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## Peritonitis-induced antitumor activity of peritoneal macrophages from uremic patients

Bohdan Turyna<sup>1</sup>, Aleksandra Jurek<sup>1</sup>, Kamil Gotfryd<sup>1</sup>, Agnieszka Siaszkiewicz<sup>1</sup>,  
Piotr Kubit<sup>2</sup> and Andrzej Klein<sup>1</sup>

<sup>1</sup>Department of General Biochemistry, Faculty of Biotechnology, Jagiellonian University;

<sup>2</sup>Department of Nephrology and Dialysis, Rydygier Hospital, Cracow, Poland

**Abstract:** The macrophages belong to the effector cells of both nonspecific and specific immune response. These cells generally express little cytotoxicity unless activated. The present work was intended to determine if peritoneal macrophages collected from patients on Continuous Ambulatory Peritoneal Dialysis (CAPD) during episodes of peritonitis were active against human tumor cell lines without further *in vitro* stimulation. We also compared macrophage antitumor potential with effectiveness of drugs used in cancer therapy (taxol and suramin). Conditioned medium (CM) of macrophages collected during inflammation-free periods did not exhibit cytostatic and cytotoxic activity against both tumor (A549 and HTB44) and non-transformed (BEAS-2B and CRL2190) cells. Exposure of tumor cells to CM of macrophages harvested during peritonitis resulted in significant suppression of proliferation, impairment of viability and induction of apoptosis, in contrast to non-transformed cells, which remained unaffected. The efficacy of CM of inflammatory macrophages as an antitumor agent appeared to be comparable to cytostatic and cytotoxic potency of taxol and suramin or, in the case of HTB44 cells, even higher. The results obtained suggest that activated human macrophages might represent a useful tool for cancer immunotherapy.

**Key words:** Macrophage, human - Activation - Peritonitis - Antitumor activity - Taxol - Suramin

### Introduction

Over the past decade much research has been focused on the role of immune system in malignant disease. Although much has been learned about tumor antigenicity, cytokine activity and specific immune cell types, the understanding of how malignancy and the immune system interrelate is still a mystery [2]. The macrophages belong to the effector cells of both nonspecific and specific immune response. These cells generally express little cytotoxicity unless activated [8]. Activation is a complex biological process which culminates in the formation of cells with the ability to mediate tumor cell killing, directly as a result of cell-to-cell contact or indirectly through the secretion of cytotoxic and cytostatic products [8, 24].

Peritoneal fluid obtained from patients in end-stage renal disease undergoing Continuous Ambulatory Peritoneal Dialysis (CAPD) provides a readily accessible

source for human macrophages. These cells can be collected when CAPD is performed without complications or during an intercurrent peritonitis. This renders it possible to study antitumor potential of human macrophages as related to their presence in a non-inflammatory or inflammatory environment. Peritoneal macrophages from patients on CAPD represent a population of fully functional cells [4]. It has been reported that these cells can be induced to express antitumor activity against tumor cells, especially if collected during a period of peritonitis [5].

The present work was intended to determine if peritoneal macrophages collected from patients on CAPD during episodes of inflammation were active against human tumor cell lines without additional *in vitro* stimulation. The macrophages were isolated from peritoneal effluents during both inflammation-free periods and episodes of peritonitis. In this report we describe the influence of macrophage-conditioned medium (CM) on growth and survival of two types of human tumor cell lines: A549 (lung carcinoma) and HTB44 (renal carcinoma) in comparison with non-transformed cell lines: BEAS-2B (bronchial epithelium cells) and CRL2190 (renal proximal tubular cells).

**Correspondence:** B. Turyna, Dept. General Biochemistry, Faculty of Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland; e-mail: bohdan@mol.uj.edu.pl

The purpose of the present study was also to compare antitumor potential of macrophages with the effectiveness of cytostatics used in the therapy of lung and kidney tumors (taxol and suramin, respectively).

## Materials and methods

**Chemicals.** Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), F12 medium, bovine serum albumin, non-essential amino acids, insulin, hydrocortisone, 3,3',5-triiodothyronine, transferrin, taxol, suramin, Hoechst No 33258, Giemsa dye and kit for non-specific esterase were purchased from Sigma (St. Louis, US). Keratinocyte-Serum Free Medium (Keratinocyte-SFM), calf serum, recombinant epidermal growth factor (rEGF) and bovine pituitary extract were purchased from Gibco (San Diego, US). The other reagents were provided by POCh (Gliwice, Poland).

**Macrophage isolation and culture.** Human macrophages were harvested from peritoneal dialysate effluents (PDEs) of patients in end-stage renal disease undergoing CAPD treatment. PDEs were collected during both inflammation-free periods and episodes of peritonitis. Non-infective PDEs from 2-3 patients were pooled because of difficulties in obtaining appropriate number of cells from one individual. A total cell population increased markedly during peritonitis and sufficient numbers of cells were isolated from PDEs of individual patients. Complete dialysate effluent volume was centrifuged at 1500 rpm for 10 min at 4°C. The cell pellet was washed with 0.9% NaCl and centrifuged again at 1500 rpm for 10 min. After suspension in DMEM supplemented with 10% calf serum, the cells were counted and their viability assessed by trypan blue exclusion test. The purity of isolated cells was determined by non-specific esterase [10] and May Grünwald-Giemsa [25] staining. The viability of the cells exceeded 90%. The average percentages of macrophages in non-inflammatory and inflammatory PDEs were 50-60% and 20% of the total cell number, respectively. The cell density was adjusted to  $4 \times 10^6$ /ml of DMEM with 10% calf serum, placed in plastic dishes at total volume of 5 ml and incubated for 2 h at 37°C. The medium with non-adherent cells was then discarded and the dishes were washed twice to remove residual non-adherent cells. Approximately 90% of adherent cells were macrophages. The macrophages were cultured for 24 h in serum-free DMEM/F12 (1:1) medium supplemented with transferrin (5 µg/ml) and selenate (2 ng/ml).

**Conditioned medium (CM) preparation.** CM of peritoneal macrophages was collected following 24 h of culture, centrifuged at 1500 rpm for 10 min and concentrated by ultrafiltration through Millipore membrane of 3000 NMWL (nominal molecular weight limit). Concentration of proteins in CM was determined by Lowry's method [15].

**Target cells.** The human tumour cell lines A549 (lung carcinoma) and HTB44 (renal carcinoma) as well as human normal cell lines BEAS-2B (bronchial epithelium cells) and CRL2190 (proximal tubular cells) were used as target cells. BEAS-2B and CRL2190 cells served as a control of CM action selectivity. A549 cells were cultured in DMEM with 10% calf serum. HTB44 cells in MEM supplemented with non-essential amino acids, sodium pyruvate and 10% calf serum, BEAS-2B cells in F12 medium supplemented with rEGF, insulin, hydrocortisone, 3,3',5-triiodothyronine, transferrin and 5% calf serum and CRL2190 in Keratinocyte-SFM with rEGF, bovine pituitary extract and 10% calf serum. All cell lines were purchased from American Type Culture Collection (Manassas, US).

**Cytostatic and cytotoxic activity assays.** Target cells were seeded on 96-well plates at concentrations of  $1 \times 10^4$  cells/well (A549, HTB44 and BEAS-2B) or  $5 \times 10^3$  cells/well (CRL2190) in specific culture media (described above) with calf serum. Following 24 h of

incubation the media were removed and replaced with 200 µl/well of serum-free media containing 20 µg or 40 µg of macrophage CM proteins. The incubation was continued for the next 48 h at 37°C. Target cells incubated with media alone served as a control. The modified crystal violet staining method [10] and Bürker chamber counting method served as a measure of cytostasis by macrophage supernatants. The MTT tetrazolium assay [18] and neutral red assay [7] were used to determine the influence of supernatants on target cell viability. The cytostatic activity of macrophage CM against target cells and the effect on their viability were determined in comparison with control and expressed as percentages of control growth and control values, respectively. The antitumor activity of macrophage CM was also compared with the influence of taxol and suramin on transformed cell proliferation and viability. To this end A549 and HTB44 cells were incubated for 48 h with media supplemented with 50 nM taxol or 1 mM suramin.

CM of peritoneal macrophages were also tested for ability to induce tumour cell apoptosis. A549 and HTB44 cells suspended in specific culture media supplemented with serum were seeded at glass slides placed in the 6-well plates at a concentration of  $4 \times 10^4$  cells/well. After 24 h of culture, media were replaced with 1 ml/well of media without serum containing 200 µg of macrophage CM proteins. Following 48 h of incubation, cells were fixed in the mixture of methanol and acetic acid (Carnoy fixative) or 1% formaldehyde and stained with Giemsa dye or Hoechst No 33258 dye. Observations were carried out with Olympus IX inverted system microscope. The ability of CM to induce nuclear alterations was compared with the effect of taxol (50 nM) and suramin (1 mM).

**Statistics.** The data was expressed as mean  $\pm$  SD, analysed using Student's t-test and considered significant at  $p < 0.005$ .

## Results

### Peritoneal cells

Dialysis effluents from patients without inflammation allowed to obtain approximately  $5-10 \times 10^6$  peritoneal cells from 2-3 L of fluid. During peritonitis, the number of cells increased markedly and  $0.1-5 \times 10^9$  cells were collected from effluent of one patient. The viability of isolated cells exceeded 90%, as assessed by trypan blue exclusion test. The average percentage of macrophages in non-inflammatory PDEs was 50-60% of total cell number and decreased to 20% in inflammatory effluents, as evaluated by May Grünwald-Giemsa and non-specific esterase staining. The number of neutrophils increased significantly during the inflammatory state and their contribution to the total cell population could exceed even 90% at the beginning of peritonitis. Ability of macrophages to adhere to plastic dishes within 2 h allowed to obtain 90% pure cultures of these cells.

### Effect of CM on target cell proliferation

Transformed (A549, HTB44) and non-transformed (BEAS-2B, CRL2190) cells were exposed for 48 h to conditioned media of macrophages collected during both non-inflammatory and inflammatory states, added at two different concentrations: 20 µg and 40 µg of proteins/well. Numbers of investigated cells determined by crystal violet staining and Bürker chamber counting

**Table 1.** Effect of conditioned medium (CM) of peritoneal macrophages obtained from patients in non-inflammatory (NI) and inflammatory (I) periods on proliferation of transformed (A549, HTB44) and non-transformed (BEAS-2B, CRL2190) cell lines

	NI		I		NI		I	
	CV	BC	CV	BC	CV	BC	CV	BC
	A549×10 <sup>3</sup>				BEAS-2B×10 <sup>3</sup>			
K <sub>0</sub>	9.7±2.0	9.8±2.6	9.7±2.0	9.8±2.6	8.0±0.9	9.5±0.9	8.0±0.9	9.5±0.9
K <sub>48</sub>	31.7±3.9	37.6±4.4	31.7±3.9	37.6±4.4	16.8±2.3	24.0±2.6	16.8±2.3	24.0±2.6
CM <sub>20</sub>	31.4±3.4	38.1±3.0	25.1±1.7*	28.5±3.6*	17.0±2.1	23.5±2.0	17.1±1.8	22.5±1.5
CM <sub>40</sub>	30.5±1.0	41.1±3.5	17.9±1.7*	16.8±3.1*	17.3±2.1	24.7±3.7	16.7±1.3	25.4±2.8
	HTB44×10 <sup>3</sup>				CRL2190×10 <sup>3</sup>			
K <sub>0</sub>	7.5±1.1	8.4±1.8	7.5±1.1	8.4±1.8	3.3±0.1	3.4±0.7	3.3±0.1	3.4±0.7
K <sub>48</sub>	20.4±0.9	19.4±0.8	20.4±0.9	19.4±0.8	6.9±0.8	7.3±0.5	6.9±0.8	7.3±0.5
CM <sub>20</sub>	19.6±0.3	19.0±0.2	14.4±1.3*	13.3±0.7*	6.8±0.1	7.4±0.7	7.2±0.2	7.2±0.9
CM <sub>40</sub>	19.3±0.9	18.2±0.2	7.8±0.5*	8.6±0.7*	6.4±0.1	7.6±0.4	6.6±0.5	7.4±1.0

CV - numbers of cells determined by crystal violet staining method; BC - numbers of cells evaluated by Bürker chamber counting method; K<sub>0</sub>, K<sub>48</sub> - numbers of cells in control at the start of experiment and after 48 h, respectively; CM<sub>20/40</sub> - numbers of cells after 48 h of incubation with 20 µg or 40 µg of CM proteins; \* - values significantly different from control.

Experiments were repeated six times with similar results (means ± SD are presented); the SD values between three parallel samples within the same experiments were less than ± 1%.

methods are shown in Table 1. The influence of CM on the cell growth was expressed as relative (to control) increase in cell number (Ri) after 48 h of incubation with CM and calculated as follows:

$$\% Ri = (CM_{20/40} - K_0) / (K_{48} - K_0) \times 100\%$$

where CM<sub>20/40</sub> = numbers of cells after 48 h of incubation with 20 µg or 40 µg of CM proteins; K<sub>0</sub>, K<sub>48</sub> = numbers of cells in control at the start of experiment and after 48 h, respectively. Average Ri values are presented in Figure 1.

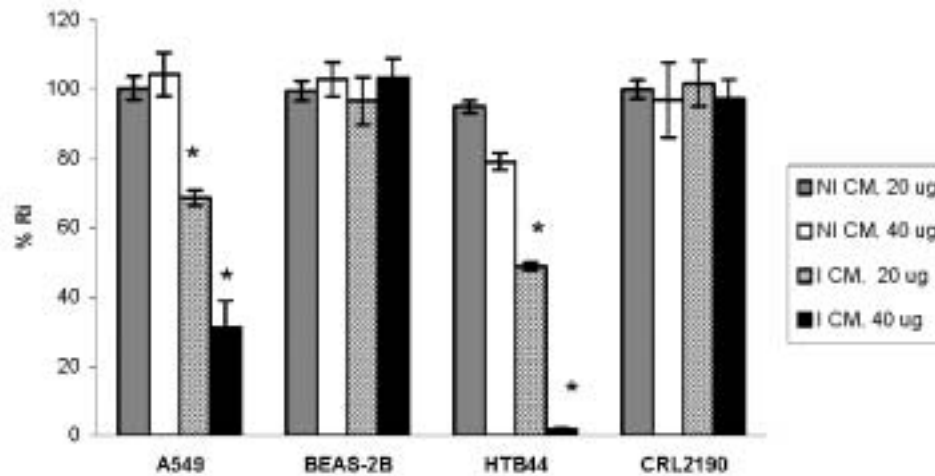
CM of macrophages collected during inflammation-free periods did not exhibit cytostatic activity against either transformed or non-transformed cells. Exposure of tumor cells to CM of macrophages harvested during peritonitis resulted in significant suppression of proliferation compared to control (Ri for A549 cells incubated with 20 µg and 40 µg of CM proteins were 69% and 31%, respectively and for HTB44 cells 49% and 2%, respectively). In contrast to tumor cells, the growth of non-transformed cells was not inhibited. The results indicated that macrophages activated during peritonitis produced substances with strong cytostatic activity, they also suggested that kidney tumor cells (HTB44) were much more susceptible to the action of these agents than lung tumor cells (A549).

The ability of inflammatory CM to suppress tumor cell growth was compared to cytostatic activity of taxol and suramin. This comparison is shown in Figure 2.

Percentage inhibitions of growth of A549 cells exposed to inflammatory CM (200 µg of proteins/ml), taxol (50 nM) and suramin (1 mM) were 69%, 83% and 68%, respectively. Exposure of HTB44 cells to these substances resulted in 98%, 79% and 63% of growth inhibition, respectively. These results showed that the highest cytostatic activity against A549 cells was exhibited by taxol, while inflammatory CM suppressed the growth of lung tumor cells to the same extent as suramin. Inflammatory CM appeared, however, to be the most effective against HTB44 cells.

#### *Effect of CM on target cell viability*

The influence of CM on target cell viability was assessed by MTT tetrazolium and neutral red staining methods and expressed as average percentages of control values after 48 h of incubation with CM. The results are shown in Figure 3. Non-inflammatory CM had no appreciable effect on viability of both transformed and non-transformed cells. CM of macrophages collected during peritonitis impaired the viability of tumor cells, in contrast to non-transformed cell, which were unaffected. The values of absorbance obtained for A549 and HTB44 cells after 48 h of incubation with the maximal used concentration of CM proteins were 76% and 21% of the control, respectively. The results indicated that during peritonitis macrophages acquired ability to release cytotoxic substances and that HTB44 cells were more susceptible to them than A549 cells. Figure 4 shows the comparison of inflammatory CM (200 µg of pro-



**Fig. 1.** The effect of peritoneal macrophage CM on target cell proliferation. Following 48 h of incubation of target cells with peritoneal macrophage CM their numbers were determined by Bürker chamber counting and crystal violet staining methods. The results are presented as a relative (to control) increase in cell number (Ri) after 48 h of incubation with CM. The values are the means  $\pm$  SD of six separate experiments. NI CM - non-inflammatory conditioned medium; I CM - inflammatory conditioned medium; \* - values significantly different from control.

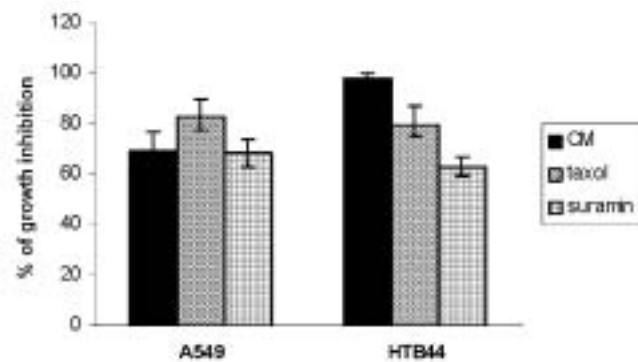
teins/ml), taxol (50 nM) and suramin (1 mM) influence on investigated tumor cell viability. The percentages of control values obtained for A549 cells incubated with CM, taxol and suramin were 76%, 64% and 70%, respectively and for HTB44 cells 21%, 56% and 37%, respectively. The results suggested that taxol was the most effective in impairing A549 cell viability, while CM influenced HTB44 cell viability more than the other two compounds.

### Induction of apoptosis

CM of peritonitis-harvested macrophages was also examined for their cytotoxic potential, especially ability to induce tumor cell apoptosis. Apoptotic cells were clearly distinguishable by characteristic morphology (cytoplasmic blebbing, cell shrinkage, nuclear condensation and fragmentation). Such morphological alterations were found in both A549 and HTB44 cells incubated with inflammatory CM (200  $\mu$ g of proteins/ml) and 50 nM taxol but not with 1 mM suramin. The results obtained for HTB44 cells are presented in Figure 5. Similar effects were observed in the case of A549 cells (data not shown).

### Discussion

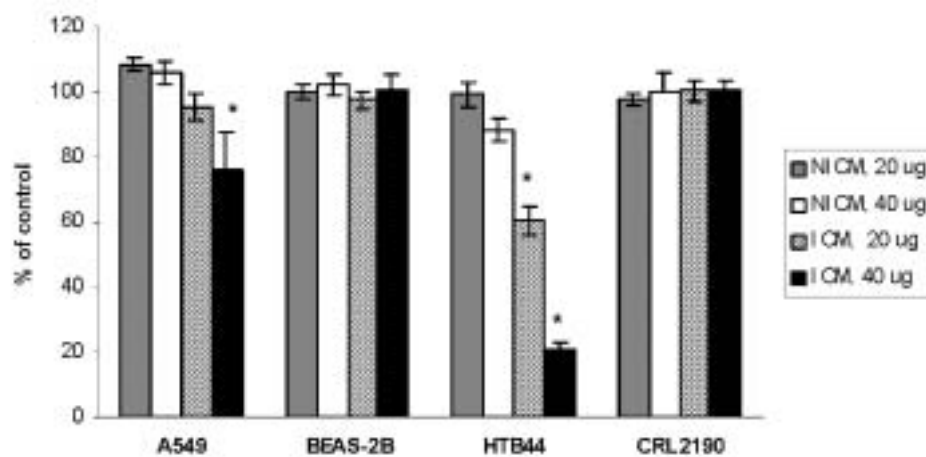
It has been reported that human peritoneal macrophages collected from renal patients on CAPD can be induced to express antitumor activity against tumor cells, especially if they were obtained during periods of peritonitis and if further stimulated *in vitro* by LPS [5]. The ability of LPS to induce antitumor activity in human peritoneal macrophages harvested during peritonitis was assumed to be due to the stimulation of the macrophages already "primed" *in vivo* in an inflammatory environment [4, 5]. It has been also shown that peritoneal macrophages



**Fig. 2.** The comparison of cytostatic activity of inflammatory CM, taxol and suramin against A549 and HTB44 cell lines. After 48 h of incubation of tumor cells with inflammatory CM (200  $\mu$ g of proteins/ml), taxol (50 nM) and suramin (1 mM), their numbers were assessed by Bürker chamber counting and crystal violet staining methods. The values are the means  $\pm$  SD of six separate experiments.

collected from patients on CAPD during inflammation-free periods can be stimulated *in vitro* to express antitumor activity [4]. The aim of the present study was to determine whether macrophages obtained from patients with peritonitis were active against human tumor cells without additional *in vitro* stimulation and, if they were, to compare this activity with efficacy of the compounds used in cancer therapy (taxol and suramin). The target models chosen were of human tumor cell lines isolated from the cases of lung and renal carcinoma. These models had the advantage of using cells originating from the kinds of tumors which are treated in cancer therapy with taxol and suramin

We showed first that CM of macrophages collected during peritonitis was active against tumor cells, in

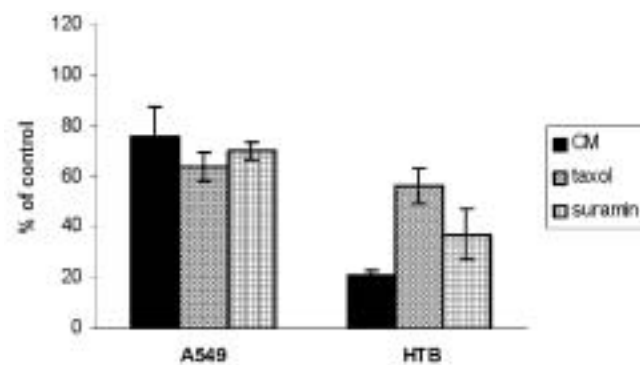


**Fig. 3.** The influence of peritoneal macrophage CM on target cell viability. Following 48 h of incubation of target cells with peritoneal macrophage CM, the values of absorbance of MTT tetrazolium and neutral red were assessed. Data shown represent the means  $\pm$  SD of six separate experiments. NI CM - non-inflammatory conditioned medium; I CM - inflammatory conditioned medium; \* - values significantly different from control.

contrast to CM of macrophages harvested during inflammatory-free periods, which did not influence the growth and viability of transformed cells. We did not notice the effect of macrophage CM on the proliferation and survival of non-transformed cells, what confirmed selective, only antitumor cell-directed activity of the evaluated supernatants. These results suggested that macrophages obtained from patients with peritonitis represented a population of fully activated rather than only "primed" cells. During inflammatory state, the macrophages acquired the capability to cytostatic and cytotoxic action and did not require further *in vitro* stimulation in order to express a potent antitumor activity.

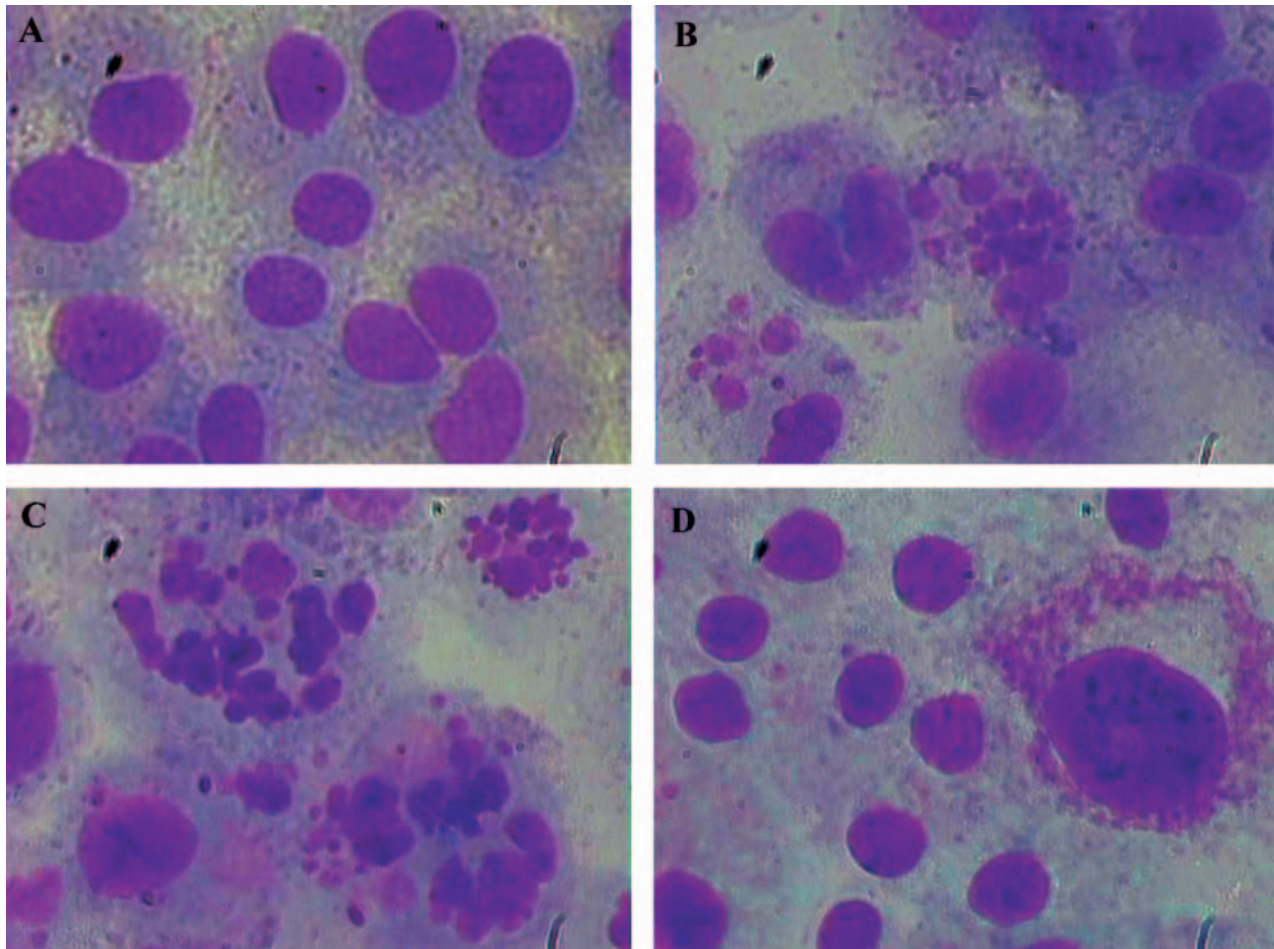
The antitumor activity of macrophages collected during peritonitis comprised the inhibition of target cell growth, impairment of cell viability and induction of apoptosis. Inflammatory CM exerted a potent cytostatic effect on both evaluated tumor cell lines. A significant suppression of growth was seen for HTB44 cells. The number of cells following 48 h of incubation with the highest used concentration of CM proteins was only 2% of control growth. Also the proliferation of A549 cells was inhibited as a result of exposition to inflammatory CM (31% of control growth). The impairment of cell viability was particularly seen in the case of HTB44 cells (only 21% of control values after 48 h of incubation with the maximal used concentration of CM proteins). A weaker but statistically significant effect was noticed for A549 cells (76% of control). Both examined tumor cell lines proved to be susceptible to agents triggering apoptosis present in CM of activated macrophages.

The results of the present study indicate that susceptibility of A549 and HTB44 cells to CM of inflammatory macrophages is different. The cells of kidney tumor were much more sensitive to CM than cells of lung tumor. Further studies are necessary to determine the agents and mechanisms underlying this difference.



**Fig. 4.** The comparison of inflammatory CM, taxol and suramin effects on A549 and HTB44 cell viability. After 48 h of incubation of tumor cells with inflammatory CM (200  $\mu$ g of proteins/ml), taxol (50 nM) and suramin (1 mM), the values of absorbance of MTT tetrazolium and neutral red were assessed. Data shown represent the means  $\pm$  SD of six separate experiments.

The cytostasis caused by macrophage CM was mediated probably by  $\text{TNF}\alpha$  and/or  $\text{IL-1}\beta$  [3, 6, 13]. The effect of macrophage anti-proliferative activity appears to be directed on the process of DNA synthesis. Many studies have employed the incorporation of [ $^3\text{H}$ ]thymidine into DNA during a period following or concomitant with exposure to cytostatic macrophages and they have clearly demonstrated that the utilization of nucleotide precursors for macromolecular synthesis is decreased by co-culture of target cells with macrophages or incubation with macrophage secretory products, resulting in a reduced DNA content [6, 11]. The cytotoxic effect of activated macrophages is primarily mediated by nitric oxide due to its ability to inhibit mitochondrial respiration and denature various essential iron-sulphur-



**Fig. 5.** Photomicrographs of HTB44 cells incubated for 48 h: **A** - in the medium alone; **B** - in the presence of inflammatory CM (200  $\mu$ g of proteins/ml); **C** - in the presence of taxol (50 nM); **D** - in the presence of suramin (1 mM). Apoptotic cells can be seen in B and C. The cells were fixed with Carnoy fixative and stained with Giemsa solution.  $\times 400$ .

containing enzymes present in target cells [16]. Other antitumor products secreted by macrophages include TNF $\alpha$ , reactive oxygen and nitrogen intermediates and FasL (CD95L), which are equally essential for macrophage cytotoxicity [13, 17]. In our experimental model in which tumor cells were exposed not to macrophages but to the macrophage-conditioned medium, the participation of reactive oxygen and nitrogen species should be excluded because of the lability of these compounds.

In the present study, we also focused on the comparison of macrophage antitumor activity with the anti-mitotic and cytotoxic potential of taxol and suramin. We demonstrated that the most effective in suppressing A549 cells growth was taxol (83% inhibition of proliferation), whereas inflammatory CM inhibited growth of these cells to almost the same extent as suramin (about 69% of growth inhibition). The greatest cytostatic effect in the case of HTB44 cells was noted for inflammatory CM (98% of growth inhibition), while taxol and suramin suppressed proliferation of these cells to a lesser extent. In our comparative study, taxol appeared to be more effective than inflammatory CM in impairment of A549

cells viability, whereas the latter was the most powerful in affecting HTB44 cells. We also found that among three compounds examined in this study only suramin did not trigger apoptosis.

The mechanism of cytostatic and cytotoxic activity of taxol and suramin is different from that of macrophage CM described above. Anti-mitotic action of taxol is due to its ability to bind to microtubules of the karyokinetic spindle. This makes the process of microtubule depolymerization, necessary for nucleus division, impossible [22, 23]. Taxol can trigger the apoptosis through induction of some transcription factors and enzymes modulating the process of programmed cell death and through interaction with proteins of Bcl-2 family [19]. Suramin influences the process of DNA synthesis through inhibition of DNA polymerase synthesis or activity and through precluding the formation of DNA-topoisomerase II complex [14]. Suramin has also ability to suppress some cyclin-dependent kinases [9]. This substance has also capability to inhibit the ATP synthesis, disturb mitochondrial electron transport chain and to impair in this way cell viability [21].

The macrophages seem to be useful for adoptive cellular immunotherapy, based on retrieving patient's own mononuclear cells, activating them *in vitro* and then infusing them back into the patient [1, 12]. The study of ovarian cancer patients, to whom  $\gamma$ -interferon was administered intraperitoneally, revealed a 32% response rate and a 23% complete response rate, defined as "the disappearance of all macroscopic and microscopic disease with negative histology". The authors attributed these encouraging findings to the effect of  $\gamma$ -interferon on peritoneal macrophage function [20]. Our results also confirmed the theory that macrophages can represent a useful tool in cancer immunotherapy.

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