A FIBROCYTE MODEL FOR MONITORING ENVIRONMENTAL CHEMICALS

Krisztián Sepp[1] and Miklós Imre Mózes[1,2,3], Marianna Radács[2], Péter Hausinger[3], Márta Gálfi[2], Zsolt Molnár[2]

[1]Endocrine Unit of First Department of Internal Medicine, University of Szeged, Szeged H-6725, Hungary
[2]Institute of Applied Health Sciences and Environmental Education, Juhász Gyula Faculty of Education, University of Szeged, Szeged H-6725, Hungary
[3]Invasive Cardiology Department, Second Department of Internal Medicine and Cardiology, Faculty of Medicine, University of Szeged, Szeged, Szeged H-6725, Hungary
e-mail: mikeleoden@gmail.com

Abstract
Human activity affects all elements of the Earth's environment and the system of relationships between them. Chlorobenzenes created during chemicalization are capable of modulating the adaptation potential of biological organisms and because of their high frequency of occurrence in the food chain, they can be used as expositors in environmental exposure models. It is necessary to develop a biological model system suitable for the investigation of environmental pollutant chemical agents, which indicates changes quickly and easily.

Introduction
Human society is the highest known complexity in terms of earthly conditions, in which the actors are able to transform the environment according to the needs of their existence through their cooperation. As a result, human activity affects all elements of the Earth's environment and the system of relationships between them [1]. Human activity (e.g. chemicalization) and its consequences - which initially served short-term needs of the society effectively, however, highly chemically stable, substances as xenobiotics synthesized during chemicalization, have already changed the accommodation patterns of biological organisms, which is why such models are necessary with which even discrete changes can be easily tracked. The chemicals burdening the environment are chlorobenzenes (ClBs) with massive chemical stability, which are deposited in the lipophilic phases of living organisms (e.g. brain and endocrine tissues) [2]. They induce a dose-dependent toxic effect in the cells of the affected tissues. Due to their high frequency of occurrence in the food chain [3], these agents can be used as expositors in environmental exposure models.
Fibrocytes are mesenchymal progenitor cells that originate from bone marrow cells, and it can also be known that they carry the mixed morphological and molecular characteristics of hematopoietic stem cells, monocytes and fibroblasts [4, 5]. They are probably precursor intermediate cells of the monocyte differentiation lineage. They show characteristics related to the mesenchymal stromal cells found in the bone marrow, they are multipotent progenitor cells that can produce different mature cell types during their differentiation [6]. *In vitro* experiments have proven that peripheral blood mononuclear cells or CD14+ monocytes are able to transform into fibrocytes during their differentiation. There are many factors that promote or inhibit fibrocyte differentiation, including cytokines, growth factors, immunoglobulins, and environmental artificial chemicals. Fibrocytes also play a key role in wound healing and tissue repair processes. They are unique cells that mediate the proinflammatory properties of macrophages and the tissue-remodelling properties of fibroblasts, so their model study can be very interesting in relation to environmental factors [5, 7].

Aim

The aim of this study was to monitor the effects of different doses of chlorobenzenes (ClB) treatments on the changes in the proliferative activity (e.g. protein production) fibrocytes, in order to develop a biological model system suitable for testing artificial homeostatic disruptor agents.

Methods

***In vivo* protocol**

Male Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55-65% and 22±2°C ambient temperature. Experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system) in groups of 10 animals for the end of the research period. Standard pellet food (CRLT/N, Charles River, Magyarország) and tap water were available *ad libitum.* Male Wistar rats were treated with combined ClB (1:1 mixture of 1,2,4- trichlorobenzene /CAS number: CAS Number: 2199-72-6, Sigma Aldrich, USA, St. Louis) and hexachlorobenzene / CAS number: 93952-14-8, Sigma Aldrich, USA, St. Louis), in 1 mL of 0.015% ethanol in distilled water was administered daily) in a dose of 0.1, 1.0 and 10.0 µg/b.w. kg via a gastric tube. The rats were exposed to ClB for 30 (n=10), 60 (n=10) and 90 (n=10) days. Control groups were set up: stress control (n=5, gastrostomy tube insertion group), absolute control (AC) (n=5, untreated group), positive control group (n=5, receives ClB solvent, 0.015% ethanol solution via gastric tube) and negative control group (n=5, receives water via gastric tube). At the endpoints of the experiment (30, 60 and 90 days), after pentobarbital anaesthesia (4.5 mg/b.w. kg, Nembutal, Abbott, USA) the animals were used for study.

***In vitro* protocol**

After anesthesia with pentobarbital (4.5 mg/kg, Nembutal, Abott, USA), from the treated and untreated experimental animals skin sample was taken.

For fibrocyte cell cultures (FC), subcutaneous connective tissue samples were enzymatically treated (trypsin: 0.2 % /Sigma Aldrich, USA, St. Louis/ for 30 minutes; collagenase /Sigma Aldrich, USA, St. Louis/: 30 µg/ml for 40 minutes; dispase /Sigma Aldrich, USA, St. Louis/: 50 µg/ml for 40 minutes, phosphate buffer /PBS-A/; temperature: 37°C/ was used to prepare the solutions) and dissociated mechanically digested (using a nylon-blutex filter with a pore diameter of 83 and 48 µm), then placed in a 6-well plate, which was filled with 50 µl with MEM-α medium supplemented with 10% FCS, 1.0 µg/mL Penicillin+Streptomycin (Sigma Aldrich, USA, St. Louis) and 1% ITS-G supplement solution (Thermo Fisher Scientific inc. Rockford, USA: bovine insulin, transferrin and sodium selenite). The plates containing fibrocytes were incubated at 37°C (5% CO_2) and the culture medium was changed every 2-3 days (sterile PBS+1.0 µg/mL Penicillin+Streptomycin /Sigma Aldrich, USA, St. Louis/) [8].

The induction of cell proliferation was performed on separate reference systems treated with 1 mg/mL benz[c]-acridine (BcA) (Sigma Aldrich, USA, St. Louis) for 168 h, because we can compare the quantitative and qualitative appearance of the tumor clones induced by it with effect of chlorobenzene. *In vivo* ClB pretreated FC were treated with acute exposure of ClB (0.1; 1.0; 10.0 µg/mL) for 168 h. The results obtained were compared with BcA. The protein content of the samples was determined using a modified Lowry method [9] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA).

Statistical analysis

To compare the means of different treatment doses (0.1, 1.0, 10.0 µg/b.w. kg) to the controls during 30, 60 and 90 days long treatments (n=5 in each group of time and dose) two-way ANOVA was run. Dose and time were used as the two factors for analysis.

Results and discussion

According to environmental aspects, if a process originating from an impact factor causes a change in the studied environmental factor or its state (e.g. cell activity, proliferative activity), in this case alteration in protein production may be triggered as direct effect. The results are presented to the absolute control group.

Number of tumor clones as a result of ClB treatments

	Fibrocyte
AC	0
BcA	47.3±4.2*

Table 1 The number of tumor clones in response to the reference absolute control (AC) and benz[c]-acridine (BcA) treatment (number of cells±S.E.M., *:p<0.001)

From the data in Table 1, it is clear that BcA significantly induced tumor clones in the model system.

Fibrocyte tumor clones after treatment			
ClB treatment	**30 days**	**60 days**	**90 days**
ClB 10.0 µg/bw. kg	0	1.32±0.02*	1.71±0.04*
ClB 1.0 µg/bw. kg	0	0	1.1±0.01*
ClB 0.1 µg/bw.kg	0	0	0.32±0.01*

Table 2 The number of tumor clones in fibrocyte (FC) cell populations as a result of each ClB treatment (number of cells±S.E.M., *:p<0.001)

When examining the results of protein production, it can be established that a significant increase (p<0.001) in protein production was observed already at 0.1 µg/bw. as a result of ClB treatments. The model is functional, because the results correlate with the increase of the numbers of tumor clones induced by benz(c)-acridine and also with increased protein production (FC: 28.11 ±2.99 mg protein/plate), compared to the AC groups (FC: 3.56±0.26 mg protein/plate).

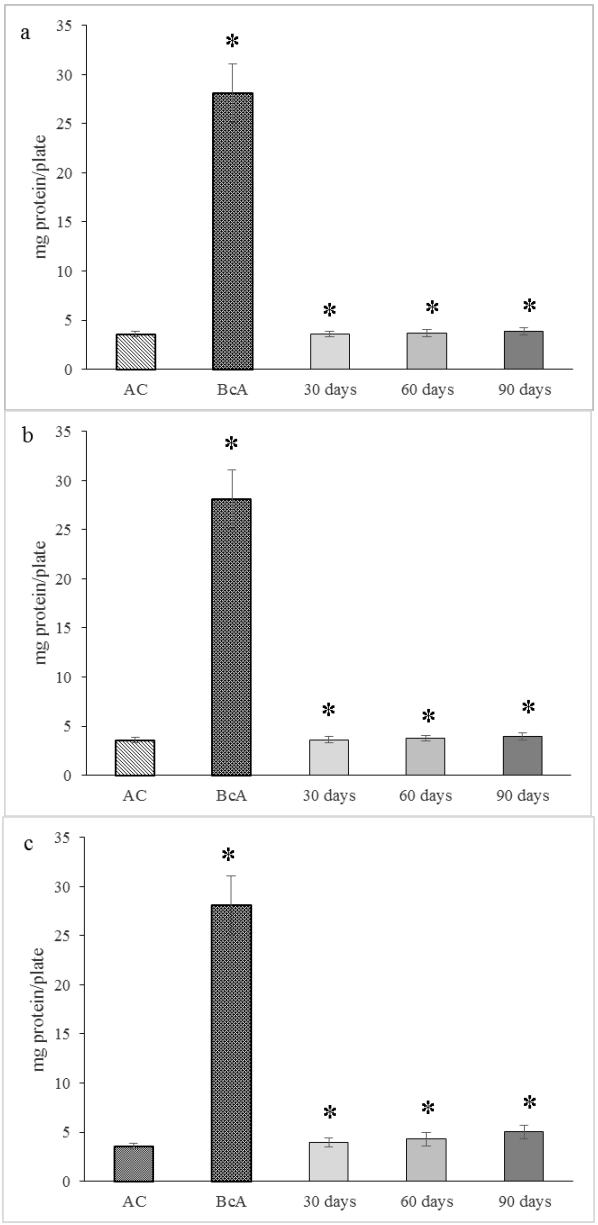

Figure 1 The effect of different ClB exposures on protein production in FC cell cultures (a: 0.1 µg/bw. kg, b: 1.0 µg/bw. kg, c: 10.0 µg/bw. kg doses, *:p<0.001, n= 5/group)

In the case of FC, compared to the AC group, ClB treatments significantly increased protein production in all experimental endpoints. The highest protein production was observed after 90 days of treatment for all three treatment doses (0.1 µg/bw. kg dose: 3.85±0.38 mg protein/plate, 1.0 µg/bw. kg dose: 3.96±0.38 mg protein/plate, 10.0 µg/bw kg dose: 5.01±0.69 mg protein/plate).

ClB can be classified as xenobiotics, which can generate changes in many physiological processes. Living systems are affected by complex environmental exposures *in vivo*, however, if these exposures are very low (subtoxic concentrations), they can exert their effects in a latent manner for a long time. As a result, it is necessary to develop a model that is suitable for evaluating and detecting the effects of subtoxic *in vivo* exposures in a complex manner.

The peripheral system elements, such as mesenchymal-derived fibrocytes could be suitable for examining the effects of ClBs. Such molecules (ClBs) are able to generate changes in the central axis [10] and in the peripheral elements [11].
Therefore we should call these agents (e.g. ClB-s) homeostatic disruptor compounds. The ClB-mix exposures resulted in a dose-dependent increase in cell proliferation.

Conclusion

According to our morphological observations, ClB treatments induced tumor clone formation in the first generations of primary cell cultures in a time- and dose-dependent manner. As a result of treatment with a higher dose of ClBs, tumor clone formation generated in FC cell cultures were detected after only 60 days. The fibrocyte cell cultures responded with a more sensitive reaction to the chemical agents included in the experiment. This means that the ClBs included in the model study exert an effect of both initiation and promotion. Thus, it can be established that ClBs exert a general effect on these model cells.

Acknowledgements

This study was supported by EFOP-3.6.1-16-2016-00008, EFOP-3.4.3-16-2016-00014, TÁMOP-4.2.4.A/2-11/1-2012-0001 and Juhász Gyula Faculty of Education's "Scientific and Artistic Activity Support Grant".

References

[1] Loeb J. Balancing human and natural worlds. Vet Rec. (2020) 187(10):375.
[2] Charisiadis P., Andrianou X. D, van der Meer T. P., den Dunnen W. F. A., Swaab D. F., Wolffenbuttel B. H. R., Makris K. C., van Vliet-Ostaptchouk J. V. Possible Obesogenic Effects of Bisphenols Accumulation in the Human Brain. Sci Rep. (2018) 8(1):8186.
[3] Mrema E.J., Rubino F.M., Brambilla G., Moretto A., Tsatsakis A.M., Colosio C. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology, (2013) 307:74-88.
[4] Krenning G., Zeisberg E.M., Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. J Cell Physiol. (2010) 631-7.
[5] de Oliveira R.C., Wilson S.E. Fibrocytes, Wound Healing, and Corneal Fibrosis. Invest Ophthalmol Vis Sci. (2020) 61(2):28.
[6] Pittenger M.F., Mackay A.M., Beck S.C., Jaiswal R.K., Douglas R., Mosca J.D., Moorman M.A., Simonetti D.W., Craig S., Marshak D.R. Multilineage potential of adult human mesenchymal stem cells. Science, (1999) 284(5411):143-7.
[7] Bellini A., Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. (2007) 87(9):858-70.
[8] Osborn A., Caruana D., Furness D.N., Evans M.G. Electrical and Immunohistochemical Properties of Cochlear Fibrocytes in 3D Cell Culture and in the Excised Spiral Ligament of Mice. J Assoc Res Otolaryngol. (2022) (2):183-193.
[9] Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, (1955) 193 265–275.
[10] Nagyeri G., Valkusz Z., Radacs M., Ocsko T., Hausinger P., Laszlo M., Laszlo FA., Juhasz A., Julesz J., Galfi M. Behavioral and endocrine effects of chronic exposure to low doses of chlorobenzenes in Wistar rats. Neurotoxicol Teratol. (2012) 34:9-19
[11] Wei Y., Zhu J. Para-dichlorobenzene exposure is associated with thyroid dysfunction in US adolescents. J Pediatr. (2016) 177:238-243.