The Targeted Immunocytokine L19-IL2 Efficiently Inhibits the Growth of Orthotopic Pancreatic Cancer

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Abstract

Purpose: Effective control of pancreatic cancer has been hampered primarily by the lack of tumor specificity of current treatment modalities. The highly specific antibody-mediated delivery of therapeutic agents to the tumor microenvironment might overcome this problem. We therefore investigated the therapeutic efficacy of the targeted immunocytokine L19-Interleukin-2 (L19-IL2), consisting of the human single-chain Fv antibody L19, which is highly specific for the extracellular domain B (ED-B) of fibronectin, and the human cytokine IL-2, in pancreatic cancer.

Experimental Design: Therapeutic effects of L19-IL-2, IL-2, and gemcitabine on tumor growth and metastasis were evaluated in orthotopic mouse models for pancreatic cancer. Immunohistochemistry was done to define ED-B expression, tumor necrosis, apoptosis, proliferation, and invasion of macrophages and natural killer (NK) cells. NK cells were depleted by i.v. injection of an anti-asialo-GM-1 antibody.

Results: ED-B is selectively expressed in human pancreatic cancer and in primary tumors and metastases of the mouse models. L19-IL-2 therapy was clearly superior to untargeted IL-2 or gemcitabine and inhibited tumor growth and metastasis with remarkable long-term tumor control. Therapeutic effects were associated with the induction of extensive tumor necrosis and inhibition of tumor cell proliferation. Immunohistochemistry revealed an increase of macrophages and NK cells in the tumor tissue, suggesting immune-mediated mechanisms. The functional relevance of NK cells for the therapeutic effect of the targeted immunocytokine L19-IL-2 was confirmed by NK cell depletion, which completely abolished its antitumor efficacy.

Conclusions: These preclinical results strongly encourage the initiation of clinical studies using L19-IL-2 in pancreatic cancer.

Adenocarcinoma of the pancreas represents the fifth leading cause of cancer related death in industrialized western countries (1). The prognosis of patients diagnosed with pancreatic cancer is extremely poor with an estimated 5-year survival rate of 1% to 4%. Surgical resection provides the only potentially curative treatment, but locally extended or metastasized disease precludes surgical treatment in most cases (2, 3). Due to poor selectivity, toxicities against nonmalignant cells, and dose limitations, currently available strategies including palliative, chemotherapeutic, and radiation treatments have little effect on the aggressive course of disease (4–7). One promising avenue to overcome these obstacles is the highly specific antibody-mediated delivery of therapeutic agents to the tumor microenvironment. In particular, vascular and/or stromal targeting represents an appealing therapeutic strategy for several reasons. First, targets, which are selectively expressed around tumor vessels and/or in the tumor stroma, are easily accessible to systemically administered antibodies. Second, markers of tumor stroma and tumor neovascularization are typically produced by endothelial cells and/or myofibroblasts, which are genetically more stable than tumor cells. Third, as neoangiogenesis is a prerequisite of tumor growth and metastasis, the selective targeting approach of L19-Interleukin-2 (IL-2) to newly forming tumor blood vessels should result in a therapeutic benefit. Currently, one of the most selective oncofetal antigens associated with neoangiogenesis and tumor growth is the extracellular domain B (ED-B) of fibronectin (6, 8). The fibronectin splice variant ED-B, a small domain of 91 amino acids, which is homologous from mouse to man, is usually absent in both plasma and tissue-fibronectin, except for some blood vessels of the regenerating endometrium and the ovaries (6, 8). However, it may become inserted in the fibronectin molecule during active tissue remodeling associated with neoangiogenesis, thereby accumulating around the neovascularization and in the stroma of malignant tumors and other tissues undergoing angiogenesis (6, 8). Recently, a human single-chain Fv (scFv) antibody fragment L19 has been generated, which displays...
picomolar binding affinity for ED-B, and has been verified to selectively target tumor neovascularature in both experimental tumor models (9) and patients with cancer (10), thus paving the way for a novel therapeutic approach targeting tumor neovascularature.

IL-2 has been characterized as one of the most potent antitumor cytokines. However, despite being approved for the clinical treatment of metastatic renal cell carcinoma, systemically applied IL-2 has failed to fulfill earlier hopes. In part, this is due to serious, potentially life-threatening side effects that limit dose escalation and prevent the application of sufficiently high doses (11, 12). In addition, the fast clearance of systemically administered IL-2 further decreases its effectiveness. However, local administration of IL-2 has been more successful and has resulted in the control of malignant effusions and remission of established lesions (11, 13–15).

In this regard, a targeted accumulation of the cytokine IL-2 at the tumor microenvironment by conjugating it to the homodimeric scFv L19 appears to be an attractive concept to enhance the therapeutic index of IL-2 and at the same time diminish its toxic side effects. This study was therefore designed to evaluate the therapeutic efficacy of the recombinant targeted immunocytokine L19-IL-2 in pancreatic carcinoma.

Materials and Methods

Human tissue samples. Nineteen pancreatic carcinoma, 15 chronic pancreatitis, and 11 normal pancreatic tissue samples were obtained from individuals who underwent surgical resection at the Department of Surgery at Charité University Hospital. This study was approved by the local ethics committee and all patients gave written informed consent prior to surgery.

Cell culture. The human pancreatic carcinoma cell lines MiaPaca (American Type Culture Collection), DanG, and the murine lymphoma cell line YAC-1 (DSMZ) were cultured as described previously (16, 17). MiaPaca cells, stably transfected with an angiopoietin-2 DNA construct, were maintained as MiaPaca wild-type cells, except for the addition of hygromycin B (400 μg/mL; Invitrogen).

Animals. Female NMRInude mice (age, ~10 weeks; weight, 21-25 g) were purchased from Bmbolgard. Animal care followed institutional guidelines and all experiments were approved by local animal research authorities.

Tumor implantation and in vivo treatment. Three orthotopic xenograft mouse models of pancreatic carcinoma were established, including two nonmetastatic models by injection of wild-type cells of the human pancreatic carcinoma cell lines DanG and MiaPaca and one metastatic model by implantation of MiaPaca cells stably transfected with an angiopoietin-2 DNA construct (MiaPaca-A2).3 Orthotopic transplantation was carried out as described (18). In brief, a median laparotomy was done under deep general anesthesia and the pancreas was exposed. Aliquots of 1 × 10^6 tumor cells were injected into the head of the pancreas. The pancreas was replaced and the abdominal wall was closed. Therapy was started 7 days (DanG), 40 days (MiaPaca), or 60 days (MiaPaca-A2) after tumor cell inoculation when solid tumors and metastases (MiaPaca-A2) had formed. Groups of 8 to 12 mice were treated with vehicle (0.9% saline) or 1.43 or 4.29 MIU/kg IL-2 equivalents as either untargeted IL-2 or L19-IL-2 (provided by Phllogen) or equimolar amounts of L19 (783 μg/kg) for 10 days or 250 mg/kg gemcitabine (Lilly) once weekly.

At the end of each therapy, the tumor volume was calculated using the formula: length × width × depth × π / 6. The area of lymph nodes of the metastatic MiaPaca-A2 tumor model was calculated by determining the largest diameter and its perpendicular diameter and computing the product of the two measurements.

Depletion of natural killer cells. Natural killer (NK) cell depletion was done as described previously (19). Briefly, mice received six injections i.p. with 50 μl anti-asialo-GM1 antibody (Wako Chemicals) every fourth day, starting 3 days before tumor cell injection. The level of NK cell depletion was monitored by flow cytometry and cytotoxicity assay.

Preparation of spleen mononuclear cells. Spleens were removed under deep general anesthesia and digested with collagenase (Worthington) for 1 h at 37°C. Subsequently, contents were forced through a 100-μm cell strainer and washed twice with HBSS. Mononuclear cells were separated from the cell suspension via Ficoll- Hypaque (Amersham) density gradient centrifugation.

AlamarBlue cytotoxicity assay. Cytotoxic activity of isolated murine spleen mononuclear cells against YAC-1 cells was examined using a 24 h AlamarBlue cytotoxicity assay (BioSource) as detailed previously (20).

Flow cytometry. Spleen mononuclear cells (1 × 10^6) were incubated with 1 μg phycoerythrin-labeled anti-NK1.1 for 15 min at 4°C. After washing with PBS, the presence of fluorescence-positive cells was analyzed on a FACSCalibur using CellQuest software (BD).

Immunohistochemical analysis. Paraformaldehyde-fixed, 4-μm-thick frozen sections of human and murine tissue samples were analyzed immunohistochemically using the avidin-biotin technique. Slides were incubated for 1 h with the following primary antibodies: biotinylated L19-IL-2 (30 μg/mL; provided by Bayer-Schering Pharma), monoclonal mouse anti-human CD31 (Dako) at 1:100 dilution, rat anti-mouse CD11b (BD Biosciences) at 1:50 dilution, biotinylated mouse anti-mouse CD161b/c/NK1.1 (PK136) antibody (Serotec) at 1:50 dilution, and biotinylated mouse anti-human pancytokeratin (C11) antibody (Santa Cruz Biotechnology) at 1:50 dilution. Proliferating cells were detected with a monoclonal mouse anti-human Ki-67 antibody (Dianova), applying the Animal Research Kit. Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay using TumorTACS In situ Apoptosis Detection Kit (R&D Systems). Both procedures were carried out following the manufacturer’s directions, except that 3-amino-9-ethylcarbazole was used as the substrate chromogen. Negative controls were included by omitting the primary antibody (Animal Research Kit) or the terminal deoxynucleotidyl transferase (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling). In the end, sections were counterstained with hematoxylin and mounted permanently. Quantitative immunohistochemical analysis for the area of vital (nonneoplastic) tumor tissue was carried out by computer-aided imaging analysis using Axiovision 4.2 software (Zeiss). A red color threshold was interactively set by the examiner for each antibody to select the red stained immunopositive area. All pixels that met the defined threshold were then transferred into a binary image and analyzed. For the quantification of proliferating, apoptotic, and inflammatory cells, 20 randomly selected measurement areas (680 × 510 μm; magnification, ×20) of each tumor specimen were analyzed. For evaluation of ED-B fibronectin expression, 10 areas (1,390 × 1,000 μm; magnification, ×10) were scanned. The results represent the positive staining as a percentage of measurement area (inflammatory cells and ED-B fibronectin) or the ratio of positive tumor cells as a percentage of total tumor cells (Ki-67 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling), respectively. For quantitative analysis of necrotic tumor area, H&E sections were scanned at low power (×2.5). Total and necrotic tumor areas were marked on the screen, calculated by the image analysis system, and the percentage of necrotic/tumor total area was determined.

Extraction of DNA and DNA PCR. Genomic DNA of mouse lymph nodes was extracted using the QiAamp DNA Mini Kit (Qiagen).
A specific 850-bp fragment of the α-satellite region of the human chromosome 17 was detected by PCR using the following primers 5′-GGGATAATTTCAGCTGACTAACCG-3′ and 5′-TTCCGTITTAGT- TAGGTCGATTAC-3′ as described (21).

Serum variables. Mouse blood samples were obtained by retro-orbital bleeding. Serum CA 19-9 levels were measured by a fully automated chemiluminescence immunoassay on an ADVIA Centaur (Bayer) and lipase levels were determined using a kinetic colorimetric assay on an ADVIA 2400 (Bayer).

Statistical analysis. Statistical differences were evaluated by two-sided Mann-Whitney U test and Fisher’s exact test and correlation was assessed by linear regression using GraphPad statistical software (GraphPad Software). Differences were considered statistically significant at $P < 0.05$.

Results

**ED-B fibronectin is selectively expressed in human pancreatic carcinoma.** We studied the expression pattern of ED-B fibronectin in human nontransformed pancreatic tissue, chronic pancreatitis, and ductal adenocarcinoma (Fig. 1A, a, c, e, and f). In parallel, serial sections from the same tissues were stained with a monoclonal antibody against the endothelial cell-specific antigen CD31 (Fig. 1A, b, d, and f). All but one carcinoma sample analyzed ($n = 19$) revealed a specific expression of ED-B fibronectin at the abluminal side of tumor blood vessels and in the tumor stroma (Fig. 1A, e and f). By contrast, nontransformed pancreatic tissue ($n = 11$; Fig. 1A, a and b) and chronic pancreatitis ($n = 15$; Fig. 1A, c and d) showed no ED-B fibronectin signal. Morphometric quantification of ED-B fibronectin in pancreatic cancer recorded the presence of ED-B fibronectin in $7.35 \pm 1.60\%$ of total tissue area compared with $<0.1\%$ in nontransformed pancreatic tissue and chronic pancreatitis (Fig. 1B). Thus, ED-B fibronectin provides the specific and selective overexpression required for an ED-B fibronectin-based targeted therapy in pancreatic cancer.

**Development of an orthotopic nude mouse model for pancreatic cancer.** To investigate the therapeutic efficacy of an ED-B fibronectin targeted therapy, we established orthotopic models for pancreatic cancer by injection of human wild-type DanG or MiaPaca pancreatic cancer cells or MiaPaca cells with stable overexpression of angiopoietin-2 (MiaPaca-A2) into the pancreas of nude mice. Orthotopic transplantation resulted in extensive local tumor growth with invasion of adjacent normal pancreatic tissue and neighboring organs. Moreover, MiaPaca-A2 tumors showed metastatic dissemination into intra-abdominal lymph nodes and liver. The immunohistochemical analysis of the orthotopically grown pancreatic tumors consistently revealed a strong cuff-like immunostaining for ED-B fibronectin around tumor blood vessels and in the stromal compartment (Fig. 1A, g and h, right). In comparison with the human situation, the extent of ED-B fibronectin expression was higher in DanG tumors (22.33 ± 0.65%; Fig. 1B) but equal in MiaPaca tumors (7.96 ± 1.32%; Fig. 1B). Thus, the observed tumor growth and immunohistochemical findings compare well with the human situation and validate the animal models as a relevant test system for therapeutic in vivo studies with the targeted immunocytokine L19-IL2.

**Targeted immunocytokine L19-IL2 exerts significant antitumor activity against established pancreatic cancer.** To determine the efficacy of L19-IL2 in pancreatic cancer, mice bearing orthotopic DanG tumors of $3.1 \pm 0.05\ mm^3$ average volume were randomized to receive vehicle or 1.43 or 4.29 MIU/kg IL-2 equivalents of either L19-IL2 or untargeted IL-2 for 10 days. Whereas IL-2 treatment had no effect on tumor growth...
in the lower dose and only a minor effect in the higher dose, administration of L19-IL-2 significantly reduced tumor volume to 21.4% (1.43 MIU/kg) and 2.7% (4.29 MIU/kg) compared with control mice (Fig. 2A). This result clearly shows that L19-IL-2 is superior to the untargeted cytokine. As shown in a separate experiment, this was not due to an IL-2-independent antitumor activity of the scFv L19 antibody fragment, because administration of 4.29 MIU/kg L19-IL-2 reproducibly led to a significant reduction of tumor load, whereas equimolar amounts of the naked scFv L19 were therapeutically inactive (Fig. 2B).

**CA 19-9 as a response marker of therapy.** Orthotopic tumors do not allow the continuous evaluation of tumor size by caliper measurements. For this reason, we established CA 19-9 as a valuable serum marker to monitor both tumor progression and therapeutic effects of the targeted immunocytokine L19-IL-2. It was possible to show a significant correlation between tumor volume and serum concentrations of CA 19-9 ($r^2 = 0.54$, $P < 0.0001$; Fig. 2C). Accordingly and in agreement with the therapeutic effects on tumor burden described above, CA 19-9 levels were significantly reduced following L19-IL-2 treatment, whereas no changes were visible in the IL-2-treated group (data not shown). These results prompted us to evaluate the time-response relationship of L19-IL-2-induced tumor growth inhibition. Interestingly, a L19-IL-2-dependent tumor growth delay in terms of 2-fold lower CA 19-9 levels when compared with controls was observed as early as day 3 after start of treatment. This difference continued to increase until the end of experiment. Here, a >15-fold increase in serum CA 19-9 levels was detectable in the control group, whereas CA 19-9 levels

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**Fig. 2.** Therapeutic effects of L19-IL-2 on orthotopic pancreatic cancer in nude mice. A to E, nude mice bearing orthotopic DanG pancreatic tumors were randomly treated. $\times$, mean ± SE tumor volume of each treatment group. Note that treatment with L19-IL-2 was significantly superior to treatment with equimolar amounts of IL-2. $*, P ≤ 0.004$, versus control; $+, P = 0.0003$, versus equimolar amounts of IL-2. $\delta$, percentage of tumor volume from vehicle-treated controls. Note that the naked L19 antibody achieved no therapeutic effect, whereas equimolar amounts of L19-IL-2 led to a significant decrease of the primary tumor volume. $*, P ≤ 0.004$, versus control and scFv L19. C, serum CA19-9 levels from mice bearing orthotopic DanG tumors and tumor-free animals were determined and CA19-9 level versus tumor volume was plotted for each individual animal ($n = 56$, $r^2 = 0.54$, $P ≤ 0.0001$). $\gamma$, CA19-9 levels were determined at the designated time points. Points, mean of control or treatment group; bars, SE. $\times$, $P ≤ 0.0007$, versus control; $+, P = 0.0003$, 1.43 MIU/kg body weight L19-IL-2 versus equimolar amounts of IL-2; $\gamma$, $P = 0.0513$, 4.29 MIU/kg body weight L19-IL-2 versus equimolar amounts of IL-2.

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**CA 19-9 as a response marker of therapy.** Orthotopic tumors do not allow the continuous evaluation of tumor size by caliper measurements. For this reason, we established CA 19-9 as a valuable serum marker to monitor both tumor progression and
were comparable with baseline levels (Fig. 2D) in animals treated with L19-IL-2.

Targeted immunocytokine L19-IL-2 does not elicit an unspecific inflammatory response. Having proven the therapeutic effect of L19-IL-2, we next ensured that IL-2 activity and subsequent inflammatory response is confined to the tumor not provoking an acute pancreatitis in adjacent nontransformed pancreatic tissue. For this purpose, lipase levels were determined as a surrogate marker of pancreatitis in mice treated with vehicle, IL-2, or L19-IL-2. However, neither treatment increased serum lipase concentrations (Fig. 2E). This finding was further corroborated by the histologic analysis of pancreatic tumors and residual nontransformed pancreatic tissue of animals treated with L19-IL-2 using conventional H&E staining and immunostaining with antibodies directed against NK1.1 (NK cells) and CD11b (predominantly macrophages), respectively. Of note, no signs of acute pancreatitis were detectable and inflammatory cells selectively accumulated in the tumor, sparing adjacent nontransformed pancreatic tissue (data not shown). Thus, L19-IL-2 treatment did not cause pancreatitis as an adverse effect.

Therapeutic effects of L19-IL-2 on pancreatic cancer are not cell type specific. To investigate whether L19-IL-2-mediated reduction of tumor growth could be reproduced in other pancreatic cancer cell lines, mice bearing orthotopic MiaPaca pancreatic tumors were assigned to treatment with the same conditions as specified above. Whereas untargeted IL-2 was not therapeutic at low dosage, equimolar amounts of L19-IL-2 reduced tumor load by 90%. At the high dosage, untargeted IL-2 achieved a 50% inhibition of tumor growth compared with an 83% reduction of tumor volume by L19-IL-2 (Fig. 2F).

L19-IL-2 treatment inhibits pancreatic cancer lymph node metastasis. Having confirmed the antitumor action of L19-IL-2 on primary pancreatic tumors, we next explored its potency in metastatic disease using the metastatic MiaPaca-A2 pancreatic tumor. Consistently, all lymph nodes infiltrated by cancerous cells and all liver metastases showed immunoreactivity for ED-B fibronectin (Fig. 3A, a), whereas noninfiltrated lymph nodes (Fig. 3A, b) and liver did not.

Based on this observation, mice bearing primary tumors and metastases were randomly treated with vehicle or L19-IL-2 at 4.29 MIU/kg. In addition to the anticipated reduction of primary tumor volume, L19-IL-2 treatment also decreased the mean lymph node area to <20% of the values obtained in vehicle-treated controls (P = 0.0120; Fig. 3B, compare a and b). To determine, if changes in lymph node area were due to infiltrating tumor cells, lymph nodes were immunostained for the presence of human cytokeratin to detect epithelial cells of human origin. This immunostaining with a pancytokeratin antibody revealed large, polymorph cells with strong immunoreactivity, indicative of tumor cells, in 80% of control mice (Fig. 3B, c) compared with 20% of L19-IL-2-treated mice. In contrast, lymph nodes of L19-IL-2-treated mice more frequently contained large quantities of small, granulocyte-like cells.
Effects of L19-IL-2 and gemcitabine. Gemcitabine is considered a standard first-line treatment for patients with advanced pancreatic cancer. We therefore compared L19-IL-2 and gemcitabine treatment in mice bearing orthotopic DanG tumors. Gemcitabine reduced pancreatic tumor volume to 28% compared with control mice. Of note, L19-IL-2 treatment compared superior with gemcitabine therapy as indicated by a 2.3-fold improved tumor reduction (Fig. 4B).

Effects of L19-IL-2 on tumor necrosis, apoptosis, and proliferation. To identify the mechanisms underlying L19-IL-2-induced tumor remission, we initially analyzed DanG pancreatic tumor samples by conventional H&E staining. One of the most intriguing features of L19-IL-2-treated tumors was extensive tumor necrosis (Fig. 5A, b). Indeed, in mice receiving L19-IL-2, necrosis comprised 42 ± 10.86% of the tumor area, whereas only 2.5 ± 2.177% of the tumor area was necrotic in untreated controls (P = 0.0159; Fig. 5A). The determination of the apoptotic index in the residual vital tumor tissue via terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay revealed no difference between both groups (Fig. 5B). By contrast, L19-IL-2-treated tumors displayed a 4.6-fold reduced fraction of proliferating cells based on Ki-67 immunostaining (P = 0.0177; Fig. 5C).

Identification of the immune effector cells mediating L19-IL-2-induced antitumor response. Apart from the extensive necrosis, H&E staining of L19-IL-2 treated tumors showed a round cell inflammatory infiltrate, which was absent in untreated controls. To further characterize the immune effector cells mediating L19-IL-2-induced tumor growth inhibition, tumor specimen were immunohistochemically assessed for NK cells and for CD11b+ cells, which include predominantly macrophages. Indeed, a >4-fold increase in CD11b+ cells was detectable in tumors of L19-IL-2-treated animals (Fig. 6A), and even more striking, NK cells were increased by >70-fold (Fig. 6B), suggesting that NK cells represent major players in the L19-IL-2-induced antitumor response in this animal model. To further substantiate this suspected role, we tested the effect of NK cell depletion via an anti-asialo-GM-1 antibody on the therapeutic efficacy of L19-IL-2. The NK depletion abrogated the effects of L19-IL-2 treatment such that tumor volume did not significantly differ from untreated controls (Fig. 6C). From these results, we conclude that NK cell–mediated mechanisms are required for the therapeutic efficacy of the targeted immunocytokine L19-IL-2.

Discussion

IL-2 is an approved drug for the treatment of metastatic renal cell carcinoma and melanoma. To specifically increase the concentration of IL-2 at the tumor site, locoregional treatment
schedules were designed and investigated. (11, 12). High concentrations of IL-2 are indeed essential for a therapeutic effect and have been exemplified by the application of IL-2 in patients with advanced pancreatic cancer via arterial or portal venous catheters in combination with polychemotherapy (23, 24). Unfortunately, all locoregional applications are limited to tumors that are accessible from the outside without having the possibility to deliver the cytokine to distant metastases. This hurdle can be overcome by a highly selective compound such as the targeted immunocytokine L19-IL-2. The most important prerequisite for a novel antibody-targeted delivery therapy like L19-IL-2 is the tumor-specific expression and accessibility of a target molecule within the body. ED-B fibronectin fulfills these requirements because (a) this molecule is specifically up-regulated during tumor angiogenesis and tumor growth (6, 25, 26) and (b) it is accessible for antibody-targeted therapies. This has been shown with the ED-B-specific scFv L19, which was successfully used for tumor imaging (10) and the delivery of various effector molecules in animal studies (26–29).

This is the first study that shows a specific overexpression of ED-B fibronectin in human pancreatic carcinoma, whereas no ED-B fibronectin was detectable in normal pancreas or chronic pancreatitis. Our immunohistochemical data strongly

Fig. 5. Effects of L19-IL-2 on tumor necrosis, apoptosis, and proliferation. DanG tumors from mice that had received either vehicle or 4.29 MIU/kg body weight L19-IL-2 were examined by conventional H&E staining (A, a and b), terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (B, a and b), or Ki-67 staining (C, a and b). Right, quantification of necrosis (A; *, P = 0.0159), apoptosis (B), and proliferation (C; *, P = 0.0177). Mean ± SE percentages of each group. Bar, 500 μm (A) and 50 μm (B and C).
supported the evaluation of the therapeutic efficacy of the targeted immunocytokine L19-IL-2 in mouse models for this devastating disease. There is increasing evidence that the ectopic or orthotopic environment has a different effect on tumor cell protein expression, tumor growth, invasiveness, angiogenesis, metastasis, drug delivery, and sensitivity to therapeutic agents (30). For this reason, we developed orthotopic mouse models for pancreatic cancer, which accurately recapitulate the tumor behavior and the clinical course of this deadly disease.

In both models, we strongly showed an ED-B fibronectin expression pattern, which is comparable with the human situation in both primary tumors and lymph node and liver metastases. Here, we report the first study that addresses the therapeutic efficacy of L19-IL-2 in a tumor grown in its natural microenvironment. In this orthotopic setting, we could clearly show that the systemic i.v. administration of clinically relevant doses of L19-IL-2 efficiently inhibited the growth of established primary pancreatic tumors and distant metastases, whereas equivalent concentrations of untargeted IL-2 had no or only minor therapeutic effects. It is noteworthy that the therapeutic effect of L19-IL-2 could be shown in two independent mouse models with different quantitative level of ED-B fibronectin expression (DanG, 22.33%; MiaPaca, 7.96%). This observation was unexpected, yet the density of ED-B fibronectin visualized...
by immunohistochemistry must not necessarily correlate with the in vivo accumulation of the targeted immunocytokine L19-IL-2 in the tumor. It may very well be that, in comparison with DanG, the apparently low target density in the MiaPaca tumor model can be compensated by a more functional vascular network leading to a higher accumulation of L19-IL-2 at the tumor site. Different levels of L19-IL-2 accumulation could also explain the induction of a complete and permanent remission in 40% of the animals, whereas untargeted IL-2 showed tumor regrowth after treatment. If our hypothesis is true, the stratification of patients who will most likely benefit from a targeted immunocytokine therapy will be possible using radioactively labeled L19. Future experiments in this direction are currently being done.

In this context, it is also important to note that, until today, the mechanisms underlying the antitumor activity of IL-2 are still not fully understood. In our study, one of the most intriguing features of L19-IL-2-treated tumors was an extensive tumor necrosis, which was most prominent in the central part of the tumor. In this region, few leukocytes were detectable. Therefore, tumor cell death caused by direct cell-to-cell contact with NK cells, macrophages, or other immune effector cells is unlikely. Indeed, recent evidence supports the occurrence of a local vascular leak syndrome provoked by lymphokines and nitric oxide produced by IL-2-activated immune effector cells (31, 32). Furthermore, direct cytotoxic effects of IL-2 (12) and activated NK cells (33, 34) on the endothelium were reported. Considering that (a) endothelial cells seem to migrate along extracellular matrix structures containing ED-B fibronectin (35) and (b) L19-IL-2 is selectively accumulating within the ED-B fibronectin-rich proangiogenic tumor microenvironment, we postulate that L19-IL-2-triggered direct and/or indirect mechanisms have a detrimental effect on both the tumor vasculature and the tumor cells themselves, which are per se insensitive to IL-2-treatment (36). Therefore, the considerable decrease of Ki-67-positive tumor cells in the tumor of L19-IL-2-treated animals can only be attributed to cytokines such as IFN-γ and tumor necrosis factor-α, which are released by the large amounts of tumor-infiltrating NK cells and macrophages. Both IFN-γ and tumor necrosis factor-α are known to directly inhibit the growth of pancreatic cancer cells (37, 38).

We could bolster this hypothesis by showing a L19-IL-2-triggered 4-fold increase of tumor-infiltrating macrophages and a >70-fold increase of NK cells. These data suggest that macrophages were not the main drivers for the therapeutic efficacy of L19-IL-2 in our study. In line with this notion, it has been established previously that depletion of macrophages could only attenuate but not abrogate the antitumor activity of IL-2, indicating the contribution of additional mechanisms (39). In our study, we could pinpoint the NK cells as the driving force, which on depletion completely abolished the therapeutic efficacy of L19-IL-2.

Our findings are in perfect agreement with published data, which report on (a) promising antitumor effects in addition to extravasation and infiltration of tumor tissues by NK cells in vitro and in vivo (40) and (b) NK cells that were identified as the primary target cell population for IL-2 in preclinical and clinical settings (41).

In conclusion, our preclinical data strongly support the initiation of clinical studies using the targeted immunocytokine L19-IL-2 in pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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