Relationships of NR4A1 Agonists Biochemical Effects with Pre-B Lymphocytes Apoptosis

DRAGOS ACATRINEI[1], CARMEN GENTIMIR (AMITITELOAIE)[1], CATALINA ZAHARIA (AVRAM)[2], CRISTINA OANA RADUCANU[1]*, GEANINA BOGZA[1], LILIANA CHELARU[2], DECEBAL VASINCU[1], OTILIA BOISTEANU[1], MARCEL COSTULEANU[1]

[1]Grigore T. Popa University of Medicine and Pharmacy Ia°i, Faculty of Dental Medicine, 16, University Str., 700115, Iasi, Romania

[2] Grigore T. Popa University of Medicine and Pharmacy Ia°i, Faculty of Medicine, 16, University Str., 700115, Iasi, Romania

Cytosporone B (Csn-B) is the known natural agonist of NR4A1/Nur77 orphan nuclear receptor. In our study, Csn-B failed to induce important apoptosis in isolated mouse pre-B cells, although used in high concentrations (100 μM) and for long time (24 h). Retinoid X receptors (RXRs) form heterodimers with NR4A1 ones, although the role of such complexes remain unidentified. 9-cis retinoic acid (9 -CRA), the agonist for RXRs, is showing a very similar effect to Csn-B concerning the apoptosis of mouse pre-B cells. The treatment with both molecules could increase in a significant, although reduced manner, the amount of apoptosis in pre-B cells. Further studies are needed to describe the mechanisms underlying such apoptotic effects.

Keywords: Cytosporone B, 9-cis retinoic acid, pre-B cells, apoptosis

The expression of multiple genes is regulated by the transcription factor NR4A1 (Nur77/TR3/NGFIB), an orphan nuclear receptor. NR4A1 is an immediate-early response gene, influencing a wide range of genomic and non-genomic biological functions. Genetic variants of NR4A1 gene were involved as risk factors in pathologic mechanisms underlying ulcerative colitis (UC) and Crohn's disease (CD). Thus, the NR4A1 expression was decreased in colon of patients with UC or CD and mice challenged with DSS. The treatment with cytosporone B (Csn-B), the known agonist for NR4A1, significantly reduced the inflammatory condition in colitis mouse model induced by DSS. NR4A1 prevents the auto-ubiquitination and oligomerization of TRAF6, interacting with TLR-IL-1R signaling through the inhibition of NF-êB activation and pro-inflammatory cytokine release. The clear conclusion is that the loss/reduction of NR4A1 activity could represent the basis for the development of inflammatory bowel diseases, considering it a potential prevention and treatment target [1].

There exists a good correlation between atherogenesis, hypercholesterolaemia and inflammatory status. One of the key inflammation regulators is NR4A1. How lipids modulate its expression in monocytes during hypercholesterolaemia remains largely unknown. The hypercholesterolaemic patients presented a low-grade inflammatory state, well correlated with an enhanced expression of NR4A1. Furthermore, NR4A1 mRNA levels were positively correlated with both total cholesterol and low-density lipoprotein cholesterol levels in plasma. The NR4A1 up-regulation in monocytes was followed by enriched acetylation of histone H3 in the gene promoter region in patients through the enhancing of p300 acetyltransferase and decreasing of HDAC7 (histone deacetylase 7) recruitment, effects induced by cholesterol. On the other hand, Csn-B totally reversed the decreased levels of IL-6 (interleukin 6) and MCP-1 (monocyte chemoattractant protein 1). Thus, the hypercholesterol-aemia-enhanced inflammatory status could be reduced by NR4A1 expression in patients with such condition [2].

There is a lot of evidence that NR4A1/Nur77 is deeply involved in multiple organs apoptosis, including the brain. These effects are mediated through Bcl-2 conformational changes and massive mitochondrial cytochrome C release. Such steps are also characteristic for cerebral cells after early brain injury following subarachnoid hemorrhage in rats. All the above effects were mimicked and enhanced by Csn-B treatment, with a maximum at 24 h after hemorrhage onset [3].

NR4A1-enhanced expression induced by adenosine A(2B) receptors activation inhibits proliferation of human coronary smooth muscle cells in culture. P2X1 receptors agonists á,â-methylene-ATP and its analog α,β-methylene-ATP inhibited cell proliferation. On the other hand, the P2X1 receptor antagonist NF449 markedly reversed these inhibitory effects. Both the above mentioned agonists enhanced the expression of NR4A1. In contrast, the P2X1 receptor antagonist reduced this effect. siRNA against NR4A1 antagonized the antiproliferative effects of adenine nucleotides agonists. It might be concluded that P2X1 receptors agonists inhibit the proliferation of human coronary smooth muscle cells through NR4A1 gene enhanced expression [4].

On the other hand, A(3) receptor agonist 2-chloro-IB-MECA increased proliferation of human coronary smooth muscle cells in culture. The agonist also enhanced the expression of early growth response genes (EGR)2 and EGR3, but not of EGR1, NR4A1, NR4A2 and NR4A3. These responses induced by 2-chloro-IB-MECA were blocked by two A(3) receptor antagonists, MRS1523 and VUF 5574. Thus, NR4A1 is not involved in human coronary smooth muscle cells proliferation induced by A(3) receptor activation, in contrast with A(2B) receptor activation [5].

Vascular endothelial growth factor (VEGF) concentrations were positively correlated with serum triglycerides, total cholesterol, LDL, VLDL, and ApoB, and negatively with HDL and ApoM concentrations. In HepG2 cells and mouse primary hepatocytes VEGF down-regulated ApoM expression and pre-β-HDL formation, meanwhile inhibiting Foxa2 and increasing NR4A1 expression. siRNA against NR4A1 reversed the inhibitory

* email: oanagatula@yahoo.com

effect of VEGF on Foxa2 expression. Moreover, the natural agonist of NR4A1 Csn-B reduced Foxa2 expression with higher potency than VEGF. The above obtained results suggest that NR4A1 stimulation enhanced by VEGF would firstly reduce the Foxa2 expression, followed by decreased expression of ApoM and pre-β-HDL formation [6].

NR4A1 and NURR1 orphan nuclear receptors form heterodimers with retinoid X receptors (RXRs). The NR4A1-RXR and NURR1-RXR heterodimers physiological roles are far of being elucidated. The above heterodimers could induce carnitine palmitoyltransferase 1A (CPT1A) in human teratocarcinoma NT2/D1 cells, human embryonic kidney (HEK) 293 cells and hepatocyte-derived HepG2 cells. This is an indirect demonstration that CPT1A, a gene involved in fatty acid β-oxidation, could be a target of RXR-NR4 receptor heterodimers [7].

Hemorrhagic shock is a severe condition which might be improved by melanocortin peptides treatment. Administration of a synthetic melanocortin 1/4 receptor agonist, Butir-His-D-Phe-Arg-Trp-Sar-NH2 (Ro27-3225), significantly increased NR4A1 in liver samples of shocked rats relative to sham animals [8].

Some new derivatives of Csn-B, as Amoitone B, demonstrated higher agonist capabilities for NR4A1. Amoitone B could be developed as a remarkable anticancer therapeutic agent. The disadvantages are the poor solubility and dissolution rate, with a low therapeutic index. To solve these problems, nanocrystals were chosen as application technology [9].

For developing T cells (thymocytes) apoptosis requires NR4A1 and Nor-1 enhanced expression as a result of TCR complex activation. Their translocation from the nucleus to mitochondria results in an association with Bcl-2 and a conformational change that exposes the Bcl-2 BH3 domain. The required phosphorylation of NR4A1 and Nor-1 is clearly followed by translocation, Bcl-2 BH3 exposure and thymocyte apoptosis, showing the importance of PKC [10].

Degenerative and hyperproliferative conditions as hypertriglyceridemia, nonalcoholic fatty liver disease, and metabolic syndrome are involving abnormal mitochondrial functioning [11].

Mitochondria is also initiating apoptosis as a result of photosensitizers activation in tumor cells, destroying them through the increased release of reactive oxygen species [12].

Enhancement of respiratory function (OXPHOS state) in isolated liver mitochondria from mice with murine melanoma is achieved after the treatment with with betulinic acid, an anti-tumoral agent with antiinflammatory, antiangiogenic, and immunomodulatory effects [13].

Androgen hormone has an important role in bladder cancer development and evolution. It was demonstrated that UM-UC-3 cell growth and cycle progression is inhibited by NR4A1 hyperexpression. NR4A1 might be a competitive inhibitor of androgen-dependent transcription activity and a competitor for binding to src-1 (coactivator for steroids). Furthermore, Csn-B significantly inhibited bladder cancer cell growth dependent on androgen [14].

Regulatory T-cell (Treg) differentiation and thymocyte deletion (negative selection) are induced by the same agonist self-antigens. An important mediator of thymocyte deletion, BIM, was removed in a mouse model of autoimmune diabetes. The deficiency did not altered effector T-cell function. In contrast, the number of Tregs was increased, paralleled by increased levels of NR4A1, CD5, GITR, and phosphorylated IκB-α. These results suggest that several autoreactive cells are diverted into the Treg pathway, escaping apoptosis [15].

Activation of NR4A family members targets genes that regulate cell cycle, apoptosis, inflammation, atherogenesis, metabolism, DNA repair and tumorigenesis. NR4A1 and NR4A3 are considered powerful tumor suppressors in acute myeloid leukemia (AML). The lack of both orphan receptors was associated with AML in mice and was also found in human patients with AML. Furthermore, myelodysplastic/ myeloproliferative neoplasms with progression to AML were characteristic to mice with NR4A1 and NR4A3 decreased expression. Aggressive lymphomas associate the same low levels of NR4A1 and NR4A3. Recently it was demonstrated that NR4A agonists could induce apoptotic effects in AML and lymphoma cells [16].

The present study aimed the effects of Cytosporone B (Csn-B), the NR4A1 natural agonist, and 9-cis retinoic acid (9 CRA), agonist of retinoid X receptors, on apoptosis of mouse pre-B lymphocytes. Such data does not exist in literature.

Experimental part

For our experiments we used mouse pre-B cells. The pre-B cells were isolated from bone marrow. To isolate them, we applied as technique peanut agglutination and fractionation by FACS, with slight adaptation of the method previously described [17, 18]. Thus, we obtained high levels of pre-B cells, using RPMI 1640 medium (Sigma-Aldrich), completed with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% heat-inactivated FBS, incubated with 5% CO_2 and at 37°C, as previously stated [19, 20].

During the growth phase, the pre-B cells were maintained at 5 x 10^5 per mL and treated with 100 μM Cytosporone B (Csn-B) or 10μM 9-cis retinoic acid (9-CRA), in triplicate. As control we used the effects of valinomycin 10 μM, a good inducer of apoptosis through mitochondrial permeability transition pore opening. To study the mitochondrial membrane potential dissipation we used 1 μM of the very sensitive marker JC-1 (Sigma-Aldrich), at 37°C for 30 min.

The Microradiance setup for the laser confocal microscopy included an inverted Nikon Eclipse TE-300 microscope, oil-immersion objectives (x60), as well as the LaserSharp software. HQ515/530 was used as emission filter for 488 nm excitation and HQ530/560 for 514 nm excitation, respectively.

The collected images (having a standard imposed resolution of 1280 x 1024) were analyzed using free ImageJ (National Health Institute, U.S.A.).

Results and discussions

The mitochondrial membrane potential (ψ_{mt}, fig. 1) of pre-B cells was not significantly dissipated by the treatment with 100 μM Csn-B for 24 h. The same effect was associated with 9 - CRA (10 μM) treatment for 24 h (fig. 2). The high ψ_{mt} was associated with almost 86.74±5.22% of the cells in the case of Csn-B, 77.81±4.69% in the case of 9 CRA, in contrast to 7.26±1.23% for valinomycin (fig. 3). The high ψ_{mt} always correspond to the bright intensity of red emission of JC-1.

The obtained results proved evidence for the fact that Cytosporone B, the natural agonist of NR4A1, did not induced important mouse pre-B cells apoptosis, although used in high concentrations (100 μM) and for long time (24 h). On the other hand, 9-cis retinoic acid, the agonist for retinoid X receptors, which dimerize with NR4A1 receptors and thus activates target transcription, is showing a very similar effect concerning the apoptosis of mouse pre-B

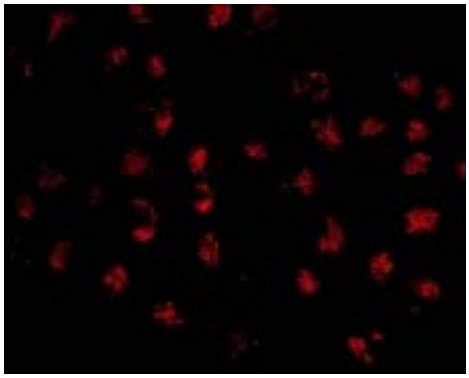

Fig. 1. Pre-B cells treated with 100 μM Csn-B for 24 h present bright intensity of red emission of JC-1, evidenced by laser confocal microscopy and JC-1 for 30 min (high ψ_{mt}). Representative image of many acquired from triplicate experiments (60X).

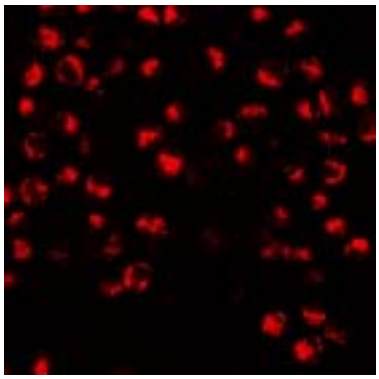

Fig. 2. The bright intensity of JC-1 red emission (high ψ_{mt}) is also showed by pre-B cells receiving 10 μM 9 - CRA for 24 h as treatment. Representative image of many acquired from triplicate experiments (60X).

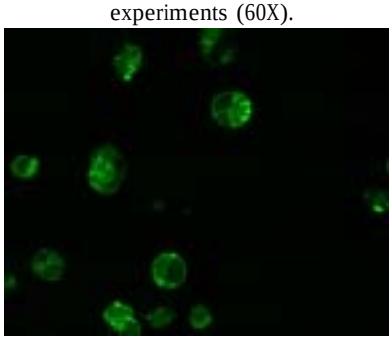

Fig. 3. Treatment with 10 μM valinomycin for 24 h of pre-B cells associates a large green emission of JC-1 (low ψ_{mt}), evident for mitochondrial membrane potential dissipation. Representative image of many acquired from triplicate experiments (60X)

cells. The concomitant use of Cytosporone B and 9-cis retinoic acid induced a small increase in apoptosis of pre-B cells, this effect being statistically significant ($p < 0.05$, personal observation, data not shown). Anyway, the control apoptotic effects induced by valinomycin were the highest ones, almost all pre-B cells being apoptotic.

There are several important differences between the grass-fed cattle and grain-fed steers (high-energy diet). The metabolic disorders and infectious diseases are obviously present in these last animals. Also, the spleen, an important immune organ, may include differential expression genes patterns under disparate regimes. The most important finding of the study was that the expression of pair genes HLA-DRA and NR4A1 was dramatically altered. Thus, there were altered the NR4A1 signaling and Ca^{2+}-induced apoptosis of T lymphocytes [21].

In a rat cardiac transplantation model there was demonstrated that donor alloantigens are massively activating lymphocyte T cells, inducing acute allograft rejection (e.g., Wistar to Lewis rats). The induced apoptosis associates high levels of NR4A1 production, with high amounts of HtrA2/Omi being released from mitochondria after the dissipation of mitochondrial membrane potential. Most CD3(+) cells presented NR4A1 immunocompetence, HtrA2/Omi-positive signal and active caspase-3. The obtained results demonstrated that acute rejection after cardiac transplantation was due to upregulated NR4A1 expression in infiltrating T lymphocytes [22].

Self-tolerance and autoimmunity preventing are both established through negative selection of thymocytes. The principal mediators of clonal deletion might be represented by Bim and NR4A1. Overexpression of NR4A1 is sufficient for cell deletion, independent of Bim and caspase activation. The combined deficiency of NR4A1 and Bim altered clonal deletion efficiency [23].

Furthermore, evidence for an essential role of negative selection in T cell self-tolerance is lacking. At least, this fact is obvious in C57BL/6 mice. The functioning of NR4A1 nuclear orphan receptor might be redundant relative to Bcl-2 association with consecutive exposing of its pro-apoptotic BH3 domain. Additionally, expression of a Bcl2 BH3 mutant transgene rescued high number of thymocytes from negative selection, with Treg increased development [24].

T-cell receptors (TCR) engagement induced NR4A1 in immature T cells. The clonal deletion induced by antigens is clearly altered in NR4A1 knockout mice. The lack of pro-apoptotic factor Bim is not enhancing the NR4A1 deletion effects. On the other hand, NR4A1 might activate several transcriptional targets as Ikzf2 and Tnfrsf9 (transcripts of the Treg signature), as well as several enzymes of the glycolytic and Krebs cycle pathways [25].

Retinoids are able to induce mouse thymocytes cell death through the activation of intrinsic mitochondrial pathway of apoptosis. The transcription-dependent apoptosis induced by retinoids was totally dependent on NR4A1 since they had no effects in NR4A1 null thymocytes. Retinoids increased the NR4A1-dependent expression of FasL, TRAIL, NDG-1, Gpr65 and Bid (apoptosis-related genes), the induction of STAT1 and caspase-8 activation [26].

Conclusions

The obtained data clearly showed that Cytosporone B, the known natural agonist of NR4A1 receptors, as well as 9-cis retinoic acid, the agonist for retinoid X receptors, did not induced important apoptosis of isolated mouse pre-B cells. The treatment with both molecules could increase in a significant, although reduced manner, the amount of apoptosis in pre-B cells. These additional effects were not so important as those induced by valinomycin, a K^+ ionophore, affecting almost all pre-B cells. Further studies are needed to discover the mechanisms underlying such apoptotic effects.

Acknowledgements: Ph.D. Student Dr. Acatrinei D. is the recipient of a grant CERO-Profil de Carierã: Cercetator Roman, POSDRU/159/1.5/S/135760, achieved at the University of Medicine and Pharmacy Grigore T. Popa, Iasi.

References

1. WU, H., LI, X.M., WANG, J.R., GAN, W.J., JIANG, F.Q., LIU, Y., ZHANG, X.D., HE, X.S., ZHAO, Y.Y., LU, X.X., GUO, Y.B., ZHANG, X.K., LI, J.M., J. Pathol., **Nov 13**, 2015, doi: 10.1002/path.4670J.
2. XIE, X., SONG, X., YUAN, S., CAI, H., CHEN, Y., CHANG, X., LIANG, B., HUANG, D., Clin. Sci. (Lond.), **129**, 2015, p. 1151-1161.

3. DAI, Y., ZHANG, W., SUN, Q., ZHANG, X., ZHOU, X., HU, Y., SHI, J., J. Neurosci. Res., **92**, 2014, p. 1110-1121.
4. HINZE, A.V., MAYER, P., HARST, A., VON KÜGELGEN, I., Purinergic Signal., **9**, 2013, p. 677-686.
5. HINZE, A.V., MAYER, P., HARST, A., VON KÜGELGEN, I., J.Mol. Cell. Cardiol., **53**, 2012, p. 639-645.
6. HU, Y.W., ZHENG, L., WANG, Q., ZHONG, T.Y., YU, X., BAO, J., CAO, N.N., LI, B., SI-TU, B., Rejuvenation Res., **15**, 2012, p. 423-434.
7. ISHIZAWA, M., KAGECHIKA, H., MAKISHIMA, M., Biochem. Biophys. Res. Commun., **418**, 2012, p. 780-785.
8. LONATI, C., SORDI, A., GIULIANI, D., SPACCAPELO, L., LEONARDI, P., CARLIN, A., OTTANI, A., GALANTUCCI, M., GRIECO, P., CATANIA, A., GUARINI, S., Anesthesiology, **116**, 2012, p. 692-700.
9. HAO, L., WANG, X., ZHANG, D., XU, Q., SONG, S., WANG, F., LI, C., GUO, H., LIU, Y., ZHENG, D., ZHANG, Q., Int. J. Pharm., **433**, 2012, p. 157-164.
10. THOMPSON, J., BURGER, M.L., WHANG, H., WINOTO, A., Eur. J. Immunol., **40**, 2010, p. 2041-2049.
11. KERTI, V., CORICOVAC, D., DUICU, O., DANILA, M., DEHELEAN, C., FAGADAR-COSMA, E., MUNTEAN, D., NOVEANU, L., Rev. Chim. (Bucharest), **65**, no. 4, 2014, p. 447
12. TAMPA, M., MATEI, C., POPESCU, S., GEORGESCU, S.R., NEAGU, M., CONSTANTIN, C., ION, R.M., Rev. Chim. (Bucharest), **64**, no. 6, 2013, p. 639
13. DUICU, O., CIURLEA, S., DEHELEAN, C., ARDELEAN, S., ANDRICA, F., MUNTEAN, D., SOICA, C., ANTAL, D., CORICOVAC, D., PINZARU, I., Rev. Chim. (Bucharest), **65**, no. 8, 2014, p. 956
14. WU, J., LIU, J., JIA, R., SONG, H., Cancer Invest., **31**, 2013, p. 654-660.
15. KRISHNAMURTHY, B., CHEE, J., JHALA, G., TRIVEDI, P., CATTERALL, T., SELCK, C., GURZOV, E.N., BRODNICKI, T.C., GRAHAM, K.L., WALI, J.A., ZHAN, Y., GRAY, D., STRASSER, A., ALLISON, J., THOMAS, H.E., KAY, T.W., Diabetes, **64**, 2015, p. 3229-3238.
16. WENZL, K., TROPPAN, K., NEUMEISTER, P., DEUTSCH, A.J., Curr. Drug Targets, **16**, 2015, p. 38-46.
17. OSMOND, D.G., MELCHERS, F, PAIGE, C.J., J. Immunol., **133**, 1984, p. 86-90.
18. ZAHARIA (AVRAM), C., ACATRINEI, D., GENTIMIR (AMITITELOAIE), C., BOISTEANU, O., RADUCANU, C.O., CHELARU, L., VASINCU, D., BOGZA, G., COSTULEANU, M., Rev. Chim. (Bucharest), **66**, no.12, 2015, p. 2040
19. ZONDA, G.I., GORIUC, A., IANCU, R.I., COSTULEANU, M., ANAL. ST. UNIV. Al. I CUZA IASI. SECT. GEN. BIOL. MOLEC., **TOM XI**, 2010, p. 15-20.
20. BOISTEANU, O., ZONDA, G.I., AVRAM, C., CHELARU, L., GENTIMIR, C., ACATRINEI, D., COSTULEANU, M., Rev. Chim. (Bucharest), **66**, no. 9, 2015, p. 1452
21. LI, Y., CARRILLO, J.A., DING, Y., HE, Y., ZHAO, C., LIU, J., LIU, G.E., ZAN, L., SONG, J., PLoS One, **10**, 2015, e0135670. doi: 10.1371/journal.pone.0135670.
22. SHI, J., WU, K., YANG, X., YAN, D., QIAN, L., ZHANG, C., YANG, C., ZHANG, C., WU, X., LIU, X. Pathol. Res. Pract., **211**, 2015, p. 633-640.
23. HU, Q.N., BALDWIN, T.A., J. Immunol., **194**, 2015, p. 2643-2653.
24. BURGER, M.L., LEUNG, KK.., BENNETT, M.J., WINOTO, A., Elife, 2014, **3**, doi: 10.7554/eLife.03468.
25. FASSETT, M.S., JIANG, W., D'ALISE, A.M., MATHIS, D., BENOIST, C., Proc. Natl. Acad. Sci. U.S.A., **109**, 2012, 179, p. 3891-3896.
26. KISS, B., TÓTH, K., SARANG, Z., GARABUCZI, É., SZONDY, Z., Biochim. Biophys. Acta, **1853**, 2015, p. 660-670.

Manuscript received: 15.06.2015