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Inhibitory effects on in vitro cell growth of human urothelial tumor cell lines under the combined administration of hematopoietic growth factors and clinically relevant antineoplastic agents

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Summary. In five of eight human transitional carcinoma cell (TCC) lines a proliferative response has been reported during exposure to interleukin-3 (IL-3), granulocyte–macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). To elucidate possible growth-modulating effects of these factors combined with clinically relevant antineoplastic agents, cells of the human TCC lines EJ28 and T24 were exposed to methotrexate (MTX), vinblastine (VBL), doxorubicin (DXR) and cisplating (CDDP) with and without single or continuous exposure to IL-3, GM-CSF and G-CSF at concentrations of 1–100 ng/ml. Compared with cells exposed only to chemotherapy, significant inhibitory effects occurred as a result of continuous exposure to IL-3 or GM-CSF at the highest activities with CDDP and MTX in the T24 and EJ28 lines; continuous G-CSF administration (100 ng/ml) in combination with MTX led to significant growth inhibition in the EJ28 line. In contrast, no significant growth modulation was found on combined administration of DXR or VBL with any one of the three colony stimulating factors tested.

Key words: Cisplatin – Doxorubicin – Granulocyte colony stimulating factor (G-CSF) – Granulocyte–macrophage colony stimulating factor (GM-CSF) – Human transitional carcinoma cell lines Interleukin-3 (LL-3) – In vitro growth modulating effects – Methotrexate – Vinblastine

The hematopoietic growth factors granulocyte–monocyte colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) are administered for chemotherapy of metastatic transitional cell carcinoma (TCC) with methotrexate (MTX), vinblastine (VBL), doxorubicin (CXR) and cisplatin (CDDP) (MVAC) to minimize or to circumvent therapy-related toxicity [11, 14]. However, the biological effects of hematopoietic growth factors on growth modulation of human TCC have not been determined in detail. The human TCC lines HTB9T, RT112, KU1, NBT2 and T24 were stimulated under the influence of interleukin-3 (IL-3), GM-CSF and G-CSF [3, 6, 16]. In contrast, these cytokines failed to induce growth modulation in the human TCC lines HTB1, EJ28 and 647V under optimal culture conditions [3, 6].

Possible interactions between antineoplastic agents and hematopoietic growth factors in TCC would mean modifying time schedules for cytokines and chemotherapy, which might have implications for experimental and clinical treatment. Therefore, to elucidate possible growth-modulating effects the cell lines EJ28 (no growth modulation on exposure to IL-3, GM-CSF or G-CSF) and T24 (significant proliferative effects on single and continuous exposure to IL-3, GM-CSF and G-CSF) were exposed in this study to MTX, VBL, DXR and CDDP with and without IL-3, GM-CSF and G-CSF.

Materials and methods

Cell lines and culture conditions

TCC lines EJ28 [T2–4 G4, minimum essential medium (MEM)-Earle medium with 10% fetal calf serum (FCS)] and T24 (Tx G3, MEM-Dulbecco medium with 15% FCS) derived from different tumor types were kindly supplied by the Tumorbank, German Cancer Research Center (Heidelberg, Germany). In each experiment, optimal growth conditions were determined. Culture media contained FCS, 1% nonessential amino acids, 2 mM glutamine and gentamycin (50 μg/ml). Both cell lines were maintained at 37°C, the EJ28 line in 5% CO_2 and the T24 line in 8% CO_2 in a humidified atmosphere. Results of cells screening for mycoplasma were negative throughout these studies.

Hematopoietic growth factors

Recombinant human IL-3, GM-CSF and G-CSF at a specific activity of 5×10^7 units per milligram protein were kindly provided

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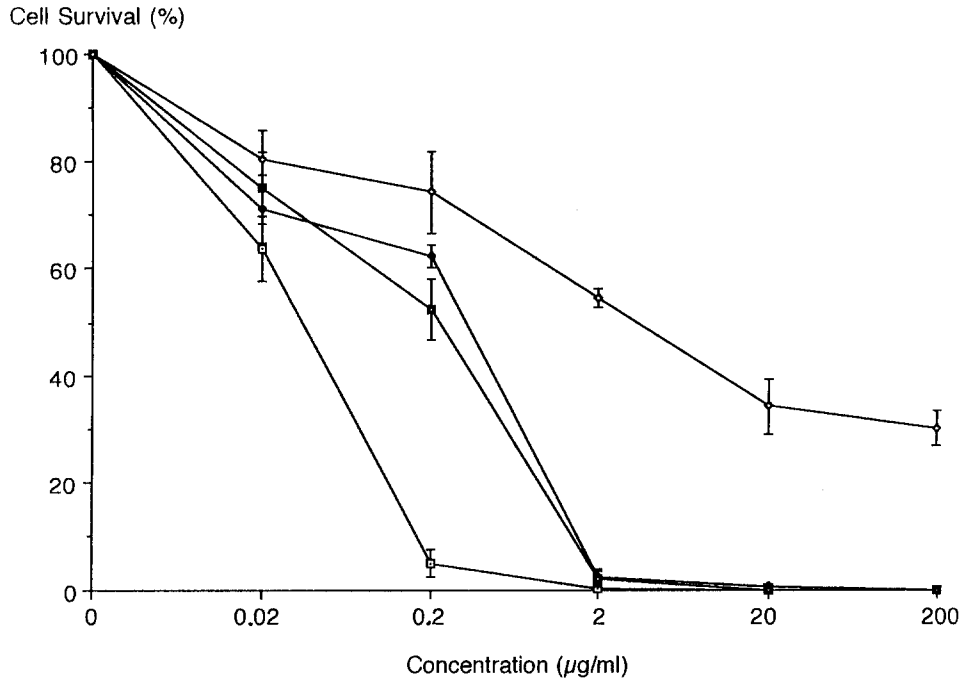

Fig. 1. Determination of IC_{30} in the human TCC line T24 after 4 h of incubation with doxorubicin (*DXR*, —⊡—), cisplatin (*CDDP*, —□—), methotrexate (*MTX*, —◇—) and vinblastine (*VBL*, —◆—) in dosages from 0.02 to 200 μg/ml

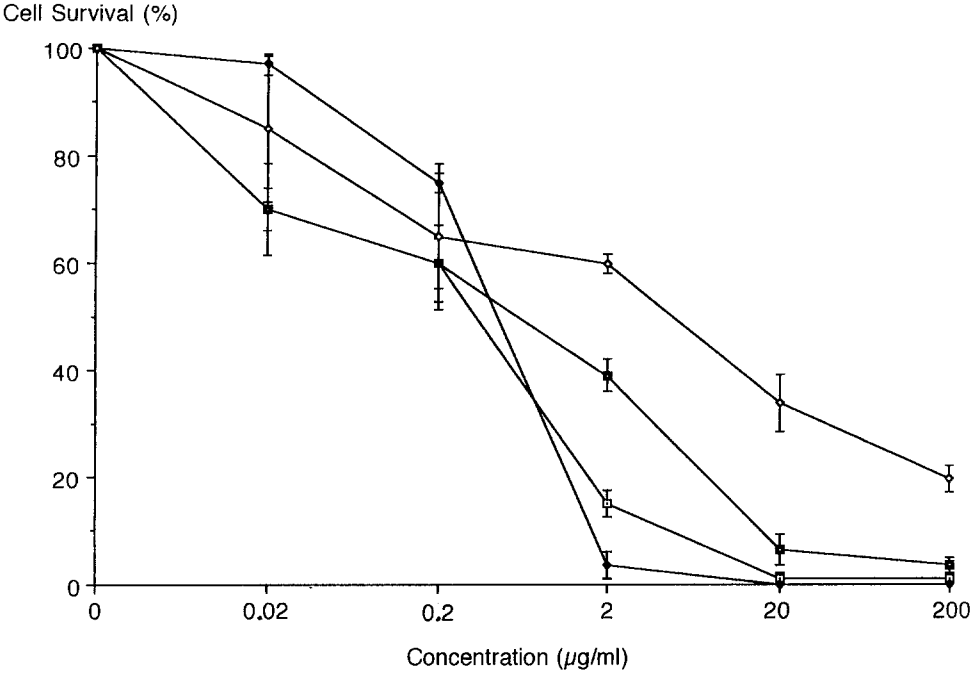

Fig. 2. Determination of IC_{30} in the human TCC line EJ28 after 4 h of incubation with doxorubicin (*DXR*, —⊡—), cisplatin (*CDDP*, —■—), methotrexate (*MTX*, —◇—) and vinblastine (*VBL*, —◆—) in dosages from 0.02 to 200 μg/ml

by Behringwerke (Marburg, Germany). Aliquots were stored at −20°C. For experimental use these factors were resuspended in adequate media with an optimal pH of 7.4 at concentrations of 1, 10 and 100 ng/ml.

Antineoplatic agents

MTX, VBL, DXR and CDDP were administered to cells of the EJ28 and T24 lines with and without IL-3, GM-CSF and G-CSF. To evaluate either stimulation or inhibition of cell growth the IC_{30} of each cytostatic agent tested was determined at day 6. Cells of the EJ28 and T24 lines were exposed in their exponential growth phase 48 h after seeding to MTX, VBL, DXR and CDDP in concentrations of 0.02–200 μg/ml over a period of 1, 4 and 24 h, respectively.

Experimental groups

To determine the growth-modulating effects of chemotherapy combined with administration of IL-3, GM-CSF and G-CSF, three experimental groups were established:

Group 1: 10^3 cells of the EJ28 and T24 lines grew under unmodified growth conditions ("untreated controls").

Group 2: 10^3 cells of the EJ28 and T24 lines were incubated with MTX, VBL, DXR and CDDP according to the dose-finding studies as already described. At the end of cells were washed twice with phosphate-buffered saline (PBS) and resuspended in cell-specific medium.

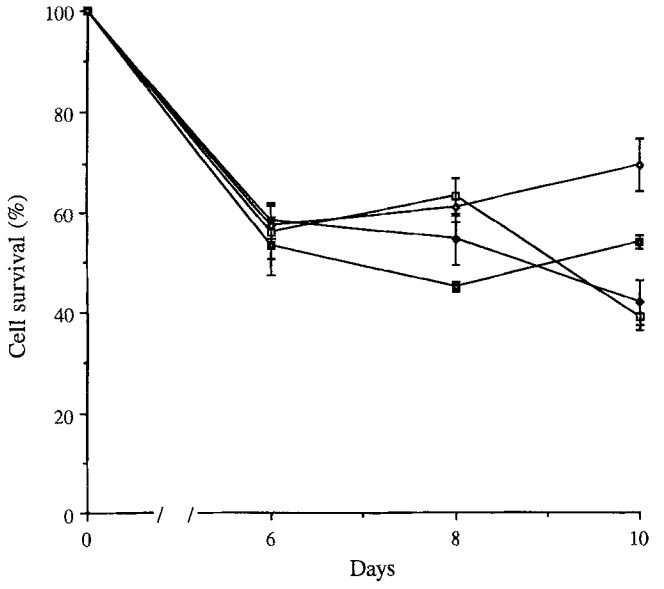

Fig. 3. Cell survival in a monolayer proliferation assay of the human TCC T24 line after combined administration of IL-3 and GM-CSF (100 ng/ml each) with MTX and CDDP (group 3) compared with cells exposed only to chemotherapy (group 2) (—⊡— MTX + GGM-CSF; —◆— MTX + IL3; —□— CDDP + GM-CSF; —◆— CDDP + IL3)

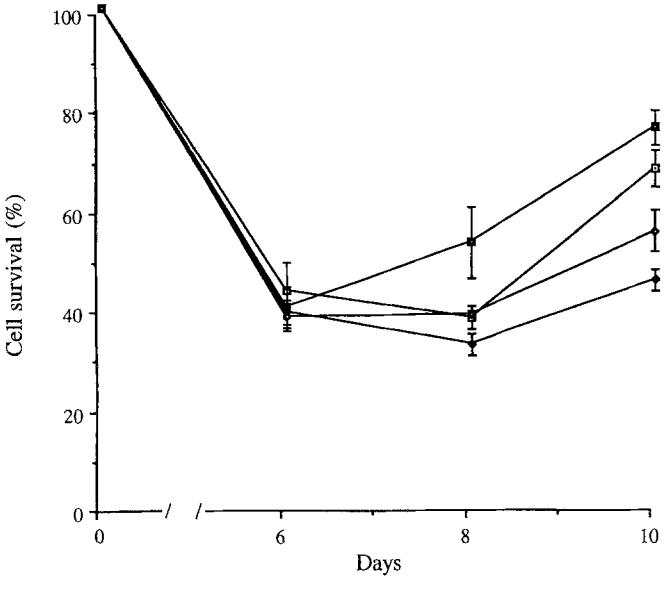

Fig. 4. Cell survival in a monolayer proliferation assay of the human TCC EJ28 line after combined administration of IL-3, GM-CSF and G-CSF (100 ng/ml each) with MTX and GM-CSF with CDDP (group 3) compared with cells exposed only to chemotherapy (group 2) (—⊡— MTX + IL3; —◆— MTX + GM-CSF; —□— MTX + G-CSF; —◆— CDDP + GM-CSF)

Group 3: 10^3 cells of EJ28 and T24 lines were exposed to 1, 10 and 100 ng/ml IL-3, GM-CSF and G-CSF (diluted in RPMI medium) respectively, as well as to 100 µl RPMI medium to exclude a growth-modulating effect due to RPMI. For "single exposure", cells were exposed to each cytokine only at the beginning of the experiment. For "continuous exposure", cytokines were added at initiation of the experiments and at days 2, 6 and 8. To elucidate the effects of combined treatment, cells exposed both singly and continuously to cytokines were incubated with MTX, VBL, DXR and CDDP as already described for group 2.

Assays for determining growth modulation

The monolayer proliferation assay (MPA) was used as described previously [6]. In the human tumor clonogenic assay (HTCA), the cloning efficiency of cells of the EJ28 line was determined. Subsequently, cells were preincubated in two 50-ml tissue culture flasks (Greiner-Labortechnik, Frickenhausen, Germany) with 100 ng/ml GM-CSF or MEM-Earl medium for 48 h, then trypsinized and transferred into plastic tubes. For exposure to antineoplastic agents 100 µl MTX (0.2 µg/ml) and CDDP (0.02 µg/ml), respectively, were added for 1 h. In the tubes the final capillary incubation mixture consited of 900 µl cell suspension diluted either in medium or in GM-CSF (100 ng/ml) and 100 µl 3% agar. From each tube 6 × 100 µl glass capillaries (500 cells per capillary) were filled and stored for 14 days at 37°C, 5% CO_2, 100% humidity. At the end of the incubation period the agar was transferred to glass slides and colony formation determined using an inverted microscope.

Statistical methods

At first, results within the experimental groups were analyzed by rank variance analysis using the Friedman test. In case of significance paired results were analyzed with the Wilcoxon and Wilcox test. Statistical significance was accepted at $P < 0.05$.

Results

In the EJ28 and T24 lines incubation for 4 h with 0.2 µg/ml MTX, 0.2 µg/ml VBL, 0.02 µg/ml DXR and 0.02 µg/ml CDDP led in the MPA to a reproducible cell survival of 70% (Figs. 1, 2). Therefore, the incubation time of 4 h and dosages of each antineoplastic agent as described were administered in further experiments in combination with IL-3, GM-CSF and G-CSF.

Between group 2 and group 3 the following growth-modulating effects were observed: In the T24 line, administration of either CDDP or MTX combined with continuous exposure to IL-3 and GM-CSF at the highest concentrations, respectively, led to a significant inhibition of cell growth from days 6 to 10 (Fig. 3). Proliferation of cells of the EJ28 line was significantly inhibited by administration of MTX with continuous exposure to IL-3, GM-CSF and G-CSF (100 ng/ml each) as well as by administration of CDDP with GM-CSF (100 ng/ml) during days 6 to 10 (Fig. 4).

In contrast, single exposure of cytokines with CDDP and MTX led to insignificant growth modulation in both cell lines investigated. Neither in T24 nor in EJ28 could significant enhancement of inhibition of cell growth be achieved by administration of DXR and VBL with combined exposure to each hematopoietic growth factor tested.

Compared with cell growth in group 1, administration of RPMI medium without cytokines in group 3 showed no influence on cell proliferation.

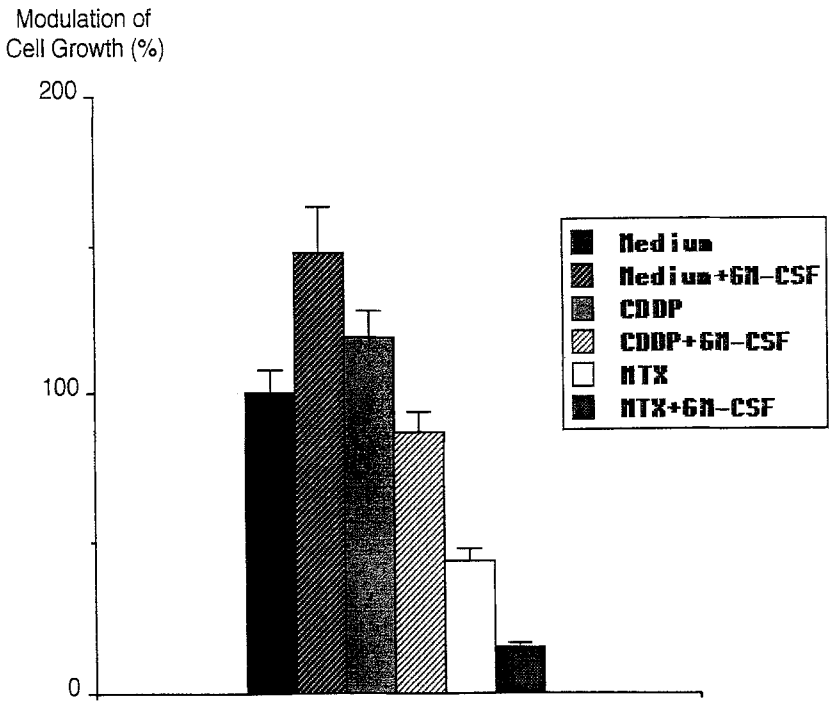

Fig. 5. Growth-modulating effects in a human tumor clonogenic assay of the human TCC line EJ28 exposed to GM-CSF, CDDP or MTX alone or to GM-CSF combined with MTX or CDDP

In the HTCA, exposure of cells of the EJ28 line to GM-CSF (100 ng/ml) led to significant growth stimulation; significant growth inhibitory effects were obtained after combined administration of GM-CSF (100 ng/ml) with CDDP and MTX, respectively (Fig. 5).

Discussion

Hematopoietic growth factors are currently in clinical use for the reduction of complications associated with cancer chemotherapy in solid tumor diseases. In spite of certain inconsistencies, results from various laboratories throughout the world provide evidence that hematopoietic growth factors can affect the growth and activity of certain types of nonhematopoietic malignant cell lines in vitro [3, 10, 12, 15]. The significance of stimulation of nonhematopoietic cells in vivo remains unclear. Nevertheless, the existence of responsiveness to hematopoietic growth factors of any magnitude in nonhematopoietic cells may have important clinical consequences, particularly with long-term administration [13].

Only a few data are available about the biological effects of hematopoietic growth factors on human bladder carcinoma cell lines. Dose-dependent proliferative effects were described in five of eight human TCC lines: HTB9T on exposure to IL-3 and GM-CSF [4], KU1 and NBT2 when exposed to G-CSF [16], and RT112 and T24 on exposure to IL-3, GM-CSF and G-CSF, respectively [6]. No growth modulation occurred on exposure to IL-3, GM-CSF and G-CSF of cells of the lines HTB1 [4], EJ28 and 647V [6]. Only under suboptimal culture conditions (1% albumin instead of 10% FCS) did the administration of IL-3, GM-CSF and G-CSF lead to a significant proliferative response in the EJ28 line [6].

The cause of induced growth modulation due to hematopoietic growth factors is not known. Ohigashi et al. [16] demonstrated that G-CSF stimulated the clonal growth of KU1 and NBT2 TCC cells by binding to its specific receptors. On the other hand, in hematopoietic tumor diseases evaluation of cytokine receptor expression failed to demonstrate a direct correlation between responsiveness to cytokines and receptor expression [7, 8, 16].

Modulation of cell growth or cell-specific metabolism is of experimental interest because of the potential enhancement of inhibitory effects in cell proliferation by associated administration of colony stimulating factors. Therefore, cells of the EJ28 and T24 lines were exposed to MTX, VBL, DXR and CDDP after determination of the IC_{30} with and without IL-3, GM-CSF and G-CSF. The IC_{30} was chosen to evaluate either stimulatory or inhibitory effects due to cytostatic agents, cytokines and their combined administration, respectively. Compared with cells exposed only to chemotherapy (group 2), significant inhibitory effects occurred on continuous exposure to IL-3 or GM-CSF at the highest concentrations with CDDP and MTX (group 3) in the T24 and EJ28 lines. Continuous G-CSF administration (100 ng/ml) led to significant growth inhibition in the EJ28 line only when it was in combination with MTX. In contrast, no significant growth modulation was found on combined administration of DXR and VBL with any one of the three colony stimulating factors tested.

In the literature only a few data are available about the growth-modulating effect in malignant cells resulting from a combination of antineoplastic drugs and hematopoietic growth factors. Adding GM-CSF to 5-fluorouracil (5-FU) in cells of the colon carcinoma cell line HTB 38 showed an increase in 5-FU cytotoxicity in six of eight experiments as measured by absolute numbers of surviv-

ing colonies and expressed as a percentage of growth inhibition when 5-FU was given 24 h after GM-CSF. However, in two experiments the absolute numbers of colonies surviving the GM-CSF/5-FU sequence were equal to or even higher than those surving 5-FU treatment only. This effect has been interpreted as being due to a strong stimulation of clonal cell growth by GM-CSF which was more than two-fold that when the cells were exposed to the factor during the entire experiment [5].

In an animal study concomitant administration of rh-G-CSF significantly enhanced the tumor-suppressing effect of MVAC therapy which led to an increase in median survival time. Therefore, rh-G-CSF may be useful for enhancing the activity of antitumor agents, and not only for alleviating granulocytopenia or preventing its development [1].

In advanced myeloid leukemia combined treatment with cytosine arabinoside (Ara C) and GM-CSF is useful for overcoming cell kinetic resistance to Ara C [9]. In an animal experiment life expectancy of leukemic mice receiving combined treatment with GM-CSF followed by Ara C was higher than that of mice treated with Ara C alone [2].

Modulation of growth inhibition by combined administration of chemotherapy with IL-3, GM-CSF and G-CSF might be considered a shift of quiescent cells from the large G_0 pool into the proliferative compartment [3, 16, 17].

From the clinical point of view any in vitro observations must be viewed with caution. Artificial in vitro systems are not the same as in vivo environments such as the tumor stroma and host response cells. However, it is of clinical interest that Logothetis [14], in a phase II study of 32 patients, reported complete and partial remission rates of 23% and 17% respectively in urothelial tumors initially refractory to MVAC, CISCA and CMV chemotherapy when these were given enhanced MVAC treatment accompanied by application of rh-GM-CSF. These remissions – described by the authors as "unexpectedly positive" – might be interpreted as evidence of better chemotherapeutic efficacy due to the higher dosages used or as an increased sensitivity of tumor cells to antineoplastic agents because of synchronization of cells in the cell cycle due to GM-CSF.

In accordance with data reported by Akaza et al. [1] and Ohigashi et al. [16], the in vitro study reported here confirmed that it might seem reasonable to administer GM-CSF or IL-3 prior to methotrexate and cisplatin as these may render cells more sensitive to chemotherapy. To determine definitively the appropriate use of these colony stimulating factors in the treatment of bladder cancer, further in vivo studies have been initiated.

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