J. Serb. Chem. Soc. 69 (12) 1005-1011 (2004)
JSCS-3229

UDC 577.15:576.385:577.164.1
Preliminary communicaton

PRELIMINARY COMMUNICATION

Superoxide dismutase activity as a function of culture aging of B-16 mouse melanoma cells

RATKO M. RADOJIČIĆ[1*], SNEŽANA D. SPASIĆ[2#], ZORICA S. SAIČIĆ[3], TAMARA B. JOVANOVIĆ[4] and JOVANA B. SIMIĆ-KRSTIĆ[4]

[1]Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade (e-mail: ratko@bf.bio.bg.ac.yu), [2]Center for Chemistry ICTM, P.O. Box 815, 11000 Belgrade, [3]Institute for Biological Research "Siniša Stanković", Department of Physiology, Bul. Despota Stevana 142, Belgrade and [4]Fullerene Science and Technology Centre, Chemical Power Sources Institute, Batajnički put 23, Belgrade-Zemun, Serbia and Montenegro

(Received 15 December 2003, revised 8 March 2004)

Abstract: The C3 clone of B-16 mouse melanoma was cultured for 1, 6 and 9 days and analysed. The changes which are not directly linked to melanogenesis in the B-16 / C3 cultures during their maturation were characterized. Early (1 day), confluent (6 days) and old (9 days) cell cultures are distinguished by their leucine aminopeptidase (LAP) and α-naphthyl acetate esterase (ANAE) isoenzyme patterns. Both quantitative and qualitative changes in LAP and ANAE isoenzyme can be observed during culture maturation. There is an increase in the activity of the enzyme copper, zinc-containing superoxide-dismutase (CuZn SOD). The increaase in the CuZn SOD enzyme activity might be related to B-16/C3 cell melanogenesis and / or to differentiation.

Keywords: B-16 mouse melanoma, isoenzymes, α-naphthyl acetate esterase (ANAE), leucine aminopeptidase (LAP), copper, zinc–containing superoxide dismutase (CuZn SOD).

INTRODUCTION

Malignant transformation is generally regarded as a disorder of differentiation. Changes in isoenzyme patterns and enzyme activities might be very sensitive markers of different stages of development.[1]

There are numerous experimental data suggesting that oxygen metabolites are potential carcinogens, because they provoke mutagenesis, tumor promotion and progression.[2,3] Protection against increased levels of reactive oxygen species (ROS) is mediated by cell antioxidants or reductants. The enzyme copper, zinc-co-

* Corresponding author.

Serbian Chemical Society active member.

ntaining superoxide dismutase (CuZn SOD, EC 1.15.1.1) converts superoxide anion radicals ($O_2^{\cdot -}$ into hydrogen peroxide (H_2O_2), thus the activity of this enzyme might be involved in developmental regulation of differentiation.[4] From previous investigations, it is known that tumors usually have lower superoxide dismutase (SOD, EC 1.15.1.1) activity, mainly manganese containing superoxide dismutase (Mn SOD, EC 1.15.1.1), than the tissues from which they originate.[6,7] Since superoxide anion radicals are generated in tumor cells, the loss of Mn SOD in cancer tissues is difficult to explain. According to Oberley's hypothesis,[8] loss of Mn SOD activity leads to non-lethal damage to tumor cells. This injury is proposed to be partially responsible for cancer phenotype.

In this study, the phenomenon of cancer cell differentiation with emphasis on the role of CuZn SOD in this process has been studied using the B-16/C3 murine melanoma cells as a model system. The C3 clone of B-16 mouse melanoma cells undergoes melanogenesis and differentiation under usual culture conditions at a specific time after plating. Early cultures are predominantly composed of amelanotic cells, while late cultures contain mostly melanotic cells. Three phenotypically distinct stages of B-16/C3 maturation have been characterized using the following markers: cell morphology, isoenzyme patterns of leucine aminopeptidase (LAP, α-aminoacyl-peptide hydrolase, EC 3.4.11.1), α-naphthyl acetate esterase (ANAE, aryl ester hydrolase, EC 3.1.1.2) and CuZn SOD activity.

EXPERIMENTAL

The C3 clone of B-16 mouse melanoma was provided by Dr. John Kreider of the Hershey Medical Center, Hershey, PA. The cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Gibco), glutamine and 0.05 M sodium bicarbonate. Stock cultures were maintained in the logarithmic growth phase by subculturing every 3 to 4 days. The cells were passed using 0.05 % trypsin plus 0.02 % EDTA (Gibco). The experimental cells were plated at an initial density of 1500 cells/cm^2 into glass flasks (11 x 4 cm) in 10 ml of medium and cultured for 1, 6 or 9 days at 37 °C. The culture medium was not changed during the experiment.

After 1, 6 or 9 days, the cells were gently scraped from the surface of the flasks and collected by centrifugation at 1200 rpm for 10 min. The pellet was suspended in 10 volumes of 0.05 M potassium phosphate buffer, pH 7.8 and homogenized at 1500 rpm (8 – 10 up and down strokes) using a glass Potter Elvehjem homogenizer with a teflon pestle. The homogenates werehen centrifuged at 4 °C at 100 000 x g for 90 min (Beckman LS). The resulting supernatants were used for protein determination.[9]

The activity of superoxide dismutase in the resulting supernatant was determined by following the inhibition of epinephrine autooxidation to adrenochrome in the presence of biological samples containing SOD.[10] One unit of SOD activity was defined as the amount of protein causing 50 % inhibition of the autooxidation of adrenaline at 26 °C. The measured SOD activity was inhibited with 4 mM KCN indicating presence of only CuZn SOD in the experimental samples. Polyacrylamide gel electrophoresis was performed by the method of Davis[11] on 7.5 % gels. The SOD activity in the gel was detected by the standard "negative staining method" as described by Beuchamp and Fridovich.[12] The gels were washed in a 2.45 mM nitroblue tetrazolium (NBT) solution for 20 min in the dark and then incubated in a solution containing 28 mM TEMED (Sigma), 0.028 mM riboflavin and 36 mM potassium phosphate, pH 7.8 for 15 min in the dark. The gels were illuminated with a fluorescent light and the presence of SOD activity corresponded to achromatic zones in a uniformly deep blue background. Staining for LAP in gels was performed by the method of Scandalious.[13] The

components for the stain were 3 ml of 1 % L-leucyl-β-naphthylamide, HCl (in 50 % acetone), 50 mg Fast Black K Salt, 100ml 0.2 M Tris-malate buffer pH 6.0. The gels were incubated in the solution at 30 °C for 1–4 h in the dark. Enzyme activity was indicated by the presence of gray to violet bands. Staining for ANAE was performed by the method of Tanskley and Rick.[14] The components for the stain were 3 ml of 1 % α-naphthyl acetate (in 100 % acetone), 120 mg Fast blue RR salt, 100 ml 0.1 M Na phosphate buffer pH 6.2. The gels were incubated in the solution at 37 °C for 40–60 min in the dark. Enzyme activity was indicated by the presence of black bands.

All the obtained data were statistically analyzed and expressed as mean ± SE. Statistical differences between the samples were estimated by Student's paired t-test.[15] The value of $p < 0.05$ was taken as the least degree of significance.

RESULTS AND DISCUSSION

The three phases of the B-16/C3 murine melanoma cell culture were observed by light microscopy. Early cultures, 1 day after plating, contained amelanotic cells (Fig. 1a). Confluent cell cultures, 6 days after plating, were predominantly composed of amelanotic cells (Fig. 1b). Old cultures, 9 days after plating, consisted of large cells producing a great amount of melanin pigment (Fig. 1c). Each of these B-16/C3 maturational stages were found to have different LAP and ANAE isoenzyme characteristics.

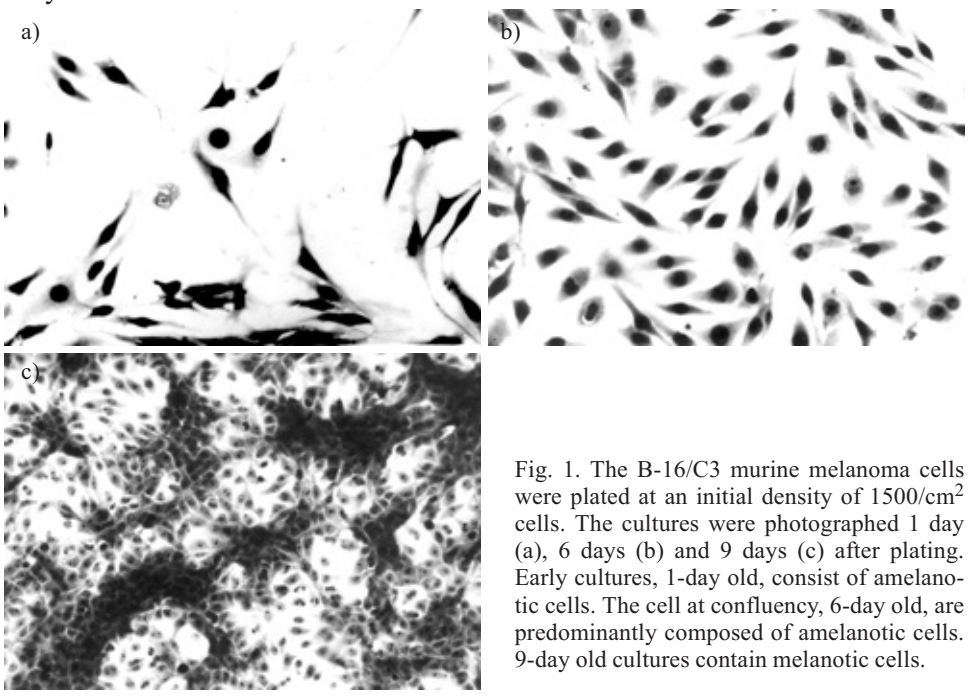

Fig. 1. The B-16/C3 murine melanoma cells were plated at an initial density of 1500/cm^2 cells. The cultures were photographed 1 day (a), 6 days (b) and 9 days (c) after plating. Early cultures, 1-day old, consist of amelanotic cells. The cell at confluency, 6-day old, are predominantly composed of amelanotic cells. 9-day old cultures contain melanotic cells.

The LAP isoenzymes of early (a), confluent (b) and old melanotic cell cultures (c) are presented in Fig. 2. The LAP in early cultures was expressed as one isoenzyme corresponding to a very weak band migrating at R_f - 0.28, while the LAP spectrum of confluent cells consists of four isoenzyme bands at R_f - 0.07, R_f -

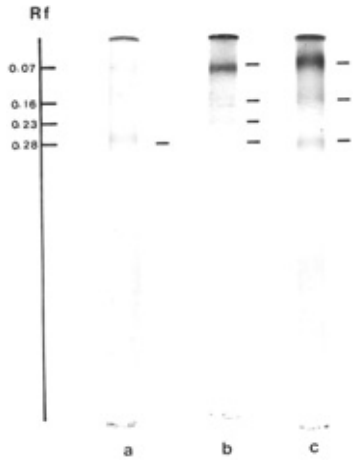

Fig. 2. Leucine aminopeptidase (LAP) isoenzyme patterns detected after staining the polyacrylamide disc gels: a) LAP isoenzymes of 1-day old cell cultures, b) LAP isoenzymes of 6-day old cell cultures and c) LAP isoenzymes of 9-day old culture.

0.16, R_f - 0.23 and R_f - 0.28. However, the isoenzyme migrating at R_f - 0.23 disappears in old cell cultures. The phenotype classes of ANAE isoenzyme spectra for the previously defined stages of maturity of B-16/C3 are shown in Fig. 3. This pattern of enzyme heterogeneity was observed in all the analyzed samples. It is evident that there is more of the ANAE isoenzyme migrating at R_f - 0.35 in confluent cells than in early cell cultures. In old melanotic cells, a new isoenzyme migrating at R_f - 0.10 was detected (Fig. 3c).

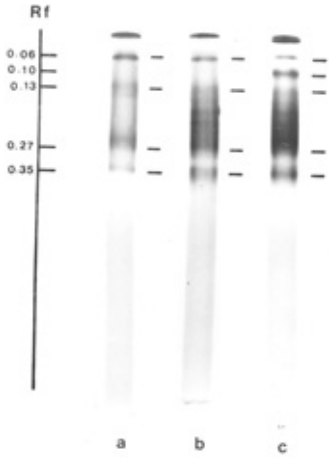

Fig. 3. α-Naphthyl acetate esterase (ANAE) isoenzyme patterns of 1-day (a), 6-day (b) and 9-day (c) old B-16/C3 cell cultures.

Fig. 4. Electrophoresis profiles of copper, zinc-containing superoxide dismutase (CuZn SOD) in B-16/C3 melanoma cells during differentiation: a) 1-day old cell cultures, b) 6-day old cell culture and c) 9-day old cell cultures.

The results show that the three distinct B-16/C3 cell culture phenotypes are accompanied by characteristic LAP and ANAE isoenzyme patterns. The B-16/C3 murine melanoma cultures are composed of cells with different melanin contents

and replicative activities.[16] The replicative activity and colony forming ability are inversely proportional to the cell size and the melanin content. Melanin pigment synthesis is thought to be essential for differentiation in both normal and malignant-transformed melanocytes.[17] Many agents which may regulate differentiation are intimately involved in melanin synthesis.[18] However, the changes in the LAP and ANAE isoenzymes during maturation of B-16/C3 cells suggest that other pathways, which are not directly involved in melanogenesis, may influence differentiation.

The band corresponding to the SOD enzyme in maturing B-16/C3 cell cultures, monitored by the "negative staining method" is presented in Fig. 4. The results indicate that the CuZn SOD activity observed in mouse melanoma B-16/C3 cells is in agreement with previous finding concerning SOD activity in B-16 murine melanoma tumors.[19] Also, it has been shown that SOD is presented and involved in the signaling pathways in human melanoma.[20]

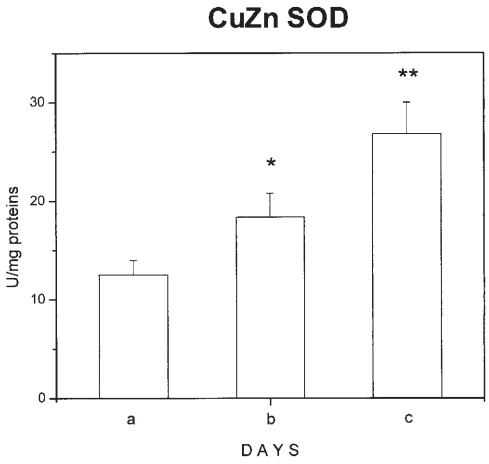

Fig. 5. Activity of copper, zinc-containing superoxide dismutase (CuZn SOD) expressed in U/mg proteins measured during the maturation of B-16/C3 cell cultures: 1-day, 6-day and 9-day old cultures. Values are presented as means ± SE. $^{*}p < 0.05$; $^{**}p < 0.02$

In this work it was found that the SOD specific activity increased significantly during B-16/C3 culture aging (Fig. 5). This SOD activity can be completely inhibited by the presence of 4 mM KCN, suggesting that this enzyme is CuZn SOD. The increase in the CuZn SOD activity with increasing culture age might be closely linked to melanogenesis and/or to differentiation.

From previous investigations, it is known that 3,4-dihydroxyphenylalanine (DOPA), which is intimately involved in melanogenesis,[21] is toxic for mouse melanoma,[22] neuroblastoma,[23] leukemia,[24] HeLa cells and human melanoma cells MM 96.[25] Since superoxide anion radicals are produced during melanin synthesis,[26] the DOPA killing effect might occur through these highly toxic species. This finding was supported by the evidence that SOD, catalase and peroxidase decrease the toxicity of DOPA.[24] Thus, the increase in CuZn SOD activity, observed during maturation of B-16/C3 cells might be induced by superoxide anion radicals produced during melanogenesis. This fact suggests that this enzyme may play an important role in preventing melanotic cell death. Namely, inhibition or loss of SOD

activity may cause an accumulation of $O_2^{\cdot -}$ which leads to damage of cellular structures and the killing of cancer cells.[27]

CONCLUSIONS

The results of this study raise the possibility that a melanogenesis independent enzyme might be involved in B-16/C3 murine melanoma cell culture maturation. The increase in CuZn SOD activity with increasing culture age supports the idea that this enzyme probably plays an important role in cell differentiation. Experiments are in progress to determine whether these melanogenesis-independent enzymes (SOD, LAP and ANAE) could influence melanin syntheses.

Acknowledgements: This research was supported by the Ministry of Science, Technologies and Development of Serbia, Grant. No. 1669.

ИЗВОД

АКТИВНОСТ СУПЕРОКСИД-ДИСМУТАЗЕ ТОКОМ МАТУРАЦИЈЕ КУЛТУРЕ ЋЕЛИЈА B-16 МЕЛАНОМА МИША

РАТКО М. РАДОЈИЧИЋ[1], СНЕЖАНА Д. СПАСИЋ[2], ЗОРИЦА С. САИЧИЋ[3], ТАМАРА Б. ЈОВАНОВИЋ[4] и ЈОВАНА Б. СИМИЋ-КРСТИЋ[4]

[1]Институт за физиологију и биохемију Биолошког факултета Универзитета у Београду, Студентски трг 16, 11000 Београд, [2]Центар за хемију, п.пр. 815, 11000 Београд, [3]Институт за биолошка истраживања "Синиша Станковић", Одељење за физиологију, 29. новембра 142, Београд, [4]Фулерен научни и технолошки центар, Институт за хемијске изворе струје, Батајнички пут 23, Београд-Земун, Србија и Црна Гора

С3 клонови B-16 меланома миша су гајени у култури 1, 6 и 9 дана и потом анализирани. Извршена је карактеризација промена B-16/C3 ћелијских култура током њихове матурације које нису директно повезане са меланогенезом. Показано је да се културе у почетној фази (1 дан), конфлуентној фази (6 дана) и касној фази (9 дана) ћелијског раста разликују на основу изоензимских спектара леуцин-аминопептидазе и алфа-нафтил-ацетат-естеразе. Током развитка ћелијских култура B-16/C3 запажене су квантитативне и квалитативне промене леуцин-аминопептидазе и алфа-нафтил-ацетат-естеразе у њиховим изоензимским спектрима. Утврђено је да постоји пораст активности бакар-цинк супероксид-дисмутазе током старења културе. Пораст активности бакар-цинк супероксид-дисмутазе може бити повезан са меланогенезом и/или диференцијацијом B-16/C3 ћелија.

(Примљено 15. децембра 2003, ревидирано 8. марта 2004)

REFERENCES

1. J. T. Chang, K. T. Chen, C. L. L. Wang, *Acta Biotechnol.* **21** (2001), 243
2. A. Wozniak, B. Wozniak, G. Drewa, T. Drewa, *Melanoma Res.* **13** (2003) 19
3. H. C. Ha, A. Thiagalingam, B. D. Nelkin, R. A. Casero, Jr. *Chim. Cancer Res.* **6** (2003) 3783
4. W. Doge, *Physiol. Rev.* **82** (2002) 47
5. G. M. Bartoli, S. Bartoli, T. Galeotti, E. Bertoli, *Biochim. Biophys. Acta* **620** (1980) 205
6. D. P. Loven, L. W. Oberley, F. M. Rousseau, R. H. Stevans, *J. Natl. Cancer Inst.* **6** (1980) 377
7. A. Varachaud, O. Berthiervergnes, M. Rigaud, D. Schmitt, P. Bernard, *Eur. J. Dermatology* **8** (1998) 90
8. L. W. Oberley, T. D. Oberley, in *Free Radicals, Aging and Degenerative Diseases* J. E. Johnson, R. Walford, D. Harman, J. Miguel (Eds) **8** (1986) 325

9. O. H. Lowry, H. J. Rosebrough, A. L. Farr, R. J. J. Randal, *J. Biol. Chem.* **193** (1951) 265
10. H. P. Misra, I. Fridovich, *J. Biol. Chem.* **247** (1972) 3170
11. B. J. Davis, *Ann. N. Y. Acad. Sci*. **121** (1964) 404
12. C. Beauchamp, I. Fridovich, *Biochim. Biophys. Acta* **317** (1973) 50
13. J. G. Scandalious, *Biochem. Genet.* **3** (1969) 37
14. S. D. Tanskley, C. M. Rick, *Theor. Appl. Genet.* **56** (1980) 209
15. P. G. Hoel, *Introduction to Mathematical Statistics* (1966) 402
16. J. W. Kreider, M. F. Schmoyer, *J. Natl. Cancer Inst.* **55** (1975) 641
17. G. B. Pierce, *Fed. Proc.* **29** (1970) 1248
18. B. Kasraee, *Dermatology*, **205** (2002) 329
19. J. N. A. Vam Balgoy, E. Roberts, *Biochem.Physiol.* **62** (1979) 263
20. R. Morandini, J. M. Boeynaems, X. Duhant, F. Jacquemotte, E. Kinnaert, G. Ghanem, *Cell. Mol. Biol.* **45** (1999), 1053
21. P. A. Riley, *Cell. Mol. Biol.* **45** (1999) 951
22. M. M. Wick, *Cancer Res.* **40** (1980) 1414
23. M. M. Wick, *Science* **199** (1978) 775
24. M. M. Wick, *Cancer Treat. Rep.* **65** (1981) 861
25. P. G. Parsons, *Biochem. Pharmacol.* **34** (1985) 1801
26. Y. Tomita, A. Hariv, C. Kato, M. Seiji, *J. Invest. Derm.* **82** (1984) 573
27. M. R. Nogues, M. Giralt, I. Cervello, D. Delcatstillo, O. Espeso, N. Argany, A. Aliaga, J. Mallol, *J. Invest. Derm.* **119** (2002) 645.