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The Effect of Vanadyl Sulphate ($VOSO_4$) on Autocrine Growth of Human Epithelial Cancer Cell Lines

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Numerous studies have focused on the growth regulation effect of vanadium compounds [21, 27]. In our preliminary investigation we have observed growth inhibition of rat hepatoma cell line H35-19 by inorganic vanadium salts [5, 14]. The aim of the present study was to determine the effect of vanadyl sulphate ($VOSO_4$) on autocrine growth and survival of tumorogenic lung (A549) and prostate (DU145) human cell lines. Additionally, non-carcinogenic human cell lines BEAS-2B (as a lung control) and PNT-2 (as a prostate control) were investigated. MTT, modified crystal violet staining, differential staining (HOECHST33258 and PI) methods and assay for anchorage-independent colony formation were used to investigate the effect of vanadyl sulphate. The results showed that $VOSO_4$ significantly inhibited autocrine growth, decreased carcinoma cells viability and increased the ratio of apoptotic and necrotic cells compared to the controls. However, it should be noted that the examined "drug" significantly decreased viability of non-carcinogenic human cell lines (BEAS-2B, PNT-2).

Introduction

In addition to various biological properties, such as being an essential growth factor of some organisms, a microelement necessary for development of young individuals, a regulator of metabolism in the thyroid and bone mineralization, and of metabolic pathways of lipids and carbohydrates [1, 7, 9] or insulin-like factor [2, 6, 11, 13], vanadium derivatives also inhibit growth of transformed cancer cells in cultures. The latter property suggests their anticarcinogenic effect [3, 8, 16, 19, 25, 27], however, not all investigators confirm this point of view [12, 21, 23, 28]. In our preliminary investigations [5, 14, 15], we have observed growth inhibition of rat hepatoma cell line H35-19, as well as some human epithelial cancer cells, by inorganic vanadium salts. An important early event in the development of the neoplastic phenotype is the induction of genes involved in autocrine growth, such as growth factors and its receptors. Therefore, an analysis of deregulation of the functional relationship between autocrine growth factors and their receptors is essential in determining the pathogenesis and growth of many tumors.

This paper presents the results of investigations on the effect of vanadyl sulphate on autocrine growth and viability of human epithelial cancer cell lines A549 (lung) and DU145 (prostate adenocarcinoma). Additionally, the study included the effect exerted by this vanadium salt on the viability of non-carcinogenic BEAS-2B (as a lung control) and PNT-2 (as a prostate control) cells.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), F12 medium, glucose, L-glutamine, trypsin, tylosine, EDTA, albumin, penicillin, streptomycin, crystal violet (N-hexamethylpararosaniline), MTT [bromide 3-(4,5-dimethyltioazo-2)- 2,5-diphenyltetrazole], HOECHST 33258 (bisbenzimid) and propidium iodide (PI) were purchased from Sigma Chemical Company (St Louis, USA). Vanadyl sulphate hydrate was obtained from Aldrich Chem. Comp. Inc. Bovine serum (FBS) was obtained from Biowest, South American Origin.

Cell Culture

The human tumor epithelial cell lines A549 (lung) and DU145 (prostate), as well as human non-tumorogenic cell

lines BEAS-2B (bronchial epithelial) and PNT-2 (prostate epithelial) were used as target cells. The A549 and BEAS-2B cells were obtained from the Institute of Immunology and Experimental Therapy Wroclaw, Poland. DU145 and PNT-2 were purchased from American Type Culture Collection (ATCC). The stock cultures for DU145, A549, BEAS-2B and PNT-2 were maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine, 0.45% glucose, penicillin (100units/ml) and streptomycin (100µg/ml). The cells were passaged 2-3 times per week using 0.05% trypsin solution with 0.02% EDTA in buffered physiological salt (PBS) without Ca^{2+} and Mg^{2+}.

Cell proliferation assays

Target cells were seeded on 96-well plates at the concentration of $3 \cdot 10^3$ cell/well in DMEM or MEM supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin in the presence of 10%FBS. Following 24h of incubation, the culture medium was replaced with serum-free DMEM/F12 (1:1) supplemented with 5% albumin, 5µg/ml transferrin, 0.3 mg/ml L-glutamine, 10 µg/ml tylosine, 2 ng/ml of sodium selenite and 1000 units/ml of penicillin and 100 mg/ml streptomycin. After subsequent 24h, the medium was replaced by serum-free DMEM/F12 containing $VOSO_4$ (in the concentration range of 0.5-30 µM). The incubation was continued for another 72h at 37ºC. The modified crystal violet staining method (CV) [10] and the MTT tetrazolium assay (MTT) [17] were used to determine the influence of the vanadium compound on proliferation of target cells. The absorbance was measured using a Tecan multiscan plate reader. Ten replicate wells were used for each experiment. The results were monitored by the Magellan 3 program. The influence of the vanadium compound was expressed as relative to the control decrease in cell growth. The parameter calculated was: %GI (% of growth inhibition) = $(A_i - A_0)/(A_c - A_0)$ x100; A_0, A_c, A_i – average values of absorbance at 540 nm (CV), 570 nm (MTT) of control sample at the start of experiment (A_0), control sample after 72h of incubation (A_c) and after 72h of incubation with $VOSO_4$ (A_i).

Assessment of cell viability

The differential staining method was used in the investigation of $VOSO_4$ effect on viability of tumorogenic (A549, DU145) and non-tumorogenic (BEAS-2B, PNT-2) cells. The cells were seeded on 24-well plates at the density of $12 \cdot 10^3$ per well in 0.8 ml DMEM or MEM with 10% FBS. After 24h, the medium was replaced by a serum-free medium (cancer cells) or a medium containing 1% of FBS (for BEAS-2B cells), and the cells were exposed to 30 µM concentration of $VOSO_4$. Following 3h, 24h, 72h and 120h of incubation, the cells were stained with HOECHST 33258 and propidium iodide (PI) (at the concentration of 5 µg/ml and 1 µg/ml, respectively). After 15 min, the investigated cultures were examined directly on the plates with an epifluorescent microscope (Olympus IX-50) equipped with appropriate filters. Two excitation filters were used: one allowing for excitation of both dyes, and the other affecting only PI. The Image J software was used for image processing (merging RGB channels, enhancing contrast and sharpening) and quantitative analysis of the processed pictures (cell counting). The software allowed for estimating the fraction of necrotic cells (PI/DNA signal), viable cells (HOECHST33258/DNA signal) and apoptotic cells (HOECHST33258/DNA signal with morphological changes). Paclitaxel in the amount of 50 nM was used as a positive control in proapoptotic examination of vanadyl sulphate [4]. Each experiment was repeated at least six times, to the quantitative analysis of the results.

Statistical analysis

The results were expressed as mean ± standard error (SEM). The differences between cells treated with vanadium sulphate and the controls were evaluated statistically using the Wilcoxon's matched pair test according to Statsoft Statistica program [18] and [24]. *P* values less than 0.05 were considered statistically significant.

Results

The effect of vanadyl sulphate (VS) on autocrine growth of two human cancer epithelial cell lines: A549 and DU145 is shown in Fig.1. The results obtained by two different methods, crystal violet (A) and MTT (B), were dependent on the type of the investigated cells. In the case of A549 cells, both methods indicated a similar effect of VS. The values of growth inhibition (%GI) of A549 cells at 15 µM and 30 µM concentrations of VS were about 50% and 100%, respectively, independently of the method used. The level of growth inhibition of DU145 cells determined by the CV method (ca. 60% and 80%, at the concentration of 15 µM and 30 µM, respectively) was much lower by the MTT test (over 100%, at both the investigated concentrations). No statistically significant differences (in comparison with the control samples) were observed at the VS concentration of 0.5 µM as determined by the CV method, while MTT indicated an approximately 10% inhibition of cell growth of A549 and DU145 cells. The IC_{50} values calculated from the CV data were 12.7 µM for A549 and 13.8 µM for DU145 cells.

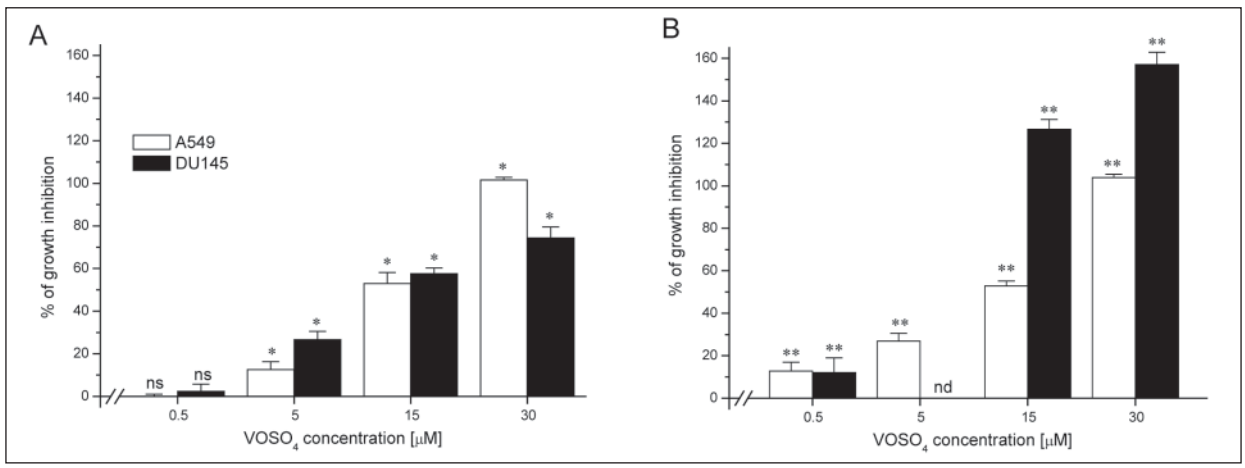

Fig. 1. The growth inhibition of cancer cell lines (A549, DU145,) by vanadyl sulphate determined by modified crystal violet staining method (A) and MTT test (B) after 72h of incubation in serum-free medium (DMEM/F12). ns – non significant ($p>0.05$) in comparison with control sample (without $VOSO_4$).* - $0.01< p <0.05$, ** $0.001< p < 0.01$.

Vanadium sulphate was also examined for its cytotoxic potential, especially its ability to induce tumor cell apoptosis. The target cells were dyed after 3h and 24h or 72h of incubation with the vanadium compound. Two cancer cell lines (A549 and DU145) investigated parallel with their appropriate non-tumorogenic equivalents (BEAS-2B and PNT-2) were viable, apoptotic or necrotic in experimental conditions. Fig. 2 shows an example of the phenomenon. Apoptotic cells were clearly distinguishable by their characteristic morphology (cytoplasmic blebbing, cell shrinkage, nuclear condensation and fragmentation). Such morphological alterations were found in all investigated cell cultures; however, in the case of control cultures, they did not exceed 10% of the total cell population.

The effect of 30μM concentration of vanadyl sulphate in comparison with the 50nM dose of paclitaxel is shown in Fig. 3. After 3h incubation with VS, both the lung (A549) and prostate (DU145) cancer cells showed a slight, but significant reduction in the percentage of viable cells and an increased amount of apoptotic cells (Fig. 3A and 3C). Under these conditions, carcinomas and their non-tumorogenic equivalents showed a comparable sensitivity to vanadyl sulphate. The cytotoxic effect of VS after 24h of incubation was similar for all the investigated cells. The percentage of viable cells was in the range of 80-85% as shown for prostate cells in Fig. 3D. Prolonging the incubation time to 72h resulted in a dramatic decrease of viability of tumorogenic and non-tumorogenic cells to about 30% (Fig. 3B). However, the percentage of apoptotic cells was higher in the case of A549 (47.3% or 36.2%) than in BEAS-2B (33.6% or 31.1%) cells. The effect of VS was comparable with that of a 50 nM dose of paclitaxel.

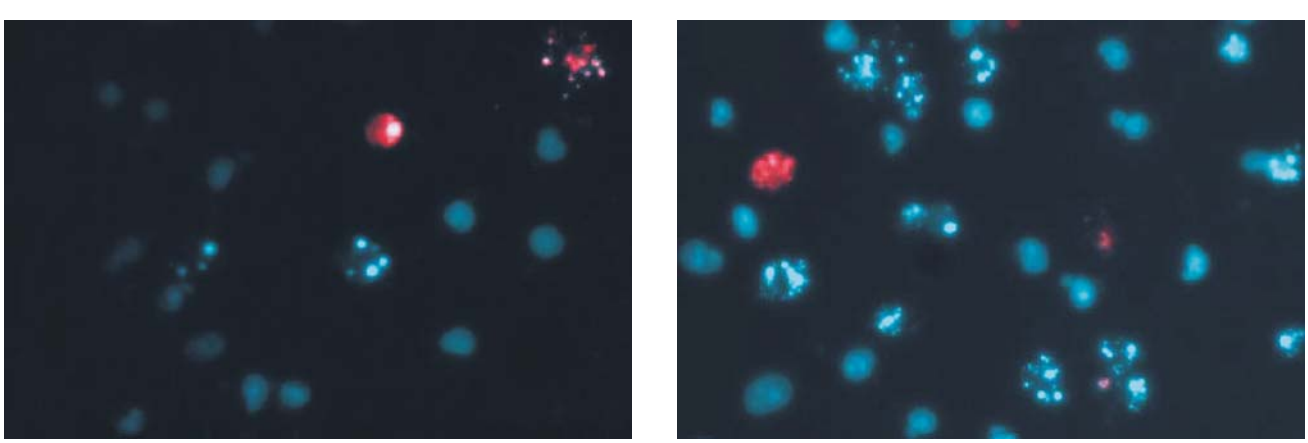

Fig. 2. An exemplary image of DU145 cells after 24h incubation with VS (A) and paclitaxel (B) dyed with HOECHST 33258/PI. Red - necrotic cells, blue – viable cells and fragmented blue – late phase apoptotic cells.

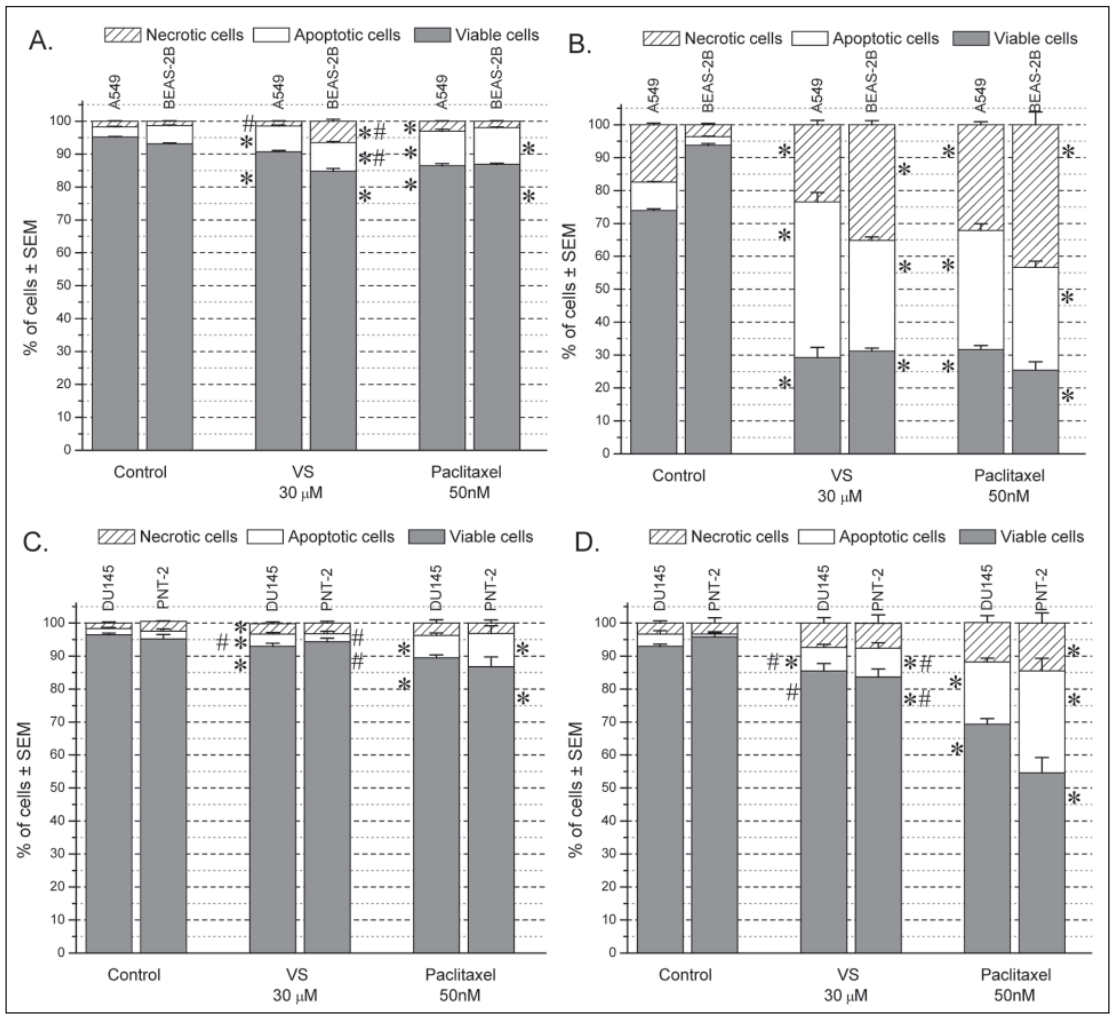

Fig. 3. Comparison of the effect of vanadyl sulphate (VS) on cell viability of tumorogenic (A549 and DU145), and non-tumorogenic (BEAS-2B, PNT-2,) cell lines determined by differential staining (HOECHST 33258/PI) after 3h (A, C), 24h (D) and 72 (B) of incubation with VS. * - $0.01 < p < 0.05$ as compared to control (without VS or paclitaxel).

Discussion

The mechanism underlying the action of vanadium compounds is not yet thoroughly understood. It has been suggested that vanadium compounds may act as phosphorus derivatives analogues. Studies on various cell lines reveal that vanadium exerts its antitumor effects thorough inhibition of cellular tyrosine phosphatases and activation of tyrosine phosphorylases. Moreover, reactive oxygen species generation is also being considered. We can only conclude that vanadyl sulphate acts as a proapoptotic factor in the examined cell lines. This property is shared with paclitaxel, but $VOSO_4$ is used in an approximately 500 times higher concentration. Vanadyl sulphate or paclitaxel affect the results in increasing the percentage of apoptotic and necrotic cells. Considering the well-known proapoptotic property of paclitaxel, a high percent of necrotic cells in the cultures indicate secondary necrosis occurrence (mainly via free radicals generation and membrane peroxidation). The thesis is confirmed by fluorescent microscope imaging, where apoptotic bodies dyed by propidium iodide appear (Fig. 2 A). At present, we are unable to answer which of the previously mentioned mechanisms is involved in apoptosis induction by vanadyl sulphate. This is in agreement with other reports. Zhang et al. [26] demonstrated that vanadate induces p53-dependent S- phase growth arrest in cultures of mouse cell lines C141. On the other hand, the same group found that vanadate ion antiproliferative activity in A549 cells results in cell cycle inhibition at the G2/M checkpoint. These studies manifest a cellular dependence in vanadium derivatives activity. Our results clearly show that the investigated vanadium compound inhibits anchorage-dependent autocrine growth of carcinogenic cells in the culture. Moreover, the data suggest that a non-specific phosphatase inhibitor

acts with a different effectiveness upon cell lines, but with a similar impact on carcinoma cells and their non-carcinogenic equivalents.

The obtained results indicate a significant and dose-dependent effect of vanadyl sulphate (VS) on autocrine growth of A549 and DU145 cells. Anchorage-dependent growth of lung and prostate carcinoma cell lines was inhibited by VS used in concentration ≥5 μM. We have not observed any stimulation of proliferation of the examined cells, which was earlier postulated by Sakai [21]. Anchorage-independent growth assay in soft agar indicates a total inhibition of colony formation for lung carcinoma cells treated by 30μM vanadyl sulphate (data not shown). Our study provides additional information on the role of non-carcinogenic cells viability (bronchial and prostate) by vanadium compound. The cytotoxic effect of vanadyl sulphate was almost the same in the case of carcinoma cells and their non-carcinogenic partners.

We have previously observed that oxidation state of vanadium affects its activity. In comparison with vanadium (IV), the orthovanadate ion [V(V)] showed a higher antiproliferative activity in carcinoma cell lines subjected to its influence [12]. The IC_{50}s values obtained for the effect of sodium orthovanadate on carcinoma cell lines varied between 7 and 10 μM for 72h of incubation in a serum-free medium. This correlates with the results obtained in the H35-19 rat hepatoma cell line [14], where differences in the effect of inorganic vanadium compounds were observed for metavanadate and orthovanadate ions. Scrivens at al. [22] described the activity of bis(peroxy)vanadium derivatives on various cancer cells and their strong dependency on ligand structure. This suggests that the type of molecular response of cells to vanadium derivatives depends on a vanadium compound and cell line. Our preliminary results have confirmed this point of view; the renal tumor carcinogenic cell line HTB-44 provides the highest opportunity for the investigated vanadium salts (data not shown in the paper). It has to be also mentioned that the investigated vanadium compound affects non-carcinogenic lung and prostate cell lines, along with carcinogenic cells.

Even though our findings indicate a restriction in vanadyl sulphate usage in cancer therapy, vanadium still may be useful as a core of organic compounds, which will hopefully exhibit a higher specificity and effectiveness in cancer cells. New organic complexes, being more potent and less cytotoxic against carcinoma cell lines, favor the future usage of vanadium compounds in anticancer therapy. Investigations of the effect of vanadium compounds on cells raises several questions, which cannot be explicitly answered as yet: whether neoplastic cells in a living organism will react to the investigated vanadium compounds in a manner similar to that observed in cultured cells, and if so, in what form should the "drug" be administered to achieve a maximally precise effect in the "neoplastic focus", without damaging normal tissues.

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