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Granulocyte macrophage colony stimulating factor pretreatment induces an increase of rat Kupffer cells with enhanced cytotoxicity *in vitro* and prevention of tumor outgrowth *in vivo*

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Male Wag rats were pretreated for 7 days with 1000 U/ml recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF). Rat Kupffer cells (KC) were isolated by a enzymatic method. We injected decreasing numbers of CC531 tumor cells in the portal system. Mean KC yield increased from 1.5 ± 0.2 to 2.2 ± 0.2 ($p < 0.05$). Mean percentage of KC-mediated cytotoxicity against CC531 increased from 20.0 ± 0.5 to 42 ± 1.0 after rmGM-CSF ($p < 0.05$). At 1×10^5 CC531 tumor cells we demonstrated prevention of formation of small foci of $CC52^+$ tumor cells. We demonstrate increased isolated KC with enhanced cytotoxic capacity after rmGM-CSF. rmGM-CSF induced prevention of minimal residual disease in the rat liver.

Key words: Cytotoxicity, GM-CSF, Kupffer cell, liver, minimal residual disease.

Introduction

The liver contains the largest population of tissue macrophages: the Kupffer cells (KC).[1] These cells were shown to be involved in the host defense against experimentally induced hepatic metastasis.[2,3] Recent investigations indicate that the growth factor granulocyte macrophage colony stimulating factor (GM-CSF) activates murine macrophage tumoricidal activity *in vivo*.[4] A second important issue concerning the therapeutic potential of GM-CSF is its ability to induce local macrophage proliferation, as was shown for peritoneal macrophages in GM-CSF transgenic mice.[5] Furthermore, it was shown that exogenous administration of GM-CSF induces an increase of KC in the murine liver.[6]

Experimental data from *in vitro* experiments in our laboratory indicate that GM-CSF also enhances human KC-mediated cytotoxicity against colorectal tumor cells (SW948), by inducing an increase of tumor necrosis factor (TNF)-α expression.[7] A recent study in patients with advanced malignancies demonstrated that systemic administration of GM-CSF induces monocyte activation, with enhanced direct cytotoxicity levels *ex vivo*, and that this therapy is tolerated well by patients involved.[8] However, no increases of the cytolytic molecules TNF-α or interleukin (IL)-1 could be measured in serum. Furthermore, antibody-dependent cellular cytotoxicity (ADCC) may play a role, by either using GM-CSF as monotherapy or in combination with the monoclonal antibody (mAb) 17-1a in the treatment of patients with colorectal cancer.[9,10]

An important drawback of these clinical studies is that the patients involved usually have advanced metastatic cancers, which are unlikely to respond to monocyte/macrophage-directed immunotherapy. Experimental evidence in our laboratory indicates that newly recruited monocytes are the first cell type that surround newly formed hepatic metastasis.[11] Any monocyte/macrophage-directed immunotherapy is therefore likely to be most effective in the earliest stages of metastatic development. This situation can be compared to the presence of minimal residual disease (MRD) in patients with colorectal cancer.[12]

Several authors described that *pretreatment* with biological response modifiers can prevent the outgrowth of experimentally induced metastasis. Herling[13] described a significant reduction of melanoma growth in rats pretreated during 24 h with divinyl ether–maleic anhydride Pyran copolymer. Holmberg[14] described that pretreatment with zymosan for 3 days before giving the tumor load resulted in a significant reduction of tumor growth in the liver, whereas no preventive effect was found for tumors induced in the kidney. Injection of *Corynebacterium parvum* or zymosan 7 days before tumor

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load (squamous cell carcinoma) significantly inhibited tumor outgrowth.[15,16] Brodt[17] described inhibition of a liver-homing clone of Lewis lung carcinoma after pretreatment with a lipophilic muramyl dipeptide. Yamashita[18] demonstrated that preoperative immunoactivation with OK-432 pretreatment, a derivative of streptococcal bacteria wall, reduced the incidence of liver metastases in rats given injections of tumor cells via the main portal vein. OK-432 has been reported to activate macrophages non-specifically in hosts.[19,20] However, activation of T lymphocytes and non-specific killer cells may also occur.[21–23]

In this study we investigated the effect of recombinant murine (rm)GM-CSF in an experimental Wag rat/CC531 hepatic metastasis model, with as primary aim the prevention of MRD in the liver. Initially, we investigated the effects of a 7 day i.p. rmGM-CSF treatment regimen on rat macrophage populations in the liver parenchyma and on rat KC-mediated cytotoxicity against the syngeneic CC531 tumor cells *in vitro*. Subsequently, we investigated the possible preventive effect of a 7 day pretreatment regimen with rmGM-CSF by injection of different amounts of CC531 tumor cells into the portal system of rats, after which the numbers of metastatic foci were counted, and compared between the control and the pretreatment group.

Materials and methods

Animals

Male Wag rats, aged 8–10 weeks (weighing approximately 240 g) were obtained from The Netherlands Cancer Institute (Amsterdam) and kept under standard conditions. The investigation protocol was approved by the ethical committee.

[^{3}H]Thymidine incorporation assay

To be certain of biologically active rmGM-CSF (Sandoz, Wien, Austria) rat KC were cultured for 7 days in a 96-well flat-bottom microtiter plate (Greiner) at 40 000 cells per well, with the presence of 0, 1, 5, 10, 50 or 100 U/ml rmGM-CSF. Cells were pulsed with 0.5 μCi [methyl-^{3}H]thymidine (specific activity 25 Ci/mmol; Amersham, Amersham, UK). Subsequently, the cells were harvested on Titertek filter paper by an automated cell harvester (Titertek; Flow, Irvine, UK). Thymidine incorporation was measured by liquid scintillation in a β-emission counter (1214 RackBeta 'Excel'; LKB, Wallac).

KC isolation

KC were enriched with a recently developed isolation procedure without the use of perfusion techniques.[24] Livers were weighed and cut into small tissue fragments (1–2 mm^3) in Geys balanced salt solution (GBSS). Liver homogenate was then incubated in 75 ml GBSS with 0.2% pronase (Boehringer-Mannheim, Germany) and 0.8 g/ml DNase (Sigma, St Louis, MO) on a magnetic stirrer at 37°C for 30 min with continuous pH registration. The pH was kept at 7.3–7.5 with 1 N NaOH. After incubation, the suspension was filtered through a gauze (pore 60 μm) and the filtrate was centrifuged at 300 *g* for 10 min. The cells were washed at least twice by resuspending the pellet in 50 ml GBSS and 0.8 μg/ml DNase. After washing, the pellet was resuspended in 5 ml GBSS and 0.8 μg/ml DNase. The non-parenchymal cells were separated from non-viable cells, remaining parenchymal cells and erythrocytes by centrifugation on a 16% Nycodenz (Nycomed, Oslo, Norway) gradient for 20 min, 600 *g* at 4°C. The interface was collected and resuspended in 5 ml GBSS and 0.8 μg/ml DNase and centrifuged at 300 *g* for 10 min. KC were subsequently enriched by counterflow centrifugal elutriation (Beckman; centrifuge J2-21M, rotor JE-6B, standard chamber) at a constant centrifuge speed of 600 *g*. After elutriation the KC were counted with a Bürker counting chamber and viability was determined by 0.25% Trypan blue dye exclusion. Purified cell types in the enriched cell suspensions were identified by immunophenotyping with the mAb ED1.

Tumor cell line

The tumor cell line CC531 is a carcinoma originating in the colon of rats exposed to methylhydralazine, syngeneic with WAG/Rij rats and well defined.[25] Tumor cells were cultured under standard incubator condition in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; Flow). Cell suspensions were prepared by enzymatic detaching of the CC531 cells with trypsin–EDTA solution (Life Technologies, Middlesex, UK) at room temperature (20°C). After centrifugation cell suspensions were resuspended in DMEM to the required concentration. The viability of the cells was assessed with Trypan blue exclusion and was always greater than

95%. For the cytotoxicity assay the tumor cells were incubated at 10^4 cell/well in 96-well flat-bottom microtiter plates (Greiner).

MTT cytotoxicity assay

Cell-mediated cytotoxicity by freshly isolated KC was measured with a modified colorimetric MTT assay as previously described.[26] KC were incubated at a constant concentration of 5×10^4/well with CC531 colon carcinoma cells at an effector to target ratio (E/T ratio) of 5 in a total volume of 100 μl/well. All experiments were performed in triplicate in a 96-well round-bottom microtiter plate (Greiner). KC and all cell concentrations of CC531 cells were incubated separately as controls. After 20 h of incubation at 37°C, the MTT assay was performed. The absorbance (OD) was recorded directly on a microplate spectrophotometer (Titertek Multiscan MCC 340; Flow) at a wavelength of 540 nm. Percentage cytotoxicity was calculated as follows:

$$\%\text{Cytotoxicity} = 1 - \frac{[\text{OD CC531 cells} + \text{KC}] - [\text{OD KC}]}{[\text{OD CC531 cells}]} \times 100$$

Induction of hepatic metastasis

An experimental hepatic metastasis model has been used as described previously.[27] A laparotomy was performed under general anaesthesia (Hypnorm) and a small loop of small intestine exposed. According to previous descriptions of the model 10^6 CC531 tumor cells in 0.5 ml DMEM were slowly injected into a mesenteric vein under microscopic vision with a 0.4×12 mm needle followed by ligation of the vein. Injection of the tumor cells results in small macroscopically visible liver metastases of 1 mm after 14 days. In this paper we introduce a modification of this model by injecting 10^6 tumor cells (two groups of six animals), 0.5×10^6 (two groups of three animals) and 10^5 tumor cells (two groups of three animals).

Immunohistochemistry

To study the numbers of liver macrophages, small snap frozen (liquid nitrogen) liver biopsies were cut into 8 μm thick cryoslides. Within 1 week after section the slides were fixed in acetone for 10 min at 4°C. Slides were then washed in phosphate buffered saline (PBS, pH 7.4) and incubated with the mAb ED1 (diluted at 1:250) ED2 (diluted at 1:250) and CC52 (supernatant undiluted) for 60 min at room temperature. The mAb ED1 recognizes all monocytes and macrophages, ED2 recognizes specifically mature tissue macrophages (KC) and CC52 is directed against CC531 tumor cells.[28] For control slides only PBS with 0.5% BSA was used. After washing with PBS, the slides were incubated with rabbit anti-mouse antibody labeled with peroxidase in PBS with 0.5% BSA and 1% normal rat serum for 60 min. Peroxidase activity was demonstrated with 0.5 mg/ml 3,3'-diaminobenzidinetetrahydrochloride (DAB; Sigma, St Louis, MO) in 0.05 M Tris–HCl buffer, pH 7.6, containing 0.01% H_2O_2 for 8 min. After washing in PBS, the samples were counterstained during a short period with Harris hematoxylin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). The numbers of KC and tumor foci were counted by in cryostat slides by light microscopy.

Experimental design

Initially, male Wag/Rij rats were treated i.p. during 7 days with 1000 U rmGM-CSF (Sandoz) diluted in PBS containing 0.1% bovine serum albumin (BSA) ($n = 5$). Control rats received PBS with 0.1% BSA only ($n = 5$). Rats were sacrificed after 7 days and KC were isolated and cytotoxicity was determined against the syngeneic CC531 tumor cell line. A small piece of liver (from each liver lobe) was snap frozen into liquid nitrogen and stored at −70°C. Immunohistochemical analysis was performed with the mAb ED1, ED2 and CC52.

Subsequently we investigated the effect of rmGM-CSF pre-treatment on the liver parenchyma of rats injected with 1×10^6 tumor cells ($n = 6$), rats injected with 0.5×10^6 tumor cells ($n = 3$) and rats injected with 1×10^5 tumor cells ($n = 3$). All rats were sacrificed after 14 days. Livers were extirpated and separated into the four lobes: the left lateral lobe, the median lobe, the caudate lobe and the right lateral lobe. Tissue was 'snap-frozen' as described and stored at −70°C. Immunohistochemical analysis was performed again with the mAb ED1, ED2 and CC52.

Statistical analysis

Analyses of differences in KC yield and percentages of KC-mediated cytotoxicity against CC531 tumor

cells between the control and the pretreatment group were performed using the Mann–Whitney test.

Results

KC proliferation, purification and yield

[^{3}H]Thymidine incorporation in rat KC increased 5.4-fold from 1178 to 6339 c.p.m. at an optimal rmGM-CSF concentration of 10 U/ml, indicating biologically active rmGM-CSF. Immunohistochemical staining with the mAb ED1 of cytocentrifuge preparations showed that the purity of KC was greater than 98%. The viability of KC, as determined by routine Trypan blue exclusion, was greater than 98% as well. The mean number of isolated KC ± SEM in rats treated with PBS alone was $1.5 \pm 0.2 \times 10^6$ KC/g liver tissue ($n = 5$) (Table 1). After treatment for 7 days with rmGM-CSF (1000 U i.p.) a statistically significant increase of KC yield was observed to $2.2 \pm 0.2 \times 10^6$ KC/g liver tissue ($n = 5$).

Rat KC-mediated cytotoxicity against CC531

Direct KC-mediated cytotoxicity against CC531 tumor cells, as determined with the colorimetric MTT assay, increased significantly from 20.0 ± 0.5 to $42.0 \pm 1.0\%$ after treatment for 7 days with rmGM-CSF ($n = 5$, $p < 0.05$) (Figure 1).

Immunohistochemistry

In rats pretreated with rmGM-CSF, we observed a gradual increase in the number of ED1$^+$ and ED2$^+$ cells, which confirmed the increase of KC yield by isolation (Table 1). ED2$^+$ cells, representing the mature macrophage population in the liver, showed

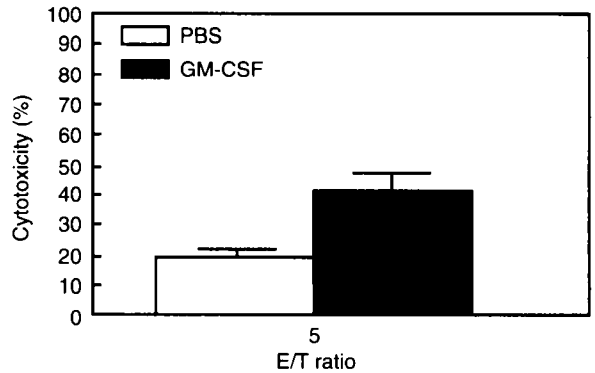

Figure 1. Enhanced rat KC-mediated cytotoxicity against syngeneic CC531 tumor cells after 7 days i.p. administration of 1000 U/ml rmGM-CSF ($n = 5$). Control rats received PBS only. Differences are statistically significant (Mann–Whitney test, $p < 0.05$).

large cell extension and were located along the sinuses. No increase of granulocytes was observed throughout the liver parenchyma.

In rats injected with 1×10^6 CC531 tumor cells we observed macroscopic metastases (varying from three to eight) of 1 mm diameter on the liver capsule in both the control ($n = 6$) and the pretreatment group ($n = 6$). This was confirmed by immunohistochemistry using the mAb CC52 as described by Heuff:[11] numerous small foci of CC52$^+$ tumor cells were seen dispersed throughout the liver parenchyma. They were surrounded by a large mononuclear infiltrate, in which we identified ED1$^+$ and ED2$^+$ macrophages, and which did not differ in size in the control compared to the treatment group. When hepatic metastases had reached a size of 1 mm the inflammatory infiltrate had disappeared. No protective effect of rmGM-CSF had occurred at this tumor load (Figure 2).

In rats injected with 0.5×10^6 tumor cells we still observed macroscopic metastases on the liver capsule in both the control and the pretreatment group. The size of the lesions was still 0.5–1 mm diameter, whereas the number of lesions (two to four) had

Table 1. Mean KC yield by isolation ($n = 5$) and the mean numbers (± SEM) of ED1$^+$ cells (monocytes and macrophages) ($n = 5$), ED2$^+$ cells (resident tissue macrophages) ($n = 5$) and granulocytes (identified by aspecific endogenous peroxidase activity) ($n = 5$) per 0.01 mm^2 as counted in cryoslides

	KC yield	ED1$^+$	ED2$^+$	Granulocytes
rmGM-CSF	2.2 ± 0.2[a]	5.8 ± 0.7	5.6 ± 0.7	0.1 ± 0.03
PBS (control)	1.5 ± 0.2	4.7 ± 0.7	4.1 ± 0.8	0.1 ± 0.02

[a]Differences are statistically different (Mann–Whitney test, $p < 0.05$).

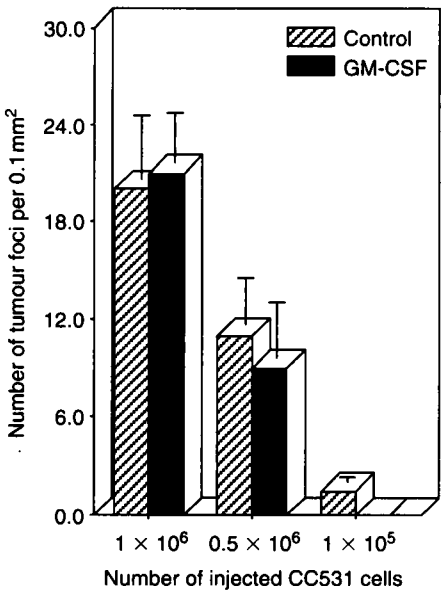

Figure 2. Numbers of $CC52^+$ tumor foci in the liver parenchyma of rats pretreated i.p. with GM-CSF (1000 U, dissolved in 0.5 ml PBS containing 0.1% BSA) and control rats (receiving 0.5 ml PBS with 0.1% BSA). The numbers of CC531-injected tumor cells were 10^6 ($n = 6$), 0.5×10^6 ($n = 3$) and 10^5 ($n = 3$). A protective effect of GM-CSF was found at 10^5 tumor cells.

decreased. This was again confirmed by immunostaining with the mAb CC52. Both the control ($n = 3$) and the rmGM-CSF ($n = 3$)-treated rats had the same size and number of $CC52^+$ tumor foci on immunohistochemical analysis. Thus, at this tumor load still no difference was observed between the control and the treatment group (Figure 2).

In control rats injected with 1×10^5 tumor cells, we observed only very few small $CC52^+$ foci (one focus per 0.1 mm^2 liver tissue of 8 μm thickness). In rmGM-CSF pretreated rats, however, no tumor foci were found ($n = 3$) (Figure 2). These data indicate that pretreatment with rmGM-CSF may prevent the outgrowth of small numbers of CC531 tumor cells from the liver parenchyma, by stimulation of KC anti-tumor activity.

Discussion

In this study we demonstrate increased numbers of rat KC, with enhanced cytotoxic capacity after i.p. administration of rmGM-CSF. An increase of isolated KC after pretreatment with rmGM-CSF ($p < 0.05$) and a gradual increase of $ED2^+$ cells *in vivo* indicates that KC may be able to proliferate locally. Moreover, an increase of $ED1^+$ cells indicates that influx of monocytes may occur as well. The concept of local KC proliferation is supported by a 5.4-fold increase of *in vitro* [^{3}H]thymidine incorporation. A local increase of KC in the liver with enhanced cytotoxic capacity after rmGM-CSF may represent an important defense barrier against metastatic tumor cells in patients with colorectal carcinoma.

Furthermore, we introduce a modification of the previously described Wag rat/CC531 hepatic metastasis model to study the preventive effect of rmGM-CSF on MRD in the liver. We demonstrate that rmGM-CSF has no preventive effect on experimentally induced hepatic metastases at high tumor load (1×10^6 and 0.5×10^6 CC531 tumor cells). However, when injecting 1×10^5 CC531 tumor cells, given after a 7 day i.p. pretreatment regimen, we demonstrate prevention of outgrowth of CC531 tumor cells. We could still detect few small foci of $CC52^+$ cells at light microscopical examination of liver tissue of control rats injected with only 1×10^5 tumor cells. This low tumor load did not lead to macroscopically visible metastatic growth, a situation very relevant to the situation in the clinic; hepatic metastases are usually only found after a follow-up of several months to years, indicating that these patients must have had clinically undetectable MRD during this interval.

The occurrence of hepatic metastasis in colorectal cancer patients is due to embolic metastatic tumor cells through the portal system.[29] Part of these metastases may be induced by surgical manipulation, since this has been shown to result in the appearance of showers of malignant cells in the circulation.[30] Activation of the immune system, before embolic cancer cells lodge into the liver sinusoids, may work prophylactically against the further outgrowth of these cells.

As far as we know, the effect of rmGM-CSF has not been investigated previously in this particular model. Hill and coworkers studied the anti-tumor effects of GM-CSF in the Lewis lung carcinoma model, in which lung metastases were already induced before initiation of treatment.[4] These authors described significant up-regulation of *in vivo* production of superoxide radicals and nitric oxide (NO), together with an inhibition of tumor growth.

The Wag rat/CC531 system has frequently been used as a hepatic metastasis model, in which the effect of several macrophage directed biological response modifiers have been investigated. The anti-tumor potential of interferon (IFN)-γ and the IFN-γ inducer 2-amino-5-bromo-6-phenyl-4(3H)-pyrimidone (ABPP) were demonstrated in this model, by injec-

tion of 0.5×10^6 CC531 tumor cells in the portal system of male Wag rats.[31,32] The tumor cells in these studies were injected, however, at the same day that treatment was initiated. Busch[33] studied the anti-tumor effects of the immunomodulatory agent levamisole in combination with 5-fluorouracil on established CC531 tumor growth in the subcutis and in the subrenal capsule. These CC531-directed studies, focusing on activation of macrophage and natural killer cell anti-tumor activity, differ from the present study, since tumor growth was induced either simultaneously with or even before initiation of the actual treatment. Brodt,[17] on the other hand, did describe that *pre*treatment with a lipophilic muramyl dipeptide was more effective both in terms of incidence of hepatic metastases and in total numbers of metastases. Moreover, Brodt also described tumor load dependency: $2-3 \times 10^5$ tumor cells were inhibited, whereas 5×10^5 were therapy refractory. Thus, different experimental hepatic metastases models have different thresholds for induction of liver tumors. Phillips and Tsao[34] described that intrasplenic injection of 1×10^5 B16 melanoma tumor cells did not induce liver tumors, but 5×10^5, the threshold for this particular model, induced 30–150 liver tumors.

To summarize, our data demonstrate an increase of isolated KC, with enhanced cytotoxic capacity, after pretreatment of Wag rats with rmGM-CSF. Furthermore, a prophylactic effect was found against induction of small metastatic lesions in the liver. These data suggest that preoperative adjuvant immunotherapy with GM-CSF may protect against the development of hepatic metastasis from MRD in colorectal cancer patients. Further studies are warranted to evaluate whether a clinical benefit in patients can be achieved.

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