THE INFLUENCE OF TNF-α ON CONCENTRATION OF SOLUBLE ADHESION MOLECULES IN CULTURES OF HT-29 CELLS EXPOSED TO INOSITOL HEXAPHOSPHATE

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Abstract: The latest studies suggest that adhesion molecules are involved in the arising of malignant changes and in distant metastasis induction. The soluble forms of several adhesion molecules, have recently emerged as novel and potentially useful tumor markers. Among a number of identified, high interest wake soluble molecules similar to the immunoglobulin – soluble intercellular adhesion molecules-1 (sICAM-1) and soluble E-cadherin (sE-cadherin). In the present work, the authors concentrate on one tumor type, colorectal carcinoma, in which distant metastases, are the main cause of failure, in spite of surgical curing of the primary tumor. It is known that TNF-α (tumor necrosis factor – alpha) serum concentration of patients with cancer is raised. The changes in soluble adhesion molecules concentrations in serum and others fluids, could be modulated by many different factors affecting cancer cells. In the case of colon cancer one of the factors is a high-fiber diet, containing an anti-cancer chemical, inositol hexaphosphate (IP6). The aim of this study was to estimate the influence of TNF-α on the concentration of sICAM-1 and sE-cadherin in the microenvironment of HT-29 malignant epithelial colorectal cells stimulated with IP6. Additionally, adhesive property of HT-29 human colorectal cancer cell line to collagen I was estimated. The HT-29 cells were treated with TNF-α (10 ng/mL and 100 ng/mL – estimation of sICAM and sE-cadherin concentration; 100 ng/mL – adhesion assay), IP6 (0.5 mM, 1.0 mM, 2.0 mM) and TNF-α in combination with IP6. The level of sICAM-1 and sE-cadherin in cultures of HT-29 cells was measured by enzyme-linked immunosorbent assay (R&D Systems), and adhesion of the cells to collagen I was investigated by Cyquant Proliferation Assay Kit. The present findings demonstrate that TNF-α and inositol hexaphosphate have an effect on the sICAM-1 and sE-cadherin concentration in cultures of HT-29 cells. IP6 at a concentration of 2.0 mM induced a decrease of sE-cadherin concentration in cultures of these cells and significantly reduced their adhesion to collagen I. TNF-α at concentration of 100 ng/mL caused the significant increase in the sICAM-1 level, but to a lesser degree in the presence of higher concentrations of IP6. However, TNF-α did not cause such a significant increase in sE-cadherin level. The sE-cadherin concentration was most likely associated with inositol hexaphosphate activity.

Keywords: sICAM, sE-cadherin, adhesion, collagen I, TNF-α, inositol hexaphosphate, HT-29 cells

Continuous improvement in colon cancer therapy is one of the most difficult challenges of contemporary medicine, especially with regard to a high metastatic potential, which is the main cause of treatment failure. Additionally, on the road of the evolution modifications of signal transmission, the pathways in colon cancer cells have strengthened, which has become a protective factor against the anticancer activity of cytokines, such as TNF-α (1, 2).

The latest studies suggest that adhesion molecules are involved in the arising of malignant changes and in distant metastasis induction. Adhesion molecules (CAMs), in human organism can occur in two main forms: first, as a transmembrane proteins playing the role of membrane receptors (mCAM) and second as soluble proteins in fluids (sCAM) (3).

Among a number of identified, high interest wake molecules similar to the immunoglobulin – intercellular adhesion molecules-1 (ICAM-1, CD 54). Previous studies have demonstrated the usefulness of ICAM-1 and soluble ICAM-1 (sICAM-1) as

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markers for early diagnosis and monitoring the effects of treatment of colon cancer. The conclusion resulted from the observation that patients with colorectal cancer showed significantly higher levels of sICAM-1 compared with control groups. Further studies in this patients group allowed to observe a strong relationship between the concentrations of sICAM-1 molecules and metastasis (3, 4).

It is known that another adhesion molecules – E-cadherins mediate cell-cell adhesion and are important in maintaining tissue architecture and consequently organ function. A tumor transformation is associated with disorders of intracellular structures function, involving adhesion-dependent signalization, in which such particles as E-cadherin play an important role. Increased concentrations of soluble E-cadherin (sE-cadherin) have been reported in patients with various cancers. There is a hypothesis that sE-cadherin causes scattering of epithelial cells and induction of invasion. Several studies have shown that loss of cell surface immunoreactive E-cadherin is associated with tumor progression and tissue invasion (1, 3).

The changes in adhesion molecules expression, and consequently, in the concentration of their soluble forms in serum, could be modulated by many different factors affecting cancer cells. In the case of colon cancer, one of the factors is a high-fiber diet, containing an anti-cancer chemical, inositol hexaphosphate (IP6). A number of in vivo and in vitro studies confirm the efficacy of IP6 in preventing the development of many types of cancer. It is suggested that IP6 can affect different types of cells by different mechanisms. It was shown that IP6 may influence the cell cycle, gene expression, as well as intracellular signaling pathways and inhibition of oxygen free radicals formation (5–7). Experimental studies with IP6 provide evidence that it may modulate the immune reaction and inflammation, inter alia, by regulating the synthesis of cytokine such as TNF-α (8, 9). Recent scientific articles have reported IP6 inhibiting influence on adhesive and migrative properties of MDA-MB 231 breast cancer cells (10).

It is known, however, that in both inflammatory diseases and the malignant tumor microenvironment the concentration of pro-inflammatory cytokines increases, including TNF-α. The action of TNF-α on the tumor tissue may be multidirectional since it is associated with activation of multiple signal transduction routes. This cytokine can modify the adhesive properties of cells and influence the concentration of soluble adhesion molecules deriving from malignant colon cells. Furthermore, TNF-α is able to modify IP6 influence on colon cancer cells (11–15).

The goal of this study was to evaluate the influence of TNF-α on the concentration of soluble intercellular adhesive molecule-1 (sICAM-1) and soluble E-cadherin (sE-cadherin) in the microenvironment of HT-29 malignant cells stimulated with IP6.

The elevated concentration of these soluble adhesive molecules suggests the intensification of the migration ability of cancer cells, and the concurrent increase in cancer cells invasiveness and metastasis. (16).

Because collagen I is a common type of collagen in the human body (17), in the work, additionally, adhesive properties of HT-29 human colorectal cancer cell line to it were estimated.

**EXPERIMENTAL**

HT-29 cell line derived from human colon cancer was used. The HT-29 cells were kindly provided by Prof. S. Szala (Maria Skłodowska-Curie Memorial Institute of Oncology, Gliwice, Poland). Inositol hexaphosphate sodium salt isolated from corn (IP6, Sigma) and recombinated human TNF-α (Peprotech), were processed following the manufacturer’s protocol.

To assess sICAM-1 and sE-cadherin concentrations in HT-29 cell line cultures 6×10^4 cells/1.9 cm^2 were seeded in 24-well culture plate (Nunc).

The cells were incubated in RPMI 1640 (Sigma) medium supplemented with 10% fetal bovine serum FBS (GIBCO BRL), antibiotics: 100 U/mL penicillin (Sigma) and 100 µg/mL streptomycin (Sigma) for 96 h. The cells were grown at 37°C in a humidified atmosphere (95%) enriched with 5% CO₂. After 4 days of incubation, a similar culture medium was introduced, additionally supplemented with 10 mM HEPES buffer (Sigma) and tested chemicals: 0.5 mM, 1.0 mM and 2.0 mM IP6, 10 ng/mL and 100 ng/mL TNF-α, and TNF-α plus IP6. Nonexposed cell line cultures as well as a culture medium without colon cancer cells served as controls. All the cultures listed were exposed to the tested chemicals for the next 96 h. Then, culture medium was collected, centrifuged and frozen at −20°C. sICAM-1 and sE-cadherin concentrations were measured in the culture medium by immunoenzymatic method ELISA using Quantikine – Human sICAM-1/CD54 Immunoassay (R&D Systems) and Quantikine Human sE-Cadherin Immunoassay (R&D Systems). All the results obtained were expressed as ng per mL based on calibration curves generated with use of analyzed adhesive molecules standards provided by manufacturer with the Quantikine kits. Absorbance was measured...
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at the wavelength of 450 nm with microplate reader MRX Revelation (Dynex, DYNEX™ Software 4.25).

Additionally, the adhesion of the exposed cells to collagen I was assessed. The cells were incubated with the tested chemicals, i.e., IP6 (0.5 mM, 1.0 mM, 2.0 mM), TNF-α (100 ng/mL), and IP6 plus TNF-α for 72 h. After treatment with 0.25% trypsin (GIBCO BRL) containing 1 mM EDTA × 4 Na (Life Technologies) and bringing cells deriving from different cultures to the same initial count, the adhesion assay on the plate covering with collagen I (Nunc) was performed. A collagen I-coated plate was blocked for 30 min. in RPMI 1640 medium with 0.1% serum, and subsequently washed in RPMI-1640 medium. HT-29 cells exposed to TNF-α, IP6 and TNF-α plus IP6 were seeded at a density of 3x10⁵ per 200 µL (per a well of a collagen I-coated microplate) in RPMI 1640 medium supplemented with 0.1% serum, antibiotics and 10 mM HEPES buffer. The cells nonexposed to the tested chemicals and culture medium without the cells served as con-

Figure 1. Effect of TNF-α and IP6 on sICAM-1 concentration in HT-29 cells cultures. Results are the mean ± SD of 3 experiments. * p < 0.05, when compared to the controls

Figure 2. Effect of TNF-α and IP6 on sE-cadherin concentration in HT-29 cells cultures. Results are the mean ± SD of 3 experiments. * p < 0.05, when compared to the controls
controls. After 1 hour incubation, culture medium was removed, and a plate was washed in PBS. Quantitative analysis of adhesion property was carried out with the use of Quantikine Proliferation Assay Kit (Cyquant). The stain was diluted with lysis buffer (1 : 300). 200 mL of 1X lysis buffer/Cyquant GR stain (R&D Systems) was added to each well. The plate was shaken at room temperature for 20 min. Next, 150 mL from each well was transferred to a microplate fitting to the fluorescence reader. Fluorescence values 480 nm/530 nm, reflecting concentrations of DNA in cells attached to the substratum, were used in statistical analysis. Fluorescence was detected using Victor Light Luminescence counter 1420 TM (Perkin Elmer).

The comparisons of sICAM-1, sE-cadherin concentrations and fluorescences in adhesion assay were performed using one-way ANOVA. When the ANOVA results were statistically significant, a single-step multiple comparison procedure (Tukey’s test) was used. All the results are expressed as the means ± SD of three independent experiments and data were considered significant when p < 0.05. Statistical analysis was carried out by Statistica PL version 10 software (StatSoft).

RESULTS

The experiments showed the presence of both soluble ICAM-1 (sICAM-1) (Fig. 1) and soluble E-cadherin (sE-cadherin) (Fig. 2) in HT-29 cell cultures exposed and unexposed to TNF-α and IP6. The control medium without the cells was negative for these adhesion molecules. Therefore, sICAM-1 and sE-cadherin were shed from HT-29 cells.

Studies have shown that 0.5 mM, 1.0 mM and 2.0 mM IP6 did not change the sICAM-1 concentrations in a significant way in comparison to culture without the compound. (Fig. 1). After TNF-α treatment at concentration of 10 ng/mL of HT-29 cells, the values of sICAM-1 in cell culture supernatants did not change (p = 0.049), but 100 ng/mL TNF-α caused a 6ñ7-fold increase of these soluble molecules compared with control culture (p = 0.0003). TNF-α at concentration of 10 ng/mL in the presence of 0.5 and 1.0 mM IP6 showed statistically significant increase in levels of sICAM-1 in the culture medium (p = 0.0002; p = 0.0006). In the presence of 2.0 mM IP6, an increase in sICAM-1 was smaller and did not differ statistically from the concentrations of these adhesion molecules in the control culture. Interestingly, it was found that TNF-α at concentration of 100 ng/mL caused the increase in the sICAM-1 level, but to a lesser degree in the presence of higher concentrations of IP6.

Similarly, as in the case of sICAM-1, TNF-α and IP6 caused dysregulation of sE-cadherin molecules in the intestinal epithelial of HT-29 cells (Fig. 2). Studies have shown that TNF-α caused a slight increase in sE-cadherin level in cultures exposed to 100 ng/mL of the cytokine (p = 0.043), but 10 ng/mL TNF-α did not influence their concentration in HT-29 cells cultures. It was observed a significant decrease in sE-cadherin in cultures supernatant
under the influence of 2 mM IP6 (p = 0.003). The addition of TNF-α at a concentration of 10 ng/mL to the cultures containing 0.5, 1.0, and 2.0 mM IP6 did not cause any increase in the sE-cadherin level. Contrary, it was observed a significant decrease of sE-cadherin concentration in cultures exposed to 1.0 mM or 2.0 mM IP6 together with 10 ng/mL of TNF-α (p = 0.0007; p = 0.0002). Taking into account the concentrations of these molecules in the control culture, TNF-α at concentration of 100 ng/mL did not significantly increase the sE-cadherin level in the presence of various IP6 concentrations either. Their level was most likely associated with IP6 activity, especially at concentration of 1.0 and 2.0 mM. The higher concentration of IP6 and the lower sE-cadherin level in the presence of TNF-α was observed. In order to determine whether TNF-α and IP6 would alter the adhesion of HT-29 cells we used collagen I as a substrate for the cell adhesion assay. As it is shown in Figure 3, 2.0 mM IP6 significantly decreased the ability of the cells to adhere to collagen I (p = 0.002), because only 67% of the cells were seeded on it, compared to control culture.

The studies have shown similar adhesion properties to collagen I of the control cells and cells exposed to TNF (100 ng/mL) or TNF-α with various concentrations of IP6.

**DISCUSSION AND CONCLUSIONS**

Action of TNF-α on healthy and modified tumor tissues can produce a variety of biological effects (11, 14, 15). In addition, TNF-α stimuli could changed cancer cells answer evoked by dietary components such as IP6. A significant anticancer activity of the naturally occurring carbohydrate IP6 has been reported against numerous cancer models, including colon cancer. Over the past few years, many scientific reports concerning mechanisms of IP6 action have been presented (18–20). Recent studies emphasize the fact that IP6 may not only act preventively and inhibit cancer development but also it can affect the process of metastasis. Vucenik et al. (21) show IP6 inhibitory action on several angiogenic responses, including growth and differentiation of endothelial cells, as well as inhibitory effects on the secretion of VEGF, a key angiogenic cytokine. Tantivejkul et al. (10, 22) studied the potential of IP6 to inhibit human breast cancer cell adhesion, migration and invasion, the key steps in cancer metastasis. They have shows that IP6 could inhibit the cell surface expression of integrins and their signaling molecule by decreasing FAK (focal adhesion kinase) autophosphorylation.

In this work, the experimental model was used as a cell line HT-29 derived from *adenocarcinoma* of the colon.

In presented study, it was taken to assess the influence of TNF-α cytokine and IP6 on the concentration of soluble intercellular adhesion molecules—ICAM-1 and soluble E-cadherin in cultures of HT-29 cells (1). The soluble forms of several adhesion molecules have recently emerged as novel and potentially useful tumor markers (16). Additionally, the adhesive properties of these cells to collagen I were assessed.

Intercellular adhesion molecules—ICAM-1 (CD54) are associated with the processes of cell migration. Regulation of ICAM-1 expression is cell specific. Up-regulation generally is by inflammatory cytokines as TNF-α, IFN-γ and IL-1 and down-regulation generally is by anti-inflammatory agents e.g., glucocorticoids. In previous reports, an expression of ICAM-1 was seen on neoplastic cells of various origins including gastric and pancreatic carcinoma. As a result of shedding of the molecules ICAM-1 from cells surface into body fluids (including blood), they appear in the form of soluble ICAM-1. The increase in their concentrations can be both an exponent of the inflammatory process or ongoing neoplastic process. Dippold et al. (4) showed that in the Caco-2 and HT-29 colonic tumors cell lines cytokine treatment resulted in an induction or enhancement of ICAM-1 expression on the cell surface, but also increased the shedding of sICAM-1. In agreement with the findings in melanoma, the shedding of sICAM-1 could be a mechanism for colonic tumors cells to evade from lymphocyte mediated cytolysis.

The experiments showed the presence of sICAM-1 both in cultures of HT-29 cells exposed and unexposed to TNF-α and IP6. This is in line with previous studies, showing the presence of soluble adhesion molecules in the biological fluids located at the contact of tumor cells. TNF-α caused 6–7-fold increase of these soluble molecules compared with control culture. Singh et al. (23) demonstrated that TNF-α induced expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells. Miyata et al. (24) examined the expression of ICAM-1 on intestinal epithelial HT-29 cells by flow cytometry. According to obtained results of experiments, stimulation with TNF-α for 5 h markedly up-regulated ICAM-1 expression on HT-29 cells, whereas stimulation with TNF-α for 30 min, did not affect ICAM-1 expression. In our studies, the stimulation of the cancer cells with TNF-α has taken 72 h and the level of sICAM-1 over this time signifi-
cantly increased. Here, we have evaluated the effect of TNF-α on sICAM-1 concentrations in cultures of HT-29 cells containing various concentrations of IP6. Interestingly, it was found that TNF-α caused an increase in the level of sICAM, but in the lowers degree in the presence of higher concentrations of IP6.

Another, the epithelial transmembrane molecule E-cadherin is the prime mediator of epithelial Ca²⁺-dependent cell-cell adhesion. The specific role played by soluble E-cadherin in the whole process of adhesion is still not clarified. Charalabopoulos et al. (25) found that increased levels in serum reflect serious dysfunction of cellular E-cadherin since they are much higher than in healthy individuals. It was been found that high concentrations of sE-cadherin in serum of patients with cancer of lung, gastric, prostate and hepatic was positively correlated with a significant advancement of disease and poor survival.

As in the case of sICAM-1, detectable concentration of sE-cadherin were present in all cultures containing TNF-α and IP6 tested in our study. TNF-α caused slight increase in sE-cadherin level in cultures exposed to 100 ng/mL of this cytokine but it did not significantly increase the sE-cadherin level in the presence of various IP6 concentrations. Their levels were most likely associated with IP6 activity. The higher concentration of IP6 and the lower sE-cadherin level in the presence of TNF-α was observed.

According to Yi et al. (26), molecular alterations of E-cadherin were seen in Caco-2 cells under the influence of 10 ng/mL of TNF-α. Down-regulated E-cadherin expression was detected via western blot and immunofluorescence staining. However, the conditions of the experiments alter from ours and the authors did not estimated concentrations of sE-cadherin in the cultures of Caco-2 cells.

Selective colonization of tumor cells to the components of the different organs is dependent in both the expression of various surface molecules on cell endothelium and from a specific composition of the extracellular matrix (ECM) in a given organ. The main component of the extracellular matrix such as collagen, fibronectin, laminin perform complex often an active role in cell migration and adhesion. (17).

To determine whether TNF-α and IP6 would alter adhesion of HT-29 cells, we used collagen I as a substrate for the cell adhesion assay. Long-term (72 h) stimulation with 100 ng/mL TNF-α did not increased the adhesion but 2.0 mM IP6 significantly decreased the ability of the cells to adhere to collagen I.

Our findings are also consistent with previous report that IP6 at concentration of 2.0 mM inhibits adhesion of MDA-MB 231 breast cancer cells, but the published results were obtained with reference to the fibronectin (10).

However, changes in cell adhesion properties could be interpreted differently, depending on the stage of invasion and metastasis the cancer therapy introduced. Our results indicate that IP6 is potentially able to inhibit tumor metastasis/dissemination to collagen I-containing organs and tissues. However, inhibition of adhesion to collagen I in the presence of 100 ng/mL TNF-α was not showed. It may suggest the opposite effect of TNF-α and IP6 on cancer cells. Recent scientific articles reported inhibition of tumor necrosis factor induced NF-kB survival pathway in HeLa cell line cultures exposed to IP6 (27).

It is likely that the terminal effect of impact of the tested molecules combination on tumor cells mainly depends on these molecules concentrations.

The present findings demonstrate that TNF-α and IP6 have an effect on the sICAM-1 and sE-cadherin concentration in cultures of HT-29 cells. IP6 at a concentration of 2.0 mM induced a decrease of sE-cadherin concentration in cultures of these cells and significantly reduced their adhesion to collagen I. TNF-α at concentration of 100 ng/mL caused the significant increase in the sICAM-1 level, but to a lesser degree in the presence of higher concentrations of IP6. However, TNF-α did not cause such a significant increase in sE-cadherin level. The sE-cadherin concentration was most likely associated with IP6 activity.

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