PANCREAS

Treatment of experimental murine pancreatic peritoneal carcinomatosis with fibroblasts genetically modified to express IL12: a role for peritoneal innate immunity

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Background: Peritoneal carcinomatosis from pancreatic cancer has a poor prognosis with a median survival of 3.1 months. This is mainly due to lack of effective treatment. Interleukin 12 (IL12) is a proinflammatory cytokine that has a potent antitumoral effect by stimulating innate and adoptive immunity.

Aim: To examine the antitumoral effect and toxicity of intraperitoneal delivery of IL12 using an ex vivo gene therapy approach in a murine model of pancreatic peritoneal carcinomatosis.

Methods: Peritoneal carcinomatosis was generated by direct intraperitoneal inoculation of the pancreatic cancer cell line Capan-1 in athymic mice. Syngenic fibroblasts were genetically modified in vitro to secrete IL12 using a polycistronic TFG murine IL12 retroviral vector coding for both p35 and p40 murine IL12 subunits. Ex vivo gene therapy involved injection of the genetically modified fibroblasts intraperitoneally twice a week for 4 weeks.

Results: Treatment of pre-established peritoneal carcinomatosis with fibroblasts genetically modified to express IL12 induced a marked inhibition of tumour growth as measured by comparison of the weights of the intraperitoneal tumour nodules in the treated and control animals (3.52 (SD 0.47) v 0.93 (SD 0.21) g, $p < 0.05$) and improved survival. This effect was associated with infiltration of the peritoneal tumour nodules with macrophages. Peritoneal lavage confirmed enhancement of the innate peritoneal inflammatory activity, with an increased number of activated macrophages and natural killer cells. Moreover, macrophages harvested from animals with peritoneal carcinomatosis and treated with IL12-expressing fibroblasts expressed an activated proinflammatory antitumoral M1 phenotype that included strongly enhanced reactive oxygen species and nitric oxide production. There was no treatment-related toxicity.

Conclusion: Multiple injections of genetically modified fibroblasts to express IL12 is an effective and well-tolerated treatment for experimental murine pancreatic peritoneal carcinomatosis via activated innate immunity and in particular activated M1 macrophages.

Peritoneal carcinomatosis is a common manifestation of cancer of the digestive tract with a poor prognosis. In a study of 100 patients with peritoneal carcinomatosis in non-gynaecological malignancies, the second most common primary tumour was pancreatic carcinoma (20%).¹ In this study, the overall median survival was 6 months. A recent French multicentre prospective study of 370 patients with peritoneal carcinomatosis showed an overall median survival of 3.1 months.² Among these patients, those with pancreatic cancer had the worst prognosis with a median survival of 2.1 months.

Multimodal therapeutic approaches including peritonectomy, intraperitoneal injection of the anticancer drug OK432, intracavitary immunotherapy, intraperitoneal chemohyperthermia and early postoperative intraperitoneal chemotherapy have been developed with limited success. The most promising results have been reported for comprehensive cytoreductive surgery combined with perioperative intraperitoneal chemotherapy. Unfortunately, preliminary data show efficacy mainly for colorectal carcinomatosis and gastric carcinomatosis.³ In current practice, patients with peritoneal carcinomatosis of pancreatic origin are offered best supportive care or chemotherapy.

Interleukin 12 (IL12) is one of the most potent proinflammatory cytokines.^{4,5} It is composed of two chains of 35 kDa (p35) and 40 kDa (p40). It directly stimulates the production of

interferon- γ (IFN γ), tumour necrosis factor- α (TNF α) and IL2 from peripheral blood T cells and natural killer cells,^{6,7} enhances the lytic activity of natural killer cells, and promotes the expansion of activated natural killer cells and activated T cells (CD4⁺ and CD8⁺ subsets).⁸ Through these functions, IL12 promotes the development of a type 1 T helper response that favours cell-mediated immunity.⁹ IL12 can also inhibit angiogenesis through IFN γ , the downstream chemokines IFN-inducible protein-10 and monokine induced by IFN γ (Mig).^{9,10} More recently, it has been shown that macrophages are required for the antitumoral effect of IL2 and IL12 in a mouse model of lymphoma.¹¹

Several phase 1 clinical trials using recombinant IL12 in the treatment of end-stage cancer showed promising results with tumour regression in some cases.¹² However, the use of IL12 administered via a systemic route is hampered by serious side effects, and its effectiveness is decreased because of its rapid degradation or elimination. These observations led us to develop alternative means of IL12 delivery by transfecting the gene into fibroblasts that will express the cytokine at the

Abbreviations: FCS, fetal calf serum; HPF, high-power field; IFN, interferon; IL12, interleukin 12; IRES, internal ribosome entry site sequences; LPS, lipopolysaccharide; Neo, neomycin; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SFM, serum-free medium; TNF, tumour necrosis factor

Figure 1 Experimental study design. Peritoneal carcinomatosis was generated by intraperitoneal inoculation of 10^5 Capan-1 cells in Swiss nu/nu athymic mice (black arrow). Ex vivo gene therapy involved intraperitoneal injections of the genetically modified fibroblasts, balb/c-interleukin 12 (IL12) or balb/c-neo twice a week starting on day 8 (white arrows). A total of nine injections were administered. Mice were killed 41 days after Capan-1 cell implantation (grey arrow).

tumour site. This ex vivo gene therapy approach mimics paracrine cytokine release in vivo and enhances the induction of tumour-specific immune responses without the troublesome side effects. Unlike most cytokines, simultaneous transfection of mammalian cells with two different genes is necessary for production of biologically active IL12.⁵ We have recently shown that experimental hepatocarcinoma can be efficiently and safely treated ex vivo with IL12 gene therapy using genetically modified fibroblasts.¹³ We applied this strategy to treat experimental pancreatic carcinomatosis. The present work shows that direct intraperitoneal injection of genetically modified fibroblasts to express IL12 induces a marked antitumoral effect via enhanced innate immunity.

MATERIALS AND METHODS

Retroviral constructs

The construction and characterisation of the polycistronic TFG murine IL12 retroviral vector coding for both p35 and p40 murine IL12 subunits have been described previously.^{13, 14} This MFG-based retroviral vector expresses cDNA encoding both subunits of IL12 with a selectable marker, neomycin phosphotransferase (Neo). Both subunits and the selectable marker Neo are promoted by the 5' long terminal repeat. The use of internal ribosome entry site sequences (IRES) allows the transcription of a single polycistronic message. The IRES sequence used was obtained from the 5' non-translated region of encephalomyocarditis virus. The control vector (MFGNeo) consisted of the same retroviral vector encoding only Neo.

Cell lines and transfection

Capan-1 is a human pancreatic cancer cell line from metastasis. It was cultured in RPMI (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum and 2 mM glutamine (Invitrogen). The syngenic fibroblasts from Balb/c mice (termed balb/c) were kindly provided by J E Gairin (Toulouse, France). Fibroblasts were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum and 2 mM glutamine (Invitrogen). IL12-producing balb/c (balb/c-IL12) cells were obtained by transfection of the cells with the plasmid DNA preparations of TFG mIL12 retroviral vector using LIPOFECTAMINE PLUS (Invitrogen) and selection with G418 (Sigma, Saint Quentin Fallavier, France) as described previously.¹³ A clone of fibroblasts expressing 2 ng/ 10^6 cells/24 h as determined by a quantitative "sandwich" ELISA enzyme immunoassay using a mIL12 ELISA kit (Genzyme, Cambridge, Massachusetts, USA) was used. IL12 production level was verified periodically. The

MFGNeo (mock) vector was also transfected into balb/c (balb/c-neo) using LIPOFECTAMINE PLUS. Balb/c-neo fibroblasts were subsequently used for treatment in the control group. Transfected cells were maintained in culture with G418 200 μ g/ml.

In vitro cell growth assay

Capan-1 cells were seeded in 35-mm dishes at a density of 100×10^3 cells/dish in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (1 ml/dish). Complemented medium (1 ml) originating from either IL12 or Neo-transfected fibroblast cultures was concomitantly added to the dishes. Culture medium was changed for fresh RPMI + 10% FCS/fibroblast complemented medium (1:1) 48 h later. Cell growth was measured at 48, 72 and 96 h after cell seeding by cell counting using a Coulter counter model ZM.

In vivo studies

Female Swiss v/v athymic mice were purchased from CERJ JANVIER (Le Genest Saint-Isle, France). All mice used for experiments were 6–8 weeks old and were housed in specific pathogen-free conditions in the animal facility of our institution, according to animal experimental ethics committee guidelines. Peritoneal carcinomatosis was generated by direct intraperitoneal inoculation of 5×10^5 Capan-1 cells. On using this experimental protocol, mice develop adenocarcinoma peritoneal nodules by day 8 after transplantation and clinical ascites from day 30. Ex vivo gene therapy involved injection of the genetically modified fibroblasts (5×10^6 cells/injection), balb/c-IL12 or balb/c-neo intraperitoneally twice a week. The total volume of cell suspension was 250 μ l in serum-free medium (SFM) in all experiments. The treatment started 8 days after Capan-1 tumour cell injection. Fibroblast injections were continued twice weekly until animals were killed 41 days after initial Capan-1 cell injection (fig 1). Blood was collected before killing by direct intracardiac puncture to measure biochemical liver tests. Data were presented as international units for aminotransferase, γ -glutamyl transferase (values <5 are arbitrarily measured at 5) and alkaline phosphatase, and as μ mol/l for bilirubin (values <1 are arbitrarily measured at 1) using a Vitros 950 (OCD, Johnson and Johnson, Rochester, New York, USA). Total ascitic fluid was collected and volume measured for each animal. A careful analysis of the peritoneal

Figure 2 Interleukin 12 (IL12) secreted by fibroblasts has no direct antiproliferative activity in vitro on Capan-1 cells. Capan-1 tumour cells were cultured using media from IL12-expressing fibroblasts or Neo-expressing fibroblasts, and cell growth was measured over 4 days (mean (standard deviation (SD)) of three experiments in triplicate).

cavity was done after a midline laparotomy. All peritoneal carcinomatosis nodules were removed and weighed to assess tumour progression. Groups of 5–6 animals were used for all experiments. In all, 20 mice were used in the experiments studying the effect of therapy on the tumour burden (10 mice treated with fibroblasts genetically modified to express IL12 and 10 control mice). Another 20 mice were used to evaluate the role of treatment on survival (10 mice in both treatment groups).

Analysis of in vivo systemic IFN γ and TNF α production

For this experiment, blood was collected from the retro-orbital sinus the day after the 7th intraperitoneal injection of fibroblasts. Mouse IFN γ ELISA set and mouse TNF α ELISA set from BD Biosciences Pharmingen (San Diego, California, USA) were used according to standard protocols.

Histology and immunohistochemistry

At 24 h after the first injection of fibroblasts—that is, 9 days after tumour challenge—mice were killed and the peritoneal carcinomatosis nodules removed for analysis. Fragments were fixed in formalin and embedded in paraffin wax for histological studies and immunohistochemical analyses. Sections of 4 μ m were coloured with haematoxylin and eosin. Mononuclear cells were counted in representative high-power fields (HPF; $\times 400$), in the periphery and within the tumour, in all animals (treated and control). Immunohistochemical examination was performed with rat anti-mouse monoclonal Mac-3 antibody (Pharmingen) at 1:100 dilution and with goat anti-mouse polyclonal anti CD31 antibody (SantaCruz Biotech, California, USA) at 1:200 dilution. Mac-3 staining was shown with EnVision (Dako, Glostrup, Denmark). CD31 staining was processed with a biotin-streptavidine-peroxidase technique (Dako) after antigen retrieval in a microwave oven in citrate buffer (pH 6) at 750 W, twice for 10 min. For all immunostainings, negative controls were created by omitting the primary antibody. Mac-3-positive cells were counted at $\times 400$ HPF in the periphery and within the tumour. CD31-positive cells were counted at $\times 200$. Histological study and immunohistochemical examination were performed by two blinded independent pathologists.

Peritoneal macrophage harvesting and culture

In specific experiments, we harvested peritoneal macrophages from animals bearing peritoneal carcinomatosis after the first injection of fibroblasts (9 days after Capan-1 injection). Peritoneal macrophages were harvested from female v/v athymic mice as described previously.¹⁵ In brief, peritoneal cells were obtained by injection of sterile 199 medium with Hank's salt into the peritoneal cavity. Collected cells were centrifuged, and the cell pellet was suspended in SFM optimised for macrophage culture (Invitrogen). For macrophage culture, cell adhesion was allowed for 2 h at 37°C with 5% CO₂ atmosphere in 96-well culture plates. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS; Invitrogen). After 2 h of adhesion, >98% of adherent cells were non-specific esterase positive and had the morphological appearance of macrophages by May-Grunwald Giemsa staining.

Flow cytometry analysis

We harvested peritoneal macrophages from animals bearing peritoneal carcinomatosis after one injection of fibroblasts (9 days after Capan-1 injection) as described above. Total peritoneal cells were counted and stained in PBS containing 2% FCS and incubated for 30 min with appropriate dilutions of various fluorochrome-conjugated mAbs. FITC-conjugated, PE-conjugated or APC-conjugated anti-Pan natural killer cells

(clone DX5), anti-CD3 (clone 145-2C11), anti-CD11b (clone M1/70), anti-CD14 (clonermC5-3), anti-CD19 (clone 1D3), anti-CD69 (clone H1.2F3) and corresponding isotype controls were used, all purchased from BD Pharmingen. Cells were extensively washed in PBS, and analysed on a FACScalibur cytometer using Cellquest software (Becton Dickinson, Mountain View, California, USA). Dead cells were excluded according to forward and side scatter characteristics. DX5⁺ CD3⁺ cells analysed in the lymphocyte gate and CD11b⁺ CD14⁺ cells analysed in the granulocyte/macrophage gate were, respectively, defined as natural killer cells and activated macrophages.

Assay for oxidising agent production

Macrophages (1.5×10^5) were placed in 96-well microplates. The oxygen-dependent respiratory burst of murine peritoneal macrophages stimulated with tumour promoter agent as described previously¹⁵ was measured by chemiluminescence in the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol, Sigma) using a thermostatically (37°C) controlled luminometer (Wallac 1420 Victor², Finland). The generation of

Figure 3 Interleukin 12 (IL12) ex vivo gene therapy of peritoneal carcinomatosis: antitumour effect and survival rates. (A) Overall, 20 mice were studied in two independent experiments, each group consisting of 4–5 animals. Mice were treated with intraperitoneal injections of balb/c-IL12 or balb/c-neo as described in fig 1. Antitumour effect was measured by comparison of weights of the intraperitoneal tumour nodules on day 41. IL12 ex vivo gene therapy induced a significant antitumour effect ($p < 0.05$). (B) Another 20 mice were used to study the effect of treatment on survival. The figure is representative of two independent experiments. IL12 ex vivo gene therapy induced a significant improvement in survival ($p < 0.002$).

chemiluminescence was monitored continuously for 30 min after incubation of the cells with luminol (66 $\mu\text{mol/l}$) in the presence of 100 nM tumour promoter agent.

Assay of nitric oxide production

Macrophages 1.5×10^5 were placed in 96-well microplates. Nitric oxide production in culture supernatants was assessed by measuring nitrite (its stable degradation product) content in culture medium. After stimulation in the presence of 100 ng/ml lipopolysaccharide (LPS), the culture medium (SFM for macrophages) was centrifuged and mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) and then incubated for 10 min at room temperature before measuring the absorbance at 550 nm in a microplate reader. NaNO_2 was used as a standard.

Statistical analysis

Statistical analysis of *in vivo* experiments was performed using the non-parametric Mann–Whitney test to compare the tumour size within the different groups. For the *in vitro* experiments, the data are expressed as mean (standard error (SE)) of three separate experiments with three replications per experiment. For each experiment, the data were subjected to one-way analysis of variance followed by the means multiple comparison method of Tukey. $p < 0.05$ was considered as the level of statistical significance.

RESULTS

IL12 has no direct *in vitro* antiproliferative activity on pancreatic cancer cells

Transfection of the fibroblasts with the plasmid DNA preparations of TFG mIL12 vector as described in Materials and methods resulted in the production of 2 ng/ 10^6 cells/24 h of IL12 in the culture media by a clone of fibroblasts. This cell clone was used for the subsequent experiments. First we assayed whether tumour cell proliferation was affected *in vitro* by IL12. Capan-1 tumour cells were cultured using media from IL12-expressing fibroblasts or Neo-expressing fibroblasts, and cell growth was measured over 4 days. There was no difference in cell proliferation between the two groups (fig 2). This confirmed that there is no direct *in vitro* antiproliferative effect of IL12 on Capan-1 cells. Next, we studied the *in vivo* effect of IL12 production in the peritoneal cavity in tumour-bearing mice.

IL12 *ex vivo* gene therapy has an antitumour effect on peritoneal carcinomatosis in a treatment model

To test our *ex vivo* gene therapy approach, we developed a murine model of pancreatic peritoneal carcinomatosis that was

generated by direct intraperitoneal inoculation of the human pancreatic cancer tumour cell line Capan-1 in athymic mice. Using this experimental protocol, mice develop adenocarcinoma peritoneal nodules as early as day 8 after tumour cell transplantation, and clinical ascites from day 30 (data from personal results, not shown).

Ex vivo gene therapy involved intraperitoneal injections of the genetically modified fibroblasts, balb/c-IL12 or balb/c-neo twice a week as described in Materials and methods (fig 1). The first injection was started on day 8 because preliminary data had shown that macroscopic peritoneal adenocarcinoma nodules are present at that time. Overall, 9 intraperitoneal injections of genetically modified fibroblasts were administered over 4 weeks and mice were killed 5 days after the last injection as shown in fig 1. Balb-c fibroblasts expressing the protein Neo have no antitumoural effect when compared with control mice that were not given fibroblast injection (data not shown). Thus, these genetically modified cells were used as controls in all experiments. Treatment with fibroblasts expressing IL12 induced a significant antitumour effect as measured by comparison of the weights of the intraperitoneal tumour nodules in the treated and control animals (3.52 (0.47) ν 0.93 (0.21) g, $p < 0.05$) as shown in fig 3A. In this experiment, two animals died from peritoneal carcinomatosis in the control group before the end point of the study compared with none in the treatment group. The survival rate was significantly improved in the interleukin-12 (IL12) treatment group (54.6 (4.9) ν 36.2 (3.0) days, $p < 0.002$; fig 3B). Figure 4 depicts two mice (one from each treatment group) from this experiment. There were no ascites in animals treated with the IL12-producing fibroblasts. On the contrary, 9 of 10 animals treated with the neo-producing fibroblasts developed ascites (mean (SD) volume was 622 (873) μl). Weight progression throughout the experiment was the same in both groups (3.1 (1.03) ν 2.5 (1.03) g in the control and IL12 groups, respectively; not significant (NS)) despite the presence of ascites only in the control group. There was no treatment-related toxicity. There was no difference in spleen (0.34 (0.19) ν 0.16 (0.05) g in the control and IL12 groups, respectively; NS) or liver weights (1.7 (0.29) ν 1.4 (0.18) g in the control and IL12 groups, respectively; NS) as well as in serum liver enzymes in the two groups (table 1). $\text{TNF}\alpha$ and $\text{IFN}\gamma$ expression was examined in the serum 24 h after intraperitoneal injection of fibroblasts expressing IL12 or neo. There was no $\text{TNF}\alpha$ expression detected in either group. There was no difference in $\text{IFN}\gamma$ expression in the two groups (132.4 (34.1) pg/ml in animals treated with IL12-producing fibroblasts ν 108.2 (47.4) pg/ml in the control animals; NS). These data show that IL12 *ex vivo* gene therapy has a strong therapeutic effect with no general or liver side

Figure 4 Interleukin 12 (IL12) *ex vivo* gene therapy of peritoneal carcinomatosis. The pictures depict two representative mice 35 days after intraperitoneal tumour cell inoculation. (A) Balb/c-neo-treated mouse (control) has ascites, Balb/c-IL12-treated mouse has no ascites. (B) Balb/c-neo-treated mouse has multiple peritoneal tumour nodules (white arrows). Balb/c-IL12-treated mouse has fewer peritoneal tumour nodules (white arrows).

Table 1 Results of intraperitoneal expression of IL12 in liver tests

	Bilirubin (μ mol/l)	AST (IU/l)	ALT (IU/l)	AP (IU/l)	γ GT (IU/l)
Balb-/c-neo (SD)	1.8 (0.4)	24 (3.5)	71.4 (17.3)	15 (0.3)	5
Balb/c-IL12 (SD)	1	17.8 (1.6)	64.4 (13.4)	14.6 (0.8)	5
Significance	NS	NS	NS	NS	NS

IL12, interleukin 12; IU, international units.

Liver tests in five treated mice and in five control mice (mean (SD)). There is no statistical difference between the two groups. Bilirubin values in the Balb/c-IL12 group were at the same level of 1. γ GT values were at the same level of 5 in all animals.

effects. To explore the underlying mechanisms, we analysed the infiltration of tumour fragments with immune cells.

IL12 induces infiltration of the tumour nodules by macrophages

To explore the mechanism of this antitumoural effect, we first did a pathological analysis of the peritoneal tumour nodules in the treated and control animals. Tumours corresponded to well or moderately differentiated adenocarcinoma. Tumour infiltration by mononuclear cells was more important in animals treated by intraperitoneal injections of IL12-producing fibroblasts when compared with control animals (401.3 (299) *v* 53.8 (9) mononuclear cells per HPF; *p* = 0.012). Mac-3 antibody staining confirmed infiltration of the tumour by macrophages in the IL12-treated animals (fig 5A,B). There was no difference in the vascular endothelial cell staining by CD31 within the tumour in the treated and non-treated animals (fig 5C,D). There was no difference in the results of histological and immunohistochemical analysis between non-treated animals and animals treated with Neo-producing fibroblasts (data not shown). Pathological analysis of the liver confirmed that neither tumour cells nor genetically modified fibroblasts migrated in the liver tissue. These results suggest a possible role for macrophages in this antitumour effect.

IL12 ex vivo gene therapy promotes peritoneal innate immunity

To further investigate whether IL12 gene therapy influences the peritoneal innate immune response, flow cytometry analyses were performed on total peritoneal cells 24 h after fibroblast injection in the context of peritoneal carcinomatosis as described in Materials and methods. Interestingly, a twofold increase in the absolute number of activated macrophages—namely, CD11b⁺ CD14⁺ cells—was observed in the peritoneal cavity of mice after one injection of IL12-expressing fibroblasts as compared with control mice (fig 6A,B). Furthermore, the absolute number of natural killer cells, defined as DX5⁺ CD3[−] cells in the lymphocyte gate, was also significantly increased after injection of IL12-expressing fibroblasts (fig 6C). Of the natural killer cells, >90% expressed the early activation molecule CD69, without any difference between the two groups (data not shown). Finally, no significant change was observed between the two groups regarding the number of B lymphocytes (CD19⁺ cells); T cell number was insignificant in this athymic mice strain. These results clearly show that IL12 ex vivo gene therapy enhances the recruitment of two major cell types of the innate immune system, macrophages and natural killer cells, which share activated phenotypes.

Figure 5 Interleukin 12 (IL12) ex vivo gene therapy induces infiltration of the tumour nodules with macrophages and does not modify endothelial cells within the tumour. (A) Immunochemical analysis of MAC3 antibody shows rare macrophages (arrows) within the adenocarcinoma proliferation in control animals. (B) Animals treated with fibroblasts expressing IL12 have numerous intratumoural macrophages with intense brown cytoplasmic staining (arrows). (C) Immunohistochemical analysis with anti-CD31 antibody showing vascular endothelial cells (arrows). There was no difference between control animals and (D) animals treated with fibroblasts expressing IL12.

Figure 6 Flow cytometry analysis on total peritoneal cells. (A) Flow cytometry analyses were performed on total peritoneal cells 24 h after fibroblast injection in the context of peritoneal carcinomatosis. Gating used for the study of CD11b⁺ CD14⁺ macrophages is shown. (B) A twofold increase in the absolute number of CD11b⁺ CD14⁺ cells was observed after one injection of interleukin 12 (IL12)-expressing fibroblasts as compared with control mice. (C) The absolute number of DX5⁺ CD3⁻ cells was also significantly increased after injection of IL12-expressing fibroblasts. Seven animals were used for these experiments.

Figure 7 Intraperitoneal injection of fibroblasts expressing interleukin 12 (IL12) increases the oxidative burst induced by phorbol myristate acetate in murine peritoneal macrophages. The generation of chemiluminescence was monitored continuously for 45 min after incubation of the peritoneal macrophages with luminol (66 μ mol/l) in the presence of 100 nM phorbol myristate acetate. Reactive oxygen species production is expressed in counts/s. The curves represent three separate experiments, each performed in triplicate. Ten animals were used in these experiments.

IL12 ex vivo gene therapy activates ROS and nitric oxide production from peritoneal macrophages

Peritoneal macrophages were harvested after one injection of fibroblasts as described in Materials and methods. They were incubated in vitro with luminol and stimulated with phorbol myristate acetate (PMA). Reactive oxygen species (ROS) production was measured by chemiluminescence. As shown in fig 7, IL12 induced a rapid, transient and significant increase in luminescence as compared with control. The observed peak occurred at 7 min. Chemiluminescence returned to the basal level after 50 min. Nitric oxide production in culture supernatants was then assessed by measuring nitrite content (its stable degradation product) in culture medium before and after stimulation by LPS (fig 8). IL12 significantly induced nitric oxide production in the absence of LPS when compared with Neo. This effect was magnified after stimulation with LPS, indicating that IL12 primes macrophages for stimulation. These in vitro data confirm that injection of fibroblasts expressing IL12 in the peritoneal cavity activates peritoneal macrophages in situ.

DISCUSSION

The data presented here are the first to show that administration of fibroblasts engineered to express IL12 is effective in treating murine peritoneal carcinomatosis from pancreatic origin. Treated mice had less ascites, a lower tumour burden and improved survival compared with control animals. These

Figure 8 Intraperitoneal injection of fibroblasts expressing interleukin 12 (IL12) increases nitric oxide production by peritoneal macrophage culture. Nitric oxide production in peritoneal macrophage culture supernatants was assessed by measuring nitrite content. Lipopolysaccharide (LPS) significantly induced nitric oxide production in both groups ($p < 0.05$). IL12 significantly induced nitric oxide production when compared with Neo ($p < 0.05$). This effect was magnified after stimulation with LPS. Ten animals were used in these experiments.

data also show strong evidence for the role of macrophages in the antitumoral effect. The peritoneal tumours of the mice treated with IL12 ex vivo gene therapy were significantly more infiltrated by macrophages. Peritoneal macrophages harvested from the treated animals were activated as shown by CD14 expression and increased ROS and nitric oxide production in culture. The prognosis of patients with peritoneal carcinomatosis of pancreatic origin is dismal. Most patients die within 6 months of diagnosis. The most promising results in the treatment of peritoneal carcinomatosis from digestive cancers have been reported for comprehensive cytoreductive surgery combined with perioperative intraperitoneal chemotherapy. Unfortunately, preliminary data show low efficacy for pancreatic carcinomatosis.³ Cytokines regulate immune responses and direct the maturation, activation and migration of inflammatory cells, but their systemic use is hampered by serious side effects and their effectiveness is decreased by their rapid degradation or elimination. These observations led to the development of alternative means of cytokine delivery using genetically modified cells.¹⁶

IL12 is one of the most potent antitumoral cytokines.⁵ It has never been used in the treatment of pancreatic peritoneal carcinomatosis. We hypothesised that transfecting the cytokine gene into carrier cells that will express IL12 at the tumour site will help mimic paracrine cytokine release in vivo, increasing the antitumoral effect and limiting the unwanted side effects. Moreover, the peritoneum is highly vascularised and should be a good candidate for immunotherapy. We have recently shown that direct liver expression of IL12 had a significant antitumoral effect in a mouse model of hepatocarcinoma.¹³

We used an ex vivo approach that offers distinct advantages over in vivo gene transfer techniques: (1) it is independent of transfection efficiency which may vary in vivo, in particular, when using liposomal gene delivery; (2) it avoids delivery of infectious viral particles to the host; (3) it does not include potential immunogens other than the nominal tumour antigen; (4) the exact expression of IL12 is known before injection and can be tailored at will depending on the number of cells injected. Fibroblasts were chosen as the carrier cells because in practice they are readily available to culture from skin biopsy specimens, transfect and select.¹⁷ We have developed a model of peritoneal carcinomatosis of pancreatic origin in athymic mice. Injection of the human pancreatic cancer cells Capan-1 induces carcinomatosis with ascites, adenocarcinoma peritoneal tumour nodules and subsequently death of the animal. A retroviral

vector with IRES that allows a balanced expression of p35, p40 and the selection marker in the transduced cells was used. We first showed that IL12 secreted by fibroblasts did not modify tumour cell growth in vitro. Therefore, its effect in vivo cannot be attributed to direct inhibition of the cell cycle. This is consistent with data showing that IL12 receptor is expressed primarily on activated T cells and natural killer cells.¹⁸ Secondly, we have shown that repeated injections of fibroblasts expressing IL12 induced a strong antitumoral effect in mice with pre-established peritoneal carcinomatosis. Mice treated with IL12 had a significant decrease in peritoneal carcinomatosis nodules and no ascites. This induced a significantly improved survival. We chose to administer repeated injections of IL12-producing fibroblasts to optimally stimulate the immune system. As with all cytokine-based therapy, repeated injections are necessary to obtain the maximum antitumour effect.¹⁶ This marked antitumoral effect was not associated with treatment-related side effects, and in particular there was no difference in liver tests between the treated and control groups. Raised liver enzymes have been a problem in clinical trials using systemic administration of IL12.¹² Moreover, weight progression was the same in the two groups despite the presence of ascites in the control group. This indicates a relative weight loss due to onset of cachexia in the control group. Systemic TNF α levels were however below detection limits in both groups. Examination of the pathology of tumour samples showed an early infiltration of the peritoneal tumour by macrophages. There was however no difference in the number of endothelial cells or neutrophils in the two groups. These data prompted us to further explore the role of macrophages in our antitumoral effect.

IL12 gene therapy induced a significant increase in the number of activated macrophages as shown using CD14 antibodies and in activated natural killer cells. These are the two major cell types of the innate immune system.

Next, we cultured in vitro peritoneal macrophages harvested from mice treated with IL12. In response to PMA that activates proteinase C, macrophages harvested from animals treated in vivo with fibroblasts genetically modified to express IL12 produced significantly more RLO in vitro than macrophages harvested from control animals. This indicates that the macrophages were primed in vivo by IL12. Furthermore, basal production of nitric oxide was increased in IL12-treated animals, suggesting induction of inducible nitric oxide synthase. This was confirmed by nitric oxide production after stimulation with LPS, which was increased ninefold in the treated animals compared with the controls. These results clearly indicate that IL12 induces an M1 polarisation of peritoneal macrophages. Activated M1 macrophages are potent effector cells that kill tumour cells and produce proinflammatory cytokines.¹⁹ The ability of IL12 to prime peritoneal macrophages for nitric oxide production may be partly accounted for by exposure of these macrophages in vivo to IFN γ produced by natural killer cells which have been shown to be activated and increased in response to administration of IL12.²⁰ There was no difference in IFN γ levels in the serum of treated animals when compared with controls, arguing in favour of a local intraperitoneal effect. IL12 could also exert its observed priming effect by enhancing the expression of receptors such as CD14 as seen in our flow cytometry experiments.

In summary, we have shown that pancreatic peritoneal carcinomatosis can be successfully treated with fibroblasts genetically modified ex vivo to secrete IL12. Our data support a pivotal role of peritoneal macrophages in this antitumour effect. This ex vivo gene therapy approach should be evaluated as a therapeutic option in patients with peritoneal carcinomatosis.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 94

Colonoscopy showed narrowing with diffuse rugged submucosal oedema but no ulcers or tumours in the sigmoid colon (fig 1). Biopsies showed no specified findings. The barium enema study showed poor extensibility of the lumen with serrated contour-like "accordion" and the extrinsic mass effect of the sigmoid colon (fig 2). Computed tomography showed circumferential wall thickening of the sigmoid colon, surrounded by inhomogeneous mesenteric fat with slightly higher attenuation than normal subcutaneous fat (fig 3). Occlusion of the mesenteric vessels was not identified. The above features on chronic presentation were consistent with mesenteric panniculitis of the sigmoid colon. Conservative treatment with total parenteral nutrition resulted in gradual improvement of symptoms and resolution of the abnormalities 3 months later.

Mesenteric panniculitis is a rare idiopathic disease characterised by chronic non-specific inflammation of the adipose tissue of the bowel mesentery. Patients may present with abdominal pain, diarrhoea, or intestinal obstruction, as the disease progresses. The appearance of the mesenteric panniculitis varies from increased attenuation in the mesentery to solid soft-tissue masses. Surgical biopsy is required to make a definitive diagnosis; however, characteristic imaging features can help in suggesting its diagnosis and distinguishing it from mesenteric lymphoma, carcinoid tumour, carcinomatosis and mesenteric mesothelioma. Medical treatments, including total parenteral nutrition, steroids and immunosuppressive agents, are usually attempted. Surgical resection may be required for retractile mesenteritis, which is the final fibrotic stage with obstructive symptoms or vascular involvement.

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