

ORIGINAL ARTICLE

NATURAL IMMUNE BOOSTER IMUREGEN SIGNIFICANTLY AFFECTS THE PROLIFERATION OF TUMOR CELLS

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Received 14th March 2019.

Revised 22nd April 2019.

Published 6th September 2019.

Summary

Background. Extracts from plant and/or animal tissues are frequently used in alternative medicine as drugs or food supplements. Such extracts may contain a complex of pharmacologically or physiologically active factors but frequently there exists no experimental confirmation as to precise mechanisms of action. This work aimed to verify if a long used bovine tissue extract Imuregen registered as a food supplement has desirable effect on tumor cells.

Methods. Two independent methodological approaches were used. Viability of cell cultures was evaluated using WST-1-based cell cytotoxicity assay. Cell growth was monitored in real time using xCELLigence cell analysis. Normal human adherent lung fibroblasts (NHLF) were used to represent non-tumor lung cells. A human non-small cell lung carcinoma cell line H1299 was used as a model of tumor cells.

Results. Our study demonstrated a direct influence on viability of the H1299 tumor cell line ($p < 0.005$) and a cytostatic/cytotoxic effect of the bovine tissue extract after 72h. of cultivation while leaving non-tumor NHLF cell line unaffected. The extract (0.1 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$, resp.) also significantly affected the viability of irradiated H1299 tumor cell line ($p < 0.005$, Co, 4Gy) compared to non-tumor irradiated counterparts. In addition to the cytotoxic effect, the extract slightly modified the generation time of the cells and substantial differences between the effects on tumor and non-tumor cell lines were observed.

Conclusion. The data presented here might suggest the extract intervenes into the proliferative cell cycle and subsequently influences the generation time of cells. Further analyses should be oriented toward the effects of animal tissue extracts on cellular systems defending against tumors and/or infections and intercellular communications that lead to influencing the fate of individual cell types.

Key words: tissue extract; tumoricidal effect; generation time; H1299 cell line; NHLF cells

ABBREVIATIONS

Co: cobalt

HPMA: N-(2-hydroxypropyl) methacrylamide

EPR: enhanced permeability and retention

NHLF: normal human lung fibroblast

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BACKGROUND

The incidence of malignant neoplasms has long continued to grow. Statistical data from the Czech Republic point to a more than doubling of the incidence of malignant neoplasms between 1980 and 2015. In contrast to the incidence, the relative and standardized rates of mortality from malignant tumors show a long-term decrease (1). The reason for the opposite trends in incidence and mortality can be seen in an improving quality of medical care, including the availability of new diagnostic approaches and therapeutic agents. In general, medical care recognizes two modalities of antitumor treatment: Local treatment consists of surgical intervention and/or radiotherapy. Systemic therapy, meanwhile, involves chemotherapy, immunotherapy, hormonal treatment, and/or biological therapy. Chemotherapy exploits the effect of mitosis inhibitors, which include, for example, vinblastine, paclitaxel, and danusertib (2). These are substances that interfere with DNA replication (e.g., folates or analogues of purines and pyrimidines), have cytostatic effects on cell growth or are inhibitory of enzymes (e.g., tipifarnib, bortezomib) (3), and/or small-molecule inhibitors of p53 protein or kinases (4, 5).

Antitumor immunotherapy utilizes cytokines (e.g., IFN- α or IL-2), monoclonal antibodies directed against cell surface receptors (e.g., cetuximab or trastuzumab), or antibodies targeted to other molecular structures of solid tumors (Reviewed at http://www.wikiskripta.eu/w/Protinádorová_terapie [in Czech].)

Targeted polymer-bound drugs are utilized in one of the specific modes of cancer treatment. Experimental studies have demonstrated that the covalent linkage of doxorubicin (Dox) to the N-(2-hydroxypropyl) methacrylamide (HPMA) polymer carrier leads to complete tumor regression and to the development of therapy-dependent long-lasting cancer resistance (6). Combined treatment of syngeneic mouse tumor models by HPMA copolymer-bound Dox conjugate and immunocomplexes of IL-2 and anti-IL-2 monoclonal antibody has synergistic antitumor activity (7). Another experimental setup utilizes HPMA copolymers with incorporated organic nitrates to deliver NO generation into solid tumors in order to increase the enhanced permeability and retention (EPR) effect (8). In an in vivo study wherein EL4 T-cell lymphoma was inoculated into mice, NO donors were shown to potentiate the EPR effect, thereby leading to increased accumulation of the polymer-bound cytotoxic Dox and a better therapeutic outcome in treating the lymphoma (9).

Another approach to treating cancers and modulating organisms' immune status utilizes extracts from animal or plant tissues. Preparations made from animal tissues or organs fall into one of the categories of nutritional supplements, which include (a) antioxidants; (b) vitamins; (c) extracts from herbs/plants – teas, non-teas; (d) extracts from fruits/seeds; (e) extracts from animal tissues; (f) extracts from fungi and yeasts; and (g) others (10). These preparations usually contain many molecular components, any one of which may be pharmacologically or physiologically active. Either the preparations as such or their isolated components can be used as food supplements for improving health status or as drugs.

Among the pioneers of animal tissue extracts therapy is the Swiss Clinique La Prairie, Montreux, Switzerland, founded by Paul Niehans in 1931. Since its inception, one of the areas in which the clinic has directed its work involves providing treatment with stem cell extracts obtained from various tissues of adult sheep and/or sheep embryos. Shortly after the founding of Niehans' clinic, a number of similar preparations were created, among which were extracts from bovine tissue originally prepared by the so-called RTN team in Prague, Czechoslovakia.

This team was initially formed and subsequently led by Bohumir Rakusan (1900–1969). The other members were Jaroslav Fanta, Osvald Zicha, Karel Zicha, Gabriel Urbanek, Alois Vystreil, and Bedrich Dolezel. The team prepared several preparations from animal tissues. Some of these were patented, such as the preparations named RTN33 – RETISIN and RTN112 – LYASTIN, both prepared from bovine tissue (11). A golden era of such tissue extracts was during the 1950s up to the 1970s.

The extracts, prepared by alcohol extraction of bovine tissue, were demonstrated to have immunomodulatory effects in laboratory experimental setups as well as in clinical practice¹². Among the study findings was a modulation of cytokines production positively influencing the induction and regulation of antitumor immunity, and which can be further supported by a positive effect to promote the activity of natural killer cells (12). These data taken together with the information on the beneficial effect of the alcohol extracts of bovine tissue in patients

suffering from carcinomas have led us to verify whether there is a possible direct effect of this extract on tumor cells. Using two independent methodological approaches, we demonstrate here that the alcohol extract of bovine tissue has a direct cytostatic/cytotoxic effect on tumor cells even as non-tumor cells are not affected. The effect is associated with lower concentrations of the preparation in the *in vitro* environment and is independent of the γ irradiation of cells.

MATERIALS AND METHODS

Cell lines and the extract

Normal human adherent lung fibroblasts (NHLF cells - ATCC® PCS-201-013™, Manassas, USA) were used to represent non-tumor lung cells. The NHLF cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and incubated at 36.8°C in an atmosphere supplemented with 5% CO₂. A human non small cell lung carcinoma cell line derived from metastatic site in lymph node (H1299 - ATCC® CRL-5803™, Manassas, USA), was used as a model of tumor cells and corresponds to the non-tumor NHLF cell model. The H1299 cells were cultivated in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and incubated at 36.8°C in an atmosphere supplemented with 5% CO₂.

The original extract of bovine tissue Imuregen® (hereinafter referred to as extract) prepared by the standard alcohol extraction was a kind gift of Uniregen Ltd., Nachod, Czech Republic. The extract is a complex mixture of low molecular weight components of bovine tissue and has been demonstrated to be nontoxic.

Standard test of viability

Viability of the cell cultures was evaluated using the WST-1-based cell cytotoxicity assay (Roche, Switzerland). The H1299 and NHLF cells were seeded into 96-well plates with density of 7×10^3 cells/well and 5×10^3 cells/well, respectively. The plates were incubated in a humidified CO₂ incubator overnight at 36.8°C. The following day, the extract was added either 1 h prior to or 1 h after gamma irradiation with ⁶⁰Co at doses of 2 or 4 Gy, respectively, and the cells were cultured for the required time (36.8°C, 5% CO₂). Subsequently, 50 μ L of WST-1/PBS (1:4) was added to each well and plates were incubated for 3 h under the same cultivation conditions. Before measuring, the plates were gently mixed and then measured using a Spectronic Helios Gamma microplate reader (Thermo Fisher Scientific) at a wavelength of 450 nm. Five replicates were run for each treatment. The relative viability of cell lines was obtained on the basis of optical density in each tested cell culture.

Real-time monitoring of cell growth

xCELLigence real-time cell analysis was used while following the instructions of the manufacturer (ACEA Biosciences, San Diego, CA, USA). Briefly, 50 μ L of growth medium was added to each well and the background impedance was measured. Next, 100 μ L of cell suspension (totaling 2×10^2 cells per well) was added and the plate was left to stand at 36.8°C until a monolayer was observed (24 h), thus allowing the cells to attach homogeneously. The cells were then treated with either gamma irradiation (4Gy), with the extract alone at final concentration of 0.1 and 1.0 μ g/mL, respectively, or with a combination of the irradiation and extract. In the case of combination, the extract was added into the cell culture 1 h after irradiation. The continuous cell proliferation was monitored every 10 min for 7 days.

Generation time calculation

A grid was drawn on the bottom of 6-well plates before measurement of the generation time. Thereafter, the cell suspensions were plated into the wells of these 6-well plates. The cells of H1299 as well as NHLF cell lines were cultivated with or without the extract (concentration 1.0 μ g/ml). The cell suspensions were seeded into the wells in three dilutions. Cells per well totaled 100, 150, and 200 for H1299 cells, and NHLF cells were seeded at 200, 300, and 400 cells per well. A total of 10 adherent cells in each well were marked at the bottom of the well after 6 h of cultivation. The dividing cells and doubling cells were identified and marked every 12 h of cultivation. The division of cells was monitored until 84 h post initiation of cultures.

Statistics

All experiments were conducted at least two times. The experiments with cell suspensions were carried out on a minimum of four parallel samples from each cell suspension. An unpaired Student's two-tailed *t*-test was applied to all measured data to evaluate statistical significance. *P* values < 0.05 were accepted as significantly different and herein are denoted by asterisks.

RESULTS

Dose-dependent effect of the extract on cell viability

A preliminary experiment served for screening the viability as a basic parameter of the direct extract effect on the cells cultivated *in vitro*. The tumor H1299 and non-tumor NHLF cell lines were cocultivated with the extract samples at concentrations of 0.1, 1.0, 10.0, and 100 µg/mL and then compared to untreated control samples. The relative viability of cells in cultures, expressed as optical density (OD), was measured 24 and 72 h after initiation of coculture (Fig. 1). Doses of the extract up to 10.0 µg/mL were well tolerated by both cell lines during 24 h of cocultivation. The highest doses of the extract decreased the viability of NHLF cells at 24 h of coculture. In contrast to the NHLF cells, the observed H1299 cells showed no increase in cell death during the same time interval (Fig. 1A). Prolonging the cocultivation to 72 h revealed a sensitivity of H1299 tumor cells to the two lowest doses of the extract. The sensitivity of NHLF cells to the highest dose of the extract is still visible, but the drop in viability already is insignificant (Fig. 1B).

Fig. 1A

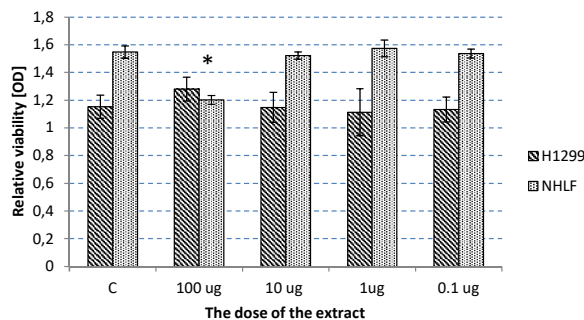


Fig. 1B

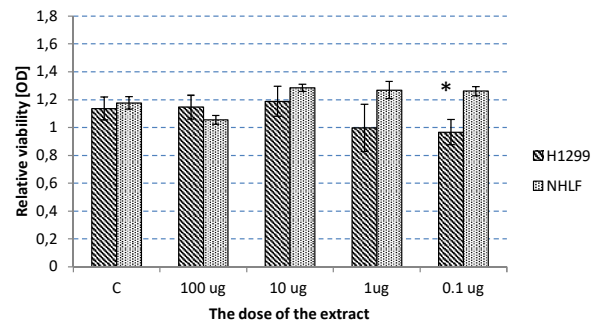


Figure 1. Dose-dependent effect on the relative viability of H1299 and NHLF cell lines calculated after 24 h (A) and 72 h (B) of cocultivation with the extract. The relative viability was expressed as an optical density of the sample. The significance of the differential effect between the cultures with the particular dose of the extract and the control culture was calculated. The asterisk (*) indicates $p < 0.05$.

Effect of the extract on irradiated cells

Due to the results of the preliminary experiment, which had demonstrated the sensitivity of H1299 tumor cells to the extract (see Fig. 2), the two lowest doses of the extract were selected for testing the effect of the extract on irradiated cells. The dose of 2 Gy of irradiation had been derived from the doses used for the single fraction typical for human exposure, while 4 Gy is a typical human LD₅₀ dose. Both tested doses of the extract significantly affected the viability of nonirradiated H1299 tumor cells in comparison with nonirradiated non-tumor NHLF cells. While irradiation of 2 Gy had no additional significant effect on the viability of H1299 cells, the viability of NHLF cells cocultivated with the extract and irradiated at the same 2 Gy dose expressed a tendency to be protected from irradiation. The combination of the higher dose of cell irradiation and the effect of the extract when added to culture at the concentration of 1.0 µg/mL affected the viability of H1299 tumor cells more than it did the viability of their non-tumor counterparts (Fig. 2).

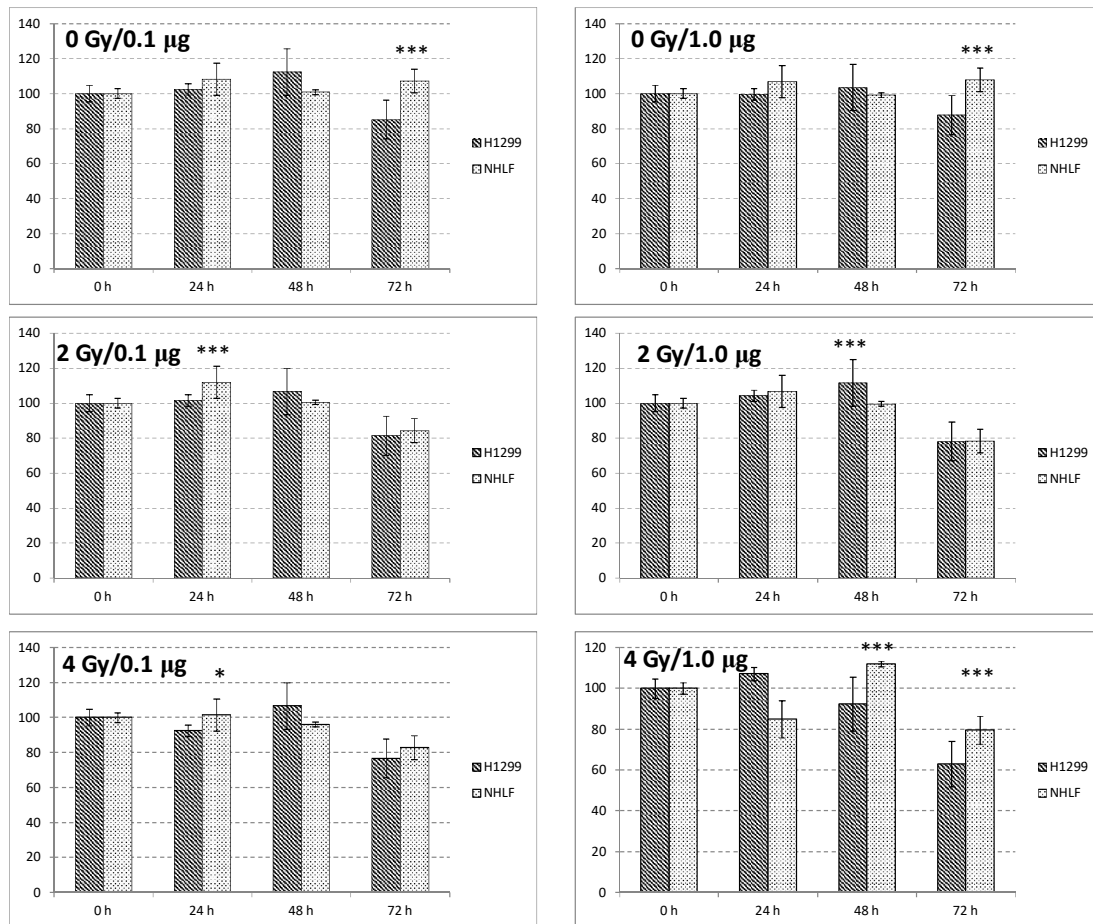


Figure 2. Effect of the extract on irradiated cells. Cells were irradiated with 2 Gy and 4 Gy of ^{60}Co . Extract was then added to cultures in the specified doses 1 h after irradiation. Differences between H1299 and NHLF cells were calculated and tested for significance at all indicated time intervals (* $p < 0.05$, *** $p < 0.005$).

Cell growth real-time monitoring

Using the xCELLigence system, NHLF and H1299 cell growth under the influence of the extract was monitored in real time and confirmed the differential effect of the extract on the two cell lines. Growth of NHLF cells was slowed without limitation under the influence of the extract at concentrations of 0.1 µg/mL and 1.0 µg/mL from the very beginning upon addition of the extract. The growth of H1299 cells was not affected during the first 3 days, but subsequently the cytotoxic/cytostatic effect of the extract strongly dominated (Fig. 3A, 3B).

Irradiation of the H1299 and NHLF cells by the dose of 4 Gy of ^{60}Co limited the growth of both cell lines. The decreased growth was observed from day 3 after irradiation in H1299 cells and from day 4 in the case of NHLF cells. The cocultivation of irradiated NHLF cells with the extract has only insignificant effect on the cell proliferation at both concentrations used. The cytostatic/cytotoxic effect of the extract was much more considerable ($p < 0.01$), and it intensified after irradiation of H1299 cells (Fig. 3C, 3D).

Dissimilar effect of the extract on generation time of tumor and non-tumor cells

Generally speaking, there are many variables at play that can influence the growth of cells. For instance, cell size and their proliferation status change in a characteristic manner within a particular medium during incubation. For this reason, we decided to provide data about the generation time within our experiments.

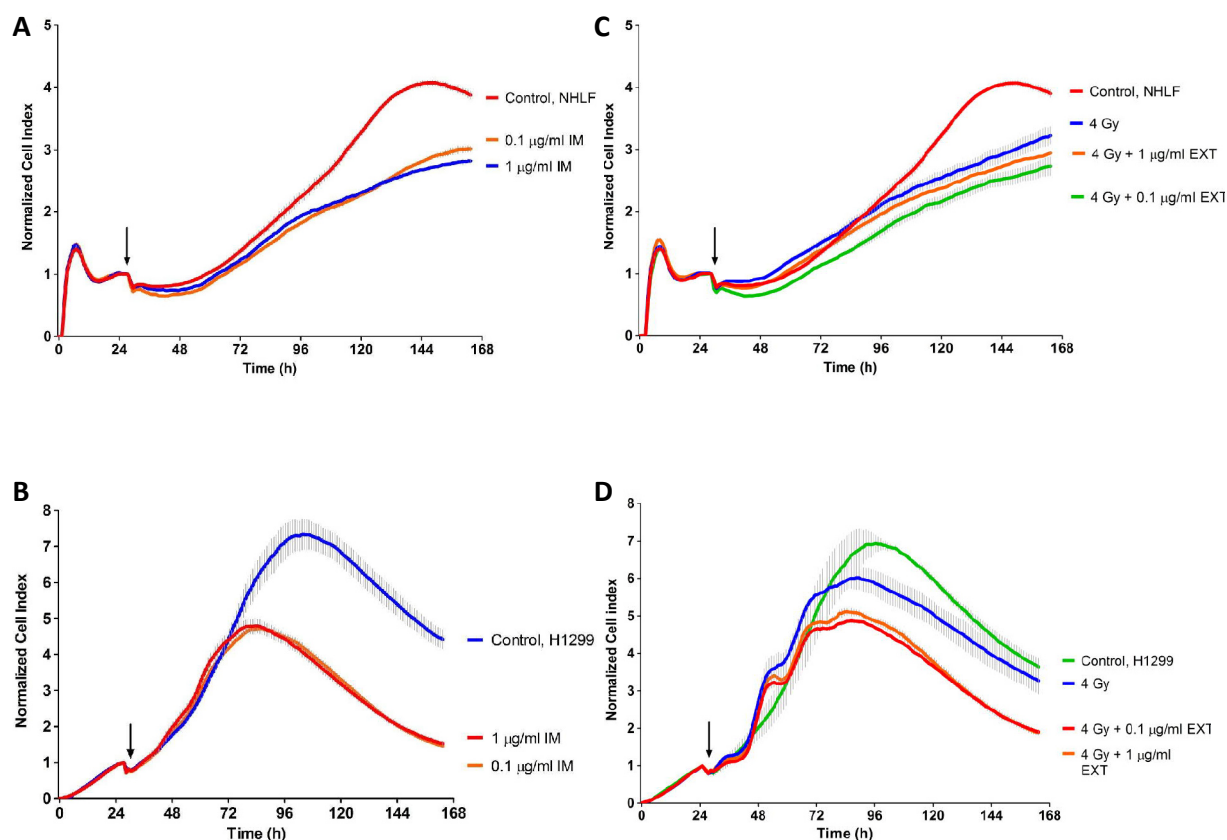


Figure 3. Representative figures of electric-impedance-based real-time monitoring of cell viability. Tested cell lines treated by diluted bovine extract were: A) non-tumor NHLF cell line, and B) tumor H1299 cell line. Both cell lines were also irradiated using ^{60}Co and cocultivated with extract in two concentrations: C) non-tumor NHLF cell line D) tumor H1299 cell line. Black arrows indicate the points of influence. Data are expressed as means \pm SEM obtained from three independent experiments.

Generation time under the influence of the extract was measured at the level of individual cells by microscopic examination. In the first experiment, we utilized only one dose of the extract, specifically 1.0 $\mu\text{g}/\text{mL}$. Addition of the extract into cell cultures influenced the generation time of H1299 cells positively but not significantly ($p = 0.88$), in that the mean generation time of treated cells was shorter than that of cells without addition of the extract to the cell culture. The influence on the generation time of NHLF cells was just the opposite, albeit again not significantly so ($p = 0.97$), as the generation time was prolonged.

A second experiment was carried out using three doses of the extract (i.e., 0.1, 1.0, and 10.0 $\mu\text{g}/\text{mL}$). The generation time of H1299 cells was reduced significantly by the two higher doses of the extract. The NHLF cells respond to the two lower doses of the extract by prolonging the generation time, while the highest dose of the extract insignificantly shortened the generation time of the non-tumor cell line (Table 1)

Table 1. Mean generation time (in hours) of H1299 and NHLF cells influenced by the extract

| Cell lines | + medium | + extract (0.1 $\mu\text{g}/\text{mL}$) | + extract (1.0 $\mu\text{g}/\text{mL}$) | + extract (10.0 $\mu\text{g}/\text{mL}$) |
|------------|---------------|---|---|--|
| H1299 | 39.6 (24–48)* | 40.8 (24–48) | 37.2 (24–48) | 33.6 (24–48) |
| NHLF | 62.4 (36–84) | 66.0 (36–84) | 68.4 (36–84) | 57.6 (36–84) |

*The generation time is expressed in hours

DISCUSSION

As defined by the Biology Online dictionary, tissue extracts are defined as “Preparations made from animal tissues or organs; they usually contain many components, any one of which may be pharmacologically or physio-logically active; extracts may contain specific, but uncharacterized factors or proteins with specific actions” (13, 14). The alcohol extracts from bovine tissue constitute one group of functionally effective tissue preparations. This group includes the products with trade names Solcoseryl, Actovegin, and/or Imuregen, the original name of which is RTN33 – Retisin. Original data demonstrate a direct anti-tumor effect of various RTN preparations when injected into cancer patients in the terminal stage of the disease (15). It had been declared by Urbanek (16) that some of the RTN preparations had direct therapeutic effects on tumor cells when tested *in vitro*. Although experimental data from *in vitro* tests is unfortunately unavailable or was not archived at all, similar direct effect of natural tissue extracts has already been reported in reputable journals (17, 18). Because the cited publications are of very old data, we decided to test the direct cytostatic/cytotoxic activity of natural tissue extract prepared by alcohol extraction from bovine tissue by current *in vitro* assays. In testing to discriminate between anti-tumor effect and any general effect of the extract in cell cultures, we chose as targets the tumor cell line H1299 and NHLF (both relatively radioresistant to 2 and 4 Gy of gamma irradiation).

Several basic and general conclusions can be derived from the data presented here. First, the alcohol extract of bovine tissue is significantly cytostatic/cytotoxic for nonirradiated normal human cells if added to cell culture at the highest dose. Nonirradiated tumor cells are insensitive to this dose of extract. The stated effect is significant only at 24 h of cell cocultivation with the extract. This suggests that limitation of the relative viability of NHLF cells by the highest dose of the extract is more likely to be a temporary block in some stage of the cell cycle than a real cytotoxic effect of the indicated dose of extract. A further conclusion relates to the effect of the extract on irradiated cells. The comparison of the viability of nonirradiated against that of irradiated cells cocultivated with the two lowest doses of the extract revealed that the non-tumor cells are less affected by irradiation than are the tumor cells. Such effect was demonstrated only in what might be termed the “therapeutic” scheme of the culture setup as opposed to what could be called the “prophylactic” scheme, in which case there was no significant effect (data not shown). A final conclusion refers to the timing of the extract’s effect on cell proliferation. Cocultivation with the extract requires some time before we see the cytostatic/cytotoxic effect of the extract on the cell cultures. In our hands, specifically, that time was 3 days. This conclusion might suggest the extract intervenes into the proliferative cell cycle and subsequently influences the generation time of cells. The data from our experiments, however, suggests that this would not be the case. The generation times of tumor and non-tumor cells were only insignificantly influenced by exposure to the extract, moreover in opposite directions.

An alternative explanation, therefore, can be that there is a direct cytotoxic effect on tumor cells with only limited influence of the extract on the growth of non-tumor cells. Such explanation can be supported by the data from the cytotoxicity testing using xCELLigence real-time cell analysis. All three conclusions drawn from the WST-1-based cell cytotoxicity assay were confirmed by the real-time cell analysis. Moreover, the curve direction of tumor cell viability started to be changed about 60 h post initiation of the cultures, whereas the slope of the curve of the non-tumor cells remained unchanged through to the end of the experiment (i.e., for 168 h). Knowing that the generation time of the tumor cells is about 35 h, direct cytotoxic effect of the extract on tumor cells better explains our data than can intervention into their cell cycle.

A final point that should be discussed is the ability of the extract to substitute for irradiation in limiting the viability of tumor cells in prolonged *in vitro* real-time cocultivation assay. The extract had only slight impact on the viability of irradiated tumor cells, and that should be compared with the substantial effect of the extract on non-irradiated tumor cells. During the same time intervals, it should be noted, no impact on the viability of non-tumor cells was recorded. If such ability of the extract will be demonstrated also in an *in vivo* system, then it might be possible to replace irradiation with all its potential side effects by completely harmless tissue extracts that leave non-tumor cells unaffected.

CONCLUSION AND PERSPECTIVES

The alcohol extract of bovine tissue Imuregen is significantly cytostatic/cytotoxic for nonirradiated non-tumor cells if added to cell culture in the supra-optimal dose. The lower doses of the extract have significant cytostatic/cytotoxic effect on the tumor cells while having only marginal effect on the non-tumor cells.

The critical evaluation of the data presented here cannot explain the published beneficial effects of bovine tissue extracts in clinical practice as reported in the literature. In the future, therefore, *in vitro* studies can be recommended with a focus on the possible modulation of metabolic processes in the cells that would influence their molecular profiles and could lead to the proven cytotoxic effect of the alcohol/ether extract. Further, future analyses should be oriented toward intercellular communications that lead to influencing the fate of individual cell types. Moreover, studies oriented toward modulation of immune responses in *in vivo* models can contribute to clarifying the value of tissue extracts for clinical practice when used as food supplements or drugs.

ACKNOWLEDGEMENT

The authors would like to kindly acknowledge prof. Ales Macela for his personal contribution and critical comments during realization of the project and for detailed reading of the manuscript. The authors would also like to thank Petr Jost for real-time impedance measurements. We would like to acknowledge, as well, the company Uniregen, Ltd., Nachod, Czech Republic, which provided us Imuregen as the basic functional component of the commercial immunomodulatory and regenerative food supplement.

AUTHOR CONTRIBUTIONS

KD performed the analysis and wrote the paper; LC, LP contributed data or analysis tools, collected the data, and performed the analysis; ZS conceived and designed the analysis, performed the analysis; KK conceived and designed the analysis, performed the analysis, and wrote the paper.

FUNDING

This work was performed within the framework of Ministry of Defence of the Czech Republic - long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding the publication of this article.

ADHERENCE TO ETHICAL STANDARDS

This article does not contain any studies involving animals performed by any of the authors.

This article does not contain any studies involving human participants performed by any of the authors.

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