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Leukemic cells modulate induction of COX-2 in human stromal fibroblasts

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The interaction of cancer cells with surrounding normal tissue cells is of utmost importance for their survival and tumor progression. For these purposes the cancer cells exploit normal tissue responses associated with inflammation and tissue repair. In the immediate tumor microenvironment one of the early stromal markers is cyclooxygenase-2 (COX-2).

In this study we evaluated the effect of leukemia cell lines on nemosis-induced COX-2 expression in stromal fibroblasts. We found that THP-1 cells were the most potent leukemic cells (IC_{50}=746) to suppress COX-2 expression. The U-937 cell line exhibited similar suppressive potency (IC_{50}=921), whereas the KG-1 cell line (IC_{50}=3519) was the least potent to affect COX-2 expression in the stromal cells.

Our study shows that human leukemic cells can actively participate in modulation of stromal inflammation via inhibition of COX-2 expression. In a co-culture model of leukemia cell lines and stromal fibroblasts, our data suggest that the tumor-stromal interactions are complexly regulated, and the straightforward association of COX-2 expression with tumor progression may require re-evaluation since some tumor cells, e.g. from hematologic malignancies, may differentially modulate inflammation and COX-2 expression.

Key words: human leukemia cells, inflammation, COX-2, nemosis

The interactions between cancer cells and their surrounding stromal tissue cells are known to be highly important for growth, survival and spread of the malignant cells [1,2]. The stroma consists of several cell types, such as fibroblasts, endothelial cells, adipocytes, and blood-derived cells. The stromal fibroblasts, however, represent the dominant cellular element [3]. It is well documented in the literature that inflammation in the stroma plays a highly important role in the pathophysiology of cancer [4]. Stromal fibroblasts are key players in this inflammatory event associated with production of proinflammatory cytokines and prostaglandins [5]. Cyclooxygenase-2 (COX-2) is the inducible rate-limiting enzyme in the generation of proinflammatory prostanoids, that are clearly implicated in progression of various human tumors [6]. There are plethora of data on how proinflammatory mediators produced by human stromal cells can affect the behaviour of cancer cells, but practically no solid data exist on the ability of tumor cells are to affect the inflammation or even to revert back such stroma to a non-inflammatory state [7].

Recently, we characterized a new biological way of mesenchymal cell activation called nemosis [8]. Nemosis is a programmed process of cell activation and subsequent death in human fibroblasts that can be triggerred by cell-cell contacts [9]. Activated fibroblasts are most distinctly characterized by induction of COX-2 expression, as well as, by production of inflammation-, cell growth- and differentiation- associated cytokines and growth factors (e.g. IL-1, IL-6, IL-8, IL-11, LIF and GM-CSF), which are similar to cytokines produced by stimulated stromal cells isolated from bone marrow [10,11]. Previously we analyzed the effect of nemosis on hematological malignancies. Our results showed that mesenchymal cells derived from a peripheral organ, for example skin, have–once undergoing nemosis–a specific effect on co-cultured leukemic cells [10]. Co-cultivation with leukemic cell lines KG-1 and THP-1 resulted in growth arrest and subsequent differentiation of these leukemic cells, whereas the U-937 leukemic cell line was unresponsive [12]. Now, based on these results, we decided to further analyze the system of co-cultured cells, and to study the possibility of a reciprocal communication between leukemia cell lines and activated fibroblasts.

The process of nemosis was employed in this study as a model of inflammation in human mesenchymal cells. We have found that the analyzed leukemic cells had capacity to significantly influence COX-2 expression in stromal fibroblasts at the early phase of the inflammatory response. The effect was less pronounced when the process of inflammation was

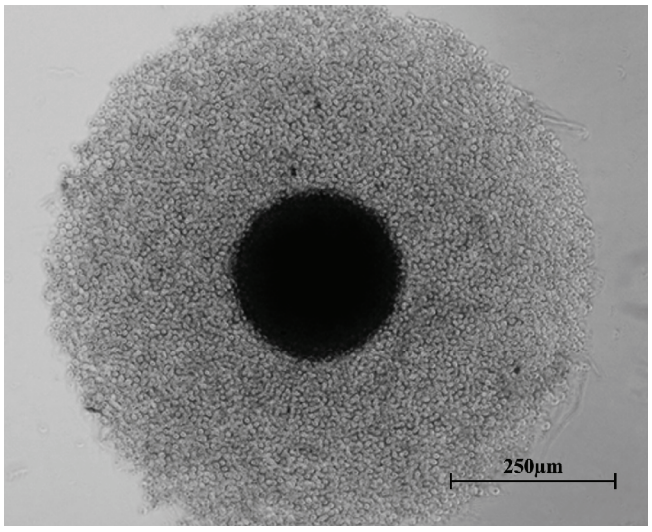


Figure 1. Co-culture of leukemic cells with the fibroblast spheroid. Phase contrast microscopy showing the central dense spheroid originally formed of MUF 7/1 dermal fibroblasts (10^4/well) surrounded by leukemic THP-1 cells after 72 h of co-culture. Scale bar represents 250 µm.

already ongoing. To our knowledge this is the first indication in literature that human leukemic cells have capacity to modulate process of inflammation in stromal cells.

Materials and methods

Antibodies. Antibodies used for immunoblotting were: goat anti-COX-2 antibody (Ab) (sc-1746), goat anti-actin Ab (sc-1615) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). The secondary anti-goat (V115A) alkaline phosphatase-conjugated was from Promega (Madison, WI).

Cell cultures and cultivation. Human dermal fibroblasts (MUF 7/1) established from neonatal foreskin were kindly provided by Dr. Miroslav Pirsel, Cancer Research Institute, Bratislava, Slovakia and were used from passages 5 to 15. THP-1, KG-1, U-937 cell lines were from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Paisley, Scotland), 50 IU/mL streptomycin and 50 µg/mL penicillin (Life Technologies, Gibco, Carlsbad, CA).

Spheroid formation was initiated as we described previously [13]. Briefly, U-bottom 96-well plates (Costar, Cambridge, MA) were treated with 0,8% LE agarose (BioWhittaker, Rockland, ME) prepared in sterile water to form thin film of nonadhesive surface. Fibroblasts were detached from culture dishes by trypsin/EDTA, and a single cell suspension (4×10^4 cells/mL) was prepared in a complete culture medium. To initiate spheroid formation, 250-µL aliquots were seeded into individual wells and the dishes incubated at +37°C in 5% CO_2 atmosphere. For the estimation of cell numbers the Bürker chamber was used.

For the cocultivation experiments the leukemic cells were cultured in 96-well plates treated with agarose at indicated concentrations. Fibroblasts were added to these cultures, and the cocultures were incubated for the next 72 h. After this period fibroblast spheroids were separated from leukemic cells by gravitational differential sedimentation and then analyzed for COX-2 expression by immunoblotting. Leukemic cell lines were also analyzed for COX-2 expression before and after cocultivation.

Immunoblotting. Cells were washed with PBS and lysed in SDS-PAGE reduced sample buffer (62,5 mmol/L Tris-HCl (pH 6,8), 2% SDS, 20% glycerol, 5% β-mercaptoethanol and 0,005% bromophenol blue) supplemented with mixture of proteases (Complete Miniprotease Inhibitor Tablets, Roche, Germany) and incubated at +95°C for 5 min. Lysates were centrifuged at 3000 rpm for 5 min to sediment particulate-insoluble material. Each sample separated by SDS-PAGE (gradient of polyacrylamide 5-15%, 3,5% stacking gel) was then transferred to nitrocelulose membrane (Schleicher&Schuell, Germany) in transfer buffer at 150 mA during 18 h. Transfer efficiency was verified by Ponceau-S staining. After blocking in 2,5% low-fat dry milk in TBS (20mmol/L Tris-HCl, 150 mmol NaCl and 0,1% Tween-20 pH=7,5) the membrane was incubated with specific primary antibodies followed by secondary alkaline phosphatase-conjugated antibody. Protein bands were visualized according to manufacturer´s recommendations.

IC_{50} was calculated using the non-linear curve-fitting algorithm in GraphPad Prism vesion 5, GraphPad Software, La Jolla, CA (www.graphpad.com).

Transduction of THP-1 cells. GFP-expression lentiviral vector (pLV-PGK/GFP, a gift from professor Seppo Ylä-Hertualla, AIV-Institute, Kuopio, Finland) was employed to transduce THP-1 cell line with GFP gene. THP-1 cells were seeded (5×10^5 cells/well) into a 6-well plate and transduced with 1:20 dilution of virus concentrate and titer of $1,4 \times 10^7$ in the presence of polybrene (8 µg/mL). After 16 h, virus-containing medium was removed, cells were washed and resuspended in normal growth medium for experimentation.

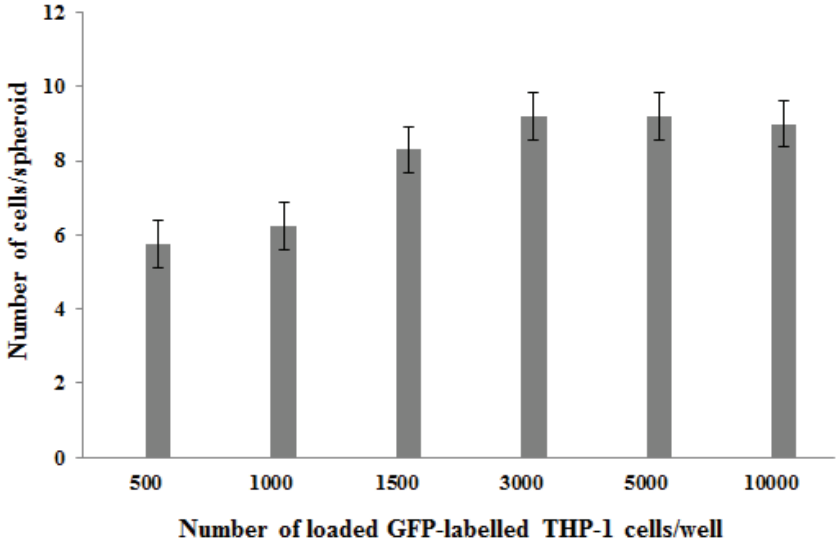
Interaction of GFP-labelled THP-1 cells with mesenchymal spheroids. Coculture of GFP-labelled THP-1 cells and fibroblasts was performed as described above. Both types of cells were incubated together for 24 h at +37°C in 5% CO_2 atmosphere. After this period, the co-cultures were transferred for the next 24 h to a 12-well plate without agarose in order to allow the spheroids to anchor onto the plastic surface. When the spheroids attached, the freely floating GFP-labelled THP-1 cells were discarded by washing with medium. The amount of THP-1 cells adhering to spheroids was analyzed by fluorescence microscopy (Olympus, Center Valley, PA).

Results

In the present study we used a similar co-culture experiment setup of fibroblast spheroids with leukemic cells as employed in our previous study [10] with a minor modification. The tested leukemic cells were first seeded into the U-well plate at indicated numbers, and afterwards the stromal fibroblasts were added to the individual wells. This way the spheroids were formed on the

cushion of leukemic cells. Within the first 3 h the fibroblasts formed a loose network and then after 8 h numerous small aggregates were visible that finally coalesced into a single spheroid by 24 h. Once the compact spheroid was formed it progressively decreased in size, and become optically denser and clearly distinguishable from surrounded leukemic cells as it is seen in Fig. 1.

Our previous results proved that distinct COX-2 expression in spheroids was triggered by homotypic interactions of the mesenchymal cells inside the aggregates [13]. Assuming that the homotypic interactions of the fibroblasts are principally the causative stimulus of COX-2 upregulation it had to be taken into account that any "foreign" cells spatially interfering with those interactions might influence the expression level of this gene. Since in this study the co-cultures of leukemic cells and mesenchymal cells were employed it was necessary to clarify interference of the leukemic cells with the clustering fibroblasts. Therefore, we investigated adherence of THP-1 cells to formed spheroids. A reason for THP-1 selection was our previous observation that clustered fibroblasts produce factors significantly affecting chemotaxis of certain but not all leukemic cell lines tested. The THP-1 cells were chemotactically most effectively attracted towards the fibroblast clusters [10]. Thus we made co-cultures of GFP-labelled THP-1 cells and fibroblasts. We then estimated the number of GFP-THP-1 cells adhered to spheroids (Chart 1) on a range of leukemic cell amounts from 5 x 10^2 up to 1 x 10^4 cells/well. Only mar-



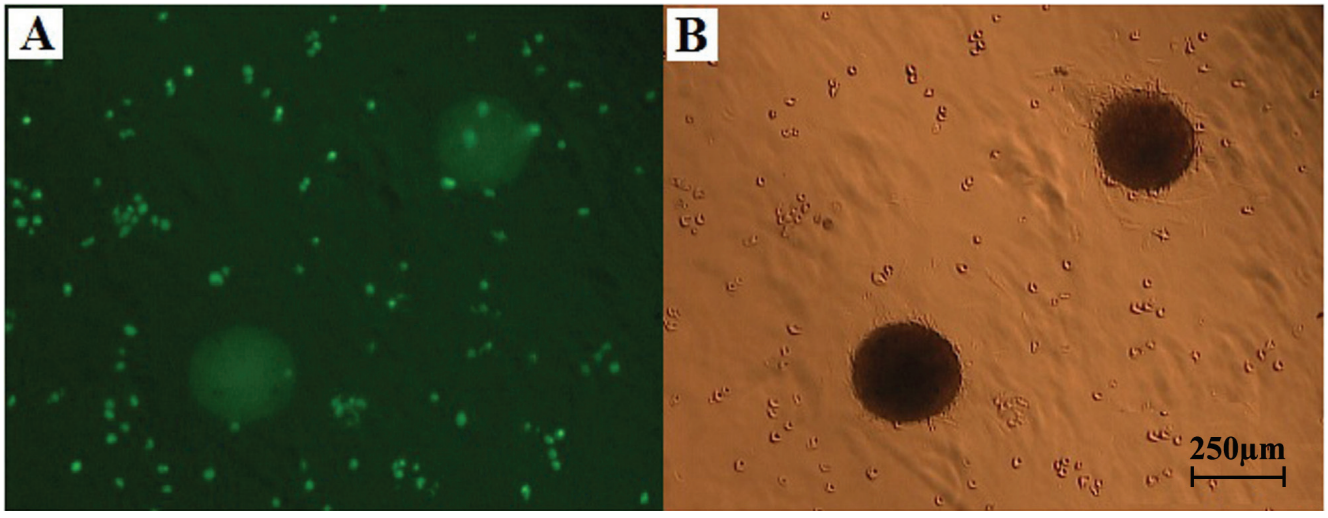


Chart 1. Interaction of GFP-labelled THP-1 cells with the fibroblast spheroids. The spheroids each representing 10^4 cells were formed for 24 h on the cushion of GFP-labelled THP-1 cells at indicated numbers in U-wells as described in Materials and methods. The spheroids were transferred to standard culture dish and let them to anchor to the surface in order to prevent their movement and freely floating leukemic cells were washed out.
Numbers of the THP-1 cells interacting with individual spheroids were estimated by counting using fluorescent microscope. Picture (A) illustrates immunofluorescence view of the culture and (B) the same field under a phase contrast.

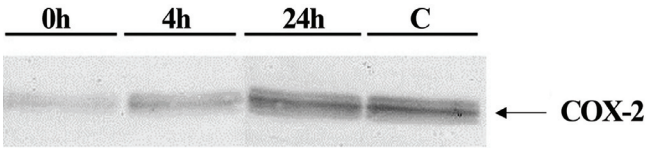


Figure 2. Ability of leukemic cells to modulate COX-2 expression. The THP-1 cells (5 x 10^3 cells/well) were added to the MUF 7/1 fibroblasts (10^4/well) forming spheroids at different time points (0 h, 4 h, 24 h). The co-cultures were incubated up to 72 h and then processed for immunoblotting. The intensities of COX-2 expression in co-cultured spheroids are compared to uninfluenced spheroids (C) representing control.

ginal levels of THP-1 cells were detected at the spheroid body (average 8 to 10 cells). Increasing numbers of loaded THP-1 cells did not have significant effect on these cells that interacted with the spheroids. The clusters after co-culture were analyzed by confocal microscopy as well, but the THP-1 cells were exclusively detected only at the surface of the spheroid and not one inside the spheroids (data not shown).

Further, we analyzed conditions when the tested leukemic cells most effectively interfered with the COX-2 expression in spheroids. Based on our previous study we knew that induction of the gene was an early event during spheroid formation. Actually, within 3.5 h after seeding the cells into culture wells 50% of the fibroblasts were committed to express COX-2 gene [13]. Therefore, we performed a time course testing of inhibitory effect of the leukemic cells on the COX-2 expression. The THP-1 cells were added on fibroblasts in a multiwell plate at 0 h, 4 h and 24 h time-points. After a 72 h incubation samples were harvested for analysis. Fig. 3 shows that leukemic cells added 4 h after initiation of spheroid formation had significantly less capacity to inhibit COX-2 expression as compared to that if the leukemic cells were added at the 0 h time point. When the leukemic cells were added after 24 h they had no effect on the ongoing COX-2 expression. Therefore, the experimentation was designed in such a way that fibroblasts were seeded onto a cushion of tested leukemic cells in order to monitor the maximal inhibitory effect of individual cell lines.

As we have shown previously the COX-2 expression in mesenchymal spheroids prepared from the other strain of human dermal fibroblasts had a characteristic temporal pattern that reached its maximal level at 60 h [13]. In the present study, we observed a similar but little earlier maximal induction of COX-2 at 48 h (Fig. 3). This remained at a comparable level for the next 24 h. The highest signal for COX-2 expression in

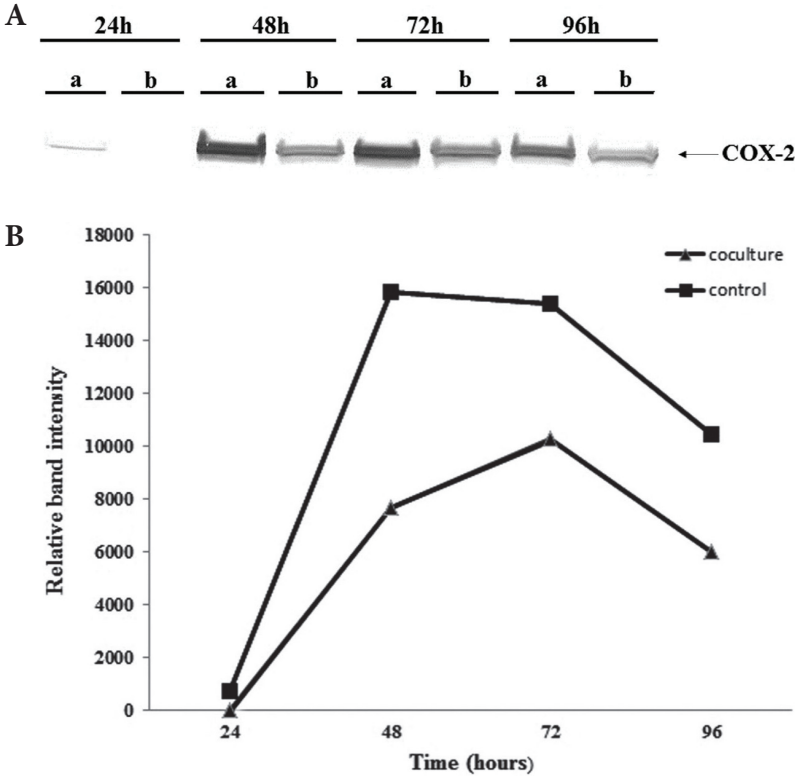


Figure 3. Comparison of time course of COX-2 expression in control spheroids and the spheroids in co-cultures. MUF 7/1 fibroblasts (1 x 10^4 cells/well) were cultured in the form of spheroids alone (a) or co-cultured (b) with THP-1 leukemia cells (1 x 10^3 cells/well) as described in Materials and methods. The samples were harvested at 24 h intervals up to 96 h and levels of COX-2 expression analyzed by immunoblotting (A). Slot (a) represents control spheroids and (b) spheroids retrieved from co-cultures at indicated time points. Intensity of bands were estimated by densitometric scanning of the immunoblot. The data were plotted into the chart (B) where curve –■– represents control spheroids and –▲– spheroids co-cultured with leukemic cells, respectively.

spheroids from co-cultures was detected in sample harvested after 72 h of incubation. Therefore the 72 h time-point was selected for further studies. This comparison against cultures of fibroblasts only, also showed that COX-2 expression in co-cultures was lower during the entire incubation period.

Since the assay is based on estimation of the COX-2 level in samples it was necessary to verify the contribution, if any, of COX-2 expression in leukemic cell lines. Samples prepared from either standardly cultured or fibroblast-co-cultured leukemic cell lines were analyzed by immunoblotting. No signal for COX-2 was found either in neat cultures (data not shown) or in 72 h co-cultured as it is evidenced in Fig.4.

In order to quantitatively compare the capability of individual leukemic cell lines to affect COX-2 in the spheroids, we analyzed series of cultures where different amounts of leukemic cells (1 x 10^2 to 1 x 10^4 cells/well) were added to a constant numbers of fibroblasts (1 x 10^4/well). The co-

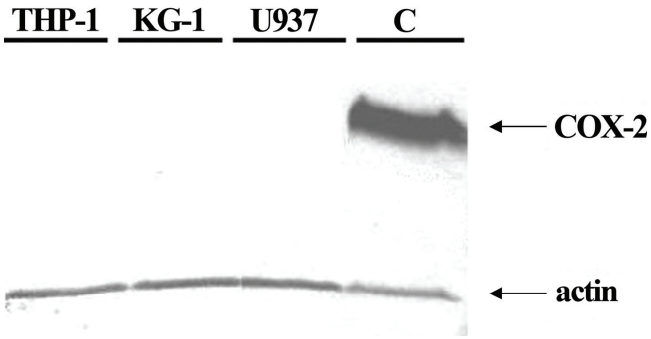


Figure 4. Assessment of COX-2 expression in analyzed leukemia cell lines after co-cultivation with fibroblast spheroids. The co-cultures were performed as described in Materials and methods for 72 h and then the leukemia cells were separated from spheroids and processed for immunodetection of COX-2. As a positive control were used MUF 7/1 fibroblast spheroids cultured simultaneously but not affected by leukemic cells (C). Positions of COX-2 and actin are indicated by arrows.

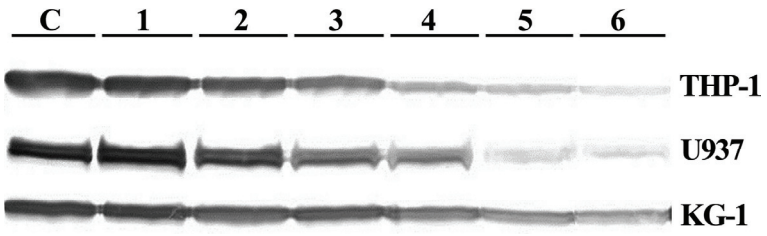


Figure 5. Comparison of COX-2 expression levels in spheroids after co-culture with the analyzed leukemia cell lines. Spheroids formed from the MUF7/1 fibroblasts 1 x 10^4 cells/well were co-cultured with indicated leukemia cells for 72 h and then processed for immunoblotting. C – control sample no leukemia cells involved, 1 – 1 x 10^2, 2 – 3 x 10^2, 3 – 1 x 10^3, 4 – 3 x10^3, 5 – 5 x 10^3, 6 – 1 x 10^4 cells/well, respectively. Intensity of the bands were estimated by densitometric scanning of the immunoblot and plotted for IC_{50} estimation as showed in Chart 2.

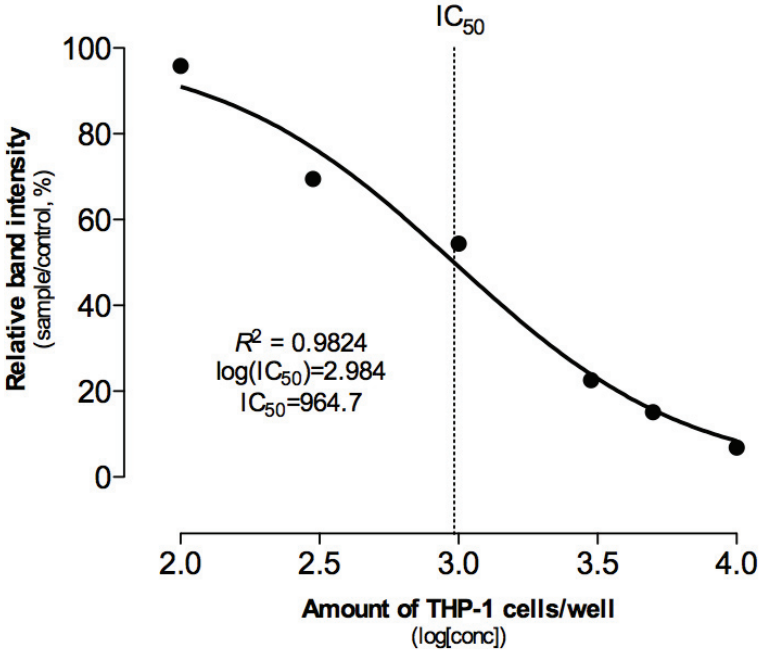


Chart 2. Calculation of IC_{50} from curve-fitting. The graph shows a log-log conversion of data, from which the IC_{50} value was evaluated.

cultures were incubated for 72 h, and were then analyzed by immunoblotting. Obtained density values for each band were plotted against the cell amounts on a log-scale graph, and IC_{50} values were calculated. The IC_{50} represents the amount of leukemic cells that quenched the signal for COX-2 in spheroids by 50%. The differences between the studied leukemic cell lines were clearly evident from the immunoblots. The THP-1 cells were the most potent, U-937 intermediate and KG-1 the least to inhibit COX-2 expression. The estimated IC_{50} values were as follows: THP-1 ~ 746, U-937 ~ 921, and KG-1 ~ 3519, respectively.

Discussion

It is well documented in the literature that COX-2 expression in the microenvironment of a potential neoplasm is able to significantly promote its progression towards malignancy [14,15]. Cancer cells have been shown to activate their neighboring stromal cells to such a supporting phenotype, as characterized for example by enhanced expression of COX-2 and high production of inflammatory prostaglandins. This process can then promote the progression of other cells to malignancy [16]. Although such processes have been mostly analyzed in solid tumors, they can take part also in the progression of certain hematopoetic neoplasms [17].

In the present study we found that human leukemic cells have ability to directly modulate induction of COX-2 expression in human fibroblasts. Although all analyzed leukemic cells were able to downregulate COX-2 expression, we observed significant differences in the intensity of this inhibition among individual leukemic cell lines. One explanation for the observed differences might be that the effect is associated with the maturation stages of individual leukemic cell lines. The cell lines were established from the specimen of the acute monocytic (THP-1), histiocytic (U-937) and acute myelogenous (KG-1) leukemia [18,19,20]. The first two cell lines were isolated from peripheral blood and pleural effusion while the last one was from bone marrow. Therefore each cell line represents different maturation stage of specific lineage precursors. This might correlate with observed differences in functionality of these cell lines to modulate COX-2 expression. This assumption needs to be confirmed by further experimentation.

Importantly, the ability of leukemic cells to modulate COX-2 expression was observed only in the early phase of inflammation development within the first couple of hours, indicating that signaling pathways triggered by leukemic cells in activated stromal cells interfered with the early phases of COX-2 expression. The most prominent pathway leading to initiation of COX-2 expression is the one involving participation of NFκB transcription factors family [21]. Therefore NFκB seems to be the first candidate gene to be analyzed in the context of observed novel cellular mechanism of COX-2 modulation.

Moreover, to assess any possible interference with analysis of COX-2 expression in spheroids, the analyzed leukemic cell lines were also tested for expression of COX-2 before and after co-cultivation with spheroids. This was also important because of insufficient and conflicting literature data on COX-2 expression in human leukemia specimens and cell lines [22-26]. None of the analyzed leukemic cell lines either as a neat standard culture or after co-cultivation for up to 72 h with spheroids exhibited positivity for COX-2. It could be speculated that the activity of COX-2 gene in leukemic cells is supressed/or under the control of an autocrine mechanism that is effective also towards other cell types if these are in intimate contact with the leukemia cells. This mechanism of COX-2 expression modulation is mediated, most probably, by a receptor(s) at cell surface since the COX-2 inhibitory activity could not be transmitted to mesenchymal cells by soluble factors present in the conditioned culture media of leukemic cells. Till now, we have screened several different leukemic cell lines (data not shown) standardly cultured *in vitro.* None of them express COX-2, as was similarly demonstrated for these three cell lines used in the present study.

Although tumor cells are in general characterized by loss of specific functions, for example as a consequence of a disrupted differentiation process, it is not known to what extent cells lose the ability to carry out their specific cellular functions. In the this study we present a novel concept of testing functionality of tumor cells to serve as a basis for further development of testing samples from leukemia patients. Our first experiments analyzing the samples of patients with different types of leukemia employing these methodological approaches suggest that the observed phenomenon of modulation of COX-2 expression can have relevance *in vivo* (Egyudova *et al*., in preparation). We propose that functional testing of the population of immune cells isolated from leukemic patients may assist in leukemia diagnosis and monitoring of therapy efficacy.

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