Retinoids in pancreatic cancer

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Summary

Prognosis of advanced, unresectable pancreatic adenocarcinoma remains dismal and has not significantly improved over the past 20 years. In a broad panel of preclinical experimental settings we have therefore evaluated the effects of retinoids on human pancreatic carcinoma cells in vitro and in vivo. We found that retinoid treatment results in inhibition of growth, induction of cellular differentiation and decreased adhesion to certain components of the extracellular matrix, all features compatible with a "less malignant" phenotype. Furthermore, retinoids act synergistically antiproliferative when combined with interferon-α. Using transient and stable genetic transfer studies we were able to identify two retinoid receptor subtypes responsible for mediating the growth inhibitory effects as well as retinoid sensitivity. In addition we observed a crucial functional interplay between the retinoid signalling pathway and the expression of a distinct protein kinase C isoenzyme, which determines the direction of the growth regulatory effects of retinoids. Based on these encouraging preclinical results we initiated a phase II clinical trial in which patients with advanced pancreatic carcinoma were treated with retinoic acid in combination with interferon-α. This therapeutic regimen was well tolerated and resulted in prolonged stable disease in approximately two thirds of the patients. In summary, these studies suggest that retinoids might be beneficial in the treatment of advanced pancreatic carcinoma patients based on their pleiotropic effects on tumor cell biology.

Key words: IFN-α, pancreas, protein kinase C, retinoids, retinoid receptor

Pancreatic cancer: The therapeutic dilemma

Human adenocarcinoma of the pancreas currently represents the fifth most common cause of cancer death in Western countries [1]. At the time of diagnosis more than 80 % of the patients have advanced regional disease or distant metastasis [2]. Therefore curative resection can not be considered for a vast majority of pancreatic cancer patients. Despite multiple clinical trials using a large panel of chemotherapeutical regimens, the prognosis of advanced pancreatic cancer has not significantly improved over the last thirty years. The median survival varies between 4 - 6 months and the five year survival rate is less than 2 % [reviewed in 3,4]. A recent survey of 27 randomized trials for advanced pancreatic cancer revealed median survivals of 1.3 to 11 months in patients receiving active treatment, with a median overall survival between 150 and 160 days under therapy [5]. The authors of this study concluded that with the objective to prolong disease-free survival or overall survival, there is currently no standard treatment available for advanced pancreatic cancer. Given that considerable toxicities were associated with the majority of the evaluated chemotherapeutic regimes it appears questionable whether the relatively small gains in survival justify the side effects, hospitalisation and the potential impairment in quality of life. It furthermore seems unlikely that conventional chemotherapy will significantly improve the current therapeutic dilemma. Based on these observations, the development of new drugs for palliative treatment of pancreatic cancer in our opinion should meet the following criteria: (i) new mechanism of action; (ii) preclinical evidence for antitumor activity; (iii) low toxicity profile; (iii) administration of the drug on an outpatient basis to minimize hospitalisation associated with treatment related procedures.

Given that conventional chemotherapy is relatively ineffective in the treatment of advanced pancreatic cancer, new therapeutic strategies focusing on inhibition of tumor cell proliferation paralleled by induction of tumor cell differentiation offer a promising therapeutic approach. In this context numerous in vitro and in vivo studies in a variety of malignancies and experimental tumor models have shown that natural and synthetic derivatives of vitamin A, biochemically summarized as retinoids, are capable of inhibiting tumor cell proliferation and inducing cellular differentiation. In subsequent clinical trials, retinoids have been demonstrated as an effective treatment modality for quite diverse malignancies such as cervical cancer, squamous cell carcinoma of the skin and promyelocytic leukemia [for review see 6]. The goal of our studies was therefore to develop an experimental treatment strategy with retinoids and interferon-alpha (IFN-α) for advanced human pancreatic carcinoma.

Preclinical studies

Retinoids regulate growth and differentiation

Our initial studies were performed using an in vitro system of a broad panel of human pancreatic carcinoma cell lines of ductal phenotype [7]. Using this in vitro system as well as xenotransplanted tumors derived from these cell lines we found that retinoid treatment results in time- and dose-dependent growth inhibition in vitro and in vivo of ductal...
but not acinar pancreatic tumor cells. Retinoid treatment induces a more differentiated phenotype in ductal tumor cells as evidenced by morphological criteria and increased expression of carbonic anhydrase II [7,8]. These findings were later on independently confirmed by other groups in a variety of pancreatic carcinoma cell lines [9,10]. The antiproliferative effects of retinoic acid can be augmented by IFN-α in some epithelial cell lines, as well as in the clinical treatment of squamous cell cancer of the skin and cervix [for review refer to Ref.11,12] and in renal cell carcinoma. In respect to pancreatic carcinoma [13], we have been able to demonstrate antiproliferative effects of IFN-α in a subset of pancreatic carcinoma cell lines and synergistic antiproliferative effects of RA with IFN-α in vitro [Rosewicz et al., unpublished data].

**Retinoids inhibit metastatic potential**

The fate of pancreatic cancer patients is critically determined by local tumor growth and infiltration as well as the presence of distant metastasis. These processes are at least partly determined by a complex interplay of tumor cells with extracellular matrix components of the basement membrane. For infiltrative growth, tumor cells have to penetrate the basement membrane of the organ; in the process of metastasis, tumor cells traverse the vascular basement membrane on their way from circulation to the target organ. Adhesion to basement membrane components therefore represents the initial step in the cascade of infiltrative growth and metastasis. Accordingly, the ability to metastasize has been correlated with the extent of tumor cell adhesion to basement membrane components. The major constituents of basement membranes are type IV collagen, heparan-sulfate proteoglycan, nidogen/entactin and laminin. Laminin has been of particular interest, because it has been shown to modulate tumor cell adhesion, growth and differentiation; furthermore the ability of tumor cells to interact with laminin has been shown to correlate with their tumorigenicity and their metastatic potential. Because the interaction of tumor cells with laminin and other basement membrane components plays a critical role in the control of local growth and metastasis of pancreatic carcinoma, we analysed the effects of retinoids on pancreatic carcinoma cell adhesion to basement membrane components, with a particular focus on tumor cell adhesion to laminin [14]. Treatment with retinoids results in a time- and dose-dependent inhibition of DAN-G cell adhesion to fibronectin and laminin, but not to collagens I, IV and VI. The adhesion of DAN-G cells to laminin could be completely blocked by anti-α5 and anti-β1 antibodies, but not by the synthetic peptide YIGSR. Flow cytometry analysis of DAN-G cells revealed no quantitative difference for α5 integrin expression in retinoid treated and untreated DAN-G cells. Furthermore, radioimmuno-precipitation showed no difference in the appearance of α6β1 integrin expression after retinoid incubation. Retinoids therefore decrease pancreatic carcinoma cell adhesion to laminin via specific alteration of the α6β1-integrin receptor function and thereby open interesting perspectives for the modulation of infiltrative growth and metastasis in pancreatic cancer [14].

**Retinoids: mechanism of action**

Over the last years much has been learned about the molecular mechanisms by which retinoids can exert such pleiotropic effects like inhibition of proliferation and induction of tumor cell differentiation. Retinoids exert these intriguing effects through interaction with specific nuclear receptors. Based on molecular cloning studies so far two families of nuclear retinoid receptors have been described, each consisting of three receptor subtypes α, β and γ: the retinoic acid receptors (RAR) which bind the naturally occurring retinoid all-trans retinoic acid (ATRA) with high affinity [15,16]; and the retinoid X receptors (RXR) whose naturally occurring, biologically active ligand is 9-cis retinoic acid, a geometric isomer of ATRA [17]. The ligand-binding domains between these two retinoid receptor families share only a 29 % sequence homology [18]. In addition, each RAR/RXR gene generates multiple isoforms by either differential use of internal promoters or alternative splicing of exons [19]. Both receptor families act as ligand dependent transcription factors, controlling gene transcription initiated from promoters of retinoid regulated genes by interacting with cis-acting DNA elements, the so called RAREs (retinoic acid responsive elements) [reviewed in 19,20]. This multiplicity of receptors and gene pathways explains the diverse effects of retinoids on a wide range of cellular processes.

**Retinoid receptor subtype specific biological functions**

Tissue specific restricted expression of RAR/RXR subtypes and isoforms during embryogenesis and in the adult organism suggests that each RAR and RXR subtype exerts a unique biological function [21]. Furthermore, disruption of the intracellular retinoid pathway via interference with specific RAR subtypes can result in carcinogenesis [22-24]. Summarized with our observation that retinoids exert antiproliferative effects and induce differentiation in human pancreatic carcinoma cells, we hypothesized that a deregulation in RAR subtype expression might be involved in the propagation of the malignant phenotype of human pancreatic cancer. In a subsequent in situ hybridisation study we therefore analysed RAR subtype expression in human pancreatic carcinoma tissue of 24 patients [25]: while there was no difference in the expression of RAR α and γ between normal and malignant tissue, we observed that approximately one third of all pancreatic tumors completely lost the expression of RARβ when compared to their nontransformed counterparts. Furthermore, the remaining tumors expressed significantly less RARβ mRNA transcripts than adjacent normal pancreatic ductal cells. Moreover, we observed a tight correlation between the loss of RARβ expression and the degree of cellular dedifferentiation [25]. These data suggested that loss or decreased expression of RARβ could either be an innocent epiphenomenon associated with malignant transformation or might indeed play a central role in the propagation of the malignant phenotype in human pancreatic adenocarcinoma. To dissect this problem we chose a reverse approach by overexpressing RARβ in the human pancreatic tumor cell line DAN-G. Overexpression of RARβ in DAN-G cells by stable transfection inhibits cellular proliferation in vitro and in vivo
purification and molecular cloning studies, PKC represents other extracellular ligands [33]. Based on biochemical mitogenic stimuli, such as hormones, growth factors and as second messengers upon cellular binding of many are diacylglycerol and arachidonic acid, which are generated of gene expression, cellular proliferation and differentiation implicated in essential cellular processes such as regulation is the interference of retinoids with Protein Kinase C (PKC). One such example of cross-talking which has recently been discovered has been commonly described as "cross-talking". One such intersection of other intracellular signalling systems essential evidence suggests that retinoids can interfere at the...phenomenon which might serve a distinct biological function.

We have established an in vitro system, in which retinoic acid exerts opposite effects on anchorage-independent growth in two cell lines, derived from an identical histological origin, a human pancreatic adenocarcinoma [34]. This system can therefore serve as a valuable model to explore the interplay of various PKC isoenzymes in the differential growth regulation by retinoic acid. RA treatment results in dose-dependent stimulation of anchorage-independent growth in AsPC1 cells and growth inhibition in Capan 2 cells. Both cell lines express an identical pattern of nuclear retinoidic acid and retinoid X receptors as determined by reverse-transcriptase PCR. Western Blotting using monospecific antibodies revealed that both cell lines express PKC isoenzymes α and ζ, while β, γ, δ, and ε were not detected. Incubation with RA in the growth stimulated AsPC1 cell line resulted in induction of PKC α expression, whereas PKC α expression was decreased by RA in the growth inhibited Capan 2 cell line. In contrast, PKC ζ expression was not affected by RA in either cell line. Incubation of AsPC1 cells with the phorbol ester TPA resulted in a time- and dose-dependent selective down-regulation of PKC α but not ζ. The dose-dependent decrease of intracellular PKC α concentration correlated well with the anchorage-independent growth rate of AsPC1 cells. Furthermore, selective downregulation of PKC α blocks subsequent growth stimulation by RA in AsPC1 cells. When PKC α concentration was decreased by stably transfecting AsPC1 cells with a PKC α cDNA antisense construct, RA stimulated growth could also be partially blocked. These data therefore suggest, that differential regulation of PKC α expression plays a central role in determining the bidirectional effects of retinoids on growth in pancreatic carcinoma cells [34].

Interaction of retinoids with Protein Kinase C (PKC)

In addition to nuclear receptors, recent experimental evidence suggests that retinoids can interfere at the intersection of other intracellular signalling systems essential for the regulation of cellular growth, a phenomenon which has been commonly described as "cross-talking". One such example of cross-talking which has recently been discovered is the interference of retinoids with Protein Kinase C (PKC). PKC is a phospholipid-dependent serine/threonine kinase implicated in essential cellular processes such as regulation of gene expression, cellular proliferation and differentiation [for review see 30-32]. PKC constitutes the natural occurring receptor for the mitogenic phorbol esters, which act as tumor promoters. The natural occurring activators of PKC in vivo are diacylglycerol and arachidonic acid, which are generated as second messengers upon cellular binding of many mitogenic stimuli, such as hormones, growth factors and other extracellular ligands [33]. Based on biochemical purification and molecular cloning studies, PKC represents a multigene family which can be divided into two subgroups, depending on whether the enzymes require calcium for activation (PKC-α, -β1, -βII, and -γ) or not (PKC-δ, ε, ζ, η and ι). These PKC isoforms differ in their cofactor requirement, substrate specificity, subcellular distribution and tissue restricted expression, suggesting that each isoform might serve a distinct biological function.

Clinical studies

Based on the results of our preclinical experiments we conducted a phase II trial of 13-cis retinoic acid and interferon-alpha in patients with advanced pancreatic carcinoma [35]. The purpose of this trial was to examine feasibility and tolerability of a combination therapy of 13-cis RA and IFN-α in patients with advanced, unresectable pancreatic carcinoma. Twenty-two patients (median age 62 years) with histologically confirmed, unresectable pancreatic adenocarcinoma of UICC stage III (5 of 22) or IV (17 of 22) were included. Patients received 1 mg/kg body weight 13-cis RA p.o. and 6 million IU IFN-α s.c. daily. Restaging by ultrasound, CT-scan and chest X-ray was performed every two months. No complete and one partial remission (PR: 4.5%) were observed. 14 patients (63.6%) demonstrated stable disease with a median duration of 5.0 months (range 2.3-17.7). Toxicity was mainly IFN-α related and predominantly hematologic (no grade 4, 13.6% grade 3). Non-hematological toxicity did not exceed grade 2 (skin,
oral mucosa) and was mainly related to 13-cis RA. Median survival of stage III cancer patients was 8.7 months (range 6.8-23.9) and stage IV patients 7.4 months (range 0.9-19.9) resulting in a median overall survival of 7.7 months (range 0.9-23.9). These data indicated that combination therapy with 13-cis RA and IFN-α is feasible and well tolerated in patients with advanced pancreatic carcinoma. Based on the median survival rates observed in this study this combination should be further investigated in phase III trials.

Future directions
Based on our preclinical studies regarding retinoid receptor subtype specific biological functions, we might be able to further optimize the clinical treatment protocol. By screening pancreatic cancer biopsies for RARy expression by in situ hybridization and RT-PCR we might be able to select a "retinoid-sensitive" subgroup of pancreatic cancer patients before initiating therapy. With the current development of synthetic retinoid receptor subtype specific agonists, we might be able to amplify the desired therapeutic effects like growth inhibition and induction of differentiation (mediated by the RARβ) and minimize undesired side effects potentially mediated by other receptor subtypes. In addition, we are currently in the process of identifying retinoid regulated genes responsible for the growth inhibitory effects observed in pancreatic carcinoma. These molecules might serve as novel therapeutic targets for antiproliferative treatment of pancreatic cancer.

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References