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Effects of cytokines on growth in vitro of primary human renal cell carcinoma

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Summary. In clinical trials different haematopoietic active cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) have been proven to alleviate myelosuppressive side effects of intensive chemotherapy in different non-urological malignancies. On the other hand, these cytokines can directly stimulate the proliferation of cells originating from some non-urological tumours. To clarify the impact of these cytokines on the proliferative behaviour of human renal cell carcinoma (RCC), 29 previously untreated RCC tumours were prepared for culturing in vitro using the cell cluster technique. The success rate for growth in vitro was 82.8% (24/29). The malignant renal cells were treated with different cytokines (GM-CSF, G-CSF and interleukin-3) in different dosages. Cell number and proliferation rates detected by immunostaining were used for treatment evaluation. A dosage-dependent stimulation of cell growth could not be observed compared to untreated cells. From the data presented in this study, proliferative stimulation of RCC by administering colony-stimulating factors in clinical trials cannot be assumed.

Key words: Renal cell carcinoma – Cytokines – Colony-stimulating factors – Proliferative behaviour

The incidence of renal cell carcinoma (RCC) ranges from 10- to 25 cases per 100000 [29]. Approximately 15000 patients will have newly diagnosed RCC in 1991 in the United States [13], a figure comparable to that in Europe. Among them, about 25–35% already have metastatic spread at the time of diagnosis [13, 16].

In local disease, radical tumour nephrectomy is the treatment of choice. This malignancy, however, is known to be unpredictable with a tendency to metastasize even years after radical surgical removal of the primary tumour. In advanced disease no commonly accepted therapy is at present available for disseminated metastases. Chemotherapy and/or immunotherapy with biological response modifiers (BRM) or combinations ofboth are the most frequently administered treatment in RCC patients with metastatic lesions [3, 13, 18, 28].

Because chemosensitivity is lower than in other neoplasms, such as testicular cancer, and because of the heamatological toxicity of chemotherapeutic drugs in higher dosages, the use of chemotherapy in metastatic RCC is limited [4, 13]. Colony-stimulating factors (CSF) are new cytokines that were recently characterized and isolated [4, 22] and that may lead to higher response rates in otherwise refractory urological tumours. Granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and interleukin-3 (IL-3) are glycoproteins involved at various levels in the maturation of haematopoietic precursor cells [4]. Clinical studies with recombinant GM-CSF, G-CSF, and IL-3 are in progress and evidence of their safety and haematological activity in vivo has been provided [14, 20, 21, 30]. G-CSF has been shown to decrease the haematological side effects of chemotherapy in bladder cancer [9, 21]. Even more data are available for GM-CSF used in other malignancies with similar results [2, 12, 20].

However, cytokines alone can increase tumour cell proliferation, as demonstrated by several studies in vivo [1, 24].

Data determining the effect of CSF on RCC are (currently) not available. This study describes the effects of GM-CSF, G-CSF, and IL-3 in different dosages on the proliferative behaviour of human RCC in vitro.

Materials and methods

Preparation of primary cell cultures according to the cell-cluster technique

Tumours from 29 previously untreated RCC patients were obtained by radical nephrectomy. A macroscopically homogeneous tumour area lacking fibrosis, necrosis, or haemorrhage was excised. One

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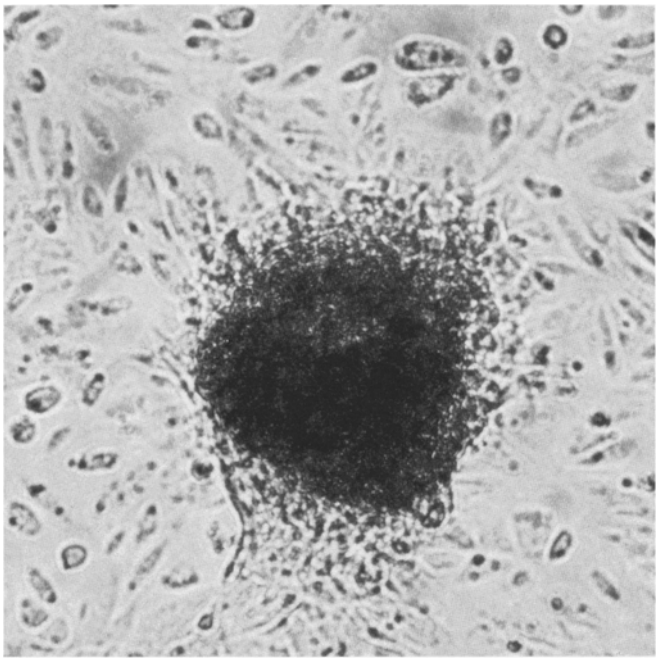

Fig. 1. Renal cell carcinoma (RCC) culture before harvesting the cells for subculturing and testing with different cytokines. One small cell cluster has grown to a confluent monolayer within 12 days of in vitro culturing. Cells are not stained, original magnification ×120

Table 1. Tumor stage and grade of the 24 renal cell carcinomas exhibiting sufficient in vitro growth

	(*n*)	(%)
Stage		
pT 1–2	5	20.8
pT 3	17	70.8
pT 4	2	8.4
Grade		
G 1	5	20.8
G 2	8	33.4
G 3	11	45.8

sample of the excised tumour tissue was taken for in vitro cell preparation; another sample was taken for histological examination as a "reference slide".

After mincing and enzymatic treatment with collagenase, the fragments of fresh tissue were centrifuged and washed twice in RPMI 1640 medium. Subsequently, the tissue clusters were resuspended in a centrifuge tube containing 2–4 ml culture medium (RPMI 1640 supplemented with 15% fetal calf serum and 1% penicillin) and vigorously dispersed using a Pasteur pipette. The suspension sedimented for 1 min, and the supernatant containing more than 90% of single cells with low viability and cell debris was decanted. The pellet containing more than 90% small clusters of approximately 10–15 cells each was resuspended and seeded in culture. The cell clusters were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in 25 cm^2 Falcon flasks (Biochrom, Berlin, FRG), to each of which 5 ml culture medium had been added. The small cell clusters attached to the flasks within 24 h and grew to an almost confluent monolayer, in most cases within 7–14 days of cell culturing (Fig. 1). The purity of the tumour cell population obtained by this in vitro preparation method was demonstrated by using immunochemical and cytogenetic techniques as recently described [25, 27].

Preparation of the in vitro slides

When the renal tumour cells had grown to a confluent monolayer (Fig. 1), the following procedure was used for cell transfer. After the medium of the primary cultures had been decanted, the cells were washed twice with warm (37°C) RPMI 1640 medium to remove cell debris. Trypsin then was added and the cells were resuspended within 3–5 min. Trypsin was inactivated by filling the flasks with culture medium. Trypsin activity should not last longer or cell damage may affect the success rate of further subculturing [25]. One part of the resuspended tumour cells was transferred to other culture flasks for further subculturing; the other part was pipetted in equal amounts into special in vitro slides chambers (Nunc, Wiesbaden, FRG), as recently outlined [26]. During the entire period of tumour cell growth on the slides, the culture medium contained various cytokines in different concentrations. At the end of the assay, the chambers of the slides were removed and the slides were taken for cell counting and immunostaining [26].

Immunostaining for cell proliferation

Gerdes et al. [10] provided the monoclonal antibody Ki-67, which binds to a human nuclear antigen associated with cell proliferation. Cell-cycle analyses demonstrated that this nuclear antigen detected by Ki-67 is only expressed in the active cell-cycle phases (G1, S, G2, M), but not in the G0 phase [11]. The Ki-67 assay is thus an easy, fast and reproducible method of determining the growth fractions of cells in vivo [27] as well as in vitro [26].

The acetone- and chloroform-fixed cells grown and fixed on the surface of the slide were exposed for 30 min to the monoclonal antibody Ki-67 (Dianova, Hamburg, FRG), which had been diluted in RPMI 1640 medium. After washing in TRIS-buffered solution (pH 7.4), the cells were incubated with rabbit anti-mouse IgG and then exposed to alkaline phosphatase anti-alkaline phosphatase (APAAP) complex after another washing [5]. Subsequently, the slides were stained with fast red and counterstained with haemalum [20]. Presence of the specific antigen was indicated by a red instead of a blue colour reaction after counterstaining [5, 26]. Cell response analysis under treatment with CSF was assessed by determining the cell numbers and the proliferation rates (PR) on the slides as recently outlined in detail [26].

Recombinant human GM-CSF, G-CSF and IL-3 were administered in vitro as lyophilized preparations at a specific activity of 50 mU/mg (Behringwerke, Marburg, FRG). Each cytokine was given in dosages from 0.1 ng/ml to 1000 ng/ml (Fig. 2). Haematopoietic-active cytokines such as GM-CSF and G-CSF are recommended at a dosage of 5–20 μg/kg per day for in vivo application in tumour patients [8]. This was the basis for the rationale for the in vitro dosages ranging from 0.1 ng/ml to 1000 ng/ml: 5 μg/kg per day in vivo = 0.1 ng/ml in vitro [15]. For each tumour, two in vitro slide chambers were used, for each dosage of cytokine. Two slide chambers without any treatment (control group) were also analysed.

Treated and untreated cells were statistically compared using Student's *t*-test [19]. A value of $P < 0.05$ was considered statistically significant.

Results

Of the 29 RCC tumours, 24 exhibited sufficient growth in vitro. The success rate was therefore 82.8%. Table 1 shows the histological grading and staging of all 24 RCCs tested in this study. Successful cell-culturing was performed not only on undifferentiated but also on low-grade RCCs. During culturing, only viable cells were fixed to the surface of the slides. Dead cells were resuspended in the

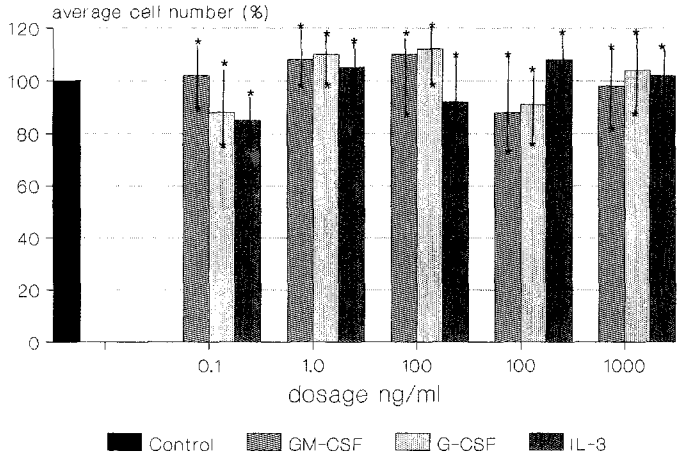

Fig. 2. Twenty four different RCCs tested in vitro with haematopoietic-active cytokines. Average cell numbers of treated groups are expressed as a percentage of untreated (control) groups. Maximal and minimal values per tested cytokine and dosage are marked with an *asterisk*

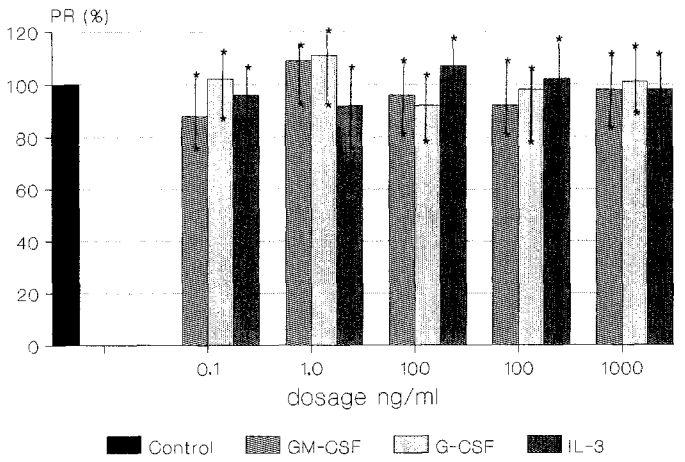

Fig. 3. Twenty four RCCs tested in vitro with the same cytokines as in Fig. 2. Average proliferation rates *(PR)* of treated groups expressed as a percentage of untreated (control) groups. Maximal and minimal values per tested cytokine and dosage are marked with an *asterisk*

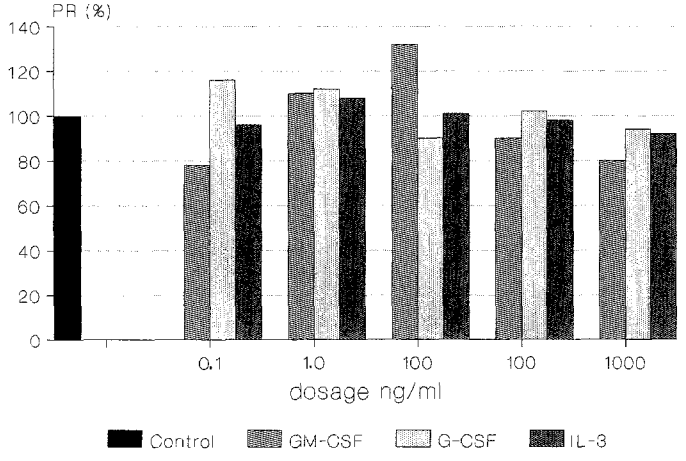

Fig. 4. Example of one RCC specimen tested in vitro with different haematopoietic active cytokines. A dosage-dependent increase of proliferation rates *(PR)* could not be achieved

culture medium and were therefore removed by the staining procedure [26].

All of the cells of each renal tumour analyzed in this study originated from the same primary in vitro cell culture. In total, 768 slides were evaluated including the control groups. The PR of each untreated group (two slides per group) did not differ more than 5%. Therefore it can be concluded that the investigated cell suspensions exhibited a standardized growth behaviour in vitro. Cell numbers of groups treated with various cytokines (GM-CSF, G-CSF, IL-3) in different concentrations were compared to the control slides and are summarized in Fig. 2. No significant difference was observed between treated and untreated groups.

The PR of the untreated tumour cells (control slides) ranged from 23 to 92%. This proliferative behaviour of RCC cells under in vitro conditions is known and has already been described [27]. Figure3 illustrates the average PR in relation to increasing cytokine dosages. For GM-CSF, a significant increase of cell proliferation was observed in 4 of 24 cases (16.7%). This increase, however, was not dosage-dependent (Fig. 4). A cytokine-concentration-dependent stimulation of tumour cell growth could not be observed in any of the specimens analysed.

Discussion

RCC represent about 2% of all human malignancies and they are the most common renal tumour (90%) [16]. Metastatic RCC is still a therapeutic challenge in urological oncology. Of RCCs 25–35% have already metastasized at time of diagnosis [16, 29]. The discussion concerning the different therapeutic strategies is still controversial. Immunotherapy, especially using BRM such as interferon and IL-2 [13], or as chemotherapy, or a combination of both [18, 28], are the most frequently chosen therapeutic regimens in metastatic RCC [3]. However, during the last decade no relevant improvement regarding therapeutic response rates and survival rates has been achieved. The causes are various and have complex connections with each other. In this context only a few aspects regarding the clinical problems in chemotherapy are mentioned.

Yagoda [31] has recently reviewed the results of 39 chemotherapeutic agents administered in clinical trials for metastatic RCC. The overall objective response rate in more than 2000 patients was only 8.8%. In phase II trials, however, higher response rates (up to 45%) were achieved using a combination of interferon and vinblastine [18, 28].

The high rate (80%) of multi-drug-resistance *(MDR)* gene expression in human RCC might be one of the main reasons for the failure of conventional approaches with chemotherapy [6]. The MDR gene codes for a 170 kDa glycoprotein (P-glycoprotein) that serves as a transmembrane efflux pump for cytotoxic drugs and thereby makes

different chemotherapeutic drugs ineffective [23]. P-Glycoprotein, however, is inhibited by several drugs that influence membrane-bound electrolyte pumps, e.g. verapamil or other calcium antagonists [23]. Clinical trials with these antagonists in metastatic breast cancer are in progress [22].

The toxic side effect of conventional chemotherapy on bone marrow is a further clinical limitation, especially in RCC in older, and therefore more sensitive, patients compared to younger testicular cancer patients. Often, the therapeutic dosages must be reduced in RCC patients due to the haematotoxic side effects [31]. Clinical studies have demonstrated the haematological activity of recombinant human CSF in non-urological malignancies [12, 13, 20, 30]. The application of these cytokines not only alleviates bone marrow toxicity caused by chemotherapy, but may also allow for further increases in dose intensity and the development of more active combinations of chemotherapeutic schedules. Tumours presently regarded as largely chemotherapy-resistant may become more amenable to therapy if dosages could be sufficiently increased in combination with cytokines such GM-CSF or G-CSF. The additional application of drugs (e.g. verapamil) inhibiting the MDR glycoprotein of the cell membrane may also enhance the therapeutic efficacy of chemotherapy in this malignancy.

Any stimulating effects of these cytokines on tumour cells should be analysed before new therapeutic strategies in clinical trials can be accepted. For instance, stimulating effects of GM-CSF on human breast cancer and osteosarcoma cell lines were recently reported [7]. In vitro preparation of malignant tissue may lead to selection of cell populations not representative of the entire tumour. The heterogeneity of RCC makes multiple sampling necessary [27]. This problem was partly overcomed by testing tumours, but the possibility cannot be excluded that selection of less sensitive cells has occurred.

Other in vitro studies have revealed inhibition by CSF in small cell lung cancer cells [32]. In vivo administration of cytokines may induce indirect effects on malignant renal cells not tested by this in vitro system. To date, no experience regarding the direct effects of these cytokines on RCC and other urological cancers has been reported.

According to the data presented in this paper there is no evidence that the cytokines GM-CSF, G-CSF and IL-3 stimulate the proliferation of malignant human renal cells in vitro. Therefore, tumour stimulation using these cytokines in clinical trials to alleviate myelosuppressive side effects of intensive chemotherapy seems unlikely for this specific neoplasm.

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