Immunoprotective Therapy with Targeted Anticancer Drugs

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SUMMARY: Doxorubicin or mitomycin C bonded to poly[N^5-(2-hydroxyethyl)-L-glutamine]-*graft*-poly(ethylene glycol) or poly[*N*-(2-hydroxypropyl)methacrylamide], non-targeted or targeted with monoclonal antibodies, do not induce expression of FasL on selected cancer cells (human colorectal cancer cell line SW 620) thus protecting effector cells of the immune system against Fas-counterattack. The pre-treatment with PHPMA-bound DOX does not only protect but in fact mobilizes defense mechanisms of the tumor-bearing hosts. The treatment with selected monoclonal antibody-targeted PHPMA-bound DOX causes a rapid and complete rejection of established tumors (mouse B cell leukemia BCL1, mouse B cell lymphoma 38C13 and mouse T cell lymphoma EL4) and generates prolonged systemic anti-tumor immunity.

Introduction

The concept behind immuno-surveillance against cancer is that tumor cells continuously develop, but that there may not be clinical evidence of their presence because the immune system recognizes the cells as foreign and destroys them.

Complex interactions of cancer and host have resulted from years of evolution, during which the mammalian immune system has developed sophisticated means to counter invading cancer while cancer cells have acquired mechanisms to increase expansive growth and survival in the hosts. We do not know yet for sure whether the physiological function of the immune system is to recognize and destroy malignant cells before they grow to tumors, or to kill tumors after they are formed. This theoretical role for the immune system is called immuno-surveillance.

However, tumor rejection does not always occur spontaneously *in vivo* indicating that defects in the generation or execution of an anti-tumor immune response may be common. Although various lines of evidence have shown that cytolytic, cytotoxic T lymphocytes, natural killer cells, lymphokine-activated killers and macrophages of the immune system have a potential to attack tumor cells, it is not yet understood how this potential is blocked. To define rational

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anti-tumor immunotherapy strategies, the reasons for the frequent lack of efficacy of the immune response to developing tumors must be elucidated. Moreover, anti-cancer reaction of the host is often hampered by the fact that the defense system of the tumor-bearing host is generally depressed either as a consequence of extensive growth of the cancer or by intensive treatment with **classic** cytostatic drugs (chemotherapy) or radiation (radiotherapy).

In a heterogeneous population of cancer cells, there are always cells resistant to **classic** chemotherapy or radiotherapy. Such classically untreatable cells represent the so-called minimal residual tumor disease, which should be eradicated by the defense system of the tumor-bearing host. We and others reported that conjugation of cytotoxic or immunosuppressive drugs to a water-soluble copolymer carrier significantly reduces their non-specific toxicity[1–6] including immunotoxicity[7,8]. Despite many data on the mechanisms of action of polymer-bound drugs obtained so far, their effect on the regulatory and effector functions of the defense systems still remains to be characterized.

Results and discussion

FasL expression on cancer cells induced by different ability of mitomycin C and doxorubicin and their polymer-bound counterparts

One of the active mechanisms by which malignant cells escape immuno-surveillance is Fas-counterattack. The Fas receptor (Fas, FasR, APO-1, CD95) and its ligand (FasL, CD95L) are transmembrane proteins. Engagement of FasL by Fas triggers a caspase-dependent apoptotic signal that culminates in programmed cell death – apoptosis[9,10]. It was initially thought that the Fas/FasL-mediated apoptosis is involved only in thymocyte clonal deletion, tolerance acquisition, immune response termination and in elimination of transformed and infected cells[11,12]. Previously, it was reported that various cancer cell lines express FasL and can kill *in vitro* lymphoid cells which are sensitive to the Fas/FasL-mediated apoptosis[13–19]. Recently, FasL expression has been demonstrated in several human malignancies *in vivo*. Among them are colorectal carcinomas[13], melanomas[14], hepatomas[16], lung carcinomas[17], pancreatic carcinomas[19], esophageal carcinomas[20], Ewing's sarcoma[21] and leukemias[22]. Fas-counterattack induced by $FasL^+$ cancer cells in Fas^+ tumor-infiltrating lymphocytes is facilitated by the frequent resistance of cancer cells to apoptosis due to non-functional[19], down-regulated or lost FasR[23] or through overexpression of the Fas inhibitory protein, cFLIP[24,25]. It was reported that treatment with anticancer drugs such as DOX, methotrexate, cytarabine, etoposide and cisplatin at therapeutic concentrations leads to the induction of CD95L[22,26] on cancer cells. In Fas-resistant tumor cells, the drug-induced up-regulation of FasL might eliminate or at least minimize any local anti-tumor immune response.

Free drugs and polymer-bound drugs differ in their intracellular accumulation and destination, in their ability to overcome multidrug resistance, in the activation of detoxification mechanisms[27] and also in their ability to induce drug-dependent apoptosis[28]. Thus, we addressed

the question of whether surface expression of FasL could be diminished if malignant cells are exposed to a non-targeted or targeted polymer-bound drug instead of to a free drug.

We have tested two drugs (DOX and MMC) conjugated to biodegradable water-soluble polymeric carriers, non-targeted or targeted with anti-CD71 mAbs, (*a*) poly[N^5-(2-hydroxyethyl)-L-glutamine] grafted to poly(ethylene glycol) to which MMC is bound through GFAL oligopeptide spacer (PHEG–PEG_{5000}–GFAL–MMC)[29] and (*b*) poly[*N*-(2-hydroxypropyl)-methacrylamide] (PHPMA) containing the oligopeptide spacer GFLG to which DOX is conjugated[30].

An increased expression of FasL was observed on the surface of SW620 human colorectal cancer cells after treatment with 50 μg/mL MMC (Fig. 1A) or 5–50 μM DOX (Fig. 2A) while no increase was observed if cells were exposed to MMC conjugated to PHEG–PEG_{5000}–GFAL (Fig. 1B) or to DOX conjugated to PHPMA, non-targeted or targeted with anti-CD71 mAbs (Fig. 2B, 2C).

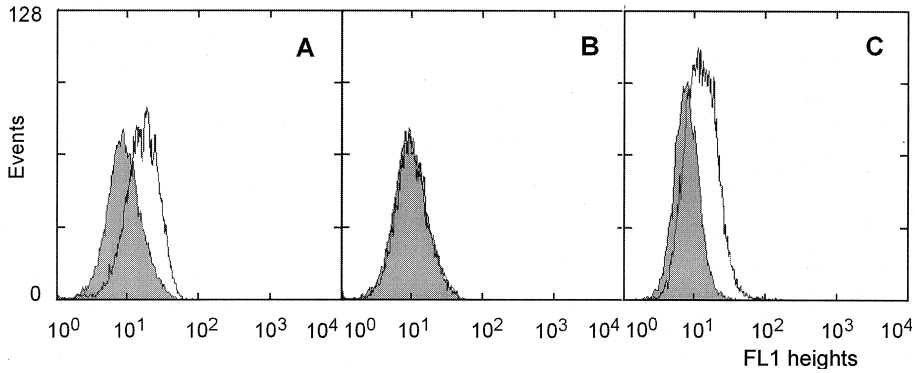

Fig. 1 Expression of FasL on SW620 human colorectal cancer cell line 48 h after exposure to free MMC or to polymer-bound MMC (PHEG–PEG_{5000}–GFAL–MMC). FasL expression was determined on the surface of SW620 cells exposed to 50 μg/mL of free MMC (Fig. 1A), 50–100 μg/mL of PHEG–PEG_{5000}–GFAL–MMC (Fig. 1B) or on SW 620 cells from MLTC (Fig. 1C). Flow cytometry analysis was performed using a FACSorter (Becton–Dickinson, USA) and anti-FasL monoclonal antibody (PharMingen, USA; clone NOK-1; 1 μg/10^6 cells). Matrix metalloproteinase inhibitor (i MMP; PharMingen, USA; clone KB8301; final concentration 10 μM) was added to the reaction mixture to avoid the shedding of FasL from the surface of $FasL^+$ cells. Cells were incubated in serum-free RPMI 1640 medium with EDTA (0.08 %) for 40 min with anti-FasL antibody conjugated with biotin. After extensive washing, the cells were incubated with streptavidin-FITC for 30 min at 4 °C and, after further washing (once with RPMI 1640-EDTA and once with PBS), 5×10^4 viable stained cells/sample were analyzed. The viability was determined by light scattering intensity and PI exclusion. While incubation of SW 620 in MLTC or cultivation in the presence of the free drug induces high expression of FasL, incubation with MMC bound to the polymeric carrier is comparable to the control.

These results suggest that the signals involved in the increase in surface expression of FasL are different if malignant cells are exposed to the free drug or to polymer-bound drug. The consequence of the fact that the polymer-bound drugs, unlike their classic free forms, do not induce FasL expression on cancer cells is that their potential use for the cancer treatment protects the effector cells against Fas-counterattack and thus spares the effector anti-tumor mechanisms of the patient's immune system.

This is in accordance with our previous *in vivo* observation that the application of polymer-bound drugs, in contrast to the free drug, protects immune effector functions represented by the cytotoxic activity of T cells and natural killer cells isolated from experimental tumors and spleens of animals transplanted with mouse T cell lymphoma or human colorectal carcinoma SW 620[31,32].

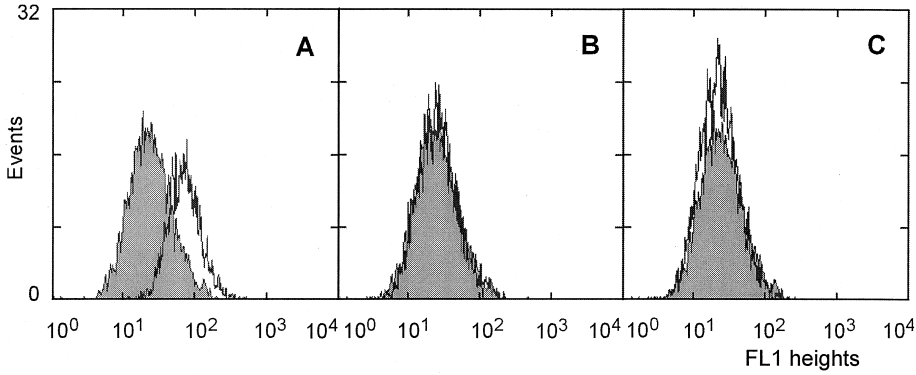

Fig. 2 Expression of FasL on SW620 human colorectal cancer cell line 48 h after exposure to free DOX or to PHPMA-bound DOX non-targeted or targeted with anti-CD71 mAbs. FasL expression was determined on the surface of SW620 cells exposed to 5 μM free DOX (Fig. 2A), 5–10 μM PHPMA–DOX (Fig. 2B) or 5–10 μM PHPMA–DOX targeted with anti-CD 71 mAbs (Fig. 2C). Flow cytometry analysis was performed as described in the legend to Fig. 1. While incubation of SW620 with free DOX induced high expression of FasL, incubation with polymer-bound DOX non-targeted or targeted with anti-CD71 mAbs was comparable to the control.

Effect of pre-treatment of experimental animals with free doxorubicin and cyclosporin A or with polymer-bound doxorubicin on the growth of mouse T cell lymphoma EL4

In mice, we have observed substantial protection of defense mechanisms of the cancer cell recipient after pre-treatment with PHPMA–DOX. The C57BL/10 strain of inbred mice was pre-treated by five daily injections of CsA (135 mg/kg), DOX (12.5 mg/kg) or of PHPMA-DOX (25 mg/kg) and, immediately after the last injection, the animals were transplanted with mouse T cell lymphoma EL4 (10–10^6 cells). The difference in the aggressivity of tumor

growth in experimental groups pre-injected with the free drugs or polymer-bound drug was significant already at a dose as low as 10^2 transplanted malignant cells (Table 1).

Table 1 Comparison of the effect of pre-treatment with free DOX and PHPMA-DOX

Pre-treatment	Number of tumor cells	Beginning of tumor growth after day	T/C [a] %	LTS [b]
No	10	never	–	5/5
DOX	10	33	–	3/5
PHPMA–DOX	10	never	–	4/4
No	10^2	21	–	3/5
DOX	10^2	19	115	4/5
PHPMA–DOX	10^2	never	–	5/5
No	10^3	17	–	0/5
DOX	10^3	14	96	0/5
PHPMA–DOX	10^3	17	120	1/5
No	10^4	14	–	0/4
DOX	10^4	12	82	0/5
PHPMA–DOX	10^4	12	101	0/5
No	10^5	10	–	0/4
DOX	10^5	7	114	0/4
PHPMA–DOX	10^5	10	92	0/4
No	10^6	7	–	0/5
DOX	10^6	5	75	0/5
PHPMA–DOX	10^6	7	101	0/5

[a] Ratio of median survival of the test group (T) to that of untreated control (C). [b] Long-term survivors.

However, the pre-treatment of mice with the polymer-bound drug, unlike with non-modified classic cytostatics, did not only protect the cells engaged in the defense anti-tumor reaction. We have observed that the administration of PHPMA–DOX mobilizes somehow the defense mechanisms and the type of defense response that follows the treatment. C57BL/10 mice five times injected with PHPMA–DOX (total dose of DOX was 25 mg/kg) before transplantation with 10^2 mouse T cell lymphoma EL4 never developed solid tumor, which was fast-growing in the controls and in the mice injected with free DOX (12.5 mg/kg). The observed mobilization of the defense system was of a limited capacity. The mice were not protected in the same way if tumor growth was induced by injection with 10^3 cells or more.

Mobilization of defense anti-tumor mechanisms in the tumor-bearing host after treatment with polymer-bound drugs

In this study, we have tested the assumption that the polymer-bound drugs mobilize in some way the defense anti-tumor response of the tumor-bearing host. First, the mice were treated with mAbs-targeted PHPMA–DOX. The group of experimental animals treated in this way (Fig. 3) was re-transplanted with a lethal dose of the same cancer cells, which caused death of all control mice, and left without any other treatment. Our data, though preliminary, demonstrate that up to 40 % of experimental mice transplanted with BCL1 B cell leukemia, treated with PHPMA–DOX–B1mAbs and re-transplanted with a lethal dose of the same malignant cells survived without any other treatment for more than 90 days. Similarly, up to 80 % of experimental mice transplanted with 38C13 mouse B cell lymphoma, treated with PHPMA–DOX–anti-CD 71 mAbs and re-transplanted with a lethal dose of the same malignant cells survived without treatment more than 30 days. Finally, 100 % of experimental mice which were cured with PHPMA–DOX–anti-Thy 1.2 mAbs from the very aggressive mouse T cell lymphoma EL4 and re-transplanted with a lethal dose of the same tumor survived more than 35 days. The reported data are still very preliminary as the experiments have not yet been finished.

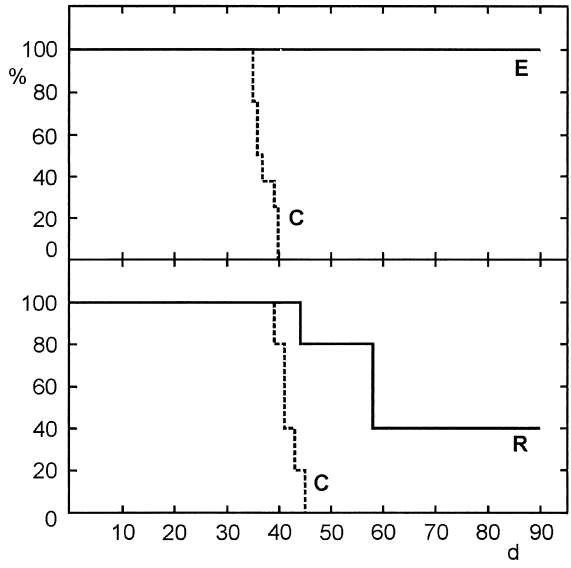

Fig. 3 *Above*: The growth of BCL1 mouse B cell leukemia (Balb/c mice were injected i.p. with 10^5 cancer cells of mouse B cell leukemia BCL1 and on days 8, 11 and 14 were treated i.v. with 100 μg of PHPMA–DOX targeted with monoclonal anti-idiotypic antibodies B1mAb (**E**). *Below*: The survival of mice after re-transplantation with the same malignant cells; 106 days after the last treatment the mice were i.p. re-transplanted (**R**) with the same dose of the same malignant cells and left without any other treatment. (**C** control)

A certain immunostimulatory activity of PHPMA-bound daunomycin was documented already in our first publication dealing with the side-effects of polymer-bound drugs. It was apparent from those data that daunomycin injected i.v. or i.p. in the form of a conjugate with PHPMA is not myelotoxic but somehow stimulates bone marrow stem cells, leading to an increase in the number of colony-forming unit-spleen[1]. On the other hand, PHPMA–DOX conjugates were not found to show immunostimulatory activity in Walker sarcoma[6].

List of abbreviations

CsA	cyclosporin A	MLTC	mixed lymphocyte-tumor culture
CTL	cytotoxic T lymphocyte	MMC	mitomycin C
DOX	doxorubicin	NK cell	natural killer cell
FITC	fluorescein isothiocyanate	PEG	poly(ethylene glycol)
GFAL	GlyPheAlaLeu	PBS	phosphate-buffered saline
GFLG	GlyPheLeuGly	PHEG	poly[N^5-(2-hydroxyethyl)-L-glutamine]
LAK	lymphokine-activated killer	PHPMA	poly[*N*-(2-hydroxypropyl)-methacrylamide]
MAb	monoclonal antibody	PI	phycoerythrin

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