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Type I interferons as radiosensitisers for pancreatic cancer

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ABSTRACT

Background: Radiotherapy is an established treatment for malignant localised disease. Pancreatic cancer however seems relatively insensitive to this form of therapy.
Methods: Pancreatic cancer cell lines MiaPaca-2 and Panc-1 were pre-treated with 3000 IU/ml IFNα or 100 IU/ml IFNβ followed by 0, 2, 4, or 6 Gray (Gy) irradiation. Colony forming assay was used to assess the effects on cellgrowth. To measure the surviving fraction at the clinically relevant dose of 2 Gy (SF2), cells were pre-treated with 1000–10.000 IU/ml IFNα or 50–500 IU/ml IFNβ followed by 2 Gy irradiation.
Results: The plating efficiency was 49% for MiaPaca-2 and 22% for Panc-1. MiaPaca-2 was more radiosensitive than Panc-1 (surviving fraction of 0.28 versus 0.50 at 4 Gray). The SF2 of MiaPaca-2 was 0.77 while the SF2 of Panc-1 was 0.70. The SF2 significantly decreased after pretreatment with IFNα 1000 IU/ml ($p < 0.001$) and IFNβ 100 IU/ml ($p < 0.001$) in MiaPaca-2 and with IFNα 5000 IU/ml ($p < 0.001$) and IFNβ 100 IU/ml ($p < 0.01$) in Panc-1. The sensitising enhancement ratio (SER) for IFNα 3000 IU/ml was 2.15 in MiaPaca-2 and 1.90 in Panc-1. For IFNβ 100 IU/ml the SER was 1.72 for in MiaPaca-2 and 1.51 in Panc-1.
Conclusions: Type I interferons have radiosensitising effects in pancreatic cancer cell lines. This radiosensitising property might lead to an improved response to treatment in pancreatic cancer. Interferon β is the most promising drug due to its effect in clinically obtainable doses.

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1. Introduction

Radiotherapy is an important treatment in cancer, especially for patients with advanced localised disease, with proven efficacy in many tumours.[1–5] Based on several randomised controlled trials studying the effect of adjuvant (chemo)radiotherapy, pancreatic and periampullary cancers are fairly radiotherapy resistant.[6–10]

Often chemotherapeutics (5-fluorouracil (5-FU), gemcitabine) are used as radiosensitisers. Besides their direct cytotoxic effects caused by incorporation of the drugs as modified nucleotides into the DNA, even low doses of these drugs can be effective in radiosensitisation. Interference with normal repair of radiation-induced DNA damage with an inappropriate progression through S phase is key in their radiosensitising properties causing late, unmanageable toxicities. A

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favourable side effect of their direct cytotoxicity is a relative increase in oxygenation, leading to an increased vulnerability for radiotherapy.[11,12] However, as mentioned, results of chemoradiotherapy are disappointing in pancreatic cancer.[6–10]

In several tumours radiosensitising properties of interferon alpha (IFNα) and interferon beta (IFNβ) have been demonstrated *in vitro*[13–17] as well as *in vivo*.[18,19] In pancreatic cancer cell lines, IFNα has already shown to act as radiosensitiser[20] and *in vivo* promising therapy results have been reported combining 5-FU, cisplatinum, and radiation therapy with IFNα alone (5-year survival rate of 55%) or followed by 2 cycles of gemcitabine (median survival 25 months) in patients with resected pancreatic adenocarcinoma.[21,22] These results are currently reinvestigated in the phase III CapRI study.[23]

Type I interferons such as IFNα and IFNβ, sort their effect through the same interferon receptor (IFNAR) with IFNβ having a higher affinity. *In vivo* studies showed that approximately 20% of pancreatic cancers express IFNARs and that expression of the interferon receptor correlates with a significant survival benefit in patients with resected pancreatic cancer.[24]

The exact mechanism by which type I interferons cause radiosensitisation is unclear. Possibly, concomitant treatment with IFNs causes an inappropriate progression of cells into S-phase, thereby interfering with repair of radiation-induced damage or increasing the proportion of lethal to sublethal damage.[17,25]

In this study we aim to gain insight in the radiosensitising abilities of type I interferons, especially IFNβ, in pancreatic cell lines. We decided to address these issues in colony forming assays, because besides the apoptotic effects of radiation and IFNs, the reproductive integrity of tumour cells (i.e. the capacity to produce an expanding colony of descendants, and therefore to regrow the tumour if left intact at the end of treatment) is of pivotal importance.

2. Materials and methods

2.1. Cell lines and culture conditions

The human pancreatic cell lines MiaPaCa-2 and Panc-1 were purchased from the American Type Culture Collection. The cells were cultured in a humidified incubator containing 5% CO_2 at 37°C. MiaPaca-2 was cultured in RPMI 1640 and Panc-1 in DMEM both supplemented with 10% FCS, penicillin (1×10^5 U/l), fungizone (0.5 mg/l) and L-glutamine (2 mmol/l). Periodically, the cells were tested for *Mycoplasma* contamination, which was not detected. Cells were harvested with trypsin (0.05%), EDTA (0.02%) and resuspended in medium. Before plating, the cells were counted microscopically using a standard haemocytometer. Tryphan Blue staining was used to assess cell viability, which always exceeded 95%. 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 (Rebif) was acquired from Serono Benelux BV (Den Haag, The Netherlands). All compounds were stored at −20 °C, and the stock solution was constituted in distilled water according to the manufacturer instructions. Doses of 1000–10.000 IU/ml for IFNα and 50–500 IU/ml for IFNβ were used.

2.3. Irradiation

Cells were exposed to gamma radiation from a ^{137}Cs source at 70.9 cGy/min at room temperature under aerobic conditions. For radiation survival studies, cells were irradiated with 0, 2, 4, 6, 8, or 10 Gray. In the combined modality treatment, the IFN treatment was given before irradiation for 72 hours. Cells were irradiated with 0, 2, 4, or 6 Gray in the presence of the drug.

2.4. Colony forming assay

Cells were plated onto poly-L-lysine coated, 60-mm Petridishes (6–12 cells/cm^2) and cultured in complete medium for 2 weeks. Poly-L-lysine (10 μg/ml; Sigma–Aldrich, Zwijndrecht, The Netherlands) inhibited cells from dispersing from the growing colonies.

Dose response curves for IFNα, IFNβ and irradiation were established for both cell lines using a colony-forming assay. Therefore, seeded cells were allowed to attach for 24 hour prior to treatment with 1000–10.000 IU/ml IFNα, 50–500 IU/ml IFNβ or 0–10 Gray irradiation. Cell lines were treated with IFNs continuously and medium plus agents were replaced every three or four days. Fourteen days after seeding, colonies were fixed with 100% ethanol and stained with hematoxicilline to allow calculation of their average colony-forming efficiency. Colonies containing >50 cells were counted automatically with the MultiImage Light Cabinet from HpH Innitech Corporation.

Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for control cultures not exposed to interferons or radiation. The surviving fraction (SF) was calculated as (mean number of colonies)/(number of inoculated cells × plating efficiency). The curve was plotted using X–Y log scatter (Graph Prism 3.0). Curve-fitting parameters α and β were determined.

2.5. Radiation enhancement by type I interferons

To asses radiation enhancement by type I interferons, cells were pretreated with IFNα 3000 IU/ml or IFNβ 100 IU/ml (doses resulting in approximately 50% decrease in surviving fraction in both cell lines) for 72 hours. Cell lines were irradiated with 0, 2, 4, or 6 Gray. Control plates without IFNs were irradiated simultaneously. Cell lines were treated with IFNs continuously and medium plus agents were replaced every three or four days. After 2 weeks, the formed colonies were fixed and stained to allow counting.

SF2 is the surviving fraction of cells that were irradiated at the clinically relevant dose of 2 Gray.

The sensitising enhancement ratio (SER) for interferon was calculated at the 37% survival level. The radiation dose that reduced the surviving colonies to 37% of the non-treated

controls was divided by the radiation dose that reduced survival to 37% after interferon pre-treatment.

2.6. Cell proliferation assay

Measurement of total DNA contents, representative for the number of cells, was performed using the bisbenzimide fluorescent dye (Hoechst™ 33258, Boehring Diagnostics, La Jolla, CA) as previously described.[26]

2.7. Measurement of DNA fragmentation (apoptosis)

10.000 cells/dish, depending on the length of the incubation period, were plated on 24-well plates and the cells were allowed to adhere overnight. The next day the cell culture medium was replaced with 1ml/well medium containing 3000 IU/ml IFN-α or 100 IU/ml IFN-β. Each treatment was performed in quadruplicate. After an additional incubation of 3 days, apoptosis was assessed using a commercially available ELISA kit (Cell Death Detection ELISAPlus, Roche Diagnostic GmbH, Penzberg, Germany). The standard protocol supplied by the manufacturer was used. Relative apoptosis was determined by calculating the ratio of the average absorbance of the treatment dishes to the average absorbance of the control dishes. The data were corrected for the effect on cell number after 3 days of treatment. Intra- and inter-assay coefficients of variation were 4.2% and 6.3%, respectively.

2.8. Statistical analyses

All experiments were carried out in duplicates and gave comparable results. For statistical analysis GraphPad Prism™ 3.0 (GraphPad Software, San Diego, USA) was used. The comparative statistical evaluation amongst groups was firstly performed by the 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 analyse the differences in surviving fraction for each dose point.

In all analyses, values of $p < 0.05$ were considered statistically significant. Data are reported as mean ± SEM. Statistical analysis was made after logarithmic transformation.

3. Results

In vitro, the control plating efficiency (mean ± SD) was measured and amounted to 49 ± 3% for MiaPaca-2 and 22 ± 3% for Panc-1.

3.1. Effect of type I interferons on relative clonogenic survival

Both IFNα and IFNβ inhibited colony formation for both MiaPaca-2 and Panc-1 cells in a dose dependent manner (Fig. 1).

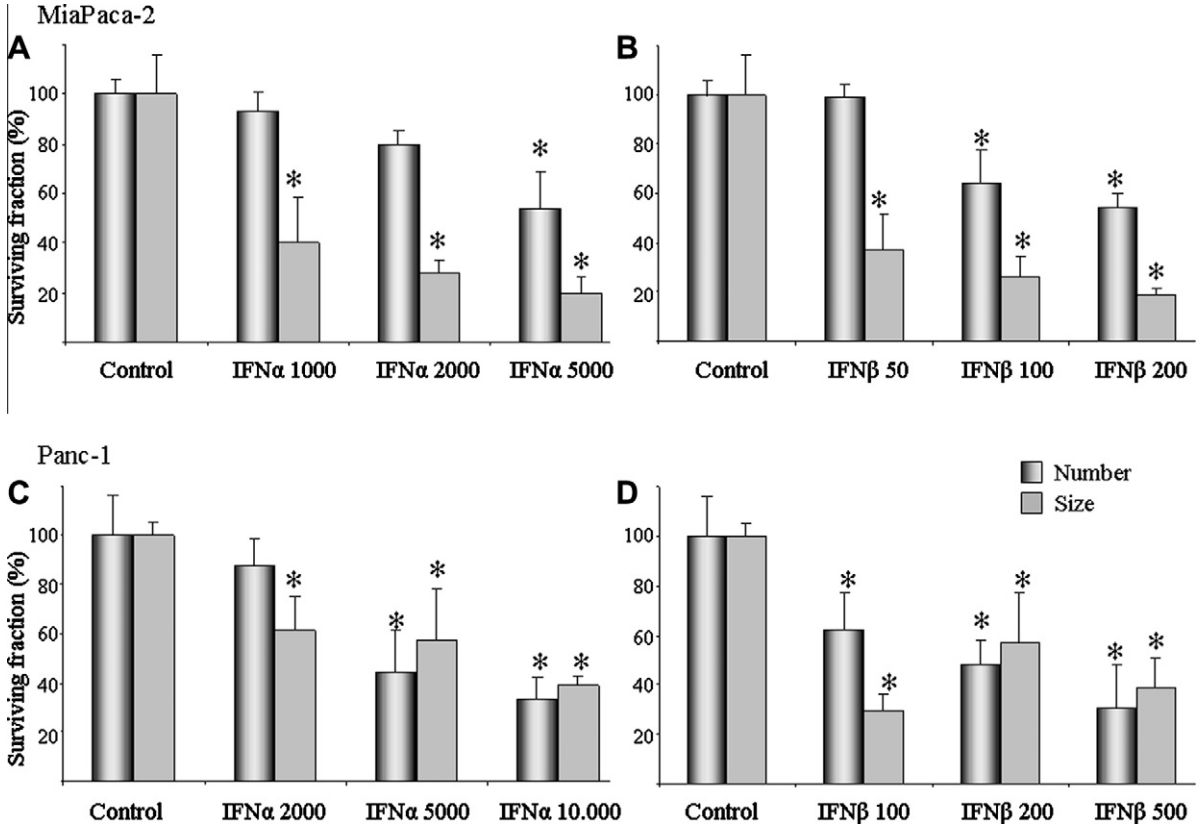

Fig. 1 – Effect of IFNα and IFNβ treatment on colony forming ability in MiaPaca-2 (A,B) and Panc-1 (C,D). Pancreatic cancer cells were treated with IFNα (A,C) or IFNβ (B,D) during fourteen days and both number and size of colonies were counted automatically. Data are the mean ± SEM. *$p < 0.05$ versus control.

For all doses analysed, MiaPaca-2 was significantly more vulnerable to both IFNα and IFNβ than Panc-1.

Besides inhibiting colony formation, type I interferon had growth inhibitory effects on both cell lines; after interferon treatment, colonies were significantly smaller than colonies from untreated cells (Fig. 1). This finding is consistent with Vitale's manuscript[27] demonstrating both growth inhibitory as well as pro-apoptotic properties of type I interferons with IFNβ being the more potent pro-apoptotic drug than IFNα, where apoptosis only occurs in higher doses.

3.2. *Effect of radiation on relative clonogenic survival*

MiaPaca-2 was more radiosensitive than Panc-1 for 4, 6 and 8 Gray (Fig. 2a). The surviving fraction at 2 Gy (SF2) of MiaPaca-2 was 0.77 while the SF2 of Panc-1 was 0.70. At 4 Gy however, the SF was 0.28 for MiaPaca-2 and 0.50 for Panc-1. The size of the colonies was not considerably influenced by radiotherapy (Fig. 2b).

3.3. *Effect of type I Interferons on apoptosis*

Significant apoptosis corrected for DNA content occurred after 72 hours (Fig. 3). Apoptosis increased to 802 ± 219% for IFNα 3000 IU/ml and 575 ± 119% for IFNβ 100 IU/ml. In Panc-1 apoptosis after 72 hours was 154 ± 14% for IFNα 3000 IU/ml and 139 ± 7% for IFNβ 100 IU/ml.

3.4. *Surviving fraction at 2 Gray (SF2)*

The SF2 of MiaPaca-2 was 0.77 while the SF2 of Panc-1 was 0.70.

Pre-incubation with IFNα for 72 hours at doses of 1000, 2000 or 5000 IU/ml changed the SF2 in MiaPaca-2 to 0.56, 0.47 and 0.18, respectively. In Panc-1 pre-incubation with IFNα at doses of 2000, 5000, or 10.000 IU/ml changed the SF2 to 0.66, 0.22 and 0.18, respectively.

Pre-incubation with IFNβ for 72 hours at doses of 50, 100, or 200 IU/ml changed the SF2 in MiaPaca-2 to 0.70, 0.33 and 0.22, respectively. In Panc-1 pre-incubation with IFNβ at doses of 100, 200 or 500 IU/ml changed the SF2 to 0.48, 0.24 and 0.23, respectively.

The decrease of SF2 compared to the SF2 without pre-incubation was significant after IFNα 2000 IU/ml ($p < 0.01$) and IFNα 5000 IU/ml ($p < 0.001$) and IFNβ 100 IU/ml and IFNβ 200 IU/ml ($p < 0.001$) in MiaPaca-2. In Panc-1 concentrations of IFNαα 2000 IU/ml ($p < 0.01$), IFNα 5000 IU/ml ($p < 0.001$) and IFNα 10.000 IU/ml ($p < 0.001$) and IFNβ 100 IU/ml ($p < 0.001$) IFNβ 200 IU/ml ($p < 0.001$) IFNβ 500 IU/ml ($p < 0.001$) resulted in significant improved radiosensitivity (Fig. 4).

3.5. *Effect of type I interferon on radiosensitivity*

The radiotherapy dose required to reduce the surviving fraction to 37% was 3.98 Gy in MiaPaca-2 for the non-treated controls. After IFNα 3000 IU/ml, the required dose was 1.85 Gy

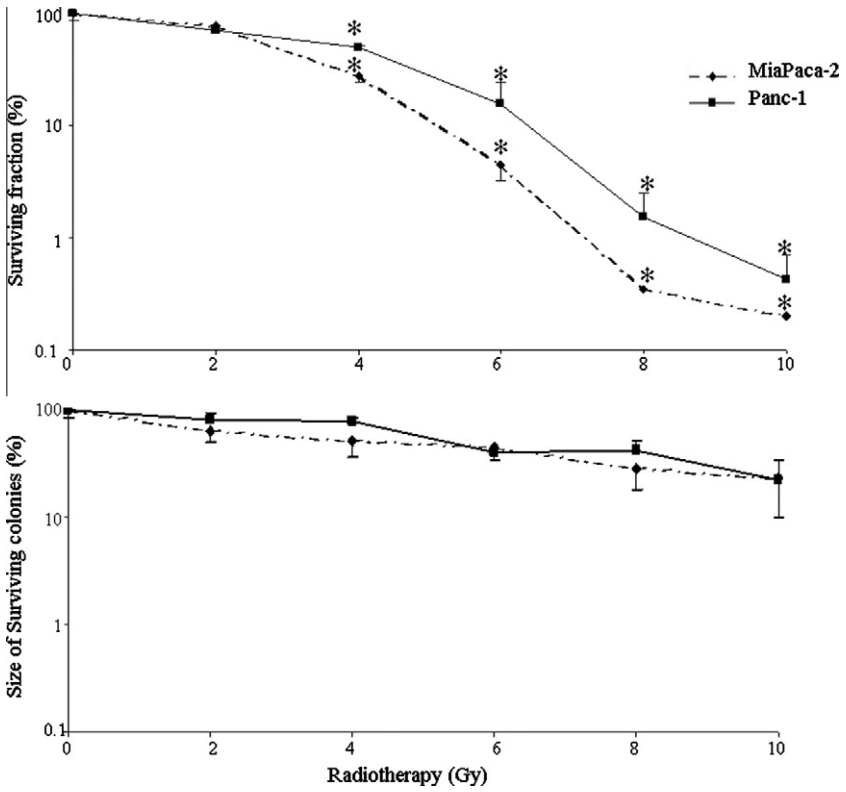

Fig. 2 – Effect of irradiation on colony forming ability in MiaPaca-2 and Panc-1. Pancreatic cancer cells were treated with irradiation during 14 days and both number and size of colonies were counted automatically. Data are the mean ± SEM. *$p < 0.05$ versus control.

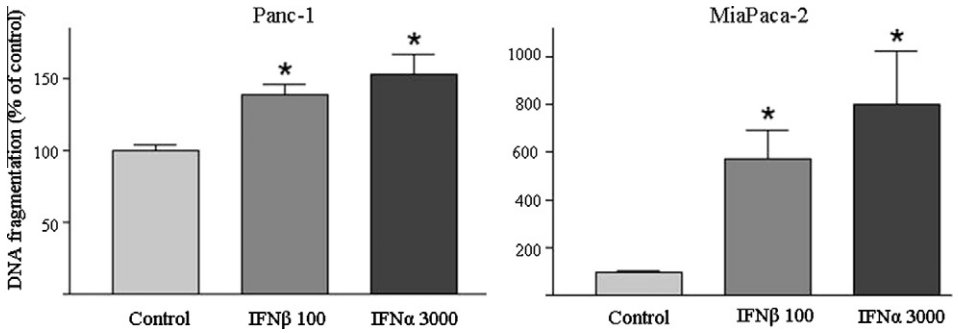

Fig. 3 – Effect of 3000 IU/ml IFNα and 100 IU/ml IFNβ treatment on apoptosis (DNA fragmentation) in MiaPaCa-2 and Panc-1 cell lines. Cells were incubated for 3 days without (control) or with the drugs indicated. Values are absorbance units and are expressed as percent of the control. Data are the mean ± SEM. *$p < 0.05$ versus control.

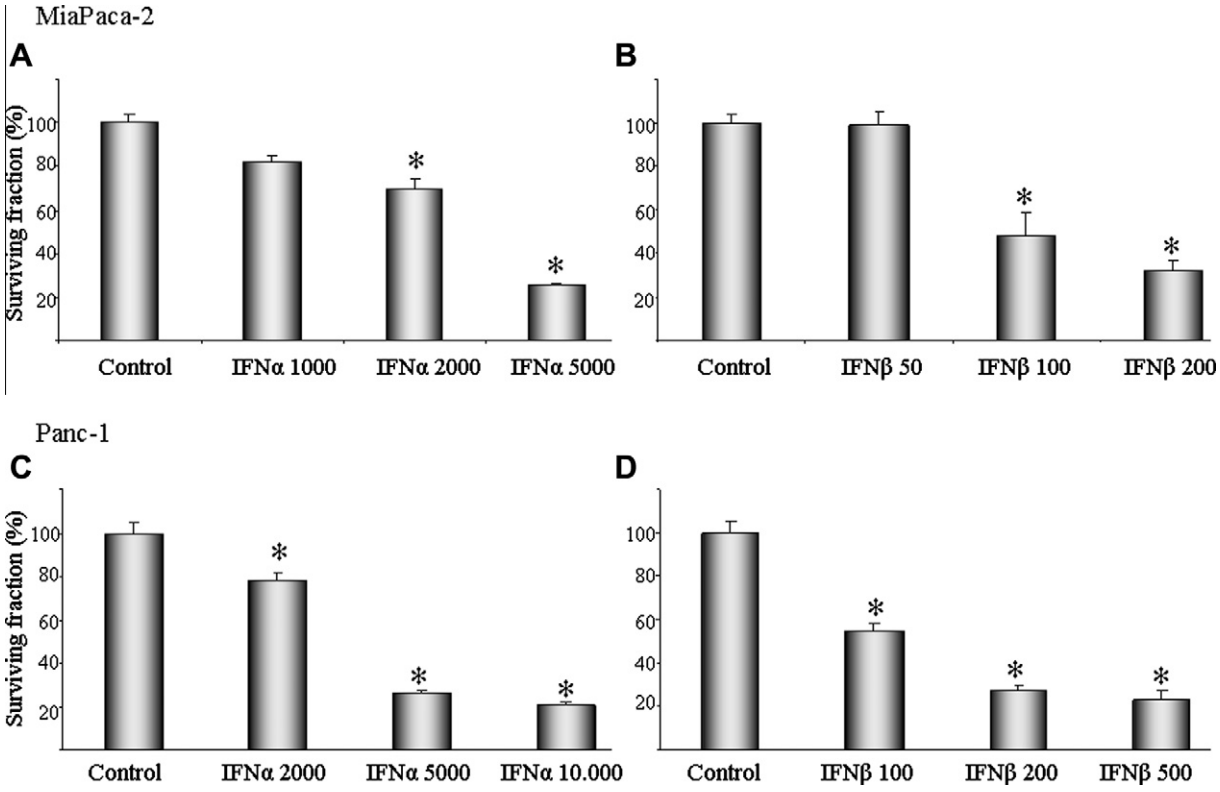

Fig. 4 – Effect of combined treatment with type I interferons and 2 Gy irradiation on colony forming ability in MiaPaCa-2 (A,B) and Panc-1 (C,D) cell lines. Cells were pretreated with IFNα (A,C) or IFNβ (B,D) for 72 hours followed by irradiation with 2 Gy. The formed colonies were automatically counted after 2 weeks. Data are the mean ± SEM. *$p < 0.05$ versus control.

and after IFNβ 100 IU/ml, the required dose was 2.32 Gy. This leads to a sensitising enhancement ratio (SER) of 2.15 for IFNα 3000 IU/ml and a SER of 1.72 for IFNβ 100 IU/ml in MiaPaca-2.

In Panc-1 the required radiotherapy dose to reduce the SF to 37% was 4.65 Gy for the non-treated controls. After IFNα 3000 IU/ml, the required dose was 2.45 Gy and after IFNβ 100 IU/ml, the required dose was 3.07 Gy. This leads to a sensitising enhancement ratio (SER) of 1.90 for IFNα 3000 IU/ml and a SER of 1.51 for IFNβ 100 IU/ml in Panc-1.

The shape of the survival curves changed after treatment resulting in an increased steepness of the survival curve with an increase of the α-component after curve fitting (Fig. 5). For MiaPaca-2 this increase was significant for both IFNα as IFNβ compared to the non-treated controls. In the Panc-1, we found no significant difference between the α-component after neither IFNα nor IFNβ and the non-treated controls. In this cell line, after comparison of the radiosensitivity between groups using the Newman–Keuls test, only the SF after 6 Gy of irradiation combined with IFNα or IFNβ was significantly lower than after irradiation alone. All other SFs were similar after treatment or no treatment.

4. Discussion

Our *in vitro* study shows a radiosensitising effect of type I interferons on pancreatic cancer cell lines MiaPaca-2 and

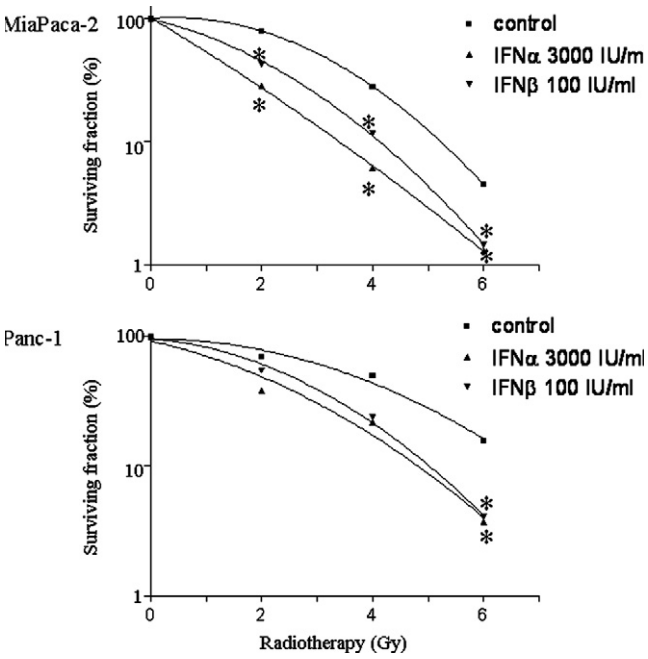

Fig. 5 – Effect of combined treatment with 3000 IU/ml IFNα and 100 IU/ml IFNβ and irradiation on colony forming ability in MiaPaCa-2 and Panc-1 cell lines. Cells were pretreated with IFNα or IFNβ for 72 hours followed by 0, 2, 4 or 6 Gy irradiation. The formed colonies were automatically counted after 2 weeks. Data are the mean ± SEM. *$p < 0.05$ versus control.

Panc-1. Miapaca-2 was 2.15 times more sensitive to radiotherapy after treatment with IFNα 3000 IU/ml and 1.72 times more sensitive after IFNβ 100 IU/ml. The sensitivity to radiotherapy of Panc-1 increased 1.90 times after treatment with IFNα 3000 IU/ml and 1.51 times after IFNβ 100 IU/ml. Radiosensitisation was dose dependently, with higher doses of interferons resulting in a higher radiosensitivity, i.e. a lower surviving fraction at 2 Gray (SF2). Both overall radiosensitivity and sensitivity to interferon therapy was higher in MiaPaca-2 than in Panc-1. Previously, IFN receptor expression (mRNA and protein) was shown by Vitale et al.[27] with MiaPaCa-2 expressing more plasma membrane located IFN receptors than Panc-1. Furthermore, treatment with type I interferons results in more apoptosis in MiaPaca-2 than in Panc-1, although only extremely high doses (>1000 IU/ml) of IFNα cause apoptosis.[27]

Tumours are most vulnerable for radiotherapy in case of adequate vascular supply (proper oxygenation) and appropriate cell cycling.[11,28] To enhance the effect of radiotherapy, effort has been made to find radiation sensitisers. Because hypoxic cells are known to be up to three-fold more resistant to radiotherapy than well-oxygenated cells,[29] several strategies have been investigated to find ways to sensitise hypoxic cells to radiation. These strategies focus at reducing tumour hypoxia by increasing the delivery of oxygen to the tumour, administering oxygen mimetics and mimicking the effect of oxygen in the radiochemical process or by selective destruction of hypoxic cells, for instance by tirapazimine.[30,31] Trials show that reducing hypoxia by any means, leads to a better locoregional control and an improved survival, especially in head and neck cancer and cervical cancer.[30]

Resistance to apoptosis is the key factor for poor responses to therapies in pancreatic cancer. Ionising radiation alone causes a range of lesions in the DNA of target cells such as base damage, single-strand and double-strand breaks. Double-strand breaks are generally considered the lethal event but can be repaired by DNA repair mechanisms. Inadequately repaired DNA damage causes activation of the mitochondrial pathway of apoptosis by p53 resulting in activation of the caspase cascade. Membrane damage activates the stress-activated protein kinase pathway leading to activation of the mitochondrial pathway as well as a direct activation of caspases. Furthermore radiotherapy activates apoptosis due to stimulation of the death receptor pathway, consisting of the tumour necrosis factor (TNF) receptor superfamily (for instance TRAIL-R1 and TRAIL-R2). Activation causes direct activation of the caspase cascade. These cell death pathways are regulated by numerous signalling molecules, such as nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K), inhibitor of apoptosis proteins (IAPs) and members of the Bcl-2 protein family.[32,33]

Pancreatic cancer cells have developed multiple resistance mechanisms to therapy-induced apoptosis. The mitochondrial pathway of apoptosis is less activated due to inactivating p53 mutations, present in >70% of the pancreatic cancers. If activated, this pathway has a diminished ability to activate the caspase cascade. Furthermore, pancreatic cancer cells overexpress anti-apoptotic proteins, have inactivated pro-apoptotic genes and express decoy receptors to prevent activation of the death receptor pathway.[34,35]

In pancreatic cancer cell lines, IFNα has already shown to be able to avoid these resistance mechanisms and act as radiosensitiser[20] The exact mechanism by which IFN exerts this radiosensitising activity is unclear. Both IFNα and IFNβ have direct anti-tumour effects including apoptosis, cell damage, upregulation of cancer antigens and a growth inhibitory effect with accumulation of cells in S phase. Indirect anti-tumour effects are caused by modulation of the immune system, mainly through activation of T-cells, macrophages and natural killer cells, and anti-angiogenesis activity by downregulation of the vascular endothelial growth factor (VEGF) receptor and an alteration in the expression of various oncogenes.[27,36–39]

In pancreatic cancer cell lines, type I interferons cause both direct apoptosis (at lower doses in IFNβ) and radiosensitisation. The exact mechanism for enhancing radiosensitivity is unclear. Apparently the accumulation of cells in S phase caused by interferons, which is the most radioresistant part of the cell cycle, does not prevent radiosensitisation. A possible mechanism is the inability of cells to accumulate sublethal DNA damage with interferon interfering in the repair of this kind of DNA damage. Furthermore alteration in oncogene expression levels might sensitise radioresistant cells to radiotherapy. Because not only the pro-apoptotic effect of IFNs combined with radiotherapy but especially the reproductive capacity of the treated cells is related to treatment efficacy, we chose colony forming assays for our study.

Beneficial effects of IFNα combined with chemoradiotherapy were already demonstrated in phase II studies in patients with resectable pancreatic cancer.[21,22] However, treatment is associated with considerable toxicity[21,22,40] and the optimal combination therapy considering efficacy as well as tolerability is yet to be determined.

In conclusion, our *in vitro* study shows that both IFNα and IFNβ have radiosensitising effects in pancreatic cancer cell lines that are not based on immunomodulatory properties. Radiosensitising effects are dose dependent, and lower doses of IFNβ than IFNα cause similar radiosensitisation. The effect of interferon seems related to the receptor status. The radiosensitising property of type I interferons might lead to an improved response to treatment in pancreatic cancer with interferon β being the most promising drug. Therefore, further clinical trials involving combination therapy of type I IFNs and radiotherapy are promising.

Conflict of interest statement

None declared.

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