area at 0 time; Right panel: Pictures of the scratch at 0 time and 8 and 24 hours after the scratch in 3 human pancreatic cancer cell lines. Original magnification x50;

B. Percentage of cell migration after 8 hours of incubation with 10nM IGF1, IGF2 or insulin in 3 human pancreatic cancer cell lines. Values are expressed as the percentage of wound closure compared to 0 time and represent the mean ± SEM of at least 3 independent experiments in triplicate, ***$p<0.001$ versus control.

In addition, to validate the scratch assay, the Cytoselect ™Wound Healing Assay was used in the BxPC-3 cell line. No significant differences were observed between the two assays regarding basal and IGF1 stimulated cell migration (data not shown).

3.4 Inhibition of growth factor stimulated proliferation by IFN-β

We evaluated the effect of two different concentrations of IFN-β on the IGF1 (10nM)-, IGF2 (10nM)- and insulin (10nM)-stimulated proliferation after 3 and 7 days of incubation. Figure 4 illustrates the effects of 100 and 1000 IU/ml IFN-β, the maximal stimulatory effect of IGF1, -2 and insulin, as well as the combined effects of the compounds after 7 days of incubation. In all three cell lines, with the exception of the insulin-stimulated cell growth in the PANC-1 cell line, both 100 and 1000 IU/ml of IFN-β were capable of reducing the growth factor stimulated cell proliferation significantly. In addition to the dose-dependent effect of IFN-β there was also a time-dependent effect on basal proliferation as well as growth factor stimulated proliferation (data available on request).

3.5 Inhibition of growth factor stimulated migration by IFN-β

We also evaluated whether INF-β was able to inhibit growth factor stimulated migration. Given that only in the BxPC-3 cell line there was a significant increase of growth factor stimulated migration, the effect of IFN-β on growth factor induced migration was assessed only in this cell line. Figure 5 shows the effects of 100 and 1000 IU/ml IFN-β on basal and growth factor stimulated cell migration after 8 hours of incubation. Similar to the experiments described in figure 3b, at a concentration of 10nM, all growth factors stimulated the cell migration in a statistically significant manner ($p<0.001$). Furthermore, there was a significant inhibition of basal cell migration by 1000 IU/ml IFN-β ($p<0.001$). Both 100 and 1000 IU/ml IFN-β were able to reduce IGF1 ($p<0.01$ and $p<0.001$, respectively) and IGF2 ($p<0.05$ and $p<0.001$, respectively) stimulated cell migration significantly. Regarding the insulin stimulated cell migration, only the higher concentration of 1000 IU/ml IFN-β was capable of reducing the cell migration significantly ($p<0.01$).

4. DISCUSSION

Pancreatic cancer is a highly invasive malignancy with the potency to metastasize early. The highly aggressive nature of this disease can be explained by the overexpression of several factors, including insulin and insulin-like growth factors[3,4]. Previous research has shown that the IGF1R is overexpressed in substantial part of pancreatic cancer specimens and associ-

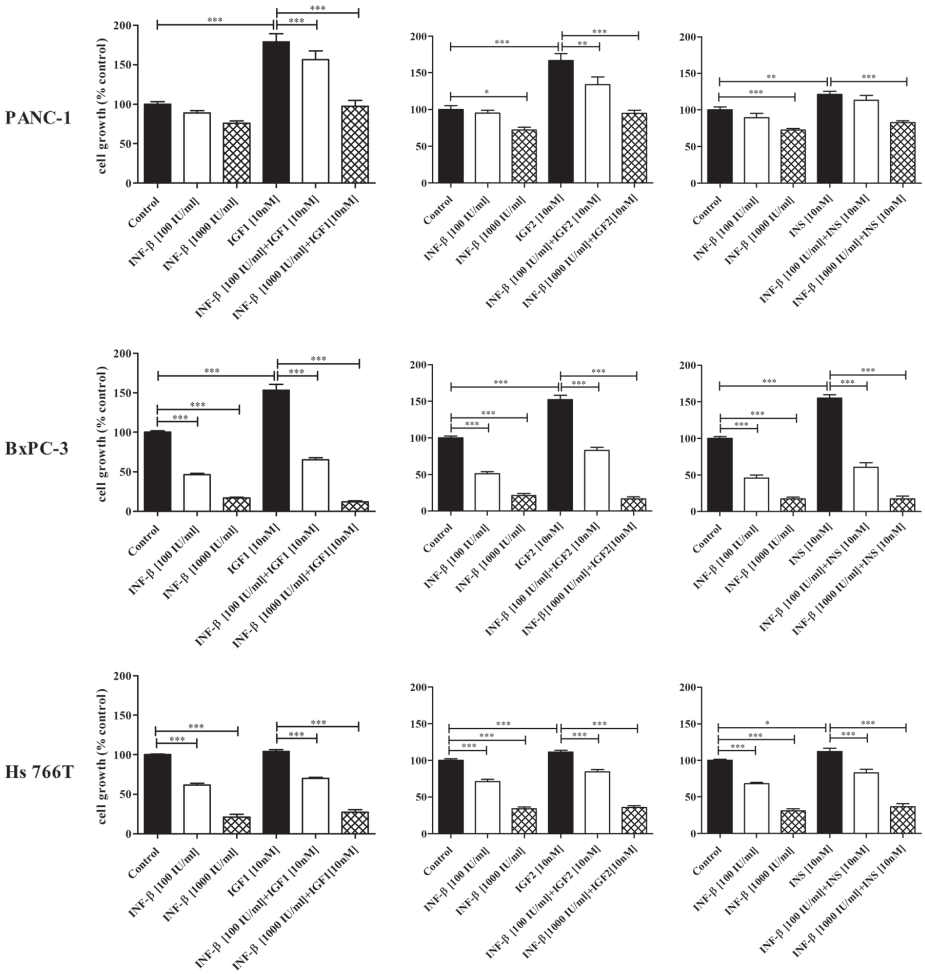


Figure 4: Effects of IFN-β treatment on cell proliferation after 7 days of incubation with and without the growth factors IGF1, IGF2 and insulin. The cells were incubated for 7 days with 100 IU/ml IFN-β, 1000 IU/ml IFN-β or 10nM IGF1, IGF2 or insulin alone, or with the combination of 100IU/ml or 1000 IU/ml IFN-β and 10nM of IGF1 IGF2 or insulin. Values are expressed as the percentage of control and represent the mean ± SEM of at least 2 independent experiments in quadruplicate * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus control.

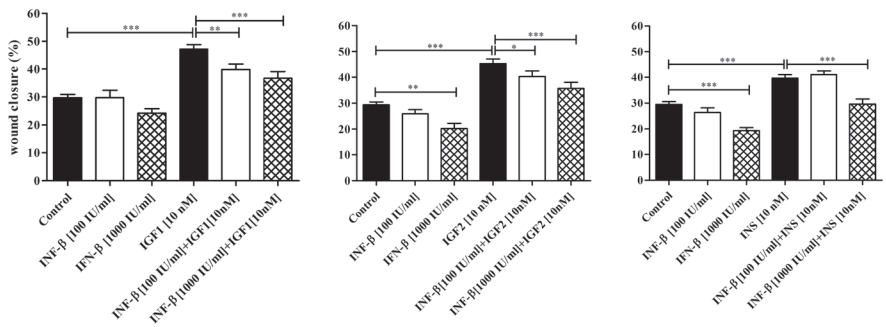


Figure 5: Percentage of cell migration after 8 hours of incubation with 100 IU/ml IFN-β, 1000 IU/ml IFN-β or 10nM of IGF1,-2 or insulin alone, or with the combination of 100IU/ml or 1000 IU/ml IFN-β and 10nM of IGF1,-2 or insulin in the BxPC-3 cell line. Values are expressed as the percentage of wound closure compared to 0 time and represent the mean ± SEM of at least 3 independent experiments in triplicate, * p<0.05, ** p<0.01 and ***p<0.001 versus control.

ated with invasive and more proliferating tumors[5]. Besides that, it becomes more clearer that high levels of insulin can stimulate tumor growth. Moreover, several epidemiological studies associated diabetes mellitus, with the accompanying hyperinsulinism, and the use of insulin with an increased risk of certain cancers, as well as all-cause mortality[12-14,23]. Additionally, given that insulin and IGFs may interact with each other's receptors, the role of insulin and the insulin receptor should not be neglected. Nevertheless, to the best of our knowledge, there are no studies yet comparing the effects of IGF1, IGF2 and insulin on both cell proliferation and migration of pancreatic cancer cells.

In the present study we first evaluated the mRNA expression of the growth factors and their receptors. Among the 3 human pancreatic adenocarcinoma cell lines, there was a considerable variability in expression of the IGF1R, IRA and IRB receptors. None of the cell lines expressed detectable levels of IGF1 mRNA and only the PANC-1 and Hs 766T cell lines expressed IGF2 mRNA.

Overall, the cell proliferative effects of IGF1, -2 and insulin were time- and dose dependent. However, in the Hs 766T cell line these effects compared to the other two cell lines, were much less pronounced, which might be explained by several reasons. First, in contrast to the other cell lines, IGF1R mRNA and protein expression is relatively low. Secondly, the IGF2 mRNA expression of this cell line is fairly high which can cause the IGF1 and insulin receptors to be already partially saturated. The addition of IGF1, IGF2 and insulin

will, therefore, be minimally effective. Finally, the role of the family of IGF binding proteins (IGF-BPs) should not be ignored as they are capable of binding free circulating IGFs and thereby opposing the cell proliferating effects of IGFs. It has been described that in certain circumstances, like after serum starvation, IGF-BPs are also capable of stimulating cell growth[24]. Furthermore, IGF-BP3 and -5 are frequently overexpressed in pancreatic cancer and correlated with pancreatic cancer cell growth and a poorer survival.[4,25-27] However, in the present study the role of IGFBP's was not evaluated.

In healthy subjects with normal insulin sensitivity, after food intake peripheral insulin concentrations rise to approximately 0.5 to 1.0 nM. It is estimated that peri-acinar concentrations of insulin are at least 20-fold higher than in the peripheral circulation, and should peak at 10-20 nM[23]. Although more than 95% of circulating IGFs are bound, in total circulating concentrations of IGFs are much higher than the concentrations of insulin. Generally, the normal IGF concentrations in adulthood can vary between 4nM and 70nM. However, this range strongly depends on factors like age, gender, diet and the used technique to determine the concentration. Besides that there is a large biological variation between individuals which makes it difficult to determine a generally applicable reference range. Therefore, given this wide spread in physiological concentrations, regarding the different growth factors, a fixed concentration of 10nM was used to determine the IGF1, IGF2 and insulin stimulated migration, as well as the potential inhibitory effects of IFN-β on the growth factor stimulated proliferation and migration. However, at this concentration the growth factors are capable of binding each other receptors and therefore it is not possible to fully correlate the effects of the growth factors with their receptors. Nevertheless, this data does imply that, regardless of receptor type or level of expression, an IGF1-, IGF2- and/or Insulin-stimulated effect can be exerted. Besides that, cancer cells themselves are capable of producing growth factors, thereby creating an environment with elevated concentrations of the growth factors enabling themselves to proliferate and migrate[4,25].

With the scratch assay method in vitro cell migration was evaluated. This method allows to study cell migration in vitro, which enables cell-cell interaction and to some extent mimics cell migration in vivo. Although the scratch assay method is a validated method to study cell migration we validated this method with the Cytoselect ™Wound Healing assay, since in the scratch assay results can be compromised by the release of factors from damaged cells. However, we did not observe any differences between the two methods. As such, the scratch assay was a suitable method to study cell migration. Furthermore, by analyzing the

growth factor stimulated migration 8 hours after scratch we excluded the possibility of the involvement of cell proliferation.

Only in the BxPC-3 cell line a significant increase in cell migration was observed after stimulation with the different growth factors. No significant increased cell migration was observed in the PANC-1 and Hs 766T cell line. Considering the relatively low IGF1R expression and the less pronounced effects of the growth factors on cell proliferation after 3 and 7 days in the Hs 766T cell line, the absence of stimulated cell migration after 8 hours could therefore be argued. Nevertheless, the absence of cell migration in the PANC-1 cell line is unexpected. It is known that cancer cell proliferation and migration are processes mediated by several molecules in different signaling cascades. AKT and its upstream regulator p13K are primarily involved in malignant cell proliferation, whereas ERK and the Ras/RAF/MAP kinase signaling pathways are more broadly involved in cellular functions like survival, proliferation, apoptosis and cell motility[3,28,29]. The epithelial-mesenchymal transition (EMT) is one of the initiators of the metastatic cascade and proteins involved in this EMT (e.g. Notch-2, Snail, N-Cadherin, Zeb, Vimentin and Slug) are also frequently overexpressed in pancreatic cancer[30-32]. As nicely investigated and described by Subramani et al., silencing the IGF1R resulted in an inhibition of proteins favoring pancreatic cancer EMT, additionally silencing of the receptor resulted as well in a downregulation of the active forms of AKT, P13K and mTOR by the upregulation of the tumor suppressor gene PTEN. Furthermore, by silencing of the receptor an effective inhibition of the active form of ERK was found. The authors concluded that the reduced capacity of the pancreatic cancer cells to proliferate and migrate was due to the suppression of key molecular pathways affected by the knockdown of the IGF1R. However, these effects became apparent after silencing the receptor for at least 48hours. Additionally, the reduction in cell migration became statistically significant 72 hours after the scratch. Therefore, it can be hypothesized, that with respect to this cell line, 8 hours of incubation with the different growth factors may not have been sufficiently long enough to detect enhanced cancer cell migration.

As demonstrated in previous research, INF-β is a very potent molecule in inhibiting cell proliferation and in inducing apoptosis in pancreatic cancer cells[18,33]. In addition, it is demonstrated that IFN-β is capable of modulating the IGF system by downregulating the expression of IGF1R and IGF2 mRNA in neuro-endocrine tumor cells[16]. However, despite these potent effects, there are no studies yet that evaluated the effects of IFN-β with respect to the IGF and insulin system in human pancreatic cancer cells. Therefore, in addition to the effects of insulin and insulin-like growth factors on pancreatic cancer cell proliferation and

migration we investigated the inhibitory effects of IFN-β on the growth factor stimulated proliferation and migration as well. As shown in previous research, and reaffirmed in this study, IFN-β is a potent inhibitor of pancreatic cancer cell growth. Moreover, after 3 days of incubation with the different growth factors, 100 IU/ml of IFN-β was capable of inhibiting IGF1-, IGF2- and insulin-stimulated proliferation in two out of three cell lines, whereas a 1000 IU/ml of IFN-β was capable of inhibiting each growth factor stimulated proliferation in all three cell lines. These effects became even stronger after 7 days of incubation with both concentrations of IFN-β and the different growth factors. Besides the effect of IFN-β on growth factor stimulated proliferation we also studied the effect of IFN-β on growth factor stimulated migration. Since no significant effect of the growth factors was observed in the Hs 766T and PANC-1 cell line, we only evaluated the effect of IFN-β in the BxPC-3 cell line. In this cell line after 8 hours of incubation IFN-β (1000 IU/ml) significantly reduced basal migration as well as the migration stimulated by either IGF1, IGF2 or insulin. Additionally, 100 IU/ml of IFN-β inhibited both the IGF1, as well as the IGF2, stimulated migration significantly, which can be due to the fact that IFN-β is capable of down regulating the IGF1R[16]. Nevertheless, the inhibitory effects of IFN-β on cell migration are less pronounced as compared to the effects on cell proliferation, indicating that the associated signaling pathways are predominately involved in cell proliferation rather than in cell migration[34].

As clearly demonstrated in this study, insulin as well is capable of inducing cancer cell proliferation and migration. Besides that, it is known that IGFs and insulin can interact with each other receptors. Therefore, one can argue that targeting only the IGF1R will be insufficient in cancer treatment. Recently a new drug, OSI-906, has been developed that selectively inhibits autophosphorylation of both IGF1R and IR. OSI-906 hinders activation of downstream pathways and thereby inhibits migration, proliferation and survival in a variety of tumor cell lines[35-37]. Currently, OSI-906 is tested in advanced clinical studies. In future research, it would be interesting to test whether the anti-cancer effects of OSI-906 and IFN-β reinforce each other.

In conclusion, this study is the first that demonstrates the potent stimulatory effects of IGF1, -2 and insulin with respect to pancreatic cancer cell proliferation and migration. These results are in line with the highly aggressive nature of this disease and confirm the potential malignant potencies of insulin. In addition, we showed that IFN-β potently inhibits IGF1, IGF2 or insulin stimulated tumor cell growth, as well as the migration stimulated by these growth factors. In addition to the potent anti-tumor effects of IFN-β, these results therefore further favor the use of INF-β as part of the treatment options for patients with pancreatic cancer.

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4

Interferon-β mono- and combination therapy in the treatment of pancreatic cancer *in vivo*

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Manuscript in preparation

ABSTRACT

Background: Pancreatic cancer is the fourth leading cause of cancer related death in the Western world. Although multiple treatment strategies have been attempted, survival has barely been improved in the last 30 years. Recently, potent anti-tumor effects of interferon-β (IFN-β) have been demonstrated *in vitro*. However, the effect of IFN-β alone and in combination with a chemotherapeutic agent on the growth of pancreatic cancer *in vivo* is unknown. Additionally, there are no methods available yet to predict the effects of therapy before start of the treatment. In the present study we evaluated the effect of IFN-β mono- and combination therapy with gemcitabine *in vitro*, *in vivo* and *ex vivo*.

Methods: The anti-tumor effects of IFN-β alone and combined with a suboptimal concentration of gemcitabine were evaluated in an *in vitro* cell proliferation assay, in a heterotopic subcutaneous mouse model and in an *ex vivo* precision cut tissue slice model. For all experiments the human pancreatic cancer cell line BxPC-3 was used.

Results*: in vitro*: IFN-β pre-treatment significantly enhanced sensitivity of BxPC-3 cells to the inhibitory effect of gemcitabine. *In vivo*: After 30-days of treatment with the combination of IFN-β and gemcitabine, there was a significant reduction by 55% of tumor volume (compared to vehicle treated animals). Both compounds tested alone, at the dosage used, did not significantly influence tumor growth. Additionally, tumors of mice treated with the drug combination showed a significant reduction in the number of proliferating cells, whereas there was a clear trend towards an increased apoptosis (Caspase-3 cleavage). Again, no statistically significant differences were found in tumors of mice treated with IFN-β or gemcitabine monotherapy. *Ex vivo:* In tissue slices, combined IFN-β and gemcitabine treatment resulted in a visible loss of tissue integrity.

Conclusion: While, at the dose tested, no effect of IFN-β alone on cancer growth *in vivo* was observed, this study demonstrates for the first time a potent chemosensitizing effects of INF-β when combined with gemcitabine *in vitro, in vivo* and *ex vivo*, already at suboptimal concentrations of gemcitabine. Therefore, the use of IFN-β as adjuvant treatment of pancreatic cancer seems promising and needs to be further explored.

1. INTRODUCTION

Patients diagnosed with pancreatic cancer have one of the most dismal prognoses. The overall 5-year survival rate is less than 6%, with a median survival of 4-6 months. The treatment of choice is surgery followed by adjuvant chemotherapy. However, at time of diagnosis the majority of patients present with locally advanced or metastatic disease and chemotherapeutic response rates are often disappointing[1,2]. Although multiple chemoradiation treatment strategies have been attempted, over the last 30 years pancreatic cancer mortality rates have barely been improved. Over the past decade research has focused on other treatment strategies, including biological response modifiers such as interferons (IFNs).

Type I interferons (i.e. IFN-α and –β) are cytokines that have a wide range of potential anti-cancer effects. They are capable of inhibiting cell proliferation, inducing apoptosis, block cell cycle and, importantly, are able to sensitize tumor cells for chemo- and radiotherapy.

A few years ago several clinical studies[3-7] demonstrated promising results indicating that there might be a role for adjuvant IFN-α therapy. Nevertheless, in all studies the majority of patients experienced grade 3 or 4 treatment toxicities and, therefore, the use of IFN-α in the treatment of pancreatic cancer remains controversial.

Recently, several *in vitro* studies showed promising results regarding IFN-β therapy. First, the study of Vitale et al.[8] demonstrated in human pancreatic cancer cells that IFN-β induces a more potent and early apoptosis and cell cycle arrest compared to IFN-α. Furthermore, other research groups demonstrated strong chemo- and radiosensitizing effects of IFN-β, even in cell lines that are non-responsive to IFN-α[9-11]. Furthermore, a study by our research group evaluated the effects of IFN-α and –β in eleven human pancreatic cancer cell lines and demonstrated that IFN-β is able to induce already at low concentrations very potent anti-tumor effects, an effect being less dependent on IFN receptor expression[12]. In addition, it has been described that IFN-β has a higher binding affinity for the receptor complex compared to IFN-α[13], which is of significance since only a small proportion of the pancreatic cancer patients have a high expression of the binding subunit of the type I interferon receptor[14]. More recently, our research group demonstrated that IFN-β is capable of inhibiting Insulin-like Growth Factor (IGF)- and insulin-stimulated pancreatic cancer cell proliferation and migration (Booy et al. American Journal of Cancer Research, provisionally accepted). However, surprisingly there are no *in vivo* and/or clinical studies that investigated the use of concomitant adjuvant IFN-β therapy in the treatment of pancreatic cancer[15].

In the present study we therefore evaluated the effect of IFN-β alone and in combination with gemcitabine in a heterotopic pancreatic cancer mouse model. Additionally, in order to try to predict the effects *ex* vivo, we evaluated the effects of IFN-β mono- and combination therapy in a pancreatic cancer xenograft tissue slice model.

2. MATERIALS AND METHODS

2.1 Cell lines and culture conditions

The human pancreatic cancer cell line BxPC-3 was obtained from the American Type Culture Collection (Rockville, MD, USA). Before use the cell line was allelotyped and the DNA (STR) profile corresponded with the profile of the ATCC. The cells were cultured in a humidified incubator at 5% CO_2 and 37°C. The culture medium consisted of RPMI 1640 supplemented with 5% FCS, penicillin ($1x10^5$ U/L) and L-glutamine (2 mmol/l). Cells were confirmed as mycoplasma-free. Cells were harvested with trypsin (0.05%)-EDTA (0.53 mM) solution. Before plating or injection, cells were counted microscopically using a standard haemocytometer. Trypan blue staining was used to determine cell viability. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

2.2 Drugs and reagents

Human recombinant IFN-β-1a was acquired from Serono Inc. (Rebif, Rockland, MA, USA) kept at 4°C and diluted in 0.9% NaCl at time of use. Gemcitabine Actavis (Actavis Group PTC, Hafnarfjordur, Iceland) was kept at room temperature and made fresh in 0.9% NaCl at the day of use.

2.3 Cell proliferation assay

After trypsinization, the cells were plated in 1 ml of medium in 24-well plates at the correct cell density. The plates were placed in a 37°C, 5% CO_2 incubator and cells were allowed to attach overnight. The next day, cells were pre-incubated for 24 hours with 1000 IU/ml of IFN-β. Hereafter, the medium was refreshed and incubated for another 24 hours, or for the remaining time of the experiment (3-days), with increasing concentrations (0.05- 5.0 ng/ml) of gemcitabine. Each treatment was performed in quadruplicate. After 3 days of treatment, the cells were harvested for DNA measurement. The measurement of total DNA contents, as previously described, was performed using the bisbenzimide fluorescent dye (Hoechst 33258) (Boehring Diagnostics, La Jolla, CA)[16].

2.4 Animals and heterotopic injection of tumor cells.

Male athymic Balb/C nude mice (Harlan laboratories, UK ltd) of eight weeks old were used in this study. The animals were kept in a barrier facility under HEPA filtration. Mice were maintained on a daily 12-h light/12-h dark cycle in cages with autoclaved bedding, water and autoclaved laboratory rodent diet were given *ad libitum*.

For tumor cell injection, cells were harvested from culture flasks, as described earlier, and dissolved in phosphate buffered saline (PBS). Only single-cell suspensions of greater than 90% viability were used for injection. Tumor cells ($1x10^6$/100µl PBS) were subcutaneously injected at the flank of each mouse after which the mice were randomly divided into four groups (n=8 each). A separate group of five mice, which did not receive any treatment, were used for the tissue slice experiments. All animal studies were done in accordance to protocols approved by the committee on animal research of the Erasmus Medical Center, Rotterdam, the Netherlands.

2.5 Therapy and assessment of tumor size

Tumor size and body weight was measured twice weekly. Tumor volume were evaluated as $(\text{length x width})^{1.5}x\ (\pi/6)$. When the tumor volume reached ~$150mm^3$ treatment was started. Mice in the control group received five times a week, on consecutive days, an intraperitoneal (i.p.) injection of 100µl of 0.9% NaCL. Mice in the IFN-β monotherapy group received upon start of the treatment, 5 times a week, on consecutive days, an injection of $1.5x10^5$ IU of IFN-β intraperitonealy. Mice randomized to the gemcitabine monotherapy group received two times a week an i.p. injection of 40mg/kg gemcitabine. Mice in the IFN-β and gemcitabine combination group received upon start of the treatment, 5 times a week, on consecutive days, an injection of $1.5x10^5$ IU of IFN-β intraperitonealy and, on day 2 and 4, an i.p. injection of 40mg/kg gemcitabine.

2.6 Necropsy procedures

Mice, under isoflurane anaesthesia, were sacrificed by cervical dislocation after 4 weeks of treatment, or when tumor volume reached $2000mm^3$ ($1500mm^3$ for tumors of mice used in the tissue slice experiments) or when the wellbeing (i.e. weight loss, lethargy, tumor ulceration) of the mice could no longer be maintained.

During necropsy, tumors were resected and tumor weight and volume were measured. Tumors were divided into three parts and subsequently snap frozen in liquid nitrogen, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands) for cryosectioning

and fixed in freshly prepared 4% formaldehyde solution, and prepared for paraffin sectioning. Furthermore, mice organs (pancreas, liver, lungs, brain, heart, spleen and kidneys) were harvested, weighted and fixed in 4% formaldehyde.

2.7 Tissue slicing and slice culture

When tumors reached a volume of 1500 mm^3, mice were sacrificed and necropsy was performed as described above, however, tumors were not divided for histology. After resection, tumors were washed twice with Hanks' Balanced Salt Solution (HBSS) supplemented with penicillin ($1x10^5$ U/L), streptomycin(1000 IU/ml) and fungizone (30µg/ml).

After the vibrocheck (measurement of vertical deflection) was performed, tumor specimens, buffered in ice-cold HBSS, were cut, with stainless steel razor blades, into slices of 200µm using the Leica vibrating blade microtome VT1000 S (Leica, Wetzlar, Germany). After slicing, samples were washed once more and transferred into six-well multiplates containing 5ml of culture medium consisting of Dulbecco's Modified Eagle's Medium: nutrient mixture F-12 (DMEM/F-12), supplemented with penicillin($1x10^5$ U/L), streptomycin (1000IU/ml) and 10% FCS. Consecutive slices were used for the experiments (minimum of 15 slices per tumor).

Tissue culture plates were placed in a humidified incubator at 5% CO_2 and continuously shaken (60 rounds/min) at 37°C up to 4 days post slicing. Media and supplements were obtained from GIBCO Bio-cult Europe (Invitrogen, Breda, The Netherlands).

After 24 hours, media were refreshed and slices were incubated with IFN-β (1000 IU/ml), gemcitabine (1ng/ml), or with the combination of IFN-β and gemcitabine. At baseline and after 72 hours of incubation tissue slices were harvested fixed in freshly prepared 4% formaldehyde solution and prepared (in upright position) for standard paraffin sectioning.

2.8 Immunohistochemistry

The formalin fixed and paraffin embedded sections (5µm thick) were treated for immunohistochemistry as described previously[14]. Briefly, sections were deparaffinised and rehydrated, followed by heat induced epitope retrieval (HIER), rinsed *(TRIS/Tween 0.5% (pH 8.0))* and blocked (hydrogen peroxide 3% in PBS) for 15 minutes before incubation with the primary antibodies for Caspase-3 and Ki-67 (both overnight at 4° C). For negative controls, the primary antibody was omitted. The Dako Real EnVision Detection System kit (Dako Detection System, Dako Denmark, Glostrup, Denmark) was used to visualize the bound antibody after which the slides were counterstained with haematoxylin and coverslipped.

The rabbit monoclonal Cleaved Caspase-3 (Asp175) antibody (cell signalling technology, Beverly, MA, USA) was used at a dilution of 1:750. The mouse monoclonal Ki-67 antibody (Dako Detection System) was used at a dilution of 1:400.

2.9 Immunohistochemical analysis

All sections were evaluated and counted independently by two investigators (SB and LJH). Apoptosis was assessed by counting the total number of caspase-3 positive tumor cells per high-power field (HPF x40 objective). Cell proliferation was assessed by the evaluation of Ki-67 positive tumor cells per HPF (HPF x40 objective). For the analysis a minimum of 3 HPF were used to evaluate cells with a positive and negative staining.

2.10 Statistical analysis

For statistical analysis GraphPad Prism 5.0 (GraphPad software, San Diego, CA) was used. All *in vitro* experiments were carried out at least thrice. The comparative statistical evaluation among groups was performed by a one-way ANOVA test. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. Regarding the *in vivo* experiments the difference between groups were evaluated by the Mann-Whitney *t*-test. In all analyses, values of $p<0.05$ were considered statistically significant. Data are reported as mean ±SEM.

3. RESULTS

3.1 Effects of IFN-β mono- and combination therapy *in vitro*

Before gemcitabine treatment pancreatic cancer cells were pre-incubated with IFN-β. Thereafter, cells were incubated with increasing concentrations of gemcitabine for 24-hours. The effects of gemcitabine treatment alone on the inhibition of cell proliferation were dose-dependent. After 24-hours of gemcitabine treatment, pre-incubation of pancreatic cancer cells with IFN-β resulted, already at low concentrations of gemcitabine (up to 0.05 ng/ml), in a statistically significant more potent inhibition of cell proliferation, compared to cells treated with gemcitabine alone (inhibition of cell proliferation by concentrations ≥ 0.5 ng/ml). Data are shown in Figure 1.

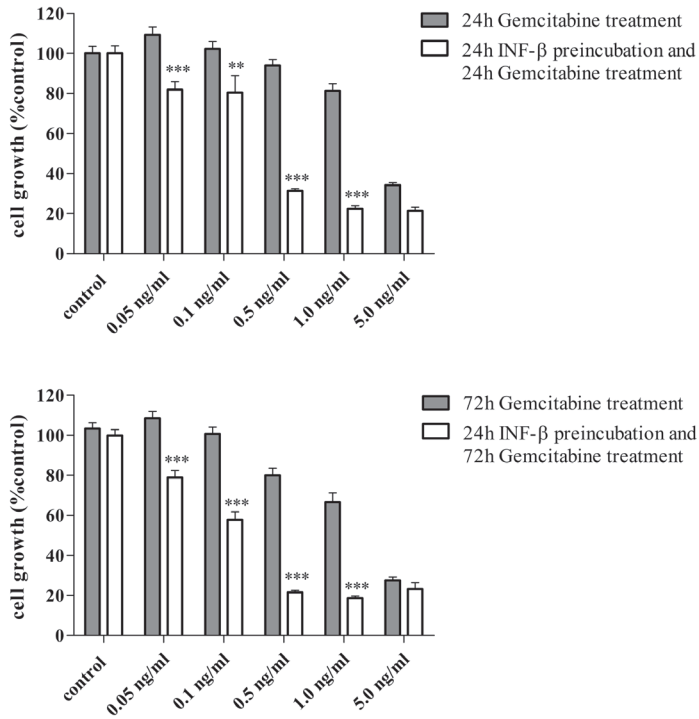

Figure 1. Effects of treatment with gemcitabine alone (grey bars) or after 24 hours of pre-incubation with IFN-β (white bars) on human pancreatic cancer cell line BxPC-3 cell proliferation. The cells were pre-incubated without or with 1000 IU/ml of IFN-β and subsequently with increasing concentrations of gemcitabine during 24 hours (upper figure) or 72 hours (lower figure). After 3 days of treatment cells were harvest for DNA measurement. Values are expressed as the percentage of control and represent the mean ± SEM of at least 2 independent experiments in quadruplicate. **p<0.01 and ***p<0.001 *versus* gemcitabine alone treatment.

3.2 Effects of IFN-β mono- and combination therapy *in vivo*

The effects of IFN-β therapy, alone, or in combination with gemcitabine, were investigated in a heterotopic subcutaneous pancreatic carcinoma mouse model. Treatments with gemcitabine or IFN-β alone, or in combination with gemcitabine, were well tolerated as determined by maintenance of body weight. After 4 weeks of treatment no significant differences were found in body weight between the treatment groups (Table 1).

Table 1. Treatment of subcutaneous heterotopic human pancreatic tumors in nude mice.

	Completion	Tumor volume (mm^3)		Tumor weight (g)		Body weight (g)	
Treatment groups	**of treatment**	Median	Range	Median	Range	Median	Range
Control	5/8[a]	798	524-902	576	373-790	28	25-30
IFN-β	7/8[a]	497	227-1179	468	161-852	27	25-29
gemcitabine	5/7[a+b]	518	210-1225	780	248-1121	25	25-30
IFN-β + gemcitabine	6/8[a]	293[c]	147-641	418	161-559	27	25-27

[a] Number of mice that completed 4 weeks of treatment. Mice were sacrificed before the end of treatment if the wellbeing of the animal could not be maintained (in all these mice this was due to ulceration of the tumor).

[b] One mice died before the start of the treatment.

[c] $P < 0.05$ *versus* control

However, in the group of mice treated with gemcitabine, one mice was found dead before start of the treatment and one mice at the end of the total treatment cycle. Due to organ and tumor lysis necropsy was not possible anymore.

The time course of the growth of tumor volume is depicted in figure 2a. Compared to the control, after 4 weeks of treatment with IFN-β and gemcitabine, a significant reduction

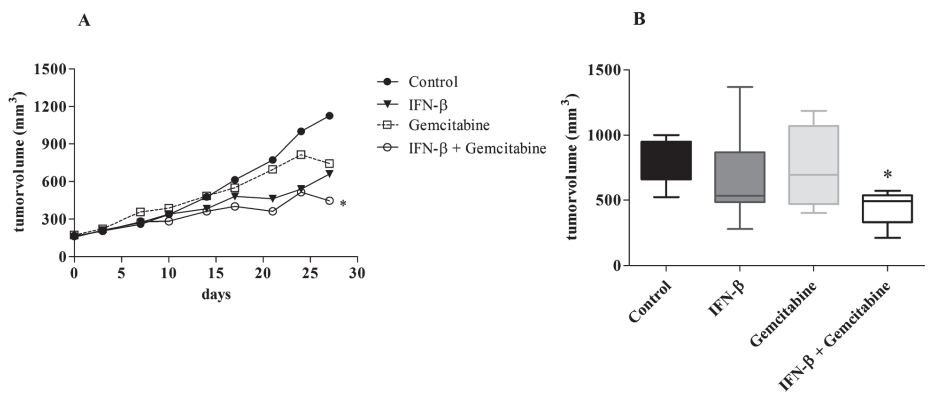

Figure 2: 2a: Time course change in estimated tumor volume of the subcutaneous injected BxPC-3 tumor cells in nude mice. Mice received intraperitoneal injections of 0.9% NaCL (closed circles), $1{,}5x10^5$ IU of IFN-β (closed triangle), 40mg/kg gemcitabine (open squares) or the combination of $1{,}5x10^5$ IU of IFN-β and 40mg/kg gemcitabine (open circles). Figures represent the mean. * $p<0.05$ *versus* control. **2b:** After 4 weeks of treatment mice were sacrificed and tumor volume was measured. Figures represent the mean ± SEM. * $p<0.05$ *versus* control.

of tumor volume by 55% was found (Table 1 and Figure 2b). No statistically significant differences were found regarding tumor weight.

All tumors expressed Ki-67 and cleaved caspase-3. Compared to the control, no differences were found in Ki-67 and cleaved caspase-3 expression in the tumors of mice treated with IFN-β and gemcitabine alone. In tumors of mice treated with IFN-β and gemcitabine alone, and in combination, there was a statistically significant reduction in the proportion of proliferation, Ki-67 positive cells (Figure 3, left panel; $p<0.01$). Additionally, a trend towards an increased apoptosis was found, which was most evident in the tumors of mice treated with the combination of both drugs (Figure 3, right panel).

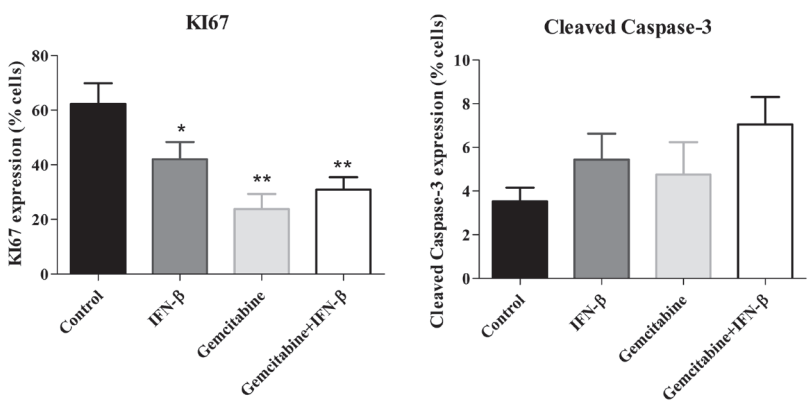

Figure 3. Immunohistochemical analysis of cleaved caspase-3 and Ki-67 expression in the subcutaneous human pancreatic tumors in nude mice that developed after the injection of BxPC-3 tumor cells. The left figure shows the percentage of Ki-67 positive cells, representing the proportion of proliferating cells, the right figure shows the percentage of positive cells expressing cleaved caspase-3, representing the proportion of apoptotic cells, in the different treatment groups. Values are expressed as the percentage of total cells and represents the mean ± SEM of at least 3 different areas within the tumor and the score of two independent investigators. * $p< 0.05$ and ** $p<0.01$ *versus* control.

3.3 Effects of IFN-β mono- and combination therapy *ex vivo*

In order to attempt to use a model that might predict the effects of the treatment with IFN-β, gemcitabine and the combination *in* vivo, an *ex vivo* precision cut tissue slice model was used.

Four xenograft tumors of untreated mice were used to create the tissue slices. Slices were incubated for 72-hours with IFN-β, gemcitabine and the combination of gemcitabine and IFN-β. After 72 hours of culture, small foci of necrosis were observed in all slices, however, in the slices treated with the combination of gemcitabine and IFN-β a clear increased loss of tissue structure and a decrease of the amount of viable cells was observed (Figure 4).

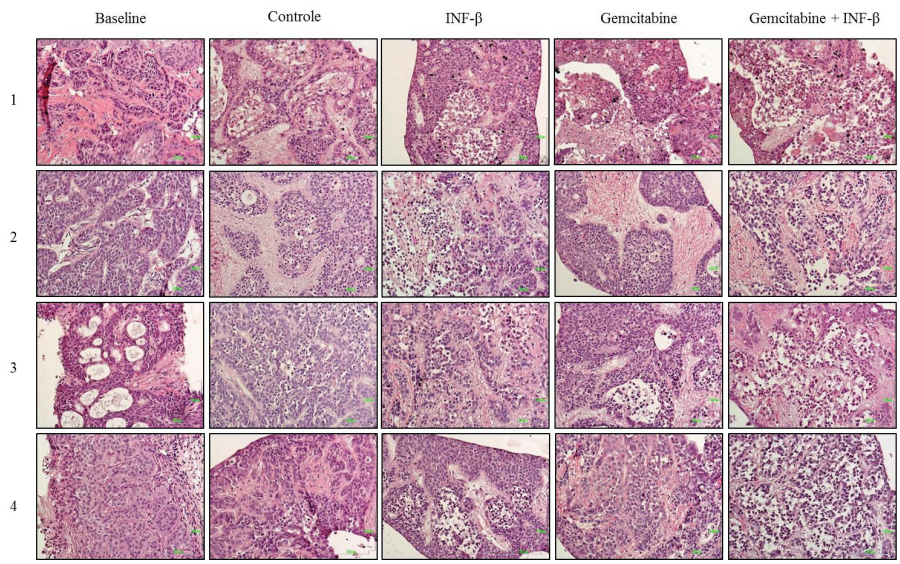

Figure 4. Haematoxylin & eosin staining of representative tissue slides of human pancreatic cancer xenograft tissue slices (mice 1-4) collected at baseline, and after 72 hours of incubation with 0.9% NaCL (vehicle), 1000 IU/ml INF-β, 1 ng/ml gemcitabine, or the combination of IFN-β and gemcitabine. Original magnification x200.

4. DISCUSSION

With an estimation of 165.100 new cases and 161.800 estimated deaths, pancreatic cancer is the fourth leading cause of cancer related death in the Western world. Despite multiple different chemo- and radiotherapeutic treatment strategies that have been attempted, the outcome of patients with pancreatic cancer remains poor with a total overall survival of less than 6%[1,2]. Although promising results have been reported regarding IFN-α therapy in the adjuvant treatment of pancreatic cancer, the use of IFN-α is disputable as treatment toxicities were notably high and the only randomized study so far did not show any significant

differences between treatment groups[3-7]. Nevertheless, several studies, including a study by our research group, demonstrated potent anti-cancer effects, already at low concentrations of IFN-β, and it has been described that IFN-β has a higher binding affinity for the receptor complex compared to IFN-α[8-13]. In this respect, adjuvant IFN-β therapy might be promising. As such, the main aim of the present study was to evaluate the effect of IFN-β, alone and in combination with the chemotherapeutic agent gemcitabine in a heterotopic pancreatic cancer mouse model.

Based on the response to type I IFNs and the amount of IFN receptors expressed we choose to use the human pancreatic cancer cell line BxPC-3[12]. First, we evaluated the effect of IFN-β in combination with gemcitabine *in vitro* and demonstrated a potent chemosensitizing effects of IFN-β. Already after 24 hours of pre-incubation with IFN-β the synergistic cell growth inhibitory effects of combined gemcitabine treatment became apparent. Such synergistic anti-tumor effects of IFN-β and gemcitabine, even in pancreatic cancer cells with low IFN receptor expression, were demonstrated as well in a more comprehensive study by Tomimaru et al., [11]. However, the *in vivo* effects of IFN-β combined with gemcitabine were not investigated in this study.

By using a heterotopic subcutaneous pancreatic cancer mouse model the effects of IFN-β, alone and combined with gemcitabine, were determined. Regarding *in vivo* research, the most frequently used concentration of gemcitabine varies between 100 mg/kg and 125 mg/kg[17,18]. Nevertheless, based on the previously described *in vitro* findings we decided to reduce the concentration of gemcitabine and used a suboptimal concentration of 40 mg/kg.

As expected, given this suboptimal treatment dose, no statistically significant decrease of tumor volume or weight was found in tumors of mice treated with gemcitabine alone. Additionally, despite the potent anti-tumor effects *in vitro,* no difference was found in the tumors of mice treated with IFN-β alone, however, there was a clear trend towards a smaller tumor volume. Although IFN-β concentrations were not measured in this study, it may be possible that the circulating concentration of IFN-β was not sufficient. This may be related to the relatively short half-life of IFNs in the circulation[19,20]. *In vitro,* the concentration of IFN-β required to reduce cell growth to 50% in a large series of pancreatic cancer cell lines ranged between 70-1000 IU/ml[12]. Regarding the BxPC-3 cell line, the concentration required to reduce cell growth with 50% *in vitro* was 114 IU/ml. These concentrations are not easily reached (4-0 IU/ml after four doses of 18 MIU IFN-β at 48-h intervals in serum of human healthy volunteers after s.c. administration)[19]. Furthermore, the potent direct anti-tumor activities of type I IFNs can be limited by the activation of several survival pathways, like the

induction of the JAK2/STAT-3 pathway, the activation of nuclear factor kappa-beta (NF-κB) and the increased expression of the epidermal growth factor receptor (EGF-R). This could results in the stimulation of cell proliferation, malignant transformation and invasion and the inhibition of apoptosis[21,22].

Gemcitabine is a cell-cycle specific inhibitor of DNA synthesis and is still the golden standard chemotherapeutic agent in the adjuvant treatment of pancreatic cancer[23]. Besides the induction of apoptosis and the inhibition of proliferation interferons are also able to induce a G0-G1 arrest, to prolong the S-phase transition and to block cells in G2/M and thereby sensitize tumor cells for chemo- and radiotherapy[24,25]. After 30 days of treatment, compared to the control, we observed a significant synergistic effect of the combined therapy of IFN-β and gemcitabine which was reflected in the reduction of tumor volume and, additionally, in a decreased proportion of proliferating tumor cells. Furthermore, a clear trend towards increased apoptosis (increased number of cleaved caspase-3 positive cells) was found in tumors of mice treated with gemcitabine and IFN-β.

Despite being the backbone in adjuvant pancreatic cancer treatment, response rates of gemcitabine are less than 20%[23]. Therefore it would be desirable to predict the response to IFN-β and gemcitabine therapy before start of the treatment and prevent unnecessary treatment toxicities. In this respect the *ex vivo* tissue slice model, already demonstrated by several other groups, seems very promising[26-29]. The advantage of this model is the ability to evaluate multiple treatments in one tumor sample. Furthermore, by the use of an automatic tissue slicer, comparable slices in size and viability can be obtained without tissue damage at baseline. In this study we were able to maintain viable slices up to 4 days of culture. This is in agreement with findings of two other studies who reported good cell viability at 72 hours even, independent of medium formulation, up to 6 days[26,28]. As nicely demonstrated by the HE-stained slides, a clear decrease in tissue structure was observed in the slices treated with the combination of gemcitabine and IFN-β. This was not observed in the vehicle treated slices, indicating that the loss of tissue structure is very likely an effect of the treatment. Despite the fact that these results seems promising, some major challenges (i.e. number and size of available tumors ,determination of compound concentration and validation of the effects) needs to be elaborated in the future before this technique can be used in the clinical setting. Nevertheless, for future pancreatic cancer research this technique seems promising.

Our study has its limitations as well. Firstly, the mouse model used was a heterotopic subcutaneous model. Although heterotopic models are often used in cancer research, it will

be important to evaluate the effects of IFN-β and gemcitabine in an orthotopic model as well. Especially since type I interferons are known to have immunoregulatory activities and interact with cells representing the host immune response. However, the role of IFN-β regarding cancer immunoediting could not be studied in the model of athymic mice or in the *ex vivo* tissue slice model. Currently, much research efforts are focused on the development of immunotherapeutic strategies[30,31] and therefore it will be interesting to evaluate the immunomodulatory host effects of IFN-β combination therapy. As mentioned previously, a challenge of IFN therapy remains the relatively short half-life of IFNs in the circulation. However, advantage can be gained from PEGylated forms of IFN-α and –β, in which lower and less frequent doses are required, compared to the conventional IFNs. The PEGylated form of IFN-α has already proven to be effective in the treatment of grade III melanoma[32] and metastatic renal cell carcinoma patients[33]. Recently, a PEGylated form of IFN-β has been developed which, 20 hours after a single s.c. dose of 3.0 MIU/kg, resulted in monkeys in a serum concentration of 100 IU/ml. Currently this form of PEGylated IFN-β is tested in a phase III clinical trial (ADVANCE) in patients with multiple sclerosis[34,35].

In conclusion, to the best of our knowledge, this is the first study that determined the effects of IFN-β alone and in combination with gemcitabine on human pancreatic cancer cells in three different experimental models. Although *in vivo* no statistically significant effects on tumor volume were found by IFN-β or gemcitabine monotherapy, a potent synergistic anti-tumor effect of the combination treatment with IFN-β and gemcitabine was observed *in vitro, in vivo* and *ex vivo*. These anti-tumor effects were already present at low concentrations of gemcitabine. However, in order to demonstrate the potent anti-tumor activities of combined gemcitabine/IFN-β therapy in the clinical setting, prospective studies are necessary.

ACKNOWLEDGMENTS

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5

Type I Interferon receptor expression in human pancreatic and periampullary cancer tissue

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ABSTRACT

Objectives: Interferons (IFNs) have several anticancer mechanisms. A number of clinical trials have been conducted regarding adjuvant IFN-alpha therapy in pancreatic cancer. Type-I IFNs exert their effect via the type-I IFN receptor (IFNAR-1, IFNAR-2c). The aim of the present study was to determine the type-I IFN receptor expression in pancreatic and periampullary cancer tissues and to study its relation with clinicopathological factors.

Methods: Receptor expression was determined by immunohistochemistry in paraffin embedded cancer tissue of 47 pancreatic and 54 periampullary cancer patients.

Results: 91.5% of the pancreatic and 88.9% of the periampullary tumors showed expression of IFNAR-1, of which 23.4% and 13.0% were strongly positive, respectively. Regarding IFNAR-2c expression, 68.1% of the pancreatic and 68.5% of the periampullary tumors were positive, of which 4.3% of the pancreatic and none of the periampullary tumors had a strong expression. No statistically significant associations were found between type-I IFN receptor expression and clinicopathological factors or survival.

Conclusions: Type-I IFN receptors are expressed in pancreatic and periampullary cancer tissue although with great inter- and intratumoral variability. A small proportion of both tumors showed a strong expression of the IFNAR-1, only a very small percentage of the pancreatic tumors showed strong expression of the IFNAR-2c.

1. INTRODUCTION

Pancreatic cancer, with 165.100 estimated new cases and 161.800 estimated deaths, is one of the most devastating malignant diseases in the western world[1]. It carries a dismal prognosis with an overall 5-year survival rate of less than 6%. Surgery is the only curative therapy but, due to locally advanced or metastatic disease, only 15-20% of the patients are eligible for resection at time of presentation. After successful surgery, prognosis remains poor mostly due to the aggressive local growth and rapid development of metastasis. Adjuvant therapy is necessary to improve survival but up till now chemo- and radiotherapy as additional treatments have proven to be of limited benefit[2]. Therefore research has focused on other treatment modalities, like adding biological modulators such as type I interferons.

Interferons (IFNs) are known to have anti-proliferative, antiviral and immunoregulatory activities. They are able to induce apoptosis and exert cell cycle blocking. Besides that they are able to sensitize tumor cells for chemo and radiotherapy[3,4]. Type I IFNs (e.g. IFN-α and IFN-β) act via the type I IFN receptor (IFNAR) complex of which IFNAR-1 and IFNAR-2c are the most important subunits[5].

Although there was little in vitro knowledge on the mechanisms of action of interferons in pancreatic cancer, several clinical studies have been conducted regarding IFN-α as additional adjuvant treatment. Investigators from the Virginia Mason Medical Center included 43 patients to interferon based combination therapy (5-FU, cisplatin, interferon-α, and radiotherapy) and reported a tremendous increase in the 2 and 5-year survival (respectively 64% and 55%) [6]. Several other institutions, all single treatment-arm studies, hoped to confirm these encouraging data but only three of them reported similar findings. Besides that, in the majority of the patients grade 3+ toxicities were observed[7-11]. The only phase III trial evaluating this adjuvant regimen was the CapRI (Combined Chemoradioimmunotherapy for Pancreatic Adenocarcinoma) trial[12]. This study showed a longer median survival in the experimental arm, although this survival benefit was not statistically significant. This difference in median survival of 3,6 months implies that some patients probably benefited from the experimental treatment, even though 85% of these patients experienced grade 3 or 4 toxicities.

In order to accomplish a direct effect of type I IFNs, the presence of the type I IFN receptor is necessary[3,5]. Surprisingly; in none of the patients treated with adjuvant IFN-α in clinical trials, the tumoral type I IFN receptor expression was known. Furthermore, no clear distinctions were made regarding cancer origin. Periampullary cancers, like distal bile duct and ampullary carcinomas, arise in the same anatomical area and are subjected to the

same surgical procedure as pancreatic cancers. Nevertheless, these cancers have different prognosis and therefore the distinction between pancreatic and periampullary cancers is of upmost importance [13,14]. Knowledge of type I IFN receptor expression in pancreatic and periampullary cancer tissue is of great importance and may prevent unnecessary, often toxic, treatments in these cancer patients. Therefore, this study evaluated the type I IFN receptor expression in tissue of pancreatic as well as in tissue of periampullary cancer patients. Additionally, we determined whether the level of IFN receptor expression was associated with clinicopathological factors and outcome.

2. PATIENTS AND METHODS

2.1 Patient population

Paraffin embedded cancer specimens of 168 patients with pancreatic or periampullary (ampullary or distal bile duct) cancer, who underwent intentional curative surgery in the Erasmus Medical Centre between the period of January 2000 and January 2007, were obtained, allowing a follow-up of at least five years. Pancreatic head specimens were carefully selected and revised by an experienced pathologist (KB). Tumor origin was based on the anatomical relationship of the center of the tumor mass, and presence of precursor lesions, to the ampulla, common bile duct or pancreas[14]. After revision cancer specimens of 20 patients were excluded due to indefinable tumor origin. A total of 72 pancreatic cancer patients and 76 periampullary cancer patients remained of which 25 tissue blocks of pancreatic cancer patients and 22 tissue blocks of periampullary cancer patients were not available for adequate analysis (Fig. S1 and Supplemental Legend, supporting information, at http://links.lww.com/MPA/A321).

In the period between January 2000 and January 2007, 31 patients of our series (14 pancreatic cancer and 17 peri-ampullary cancer patients) were randomized to the treatment arm of a trial combining adjuvant intra-arterial chemotherapy in combination with radiotherapy. Patients received 6 cycles of 5 treatment days, with an interval of 4 weeks between each cycle, of intra-arterial mitoxantrone (10mg/m^2, day 1), folinic acid (170 mg/m^2 day 2 and 4), 5-fluorouracil (600mg/m^2 day 2 and 4) and cisplatinum (60 mg/m^2 day 5). Two weeks after the first cycle of chemotherapy, 6 weeks of radiotherapy (total dose of 54 Gray (Gy) in single doses of 1.8Gy/day, 5 days a week) was started. The results and details of this trail have been described elsewhere[15]. Clinical and pathological information was derived from medical records. Tumors were staged according to the 7th edition of the

American Joint Committee Guidelines on cancer staging issued in 2009. Tumor histology was classified as well, moderately and poorly differentiated. At the time of the present report, the median follow-up duration of the pancreatic cancer patients was 13 months (ranging from 1 to 115 months), the median follow-up duration of the periampullary cancer patients was 21 months (ranging from 4 to 141 months). The study was approved by the medical ethical committee of the Erasmus Medical Center.

2.2 Immunohistochemistry

Formalin fixed, paraffin embedded sections (5µm thick) were deparaffinized and rehydrated, followed by heat induced epitope retrieval (HIER) using the PT Link, pretreatment module for tissue specimens (Dako Denmark, Glostrup, Denmark), rinsed with TRIS/Tween 0.5% (pH 8.0) and blocked for 15 minutes in hydrogen peroxide 3% (final concentration) in phosphate buffered saline (PBS). After blocking the sections were washed with TRIS/Tween 0.5% and incubated with the primary antibodies for IFNAR-1 and IFNAR-2c (both overnight at 4° C). Negative control for the immunohistochemistry included omission of the primary antibody. To visualize the bound antibody a two-step procedure was conducted using the Dako Real EnVision Detection System kit (Dako Detection System, Dako Denmark, Glostrup, Denmark). Briefly, the sections were incubated for 30 minutes with three drops of horseradish peroxidase rabbit/mouse and visualized with freshly prepared DAB+ Chromogen twice for 5 minutes in the dark. Slides were counterstained with hematoxylin and coverslipped. The mouse monoclonal anti-IFNAR-1 antibody (Sigma Aldrich, Saint Louis, MO, USA) was used at a dilution of 1:4800. The specificity of the IFNAR-1 antibody was demonstrated by western blotting (Fig. S2 and Supplemental Legend, supporting information, at http://links.lww.com/MPA/A321). The anti-IFNAR-2c mouse monoclonal antibody (kindly provided by Dr. E. Croze, International review of investigational science, Lafayette, CA, USA) was used at a dilution of 1:800. The use and specificity of the IFNAR-2c antibody has been described previously[3,16] and demonstrated by western blotting (Fig. S2 and Supplemental Legend, supporting information, at http://links.lww.com/MPA/A321).

2.3 Immunohistochemical analysis

Slides were examined and scored by a pathologist blinded to both clinical and pathological data. The semi-quantitative analysis of the stained slides in this study was done based on the Immunoreactivity score (IRS) described by Remmele and Stegner[17] and according to the modification of McCarty et al[18-20]. Briefly, the IRS is calculated by the product of percentage

of positive cells (>80%: 4; 51-80%: 3; 10-50%: 2; <10%: 1 and 0%: 0) and the intensity of the staining (strong: 3; moderate: 2; mild: 1 and no staining: 0). A score of 0-1(IRS 0) was considered as negative, 2-3 as weak positive (IRS 1), 4-8 moderate positive(IRS 2) and 9-12 strongly positive (IRS 3) (Table S1, supporting information, at http://links.lww.com/MPA/A323).

2.4 Statistical analysis

Data were analyzed using SPSS version 20.0 for windows. To evaluate differences in distribution of clinicopathological parameters and to determine the relationship between these parameters and the expression of the IFNAR-1 and IFNAR-2c, linear regression, the chi-square or fisher exact test, when appropriate, was used. Overall survival (OS) was defined as the number of months from resection to death of any cause. The probability of overall survival was calculated according to the Kaplan-Meier method and compared with the log-rank test. For univariate and multivariate analysis the Cox proportional hazard model was used. Only variables with a p-value of 0.10 or less on the univariate analysis were incorporated into the multivariate model. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Patient population

For analysis 47 pancreatic cancer paraffin embedded blocks and 54 periampullary cancer paraffin blocks (35 ampullary cancer and 19 distal bile duct cancer patients) were available (Fig S1, supporting information).

The mean age of patients with pancreatic cancer was 64 years, ranging from 33 to 79 years. Regarding the periampullary cancer patients the mean age was 65 years, ranging from 39 to 81 years. Patient and tumor characteristics were similar among the pancreatic and periampullary cancer patients except for a significant difference in primary tumor staging (more T3 pancreatic cancer tumors and more T4 periampullary cancer tumors; $p<0.001$), a borderline significant difference in resection margins (more R1 resections in pancreatic cancer patients; $p=0.05$) and a surprisingly modest difference in observed perineural invasion (more perineural invasion in periampullary cancer patients; $p=0.05$) (Table 1)

Table 1. Patients profiles

Clinicopathological factors	Pancreatic cancer n=47(46.5%)	Periampullary cancer n=54 (53.5%)	p value
Age (range), yrs	64 (33-79)	65(39-81)	0.76
Gender			0.68
Male	27 (57.4)	34 (63.0)	
Female	20 (42.6)	20 (37.0)	
Adjuvant therapy			1.00
Yes	14 (29.8)	17 (31.5)	
No	33 (70.2)	37 (68.5)	
Type operation			0.16
Whipple	12 (25.5)	13 (24.1)	
PPPD	32 (68.1)	41 (75.9)	
Other	3 (6.4)	0 (0.0)	
Location			n.a.
Pancreatic head	44 (93.6)		
Pancreatic body	1 (2.1)		
Pancreatic tail	2 (4.3)		
Ampulla		35 (64.8)	
Distal bile duct		19 (35.2)	
Diameter (cm)	2.63	2.35	0.15
Primary tumor (pT†)			0.001*
T1	1 (2.1)	4 (7.4)	
T2	6 (12.8)	11 (20.4)	
T3	38 (80.9)	25 (46.3)	
T4	2 (4.3)	14 (25.9)	
Grade			0.71
Well differentiated	2 (4.3)	4 (5.7)	
Moderately differentiated	33 (70.2)	33 (68.6)	
Poorly differentiated	12 (25.5)	16 (25.7)	
Regional lymph nodes (pN†)			0.84
N0	17 (36.2)	4 (33.3)	
N1	30 (63.8)	36 (66.7)	

Table 1. Patients profiles (continued)

Clinicopathological factors	Pancreatic cancer n=47(46.5%)	Periampullary cancer n=54 (53.5%)	p value
Resection margins			0.05
R0	33 (70.2)	47 (87.0)	
R1	14 (29.8)	7 (13.0)	
Stage†			0.50
0-I	3 (6.4)	6 (16.7)	
II-III	44 (93.6)	30 (83.3)	
Vascular invasion			0.31
Positive	11 (23.4)	8 (14.8)	
Negative	36 (76.6)	46 (85.2)	
Perineural invasion			0.05
Positive	32 (68.1)	26 (48.1)	
Negative	15 (31.9)	28 (51.9)	
IFNAR-1 expression			0.59
Negative	4 (8.5)	6 (11.1)	
Positive, mild	12 (25.5)	14 (25.9)	
Positive, moderate	20 (42.6)	27 (50.0)	
Positive, strong	11 (23.4)	7 (13.0)	
IFNAR-2c expression			0.19
Negative	15 (31.9)	17(31.5)	
Positive, mild	12 (25.5)	22 (40.7)	
Positive, moderate	18 (38.3)	15 (27.8)	
Positive, strong	2 (4.3)	0 (0.0)	
IFNAR-1 + IFNAR-2c			0.08
Negative	23 (48.9)	27 (50.0)	
Positive, mild	9 (19.1)	19 (35.2)	
Positive, moderate	14 (29.8)	8 (14.8)	
Positive, strong	1 (2.1)	0 (0.0)	
Cancer related death			0.12
Yes	33 (71.7)	37 (84.1)	
No	5 (10.9)	3 (6.8)	
Complications	4 (8.7)	2 (4.5)	
Unknown	4 (8.7)	2 (4.5)	

† Based on American Joint committee on cancer. AJCC cancer staging manual. 7th Ed.

* P-values were calculated by linear regression, chi-square or fisher exact test

3.2 Interferon receptor expression

Both IFNAR-1 and IFNAR-2c were heterogeneously expressed within pancreatic and periampullary cancer tissue. For each receptor, both cytoplasmic and membrane staining was observed (Fig. 1a).

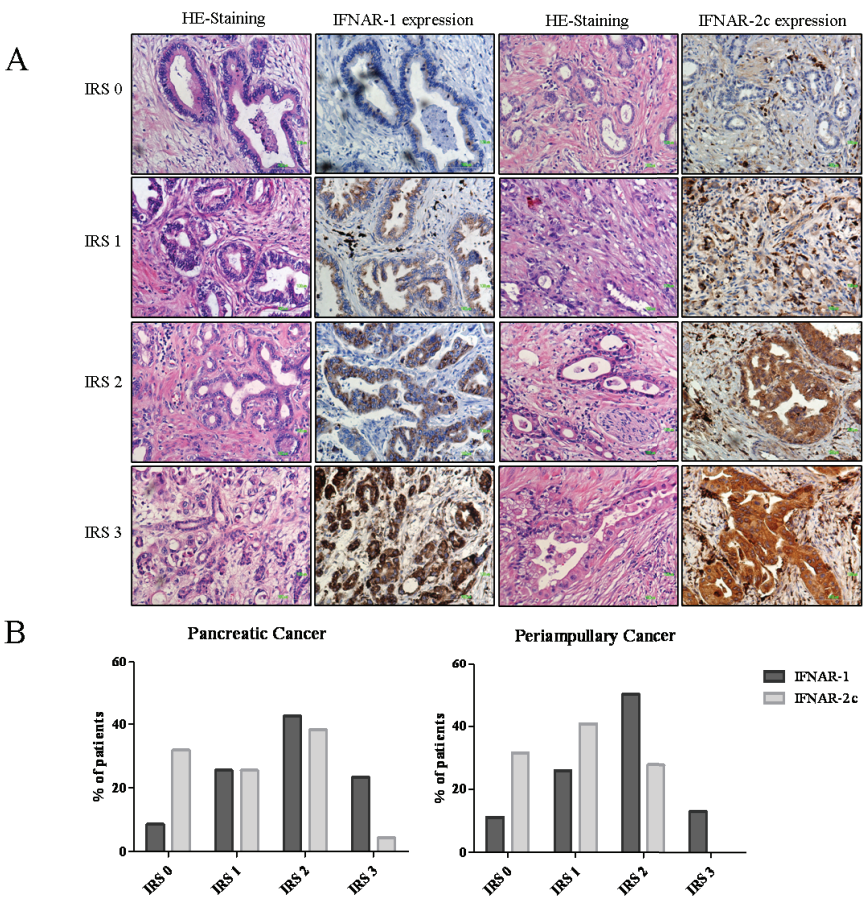

Figure 1: Immunohistochemical analysis of IFNAR-1 and IFNAR-2c expression in pancreatic and periampullary cancer tissue. **1a**: Examples of tumors from pancreatic cancer patients with IRS0, IRS1, IRS2 or IRS3 staining of IFNAR-1 and IFNAR-2c. The tumors show heterogeneous expression of the IFNAR-1 and IFNAR-2c receptors. **1b:** Distribution of IFNAR-1 (dark-grey bars) and IFNAR-2c (light-grey bars) immunopositivity in tumors of 47 pancreatic cancer patients (left) and 54 periampullary cancer patients (right).

Regarding the IFNAR-1 expression, in the pancreatic cancer patients group 8.5% (4/47) of the tumors had no expression, 25.5% (12/47) had a weak expression, 42.6% (20/47) had a moderate expression and 23.4% (11/47) of the tumors had a strong expression. In the group of patients with periampullary cancer this was 11.1% (6/54), 25.9% (14/54), 50.0% (27/54) and 13.0% (7/54) respectively. The expression of the IFNAR-2c in tumors of pancreatic cancer patients was in 31.9% (15/47) negative, 25.5% (12/47) weak, 38.3% (18/47) moderate and in 4.3% (2/47) strong. In the group of patients with periampullary cancer this was 31.5% (17/54), 40.7% (22/54), 27.8% (15/54) and 0% (0/54), respectively (Fig.1b). No significant correlation was observed between IFNAR-1 and IFNAR-2c expression. Additionally, it is notable that the immune cells, probably macrophages, surrounding the tumor had a very strong staining for both receptors, in particularly the IFNAR-2c receptor (Fig. 2). Only one tumor of a pancreatic cancer patient was found with a very strong IFNAR-1 and IFNAR-2c expression (IRS 3) (Fig.2).

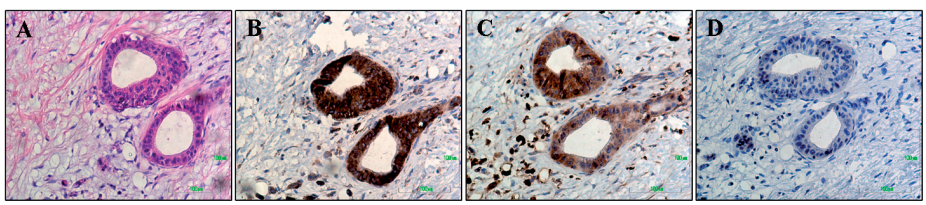

Figure 2: Representative case of pancreatic cancer tissue with high IFNAR-1 (panel B) and IFNAR-2c (panel C) staining (both IRS3). Hematoxylin & eosin (HE) staining (A) and negative control (D). Original magnification x200

3.3 Clinicopathological associations

IFNAR-1 and IFNAR-2c receptor expression was associated with age, gender, tumor location (pancreatic head, ampulla or distal bile duct), tumor diameter, tumor grade (well, moderately and poorly differentiated), tumor (T) and lymph node (N) staging, resection margins, tumor stage, vascular invasion and perineural invasion. No significant correlations were found between the above-mentioned factors and receptor expression in tumors of both pancreatic as periampullary cancer patients (data not shown). Since the type-I IFN receptor complex is composed of the IFNAR-1 and IFNAR-2c we also determined the associations between any of these factors and the combination of IFNAR-1 and IFNAR-2c. None of the clinical or pathological factors were associated with the combination of IFNAR-1 and IFNAR-2c. Furthermore, no statistically significant differences in IFNAR-1 and/or IFNAR-2c receptor expression were found between the pancreatic and the periampullary cancer patients.

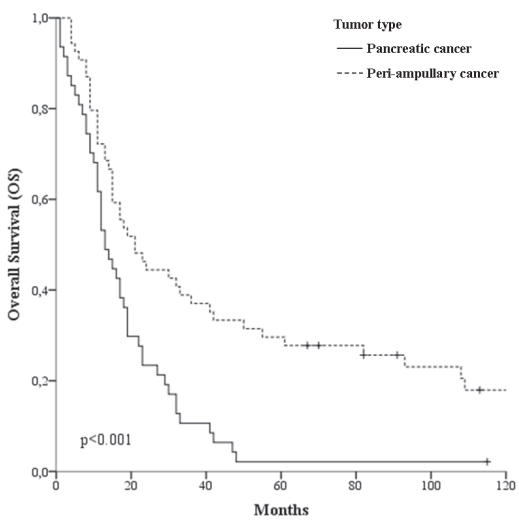

Figure 3. Kaplan Meier curves of overall survival (OS) of patients with pancreatic cancer (straight line) and periampullary cancer (dotted line) show a significant survival benefit favoring patients with periampullary cancer ($p<0.001$).

Table 2a. Univariate and multivariate analysis of various prognostic parameters for survival in 47 pancreatic cancer patients.

	Univariate analysis		Multivariate analysis	
Prognostic parameters	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age	1.04 (1.01-1.08)	0.024*	1.03 (0.99-1.06)	0.117
Adjuvant treatment	0.63 (0.32-1.25)	0.190		
Tumor location[a]	1.22 (0.37-3.97)	0.746		
Tumor diameter	1.30 (0.90-1.89)	0.162		
Grade		0.732		
G1	0.70 (0.15-3.23)	0.651		
G2	0.76 (0.38-1.53)	0.321		
G3	1.00			
Primary tumor		0.332		
T1	0.12 (0.01-1.39)	0.089		
T2	0.39 (0.08-2.02)	0.264		
T3	0.47 (0.11-2.01)	0.308		
T4	1.00			
Regional Lymph nodes	1.74 (0.92-3.29)	0.090	1.60 (0.79-3.26)	0.192
Resection margins	1.05 (0.55-2.01)	0.884		
Vascular invasion	1.36 (0.69-2.70)	0.376		
Perineural invasion	0.96 (0.52-1.80)	0.908		
IFNAR-1 score	0.91 (0.83-0.99)	0.033*	0.92 (0.83-1.03)	0.137
IFNAR-2c score	0.94 (0.84-1.05)	0.254	1.05 (0.90-1.20)	0.604

[a] head vs other
* Statistically significant

3.4 Survival analysis

5 years after intentional curative resection, 46 deaths (98%) were observed in the group of pancreatic cancer patients compared to 38 (70%) deaths in the group of periampullary cancer patients. A significant better overall 5-year survival was found in the periampullary cancer group versus the pancreatic cancer group (median survival was 21 vs. 13 months; log-rank p<0.001) (Fig.3).

Factors associated with survival were included in the univariate analysis. Variables with a p-value of 0.10 or less were also incorporated into the multivariate analysis. In the group of 47 pancreatic cancer patients univariate analysis revealed that age (p=0.024) and

Table 2b. Univariate and multivariate analysis of various prognostic parameters for survival in 54 periampullary cancer patients.

	Univariate analysis		Multivariate analysis	
Prognostic parameters	**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age	1.01 (0.98-1.05)	0.369		
Adjuvant treatment	0.59 (0.30-1.15)	0.123		
Tumor location[a]	0.81 (0.43-1.52)	0.512		
Tumor diameter	1.31 (0.96-1.78)	0.085	1.06 (0.73-1.56)	0.752
Grade		0.155		
G1	0.76 (0.25-2.30)	0.625		
G2	0.76 (0.28-1.01)	0.055		
G3	1.00			
Primary tumor		0.033*		0.321
T1	0.10 (0.01-0.81)	0.030*	0.29 (0.03-3.19)	0.312
T2	0.34 (0.14-0.84)	0.020*	0.55 (0.16-1.90)	0.346
T3	0.69 (0.34-1.39)	0.301	1.18 (0.45-3.11)	0.742
T4	1.00		1.00	
Regional Lymph nodes	1.73 (0.89-3.33)	0.104		
Resection margins	1.79 (0.74-4.29)	0.194		
Vascular invasion	2.38 (1.05-5.44)	0.039*	2.11 (0.63-7.07)	0.227
Perineural invasion	1.83 (0.99-3.36)	0.053	1.42 (0.63 -3.24)	0.402
IFNAR-1 score	1.01 (0.93-1.09)	0.906	1.00 (0.90-1.11)	0.941
IFNAR-2c score	0.97 (0.86-1.09)	0.644	0.96 (0.83-1.12)	0.607

[a] ampulla vs distal bile duct
* Statistically significant

IFNAR-1 receptor expression (p =0.033) were significant associated with overall survival. After multivariate analysis no significant correlations were found (Table 2a). In the univariate analysis of prognostic factors for survival in 54 periampullary cancer patients primary tumor stage (T) (p=0.033) and vascular invasion (p=0.039) were significantly associated with survival. After including the eligible prognostic factors into the multivariate model no significant associations were found (Table 2b). Furthermore, Kaplan-Meier survival analysis was preformed but showed no statistically significant survival benefit of IFNAR-1 or IFNAR-2c receptor expression in both pancreatic and periampullary cancer patients (figure not shown).

4. DISCUSSION

Type I IFNs (e.g. IFN-α, -β) are cytokines that are able to modulate several anti-tumor defense processes like inducing apoptosis, cell cycle blocking and sensitizing tumor cells for chemo- and radiotherapy[3,4,21,22] Currently, IFN-α have been used clinically for the treatment of several malignancies like chronic myeloid leukemia, metastatic melanoma, renal cell carcinoma and Kaposi sarcoma[23,24] IFN-β is only used in the treatment of multiple sclerosis[25].

Type I IFNs act via the type I IFN receptor complex which is composed by two subunits; IFNAR-1 and IFNAR-2 of which there are three isoforms that are differently spliced from a common gene. IFNAR-2a is the soluble form and can act as a dominant negative regulator of free IFNs, IFNAR-2b is a shorter form lacking regions of the cytoplasmic domain and unable to activate JAK-STAT signaling once the receptor binds IFNs. IFNAR-c contains the entire cytoplasmic domain and along with IFNAR-1 make up a functional IFN receptor complex, binding IFNs and inducing JAK-STAT signaling[5,26].

A number of clinical studies have been conducted regarding adjuvant IFN-α therapy in the treatment of pancreatic cancer[6-8,10,12,27] The study of Picozzi et al. reported a remarkable 2- and 5-year survival of respectively 64% and 55%. Three studies showed similar findings, but they were all non-randomized studies[7,9,10]. Furthermore in all studies the majority of treated patients suffered from severe treatment toxicities. Although the importance of IFN receptor expression for the anti-cancer effect of IFNs has been described for fibrosarcoma, melanoma, breast cancer and hepatocellular cancer cells, IFN receptor status was not known in any of the patients receiving adjuvant IFN-α treatment [3,28]. In addition, in a recent study we demonstrated, in a panel of 11 human pancreatic cancer cell lines, that the maximal inhibitory effect of IFN-α is positively correlated with the IFNAR-1 mRNA and IFNAR-2c mRNA and protein expression[29] As a result, it is possible that the lack of IFN receptor expression resulted in a non-response to adjuvant IFN-α treatment.

The aim of this study was therefore to determine the type I IFN receptor expression in tissue of pancreatic as well as in tissue of periampullary cancer patients (ampullary and distal bile duct cancer), since the latter is accounting for 53% of the surgical operable pancreaticoduodenal tumors[30]. Furthermore, we correlated the interferon receptor status with clinicopathological factors and overall survival.

As described in literature, our study confirms that periampullary cancer patients have a significant favorable survival compared to pancreatic cancer patients. Furthermore, primary tumor stage is of prognostic value in patients with pancreatic cancer. In our study we found a trend towards a better prognosis in patients with a lower tumor stage, however, this did

not reach statistical significance. This might be explained by the relative low number of patients in the pT1 and pT4 group. Although we found a considerable inter- and intratumoral variability of type I IFN receptor expression in the pancreatic and periampullary cancer tissue, no statistically significant differences were found in the level of interferon expression between the pancreatic and periampullary cancer patients. The survival benefit of periampullary cancer patients can therefore not be explained by a discrepancy in interferon receptor expression.

In the group of pancreatic cancer patients 91.5% of the tumors had a positive expression of the IFNAR-1, of which 23.4% was strongly positive. Regarding IFNAR-2c expression, 68.1% of the tumors showed a positive receptor expression, of which only 4.3% was strong positive. In the group of periampullary cancer patients, positive IFNAR-1 expression was found in 88.9% of the tumors of which 13% was strong positive. Regarding IFNAR-2c expression, 68.5% of the tumors in these patients positively expressed this receptor subunit, but none showed strongly elevated IFNAR-2c expression. Finally, expression of the IFNAR-1 and IFNAR-2c subunits did not correlate with any clinical, pathological or prognostic factors evaluated in pancreatic or periampullary cancer patients. Nevertheless, determination of the expression levels of type I IFN receptors in pancreatic cancer can be of value for the selection of patients that might benefit from adjuvant IFN-α therapy. On the other hand, in our above- mentioned recent study we demonstrated that the direct anti-tumor effects of IFN-β compared to IFN-α are much more potent and less dependent on the level of receptor expression[29]. Moreover, van Weerd et al. described recently that IFN-β is able to exert its direct effects via the IFNAR-1 receptor independently of the IFNAR-2c receptor[31]. Taken these observations together and the fact that more tumors of pancreatic and periampullary cancer patients showed a positive, as well as a stronger positive, expression of the IFNAR-1 receptor, compared to the IFNAR-2c receptor, adjuvant IFN-β therapy might be promising. In addition, it should be realized that besides the direct anti-tumor effects, type I IFNs are also known to have several indirect effects, like the activation of the immune system (cytotoxic t-lymphocytes, NK-cells and monocytes), the regulation of cytokine production, as well as the inhibition of angiogenesis (inhibition of endothelial cell proliferation and VEGF transcription and secretion)[32]

Two previous studies investigated IFNAR-2 expression in pancreatic cancer tissue. The study of Ota et al.[33] showed expression of the IFNAR-2 in 25% of the tumors, in the study of Saidi et al.[34] IFNAR-2 expression was found in 24% of the tumors, which are both lower percentages compared with our study. This discrepancy could be due to the use of differ-

ent scoring systems and/or the antibodies that were used. Both studies used a polyclonal antibody to stain and determine the IFNAR-2 receptor. This may be associated with less distinctiveness and a lower target to background ratio compared to the monoclonal antibody used in our study. In addition, in both studies it was not indicated which isoform of the IFNAR-2 receptor was detected with the antibodies that were used.

Evidently, our study has its limitations as well. This study is of retrospective nature which may have caused incomplete and inaccurate documentation. Another limitation can be found in the semi-quantitative immunohistochemical analysis of the tissue sections, which was subjective and thereby limited the comparability. Furthermore, the number of patients in our series is relatively low.

In conclusion, this is the first immunohistochemistry-based study that determined the IFNAR-1 and IFNAR-2c receptor expression in pancreatic and periampullary cancer tissue. IFNAR-1 and IFNAR-2c receptors are expressed in human pancreatic and periampullary cancer tissue although with great inter- intratumoral variability. Only in a small proportion of patients in this study the tumors strongly express the IFNAR-1 and/or IFNAR-2c. In order to demonstrate the relationship between interferon receptor expression and the anti-tumor activities of type I IFNs in pancreatic and periampullary cancer patients, prospective studies are necessary.

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6 General discussion

1. PANCREATIC CANCER

Pancreatic cancer has a dismal prognosis with mortality rates almost equaling the incidence and is characterized by its insidious onset and late diagnosis[1]. Surgery is the only curative therapy, but at time of presentation over 80% of the patients are diagnosed with locally advanced or metastatic disease, excluding them from curative resection. Nevertheless, even after intentional curative radical surgery, due to the high recurrence rate, prognosis remains poor resulting in an overall 5-year survival rate of less than 6%[2]. With the evaluation of the efficacy of adjuvant chemo- and radiotherapy in several clinical trials, an attempt have been made to improve survival. However, since the introduction of gemcitabine survival rates have barely been improved and the role of adjuvant treatment in pancreatic cancer remains an area of conflict[3-5].

It is stated that characteristics of pancreatic cancer such as the tumor stroma, restricted vasculature and a hypoxic environment may prevent delivery of chemotherapy to tumor cells, thereby explaining the limited benefits of adjuvant treatment[6]. Therefore, in order to improve outcome other treatment strategies such as neoadjuvant therapies[7], immune modulating therapies[8], tumor-stroma targeting therapies[9] and the addition of biological response modifiers like interferons[10-14], have been attempted. The last decade several clinical studies provided some evidence that there might be a role for adjuvant IFN-α therapy in the treatment of pancreatic cancer. Despite encouraging data that were reported, the study design of most of the conducted studies was not very refined and in all studies treatment toxicities were notably high[10-13]. Additionally, the only phase III trial evaluating the use of adjuvant IFN-α, the CapRI trial (Combined Chemoradioimmunotherapy for Pancreatic Adenocarcinoma), did not demonstrate a significant survival benefit for patients in the experimental arm. Nevertheless, there was a difference in median survival of 3,6 months which implies that probably some patients benefited from the experimental treatment[14]. However, before start of these studies, certain aspects of interferon therapy, such as the relevance of the interferon receptors and the use of other type I interferons, such as IFN-β, were not addressed. Therefore, the main aim of this thesis was to further elucidate the role of interferon therapy in the treatment of pancreatic cancer.

2. INTERFERONS

Type I IFNs (e.g. IFN-α, IFN-β and IFN- ω) are cytokines that are known to have antiproliferative, antiviral and immunoregulatory activities. Additionally, they are involved in cell differentiation and anti-tumor defense processes. These anti-tumor processes can either be

direct, by the stimulation of apoptosis and blocking of cell cycle, or indirect by the activation of natural killer cells, T-cells and macrophages. Furthermore, type I IFNs are able to sensitize tumor cells for chemo- and radiotherapy by inducing a cell cycle arrest at the G1 phase, or by the prolongation and accumulation of cells in the S-phase[15-17]. Type I IFNs act via the type I IFN receptor complex of which IFNAR-1 and IFNAR-2c are the most important subunits[18,19].

In the early days, IFN-α was approved and used in the treatment of hairy cell leukemia, chronic myelogenous leukemia, multiple myeloma, malignant cutaneous melanoma, Kaposi's sarcoma, gastroenteropancreatic neuroendocrine tumors and renal cell carcinoma[20-26]. However, due to the increased understanding of molecular mechanisms of various types of cancers nowadays IFN-α is primarily used in the treatment of metastatic renal cell carcinoma and malignant melanoma. At present, IFN-β is only approved in the treatment of relapsing remitting multiple sclerosis (RRMS).

3. INTERFERONS IN THE TREATMENT OF PANCREATIC CANCER

Although clinical studies regarding adjuvant IFN-α therapy in the treatment of pancreatic cancer have already been conducted, several aspects of interferon therapy were not addressed. Despite promising results reported regarding IFN-β therapy, very few studies investigated the effect of IFN-β[15,27-29]. Furthermore, the relationship between the amount of type I IFN receptors expressed and the anti-tumor effects of IFN-α and IFN-β in pancreatic cancer cell lines is not established. Therefore, as described in **chapter 2**, we first determined the anti-tumor potencies of IFN-α and –β in a large panel of pancreatic cancer cell lines and assessed the correlation between the responsiveness to type I IFNs and the expression of IFNAR-1 and IFNAR-2c. In this study a considerable variability in both type I interferon receptor expression and in the response to type I IFNs was demonstrated. However, the anti-tumor activity (i.e. induction of apoptosis and inhibition of cell proliferation) of IFN-β was significantly more potent, already at low concentrations, compared to with IFN-α. The maximal inhibitory effect induced by IFN-α was significantly correlated with the level of IFNAR-1 and IFNAR-2c receptors expressed. On the other hand, this was not the case for IFN-β. This can be explained by the fact that IFN-β has a 10-fold higher binding affinity with the receptor complex compared to IFN-α and, additionally, is capable of inducing functional signal transduction via the IFNAR-1, independently of the IFNAR-2c receptor in which a 104 unique set of genes is induced[30,31]. Nevertheless, the potent anti-tumor activities of IFN-β were clearly shown and it is demonstrated that the expression of type I IFN receptors in

pancreatic cancer can be of predictive value in the responsiveness to the growth inhibitory effects of IFN-α.

Given these observations, it is surprising that in none of the patients treated with adjuvant IFN-α in clinical trials, before start of treatment in the resection specimens, the tumoral type I IFN receptor expression was assessed. The knowledge of type I IFN receptor expression is of importance and may prevent unnecessary, often toxic, treatments in these cancer patients. Therefore, in **chapter 5**, the type I IFN receptor expression (i.e. IFNAR-1 and IFNAR-2c) was determined in tissue of pancreatic cancer, as well as in tissue of periampullary cancer patients (ampullary and distal bile duct cancer), since the latter is accounting for 53% of the surgical operable pancreaticoduodenal tumors and are known to have different prognosis[32-34]. Although our study was of retrospective nature and the number of patients were relatively low, this study did demonstrate, for the first time, that both IFNAR-1 as IFNAR-2c receptor are expressed in human pancreatic and periampullary cancer tissue. However, interferon receptors were expressed with great inter-and intratumoral variability and, unfortunately, only a small proportion of the tumors strongly expressed the IFNAR-1 and/or IFNAR-2c. These observations may explain the differences found in the clinical studies regarding adjuvant IFN-α therapy. However, as depicted previously, the anti-tumor effects of IFN-β are more potent and less dependent on the level, and subunit, of receptors expressed. Additionally, it has been demonstrated that IFN-β can induce functional signal transduction, *via* the IFNAR-1, independently of the IFNAR-2c receptor. On this basis, adjuvant IFN-β therapy seems more promising as more tumors of pancreatic and periampullary cancer patients showed a positive, as well as stronger positive, expression of the IFNAR-1 compared to the IFNAR-2c receptor. Nevertheless, since at present it is unknown what the relationship between interferon receptor expression and the anti-tumor activities of type I IFNs in pancreatic and periampullary cancer patients is. Therefore, prospective studies are necessary.

In cancer tissue general, thus including pancreatic cancer, growth factors and their receptors, like insulin and insulin-like growth factor (IGF) receptors are frequently overexpressed[35-37]. In pancreatic cancer, the overexpression of the IGF1 receptor (IGF1R) is even associated with more proliferating and invasive tumors, leading to a poorer survival[38]. Additionally, it is becoming more evident that high levels of insulin can stimulate tumor growth. As such, the role of insulin and the insulin receptor, which is also closely related to the IGF-system and can interact with each other's receptors, should not be neglected[39-41]. Furthermore, as described in previous research, IFN-β is capable of modulating the IGF-

system in neuro-endocrine tumor cells[42]. However, no studies yet evaluated the effects of IFN-β with respect to the IGF and insulin system in human pancreatic cancer cells. Therefore, depicted in **chapter 3**, we investigated, in addition to the effects of insulin and insulin-like growth factors on pancreatic cancer cell proliferation and migration, the inhibitory effects of IFN-β on IGF- and insulin-stimulated proliferation and migration. We demonstrated that both IGF-1, IGF-2, as well as insulin, have potent stimulatory effects on pancreatic cancer cell proliferation and migration. Importantly, IFN-β is able to inhibit the IGF1-, IGF-2- and insulin-stimulated cell growth as well as migration. However, IFN-β is not able to inhibit basal cell migration and the inhibitory effects of IFN-β on growth factor stimulated cell migration were less pronounced compared to the effects on growth factor stimulated proliferation. One of the explanations might be that associated interferon signaling pathways are predominately involved in cell proliferation, rather than in cell migration. Moreover, in the present study several aspects of the insulin and insulin-like growth factor systems, like for example the role of the family of IGF binding proteins and the involvement of different molecular pathways in cancer cell proliferation and migration, remain underexposed. Nevertheless, results from this study further favor the use of IFN-β as part of treatment options for patients with pancreatic cancer.

Generally, before the start of clinical studies, treatment effects have to be evaluated *in vivo* as well. Therefore, in **chapter 4**, the effects of IFN-β alone, and combined with the golden standard chemotherapeutic agent in pancreatic cancer gemcitabine, were evaluated in a heterotopic subcutaneous pancreatic cancer mouse model[4]. Additionally, the effects of combined treatment were first evaluated *in vitro* and demonstrated a potent synergistic effect of IFN-β combined with gemcitabine. On the basis of these results, it was decided to lower the concentration of gemcitabine *in vivo* to a suboptimal dose. After 30 days of treatment, tumors of mice treated with the combination of IFN-β and gemcitabine were statistically significantly smaller compared to the control. Additionally, we demonstrated in these tumors by immunohistochemistry, a statistically significant lower proportion of proliferating and a clear trend towards an increased proportion of apoptotic cells. The combined treatment was well tolerated. In contrast to the effects of IFN-β *in vitro*, *in vivo* IFN-β monotherapy showed no statistically significant inhibitory effect on tumor growth, although the percentage of proliferating tumor cells was significantly reduced. *In vitro,* for the BxPC-3 cell line the concentration required to reduce cell growth with 50% was 114 IU/ml. With respect a large panel of cell lines the required concentration ranged from 70-1000 IU/ml[43]. However, in clinical settings these concentrations seem not easily reached (4.0 IU/

ml after four doses of 18 MIU IFN-β at 48-h intervals in serum of human healthy volunteers after s.c. administration)[44]. As mentioned before, the predictive value of the IFN receptor expressed in pancreatic cancer tissue for efficacy of interferon therapy has not been established. Furthermore, although considered as the backbone in adjuvant pancreatic cancer treatment, the success of gemcitabine is disappointingly low, with response rates of less than 20%. Therefore it would be desirable, in order to prevent unnecessary treatment toxicities, to predict the response to treatment before start of therapy. In this respect, one could think of the use of molecular markers, such as microRNA (miRNA) or circulating proteins in order to predict the treatment sensitivity. In the literature several miRNA have been investigated for their role in chemoresistance in pancreatic cancer of which some selected miRNAs (-15a, -21, -34, -200b, -200c, -214 and -221) showed promising results. However, the consequence of modifications in miRNA expression and their prognostic value in the clinical setting is still unclear[45]. With regard to the prediction of the effect of interferons the presence of the circulating free interferon receptor (IFNAR-2a) seems interesting. In serum of cancer patients the presence of the IFNAR-2a receptor has been demonstrated, however, it is unknown what the clinical significance, with respect to the effect of interferon therapy, in pre-clinical and clinical setting[46-48]. Nevertheless, at present, the use of biomarkers in order to predict the effect of treatment is still wishful thinking. More promising seems the use of an *ex vivo* tissue slice model. By the use of this technique it is possible to culture with good viability, up to 4 days very thin (200μm) slices of human resection specimens, like for example pancreatic cancer (Figure 1), and to evaluate multiple treatments in one tumor sample. The use of the tissue slice model in pancreatic cancer (evaluation of gene-therapy and the influence of pancreatic stellate cells) has been described before and showed promising results[49,50]. In **chapter 4** the *ex vivo* tissue slice technique was used as well and demonstrated encouraging results. After 3 days of incubation with different therapeutic

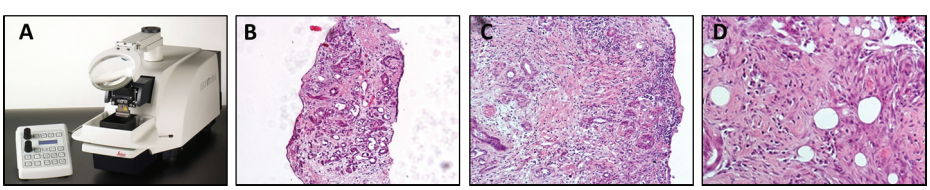

Figure 1: Tissue slicer machine (A) and hematoxylin and eosin stained coupes of tissue slices of human pancreatic cancer resection specimen (B and C) and periampullary cancer resection specimen (D) after 72 hours of culture. Original magnification x100 (B), x200 (C), x300 (D).

agents, although not yet validated, a very clear morphological difference in tissue structure was observed, particularly pronounced in the tissue slices that were incubated with the combination of IFN-β and gemcitabine. Given this pronounced effect of the therapeutic agents on the tissue structure, in future, the validation of the effects of treatment is challenging. While the use of an *ex vivo* tissue slice model to predict treatment effects seems promising there are still some major challenges, like the determination of the concentration of treatment compounds comparable between *ex vivo* as well as in the clinical setting. Furthermore, the validation of the effects of drug treatment remains challenging and needs to be elaborated before this method can be used in clinical setting.

4. CHALLENGES AND FUTURE PERSPECTIVES

Although a major part of the potential anti-cancer effects of type I IFNs, especially IFN-β, have been highlighted in this thesis, some important aspects of interferon therapy have not been addressed yet.

Nowadays it is becoming evident that the immune system is capable of detecting tumor antigens and to initiate humoral and cellular responses to track and eliminate these transformed cells and thereby inhibiting tumor development. Nevertheless, some tumor cells have already been evolved and able to escape the immunosurveillance creating tumors with reduced immune responsiveness and greater potential to grow in an inflamed microenvironment[51,52]. This concept is called cancer immunoediting and its relevance is becoming more clear[53]. As mentioned before, type I interferons are also known to have indirect effects like the activation of NK-cells, t-cells and macrophages, and additionally, it has been demonstrated that type I IFNs have crucial roles in promoting the host anti-tumor immunity[54,55]. In a clinical trial of adjuvant IFN-α therapy in the treatment of malignant melanoma it is demonstrated that patients who develop autoantibodies or clinical manifestations of autoimmunity had significant longer overall and relapse free survival compared to patients who did not develop symptoms or signs of autoimmunity[56]. Therefore, although not studied in this thesis but interesting for future research would be the interaction between pancreatic cancer cells, the immune system and interferons in for example an orthotopic pancreatic cancer model in mice with intact immune system. Additionally, it has been described that multiple tumor types, like ovarian, breast, colon, prostate, bladder, glioblastoma and hepatocellular carcinoma, express increased levels of cell surface proteins, like CD47, allowing cancer cells to avoid phagocytosis and to escape the innate immune system surveillance[57]. In addition, recent preliminary data showed that

human pancreatic adenocarcinomas express elevated levels of CD47 as well[58]. In this respect it can be hypothesized that blocking CD47 in combination with the use of type I IFNs, could enhance the immunomodulatory host effects of patients with pancreatic cancer. Currently, the recently developed humanized anti-CD47 antibody, Hu5F9-G4 is being tested in a Phase I trial (A First-in-Human Phase 1 Dose Escalation Trial of Hu5F9-G4 in Patients With Advanced Solid Malignancies; NCT02216409).

A major challenge of interferon therapy is the induction and activation of several cancer cell survival pathways, limiting the anti-cancer effects of interferons. Survival pathways induced by IFNs include the activation of nuclear factor kappa-beta (NF-κB), the dephosphorylation of specific protein tyrosine phosphatases (PTPs), the over activation of G1P3 and a decrease in cyclic adenosine monophosphate (cAMP). However, one of the main resistance mechanisms that have been extensively described includes the JAK-2/STAT-3 pathway[59,60]. In addition to phosphorylation and activation of STAT-1 and STAT-2, type I IFNs are also able to activate and phosphorylate the STAT-3 protein which can act as an adaptor of the phosphatidylinositol 3 kinase (PI3K) and activates it's downstream target Akt, important for the anti-apoptotic signals. A recently demonstrated potential strategy to counteract the STAT-3 induced escape mechanism is via the activation of peroxisome proliferator-activated receptor-γ (PPAR- γ). PPAR-γ is one of the main targets of insulin sensitizing drugs. On the other hand PPAR-γ agonists, like troglitazone, also display antineoplastic effects in several tumors, including pancreatic cancer. Additionally, in pancreatic cancer cells, it can oppose the STAT-3 dependent escape mechanism induced by type I interferons and thereby consequently increase cell death[61,62]. Furthermore, interferons can induce a stress response in which the expression and signalling activity of the epidermal growth factor (EGF) receptor is enhanced providing an escape mechanism to the growth inhibition induced by IFN-α. Besides that, EGF has been shown to have a protective effect on IFN-α induced apoptosis. In order to overcome these survival mechanisms the use of specific inhibitors may be considered, for example the farnesyl-transferase inhibitor R115777 or the tyrosine kinase inhibitor Gefitinib, which both have shown promising results enhancing the anti-tumor activity of type I IFNs in head and neck squamous cell carcinomas and human epidermoid cancer cells[63,64].

Another major challenge limiting the clinical implication of IFN therapy in the treatment of pancreatic cancer is the short half-life of IFNs. However, advantage can be gained from PEGylated forms of IFN-α and –β, in which lower and less frequent doses are necessary compared to the conventional IFNs. The PEGylated form of IFN-α has already proven to

be effective in the treatment of grade III melanoma[65] and metastatic renal cell carcinoma patients[66]. Currently, a newly developed PEGylated form of IFN-β is being tested in a phase III clinical trial (ADVANCE) in patients with multiple sclerosis. [67,68]. This PEGylated form of IFN-β reached, in an experimental model, still 20 hours after a single s.c. dose of 3.0 MIU/kg in monkeys a serum concentrations of 100 IU/ml and thereby seems very promising as additional therapy in the treatment of pancreatic cancer.

5. GENERAL CONSIDERATIONS AND CONCLUDING REMARKS

Although promising data regarding adjuvant IFN-β therapy in the treatment of pancreatic cancer have been shown in this thesis, it has also been demonstrated that monotherapy is inadequate in pancreatic cancer and combination therapies should be attempted. The considerable degree of heterogeneity of pancreatic cancer is demonstrated in this thesis as well, which makes it difficult, perhaps impossible, to find one optimal treatment strategy. For example, in the optimal setting, tumors with very rich tumor stroma need to be treated differently, for instance with a c-met inhibitor[69], compared to tumors with less tumor-stroma but an abundant expression of the IGF1R, which can possibly be targeted with the newly developed tyrosine-kinase inhibitor OSI-906[70-72]. Future prospective studies are necessary to demonstrate the beneficial effect of IFN-β, although the enhancement of chemo- and radiosensitivity is not absolutely receptor specific, it should be considered to determine the IFN receptor expression in pancreatic cancer tissue prior to therapy. In the purpose of a more patient approached medicine, the development of the tissue slice technique seems promising, and could be useful in determining the effects of interferon in combination with gemcitabine or other chemotherapeutic agents before start of treatment. On the other hand, using this technique, the effect of IFN-β on the immune system cannot be determined. Nevertheless, despite the expressed concerns and possible topics that still can be elaborated, this thesis demonstrated promising results with regard to the use of IFN-β in the treatment of pancreatic cancer and provides a sufficient basis for the next step, a future prospective clinical study.

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7 Summary & Samenvatting

SUMMARY

Pancreatic cancer is a highly aggressive malignancy with limited treatment options. Over the last 30 years survival rates have barely been approved and research has focused on other treatment modalities like biological modulators such as type I interferons (IFNs). Type I IFNs (i.e. IFN-α and –β) are known to have antiproliferative, antiviral and immunoregulatory activities. In addition, they are also able to induce apoptosis, exert cell cycle arrest and thereby sensitize tumor cells for chemo- and radiotherapy. A few years ago several *in vitro, in vivo* and clinical studies have been conducted evaluating adjuvant IFN-α therapy after curative resection of pancreatic cancer and although these studies showed promising results, the only randomized clinical trial did not demonstrate a significant survival benefit for patients receiving adjuvant IFN-α treatment. Therefore, to date, the use of interferons in the treatment of pancreatic cancer remains controversial. Nevertheless, before the start of these clinical studies, certain aspects of interferon therapy, such as the relevance of the interferon receptors, the importance of growth factors and the potential use of IFN-β were not addressed. In this thesis we aimed to further investigate the role of interferon therapy in the treatment of pancreatic cancer.

Chapter 1, the general introduction, describes the challenges regarding the treatment of pancreatic cancer and provides information about type I interferons and their use in cancer treatment. In particular, this chapter highlights the current knowledge of interferon use in the treatment of pancreatic. Chapter 1 ends with the objective and outline of this thesis.

In **Chapter 2** the anti-tumor potencies of IFN-α and –β were determined in a panel of 11 human pancreatic cancer cell lines. Additionally, the correlation between the responsiveness to type I IFNs and the expression of the interferons receptors, IFNAR-1 and IFNAR-2c, was evaluated. In this study a considerable variability between pancreatic cancer cell lines in the expression of type I IFN receptors and in the response to type I IFNs was found. We demonstrated a significant positive correlation between the inhibitory effect of IFN-α and the level of IFNAR-1 and IFNAR-2c receptors expressed, indicating that these receptors can be of predictive value for the responsiveness to IFN-α therapy. No correlation was found between receptor expression and the responsiveness to IFN-β. Interestingly, the anti-tumor activity (i.e. the induction of apoptosis and inhibition of cell proliferation) of IFN-β was significantly more potent, already at low concentrations, and less dependent interferon receptor expression, compared to the anti-tumor activities of IFN-α. In this study we therefore conclude that the use of IFN-β in the treatment of pancreatic cancer seems more promising than IFN-α.

In **Chapter 3** the effects of IFN-β with respect to the insulin and insulin-like growth factor system in human pancreatic cancer cells is evaluated. In cancer tissue in general, and thus in pancreatic cancer as well, insulin and insulin-like growth factors and their receptors are frequently overexpressed, leading to more invasive and proliferating tumors and eventually to a worse prognosis. In the *in vitro* studies described in chapter 3 we demonstrated that both IGF1, -2 and insulin have potent stimulatory effects on pancreatic cancer cell proliferation and migration. We showed that IFN-β is able to inhibit this growth factor stimulated cell growth and migration. Although the effects of IFN-β on insulin- and IGF-stimulated cell migration were less pronounced compared to its effects on proliferation, this study further favors the use of IFN-β as a part of the treatment for patients with pancreatic cancer.

Chapter 4 demonstrates the potent anti-tumor effects of IFN-β combined with gemcitabine *in vitro, in vivo* and *ex vivo*. In this study the synergistic effects of IFN-β combined with gemcitabine were first demonstrated *in vitro*. Thereafter, in a heterotopic pancreatic cancer mouse model, mice were treated with IFN-β alone or combined with a suboptimal concentration of gemcitabine. It was found that the tumor volume of mice treated for 30-days with the combination of interferon and gemcitabine was significantly smaller compared to the control. Furthermore, an *ex vivo* tissue slice model demonstrated at histological level potent effects of IFN-β and gemcitabine combination treatment as well. These positive results argue for well-substantiated research in the clinical setting.

In **chapter 5** the type I IFN receptor expression in human pancreatic and periampullary cancer tissue was determined. In order to establish a direct antitumor effect the expression of the type I IFN receptor, IFNAR-1 and IFNAR-2c is of importance. In this study we demonstrated that the IFNAR-1 and IFNAR-2c receptors are expressed in pancreatic and periampullary cancer tissue, although with great inter- and intratumoral variability. Receptor expression was not correlated with any clinicopathological factors or with survival. Furthermore, we demonstrated that, in both types of tumors, only a small percentage of the patients showed a strong expression of the IFNAR-1 and IFNAR-2c, which could possibly explain the differences found in the clinical studies regarding adjuvant IFN-α therapy. Nevertheless, prospective studies are necessary to demonstrate the relationship between the IFN receptor expression and the antitumor activities of type I IFNs in pancreatic and periampullary cancer patients.

Chapter 6, the general discussion, provides an overview and discusses the findings from the previous chapters. Moreover, the challenges and future perspectives regarding adjuvant IFN therapy are discussed. Some major issues like the induction of tumor cell

Summary

survival pathways, the role of the immune system and the short half-life of type I IFNs are pointed out in this chapter. Finally, some general considerations and remarks are given.

SAMENVATTING

Alvleesklierkanker, oftewel het pancreascarcinoom, is met een totale 5-jaars overleving van minder dan 6% de 4e kanker gerelateerde doodsoorzaak in de westerse wereld. Chirurgische resectie is de enige curatieve behandeling echter, door vroege metastasen en/of lokaal uitgebreide ziekte, komt op het moment van de diagnose maar 15-20% van de patiënten in aanmerking voor een in opzet curatieve therapie. Ondanks jaren van uitgebreid onderzoek blijkt aanvullende (adjuvante) chemo- en/of radiotherapie slechts een zeer beperkte toegevoegde waarde te hebben en is de overleving van patiënten met een pancreascarcinoom de afgelopen jaren nauwelijks verbeterd. Onderzoek heeft zich daarom gericht op het toevoegen van andere behandelingsmogelijkheden, waaronder biologische modulatoren zoals type I interferonen (IFN-α/IFN-β), aan de bestaande behandelingen. Interferonen zijn lichaamseigen eiwitten, behorend tot de groep van cytokinen, die o.a. worden afgescheiden door het immuunsysteem na contact met een virus, bacterie of schimmel. Interferonen hebben een antivirale, antiproliferatieve en immuun regulerende werking en zijn daarnaast ook in staat om apoptose te induceren en de cel cyclus te blokkeren waardoor kankercellen gevoeliger kunnen worden voor chemo- en radiotherapie. Type I Interferonen oefenen hun werking uit via de type I IFN receptor, hetgeen voornamelijk bestaat uit een signalerend deel (IFNAR-1) en een IFN-bindend deel (IFNAR-2c). De toegevoegde waarde van IFN-α therapie aan de bestaande behandeling van het pancreascarcinoom is enkele jaren geleden door verschillende onderzoeksgroepen onderzocht. De klinische studies lieten, afgezien van de grote hoeveelheid bijwerkingen, indrukwekkende resultaten zien met toegenomen overleving voor patiënten die de adjuvante IFN-α behandeling ondergingen. De enige gerandomiseerde klinische studie liet echter geen significant toegenomen overleving zien, al lijkt het er op dat in de experimentele behandelingsarm er patiënten zijn die wel geprofiteerd hebben van de aanvullende IFN-α behandeling. Desalniettemin is tot op heden het gebruik van IFN-α, in de behandeling van het pancreascarcinoom, gezien de mate van bijwerkingen, tegenstrijdig. Voor de start van deze klinische studies waren er verschillende aspecten van adjuvante IFN behandeling, zoals de relevantie van de IFN receptoren en het gebruik van IFN-β, echter niet onderzocht dan wel onderbelicht. Dit proefschrift heeft zich er daarom op gericht om de rol van interferon therapie in de behandeling van het pancreascarcinoom verder te onderzoeken.

In **hoofdstuk 1**, de algemene inleiding, worden de uitdagingen met betrekking tot de behandeling van het pancreascarcinoom omschreven en informatie gegeven over type I interferonen en hun plaats in de behandeling van verschillende vormen van kanker. Daar-

naast wordt specifiek de op dit moment bestaande kennis over het gebruik van interferon in de behandeling van het pancreascarcinoom besproken en toegelicht. Hoofdstuk 1 eindigt met een toelichting omtrent het doel en een overzicht van dit proefschrift.

Hoofdstuk 2 laat de celgroei remmende en apoptose inducerende effecten van IFN-α en –β zien in 11 verschillende humane pancreas carcinoom cel lijnen. In deze studie wordt ook de correlatie tussen de expressie van interferon receptoren (IFNAR-1 en IFNAR-2c) en de mate van respons op IFN-α en –β bepaald. Er werd in deze studies een aanzienlijke variabiliteit in receptor expressie gevonden. Het effect van IFN-α bleek significant positief gecorreleerd met het aantal receptoren (IFNAR-1 en IFNAR-2c) dat tot expressie wordt gebracht, hetgeen aanduidt dat deze receptoren van waarde kunnen zijn bij het voorspellen van de respons op IFN-α. Er werd geen correlatie gevonden tussen het aantal interferon receptoren dat tot expressie wordt gebracht en het effect van IFN-β. Het cel groei remmend en apoptose inducerend effect van IFN-β minder afhankelijk van de receptor expressie en vele malen krachtiger, al bij lage concentraties, dan het effect van IFN-α. Uit deze studie word daarom geconcludeerd dat het gebruik van IFN-β in de behandeling van het pancreascarcinoom er belovender uit ziet dan het gebruik van IFN-α.

In **hoofdstuk 3** worden de effecten van IFN-β op het insuline en de insulineachtige groeifactoren (IGF) systeem onderzocht. Bij kanker in het algemeen, en dus ook bij het pancreascarcinoom, worden insuline, IGFs en hun receptoren vaak overmatig op de tumorcelmembraan tot expressie gebracht wat kan leiden tot sterkere prolifererende en invasievere tumoren en daardoor ook tot een slechtere prognose. De studies beschreven in hoofdstuk 3 laten de sterke celgroei inducerende en cel migratie bevorderende effecten van zowel IGF-1 en IGF-2, alsmede insuline, op humane pancreascarcinoom cellen zien. De studie laat tevens zien dat IFN-β in staat is om deze groeifactor geïnduceerde celgroei en migratie significant te remmen. Ondanks het feit dat de effecten van IFN-β op de insuline- en IGF-gestimuleerde celgroei sterker waren dan het effect op de migratie, bekrachtigt deze studie de veelbelovende werking van INF-β in de behandeling van het pancreascarcinoom.

Hoofdstuk 4 laat de anti-tumor effecten van IFN-β gecombineerd met gemcitabine *in vitro, in vivo* en *ex vivo* zien. In deze studie werd eerst het synergistische effect van IFN-β gecombineerd met gemcitabine *in vitro* aangetoond. Hierna werd met behulp van een heterotoop subcutaan pancreascarcinoom muismodel het effect van de behandeling met IFN-β en een suboptimale dosis van gemcitabine aangetoond. Na 30 dagen behandeling was het tumorvolume van muizen behandeld met de combinatie van IFN-β en gemcitabine significant lager vergeleken met het tumorvolume van de controle muizen. Daarnaast werd

door middel van een *ex vivo* tissue slice model ook op histologisch niveau de effecten van IFN-β en gemcitabine combinatie behandeling aangetoond. Deze veelbelovende resultaten geven aanleiding voor een goed onderbouwd klinisch onderzoek naar de effecten IFN-β combinatietherapie in patiënten met een pancreascarcinoom.

Om een effect van IFN te bewerkstelligen is expressie van de type I IFN receptor van belang. In **hoofdstuk 5** werd daarom de type I IFN receptor expressie in tumoren van patiënten met een pancreas- of peri-ampullair carcinoom bepaald. Deze studie toonde aan dat, hoewel met enige variabiliteit, zowel het signalerende deel van de receptor (IFNAR-1) als het interferon bindende deel van de receptor (IFNAR-2c) tot expressie komt in humaan pancreas en peri-ampullair kanker weefsel. De expressie van deze receptoren was echter niet gecorreleerd met klinische of pathologische parameters, of met overleving. Verder werd middels deze studie aangetoond dat in beide type kanker maar een klein percentage van de patiënten tumoren hadden met een sterke expressie van de IFNAR-1 en IFNAR-2c receptor. Mogelijk zou dit de verschillende uitkomsten van overleving na adjuvante IFN-α therapie in de verschillende klinische studies kunnen verklaren. Prospectieve studies zijn echter nodig om de relatie tussen de IFN receptor expressie en het anti-tumor effect van interferonen in patiënten met een pancreas of peri-ampullair carcinoom aan te tonen.

Hoofdstuk 6 betreft de algemene discussie waarin de verschillende resultaten uit voorgaande hoofdstukken besproken en bediscussieerd worden. Daarnaast worden de uitdagingen en toekomstperspectieven met betrekking tot adjuvante IFN therapie besproken. Verder worden belangrijke aspecten, zoals de inductie van verschillende tumorcel overlevingsmechanismen, de rol van het immuunsysteem en de relatief korte half waarde tijd van type I IFNs in de circulatie besproken in dit hoofdstuk. Ten slotte worden er enkele algemene overwegingen en opmerkingen aangedragen.

List of publications
Curriculum Vitae
PhD Portfolio

LIST OF PUBLICATIONS

1. **Booy S**, van Eijck CH, Dogan F, van Koetsveld PM, Hofland LJ. Influence of type-I Interferon receptor expression level on the response to type-I Interferons in human pancreatic cancer cells. *J Cell Mol Med.* Mar 2014;18(3):492-502
2. **Booy S**, Hofland LJ, Waaijers AM, Croze E, van Koetsveld PM, de Vogel L, Biermann K, van Eijck CH. Type I Interferon Receptor Expression in Human Pancreatic and Periampullary Cancer Tissue. *Pancreas.* Jul 28 2014
3. **Booy S**, Hofland LJ, van Eijck CH. Potentials of Interferon Therapy in the Treatment of Pancreatic Cancer. *J Interferon Cytokine Res.* Dec 31 2014
4. **Booy S**, van Eijck CH, Janssen JA, Dogan F, van Koetsveld PM, Hofland LJ. IFN-β is a potent inhibitor of insulin and insulin like growth factor stimulated proliferation and migration in human pancreatic cancer cells. *American Journal of cancer research, provisionally accepted*.
5. **Booy S**, van Eijck CH, van Koetsveld PM, Karelse B, Dogan F, Hofland LJ. Interferon-β mono- and combination therapy in the treatment of pancreatic cancer *in vivo*. *Manuscript in preparation*.
6. **Booy S**, van Aalten SM, Groenendijk RP. Cutane metastasen van een oesophagus plaveiselcelcarcinoom. *Submitted for publication*.

CURRICULUM VITAE

Stephanie Booij was born on June 13th, 1987 in Zwolle. In 2005 she graduated from secondary school at the Bonifatius College in Utrecht. In 2006 she completed the one year course in mathematics, chemistry and physics at the James Boswell Institute in Utrecht. The same year she got admitted to the study Medicine at the Erasmus Medical Center, Rotterdam. In 2008 she started the master Clinical Research at the Netherlands Institute for Health Sciences on top of the regular medical curriculum. As a part of the Master of Science programme she attended a summer programme at the Johns Hopkins Bloomberg School of Public Health, at the Johns Hopkins University in Baltimore, United States of America. She obtained a "doctoral" degree in medicine in 2010. In 2011 she obtained her Master of Science in Clinical Research degree after which she could extend her research project into the current PhD project on the potentials of type I interferons in the treatment of pancreatic cancer at the department of Surgery (promotor: Prof.dr. C.H.J. van Eijck) and Internal Medicine (division of neuro-endocrinology, promotor: Prof.dr. L.J. Hofland). At this moment she is doing her internships and hopes to graduate as a medical doctor in 2015.

PHD PORTFOLIO

Name PhD student: Stephanie Booij
Erasmus MC department: Surgery; Internal Medicine
PhD period: 01 June 2011 – 10 June 2015
Promotors: Prof. dr. C.H.J. van Eijck, Prof. dr. L.J. Hofland

Academic Education

2008-2011 MSc in Clinical Research, NIHES, Rotterdam, The Netherlands.
Including a summer programme at Johns Hopkins Bloomberg School of public health, Johns Hopkins University, Baltimore, USA.
2006-2010 Doctorate in Medicine, Erasmus MC, Rotterdam, The Netherlands.

	YEAR	WORKLOAD (ECTS)
COURSES		
Research integrity	2013	2
Workshop Writing successful grant proposals	2011	0.5
Proefdierkunde (Artikel 9)	2011	2
English biomedical writing and communication	2011	2
PRESENTATIONS		
1e Rotterdam-Leiden pancreatic surgery meeting, Leiden,The Netherlands (oral presentation) – *potentials of interferon therapy in the treatment of pancreatic cancer.*	2014	1
ESMO 15th world congress on gastrointestinal cancer, Barcelona, Spain. (poster presentation) -*Role of IGF-IR/IR in human pancreatic cancer cells.*	2013	2
47th Annual Pancreas club meeting, Orlando, FL, USA. (poster presentation)- *Type I interferon receptor expression in pancreatic and periampullary cancer tissue.*	2013	2
Wetenschapsdagen, Internal medicine, Antwerp, Belgium. (poster presentation) – *Type I interferon receptor expression in pancreatic and periampullary cancer tissue.*	2013	1
SEOHS, Amsterdam, The Netherlands. (oral presentation) - *Expressie van type I interferon receptoren en respons op type I interferonen in humane pancreascarcinoom cellen*.	2012	1

	YEAR	WORKLOAD (ECTS)
SEOHS, Amsterdam, The Netherlands. (poster presentation) - *Type I interferon receptor expressie in humaan pancreas en periampullaire carcinomen*.	2012	1
Wetenschapsdagen, Internal medicine, Antwerp, Belgium. (poster presentation)- *Type I interferon receptor expression and response to type I interferons in human pancreatic cancer cell lines*.	2012	1
45th Annual Pancreas club meeting, Chicago, IL, USA. (poster presentation)- *Type I interferon receptor expression and response to type I interferons in human pancreatic cancer cell lines.*	2011	2
Molmed day, Rotterdam, The Netherlands. (poster presentation)- *Type I interferon receptor expression and response to type I interferons in human pancreatic cancer cell lines*.	2011	1
Annual Dutch pancreatic cancer group meeting (oral presentation)- *Interferons in pancreatic cancer*.	2011	1
CONFERENCES		
Chirurgendagen, Surgery, Velp, The Netherlands.	2014	1
Chirurgendagen, Surgery, Velp, The Netherlands.	2013	1
Wetenschapsdagen, Internal medicine, Antwerp, Belgium.	2011	1
Dutch gastroenterology symposium, Rotterdam, The Netherlands	2011	0.5
TEACHING ACTIVITIES		
Workshop and journal club (endocrinology) 2nd year medical students	2013	1
Workshop and journal club (gastroenterology) 2nd year medical students	2013	1
Workshop and journal club (endocrinology) 2nd year medical students	2012	1
Supervising internship 3rd year Biology and Medical Laboratory student	2012	4

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Het is af! And now we party!