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A New Model Applied for Evaluating a Rhenium-diselenium Drug: Breast Cancer Cells Stimulated by Cytokines Induced from Polynuclear Cells by LPS

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Abstract. *Background/Aim: New anticancer drugs are usually tested on cancer cells in culture in a standard medium. We stimulated immune polynuclear cells by lipopolysaccharides to obtain an enriched medium (EM) containing inflammatory cytokines more closely reflecting the tumor microenvironment and tested a rhenium-diselenium (Re-diSe) drug in this new model. Concentrations of cytokines were compared with a control medium (CM). Materials and Methods: Human-derived breast cancer cells were grown in culture either in CM or EM with or without Re-diSe. Assays of tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), intereukin 1 beta (IL1β), transforming growth factor-beta (TGFβ), insulin growth factor 1 (IGF1) and vascular epidermal growth factor A (VEGFA) were performed by enzyme-linked immunosorbent assays. The production of reactive oxygen species (ROS) was determined by 2,7-dichlorofluorescein test. The cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tests. Results: Concentrations of TNFα, IL6 and Il1β were observed to be significantly higher in EM than in CM. There was no difference for TGFβ, IGF1 and VEGFA. The cells were sensitive to Re-diSe, with reduced concentrations of TGFβ, IGF1, VEGFA and ROS, but the half-maximal inhibitory concentration was significantly higher in EM than in CM. Conclusion: The efficacy of the Re-diSe drug was confirmed in this model of aggressive cancer.*

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Key Words: Inflammatory cytokines, biomarkers, rhenium, selenium, cancer cells.

The cells involved in inflammation are important constituents of the tumor microenvironment (TME) (1) producing inflammatory cytokines (2), which are particularly involved in breast cancer development and progression by inducing the expression of biomarkers of oxidative stress, proliferation and angiogenesis (3). However, conventional culture media fail to mimic the TME. Herein we propose a simple model to address this problem, thereby reducing this bias in anticancer drug screening. Our *in vitro* experimental model consisted of inducing inflammatory cytokines by polynuclear cells (PNCs) in the presence of lipopolysaccharides (LPS) in order to obtain a medium enriched in cytokines (EM). We assayed the concentrations of inflammatory cytokines, tumor necrosis factor (TNFα) and interleukins (IL1β and IL6), and of transforming growth factor (TGFβ), insulin growth factor (IGF1) and vascular epidermal growth factor (VEGFA) by enzyme-linked immunosorbent assay (ELISA) in both EM and RPMI1640, a Dulbecco's modified Eagle's medium (DMEM) which was used as control medium (CM). A rhenium-diselenium (Re-diSe) compound has already been tested in a standard medium and showed a selective inhibitory effect on breast cancer cells compared with normal cells (4). The proliferative rates of the cancer cells were compared in EM and CM by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. TGFβ1, IGF1 and VEGFA and reactive oxygen species (ROS) were assayed in a final medium obtained after the culture of human-derived breast cancer cells either in EM or CM, with or without a Re-diSe anticancer drug. Furthermore, the production of ROS by the cancer cells was also determined and

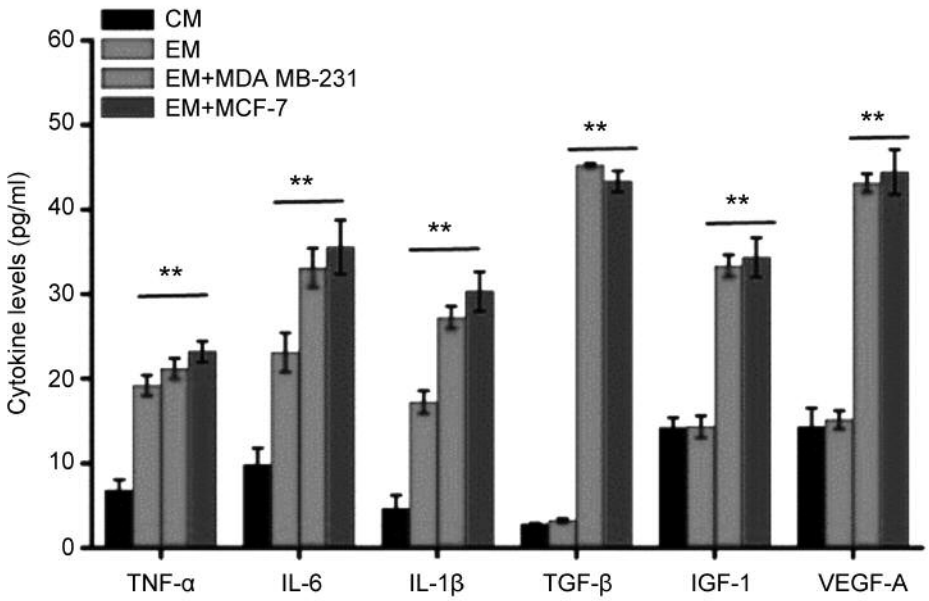

Figure 1. *Assays of tumor necrosis factor (TNFα), interleukins (IL6, IL1β), transforming growth factor (TGFβ1), insulin growth factor (IGF1) and vascular epidermal growth factor (VEGFA) in enriched medium (EM) versus control medium (CM), and between MCF-7 and MDA-MB-231 breast cancer cells in EM. **Significantly different at p<0.05 at 95% confidence level.*

performed by fluorometric quantification of 2,7-dichloro-fluorescein (DCF) formed in the presence of ROS using 2,7-dichlorofluorescein acetate (DCF-DA).

Materials and Methods

Reagents and kits. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma Aldrich, Bengaluru, India.

Cell types. Human hormone-sensitive MCF-7 and hormone-independent MDA MB-231 derived- breast cancer cells were purchased from National Centre for Cell Science (NCCS), Pune, India. PNCs were purchased from HiMedia Laboratories, Mumbai, India.

Media. The CM consisted of DMEM supplemented with 10% FBS, 10 U/ml of penicillin, 10 mg/ml of streptomycin, and 0.25 mg/ml of amphotericin-B maintained in a CO_2 incubator at 37˚C and 5% CO_2. PNCs were cultured in the presence of LPS (1 ng) for 12 h to stimulate cytokine secretion to produce EM. The EM was collected and stored at −80˚C until use.

Cell proliferation. The cell proliferation was measured by colorimeter method using MTT. Briefly 1×10^4 cells/ml of MDA MB-231 and MCF-7 cells (obtained from the National Centre for Cell Science, Pune, India) at a confluency of 80%, after 45-65 passages, were seeded in 12-well cell plates in 1 ml of CM and allowed to attach for 12 h. Then, 1 ml of EM was added and the plates were incubated for 24 h in a CO_2 incubator. After incubation, the cells were incubated with MTT reagent for 3 h. After 3 h, the medium was removed and formazan crystals produced were dissolved in dimethyl sulfoxide. The absorbance was read at 600 nm and the relative absorbance was recorded, using a multiwell plate reader.

Re-diSe treatment. The procedure of synthesis of the Re-diSe compound and its analytical structure were described elsewhere (5-7). MDA-MB-231 breast cancer cells were seeded at the cell density of 1×10^4 cells/ml (150 μl of DMEM) in 96-well plates overnight in a CO_2 incubator at 37˚C. They were then exposed for 120 h to different doses of Re-diSe (5, 10, 25, 50 and 100 μM) in EM or in CM.

Assays of biomarkers. TNFα, IL6, IL1β, TGFβ1, IGF1 and VEGFA were assayed using ELISA kits from Abcam (Cambridge, UK). They were assayed in EM and CM. They were also assayed in the final medium of cancer cells in culture in CM and EM with different doses of Re-diSe after an exposure time of 120 h.

ROS production. The production of ROS was measured by fluorimetric quantification of fluorescent 2,7-dicholorofluorescein (DCF) formed in the presence of ROS using cell-permeable non-fluorescent, 2,7-dichlorofluorescein diacetate (DCF-DA). After 120 h treatment with Re-diSe, the cells were trypsinized, pelleted, washed twice with PBS and exposed to DCF-DA (10 μM) in DMEM for 45 min at 37˚C. After incubation, the cells were analyzed using 96-well multimode reader with excitation at 480 nm and emission was recorded at 540 nm. Results were expressed as mean fluorescence intensity (MFI) in arbitrary units (a.u).

Statistical analysis. The results were expressed as the average of six independent experiments with standard deviations. The statistical analysis was performed using SPSS software (version 24; IBM, India). Concentrations of biomarkers were compared using Mann–

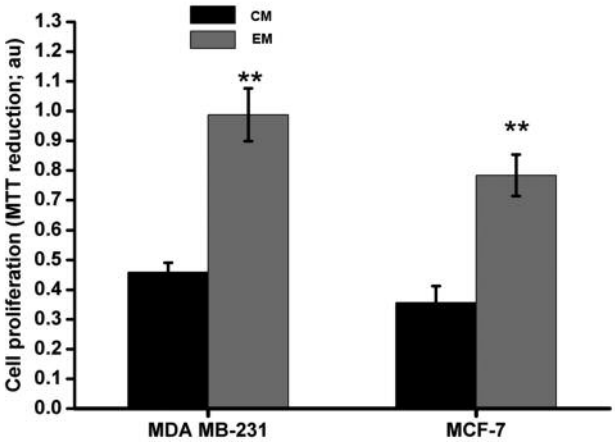

Figure 2. *Proliferative rates of MDA-MB-231 and MCF-7 cancer cells in culture in control medium (CM) versus enriched medium (EM). MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test; au: arbitrary units. **Significantly different at p<0.05 at 95% confidence level.*

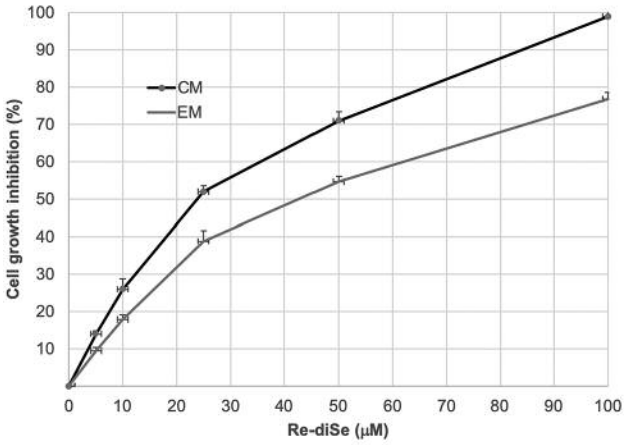

Figure 3. *Dose-dependent effects of rhenium-diselenium (Re-diSe) on MDA-MB-231 cancer cells in culture in control medium (CM) and in enriched medium (EM). All values were statistically significantly different for CM compared to EM (p<0.05).*

Whitney *U*-tests. Results were considered statistically significant with $p<0.05$ at 95% confidence levels by two-tailed tests.

Results

Model of LPS-induced cytokine production. The concentrations of inflammatory cytokines TNFα, IL6 and Il1β were significantly higher in EM compared with CM. There was no significant difference for TGFβ1, IGF1 and VEGFA (Figure 1).

After incubation of hormone-dependent MCF-7 and hormone-independent MDA-MB-231 breast cancer cells in EM, the concentrations of TNFα, IL6 and IL1β increased compared with when EM, but more remarkably so for TGFβ1, IGF1 and VEGFA.

Proliferation of MDA-MB-231 and MCF-7 cancer cells was statistically significantly higher (approximately two-fold) when the cells were cultivated in EM than in CM (Figure 2).

Effects of Re-diSe. The effects of different doses of Re-diSe for an exposure time of 120 h were studied on MDA-MB-231 cancer cells.

Cell growth inhibition was dose-dependent both in CM and in EM (Figure 3).

The half-maximal inhibitory concentration (IC_{50}) was 22.82±2.34 μM for MDA-MB-231 cells in culture in CM and significantly higher for those in culture in EM (38.68±1.98 μM). The IC_{50} values were calculated by non-regression curves of the dose–response of six independent experiments, using Graphpad Prism 6.0 software. However, the graph in Figure 3 represents the average percentage inhibition from six independent experiments.

Table I. *Levels of biomarkers in the final culture medium after incubation of MDA-MB-231 cancer cells either in control medium (CM) or in enriched medium (EM) for 12 h.*

	CM	EM	*p*-Value
IGF1 (pg/ml)	419±12	592±11	0.001
TGFβ1 (pg/ml)	956±10	1321±11	0.001
VEGFA (pg/ml)	762±12	876±17	0.001
ROS (MFI)	2980±109	3567±97	0.05

MFI: Mean fluorescence intensity (in arbitrary units); IGF1: insulin growth factor; TGFβ: transforming growth factor; VEGFA: vascular epidermal growth factor; ROS: reactive oxygen species.

The levels of the studied markers were significantly higher in medium of MDA-MB-231 cells cultured in EM than in CM for 12h, before treatments (Table I). These levels are those reported in Figure 4 at the dose of 0 μM.

Re-diSe treatment induced a dose-dependent decrease of ROS, TGFβ1, IGF1 and VEGFA but the results were affected by the type of medium culture, concentrations being significantly lower in CM than in EM (Figure 4).

Discussion

It is known that exposure of cells to LPS may induce both an inflammatory response and oxidative stress (8-12). It may induce the production of inflammatory cytokines such as IL1β, IL6, IL8, and TNFα, the overexpression of cyclooxygenase-2 (COX2) and toll-like receptor 4 (TLR4).

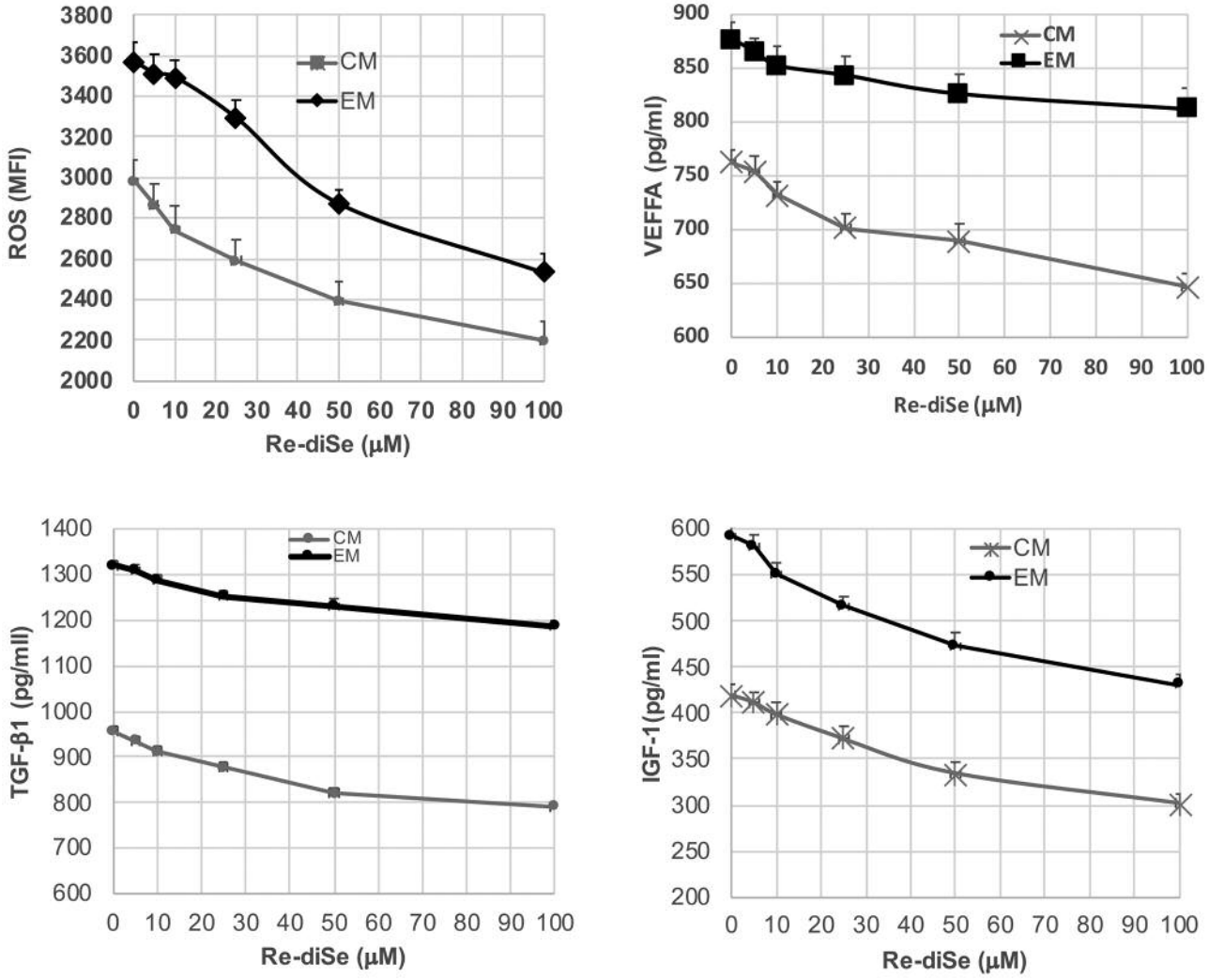

Figure 4. *Dose-dependent effects of rhenium-diselenium (Re-diSe) on the concentrations of reactive oxygen species (ROS), insulin growth factor (IGF1), transforming growth factor (TGFβ1), and vascular epidermal growth factor (VEGFA) in the final medium after incubation of MDA-MB-231 cancer cells in control medium (CM) and in enriched medium (EM). All values were statistically significantly different for CM compared to EM ($p<0.05$).*

It may also induce oxidative stress, with an increase in production of ROS, NO and malonaldehyde (MDA), higher expression of inducible nitric oxide synthase and COX2 protein, and a decrease of glutathione peroxidase. Among these cytokines, we chose to assay TNFα, IL6 and IL1β in the medium after exposure of PNCs to LPS. As expected, we observed a high amount of these substances in the medium that we considered thus as EM.

The model of PLS-induced inflammation and oxidative stress may be of great interest in testing new drugs and moreover when cancer cells are incubated with the resulting 'inflammatory' medium. Chronic inflammation has a predominant role in tumor cell survival and proliferation, angiogenesis and immunosuppression and is associated with the development and malignant progression of most cancer types (13, 14). Cancer-related inflammation (CRI) has become a potential target in the era of immunotherapy (15). Chemokines are produced not only by cells of the TME including cancer-associated fibroblasts, mesenchymal stem cells, endothelial cells, tumor-associated macrophages and tumor-associated neutrophils, but also by tumor cells themselves (16). It was shown in MDA-MB-231 cancer cells that TGFβ, TNFα and IL1β up-regulated production of IL6 and IL11 (17). It has been noted that IL6 was highly produced by triple-negative breast cancer cells, and contributed to immune suppression in the TME (18). Finally, a TME enriched chronically with inflammatory cells and the pro-inflammatory cytokines TNFα and IL1β promotes breast

cancer progression, despite the potential antitumor cytotoxic activities of TNFα (19).

It has already been demonstrated that a Re-diSe compound reduced the production of ROS, TGF-β1, IGF1 and VEGFA in hormone-independent MDA-MB-231 breast malignant cells in culture, while selectively reducing their proliferation compared with normal HEK-293 human embryonic cells (4). In this study, these decreased as a dose-dependent effect of Re-diSe drug but the results were affected by the type of medium and their concentrations remained clearly higher in EM than in CM. The IC_{50} was significant higher in EM than in CM.

Conclusion

Results from studies with standard culture medium do not take into account the TME. We propose an easy *in vitro* model for studying the effects of anticancer drugs on cancer cells stimulated by inflammatory cytokines. We demonstrated that a simple exposure of immune cells with LPS increased the amount of the main inflammatory cytokines in the culture medium. This EM increased the growth of malignant cells, with an overproduction of TGFβ1, IGF1 and VEGFA.

In this experimental model in which the cancer cells were stimulated by inflammatory cytokines, dose-dependent inhibitory effects were observed with Re-diSe drug. By reducing ROS production by cancer cells, Re-diSe acts as an anti-oxidant. It is also an anti-angiogenic factor, reducing the production of VEGFA. The reduced concentrations of TGFβ1 and IGF1 may be related to the anticancer effects of this combined rhenium and selenium drug on cell growth. Anticancer activities have also been observed in MDA-MB-231 tumor-bearing mice after repeated daily doses of 10 mg/kg for 4 weeks, at non-toxic doses for the mice (6, 20). The development of Re-diSe should be continued with the aim of entering clinical trials.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

Authors' Contributions

The new model of studying cancer cells in EM with LPS-induced cytokines was proposed by Vijay Veena. Sunali Khanna, Philippe Collery and Vijay Veena conceived the idea of evaluating a metal-based drug, Re-diSe, in this model. The full protocol was elaborated by Vijay Veena. Didier Desmaele was involved in the development of this compound and achieved its synthesis. The experiments were performed with Adhikesavan Harikrishnan and Basavegowda Lakshmi. Vijay Veena, Didier Desmaele and Philippe Collery were the main contributors to the redaction of the article.

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