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TAXANES AND GENE EXPRESSION IN BREAST CANCER CELLS

JOLANTA RZYMOWSKA[1], PIOTR MAJ[2], MACIEJ NIEMCZYK[1], TADEUSZ MALEWSKI[3] and ANDRZEJ WILKOŁASKI [4]

[1] Chair and Department of Biology and Genetics, Medical University, 6 Staszica Str., 20-081 Lublin, Poland
[2] Chair and Department of Nuclear Medicine, Medical University, 8 Jaczewski Str., 20-954 Lublin, Poland
[3] Department of Animal Genetics, PAN, 64 Wilcza Str., 00-950 Warszawa, Poland
[4] Department of Cardiology, 100 Krasnicka Str. 20-718 Lublin, Poland

Abstract: We analyzed expression of genes participating in apoptotic and oncogenesis processes under influence of paclitaxel applied in treatment of breast carcinoma. Analysis showed, that in a group of cells where paclitaxel was administered at lower dose (60 ng/mL) it caused statistically significant increase of expression of pro and antiapoptotic genes, genes coding caspases and oncogenes in comparison to control group.

Keywords: paclitaxel, breast cancer, oncogenes, apoptosis, microarrays

In metabolism of neoplasm cells important role play processes concerned with division and growth. In these cells there are observed alterations in function of genes responsible for regulation of cell cycle concerned with an increase of expression of genes participating in proliferation and growth. These changes are usually connected with a decrease of expression of genes which control growth, division and transcription processes in the cells. A very important role in transformation of normal cells into neoplasm cells play oncogenes. A great number of these genes code proteins participating in transfer of information inside the cell. Some of them code factors which influence the cellular growth (*Sis, Int*), growth factor receptors (*Erb-B, Fms*). In this group are known genes which code transcription factors (*Myb, Myc, Jun, Fos*) kinases (*Src, Yes, Abl, Raf, Mos*), GTP binding proteins (*ha-Ras, ki-Ras, n-Ras*) (1, 2). Some of these genes cause transformation of normal cell into neoplasm cell in a case when mutation appears. To the group of suppressor genes belongs *BRCA1*, which is representative of activator of transcription, involved mainly in DNA repairing system.

Genes concerned with apoptosis can inhibit (BCL-*2, BCL-XL, BCLW, MCL1, BFL-1/A1, BCL-W, BCL-G)* or activate this process (*BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B*). After initiation of signal to apoptosis these factors pass through mitochondrial membranes and cause programmed cell death (*BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B*) (3, 4). Gene *BCL-XL* inhibits apoptotic process, can be joined to APAF-1, and causes inactivation of caspases. In a case of increasing production of genes activating apoptotic process it causes an increase of permeability of mitochondrial membrane and can lead do the release of cytochrome c to the cytoplasm. This factor binds with APAF1 and can activate caspases 8 and 9 (4-6). Caspases 8 and 10 can be activated by TNF. This factor cooperates with exterior receptor on the surface of the cell. Apoptosis can be induced by passing a signal from such receptors like APO1, IGF1R, TNFR1 inside the cell. This process activates caspases. In this mechanism participates P-53, a protein which leads to an increase of products connected with apoptosis (BAX, BCLX). It can release the c cyclin (4-7). This process is connected with activation of sphingomyelin and release of ceramides (4). Ceramides inhibit activity of genes enabling survival of the cells. In the process of activation of apoptosis participate transcription factors such as NF-kB, MAPK family, ERK kinases, SAPK/JNK and caspases (8-10). This phenomenon can be induced through an increase of activity of membrane receptors connected with FAS ligands. This factors bind with signal proteins, FAS and cause degradation of the cells through activation of caspase 8 (4).

Recently, a significance has grown of multilevel analysis of gene expression performed by the array technique. Such analysis broadened the knowledge about the proliferation of neoplasm. The

array consists of stable nylon or glass ground of small size on which genes are localized (11-13).

Paclitaxel belongs to taxanes – which are a group of drugs binding with units of beta-tubulin. This protein participates in building of microtubules, which are responsible for intercellular transport, excretion processes, neurosecretion, and also in controlling shape of the cell and cell division. Microtubules are structures having ability to polymerization and depolymerization. Taxanes cause inhibition of this process which can stop cell division in G2/M phase. Paclitaxel inhibits forming of mitotic spindle and limits growth of tumor also between chemotherapy cycles. This drug limits widespread of neoplasm cells also resistant to treatment. It increases inhibition of neoplasm through activation of apoptotic process and inhibition of angiogenesis. This drug influences the phosphorylation of BCL-2, and causes acceleration of apoptosis in neoplasm cells (14).

Our analysis was connected with estimation of changes in the expression of genes participating in apoptotic and oncogenesis processes after paclitaxel treatment of breast carcinoma cells.

EXPERIMENTAL

The cancer cells were estimated from breast tissues of 36 women with histopathologically confirmed breast cancer. The group included patients with intraductal breast cancer (preinvasive breast cancer and lesions to 5.0 cm size without metastases to lymph nodes). Cytostatics were not administered to patients before surgery. Breast tissues were homogenized mechanically and chemically by using 0.01% trypsin (SIGMA). The cultures were conducted in RPMI medium (SIGMA) with addiction of 10% fetal bovine serum (FBS SIGMA), penicillin and streptomycin. After isolation, breast cancer cells were incubated under standard conditions (37^{o}C, 5% CO_2, 90% humidity of the air). To the cultures where density of the cells achieved 10000 per mL paclitaxel was administered in 60 ng/mL and 300 ng/mL for 72 h. Control culture was a suspension of the breast cells without administration of this cytostatic. 5% DMSO was added to control cultures.

The cells were homogenized in TRI (SIGMA) solution. The water fraction was transfered to the test tubes and 0.5 mL of isopropanol was added. After 10 min of incubation at room temperature the solution was centrifuged (12000 rpm) at -4^{o}C. Total RNA (TRNA) estimated after this process was controlled on the presence of impurities and DNA using a spectrophotometer (EPPENDORF). TRNA samples were stored at – 70^{o}C.

Table. The expression of pro- and antiapoptotic genes, caspase genes and oncogenes.

	ACTIVATION OF APOPTOTIC GENES			Numer of genes
	Control	60 ng/mL	300 ng/mL	
Gene expression level	12.05	26.17	9.15	37
p	0.0000001	0.09	0.0000001	
	INACTIVATION OF APOPTOTIC GENES			Numer of genes
	Control	60 ng/mL	300 ng/mL	
Gene expression level	9.62	26.13	8.68	12
p	0.000018	0.42	0.000017	
	CASPASE GENES			Numer of genes
	K	60 ng/mL	300 ng/mL	
Gene expression level	9.77	26.19	7.88	13
p	0.000008	0.0086	0.000009	
	ONCOGENES			Numer of genes
	K	60 ng/mL	300 ng/mL	
Gene expression level	10.99	25.37	7.98	39
p	0.0000001	0.0001	0.0000001	

TRNA from the cells was subjected to RT-PCR reaction to estimate cDNA. Such cDNA was hybridized in a hybridization chamber using Panorama Human Cancer cDNA Labeling and Hybridization Kit; CDLBL-HCN (SIGMA-GENOSIS). The kit included control RNA from E. coli (Panorama Armored RNA E. coli-B 1444 RNA) which enabled to calibrate expression of researched genes in relation to this gene.

The samples containing 4 µL TRNA, 4 µL mixture of nucleotides (dATP, dGTP, dTTP labeled with ^{32}P dCTP of 40 µCi activity), 6 µL reverse transcriptase and 2 µL of control RNA E. coli were filled up with solution buffer to 20.0 µL. This mix was incubated at 42^{o}C for 2,5 h. The cDNA containing labeled ^{32}P cytidine was estimated in reverse transcripion reaction. This received and purified cDNA was hybridized on nylon surface of array. Sigma-Genosis array contained sequences of genes coding pro and antiapoptotic proteins, caspases and oncogenes. Hybridization was a process of connection of cDNA with complementary sequences of genes fixed on the surface of array in the hybridization chamber in a period of 20 min at 65^{o}C at 6/min speed. After removing unconnected fragments of cDNA the array was dried on a sterile blotting paper for 2 min and placed in a cassette sensitive to radiation to collect the activity (Screen Imaging K – BioRad). After radiation of the radiosensitive material in the cassette the surface of the array was scanned with a Molecular Imager FX BioRad scanner. Activity for a single gene was counted as an average measured from two adjacent pools. The analysis was based on comparison of expression of genes in cancer cells after administration of the drug to control cells.

For statistical analysis program Statistica 6.0 was used.

RESULTS AND DISCUSSION

The analysis showed, that in a group of cells where paclitaxel was administered at higher dose (300 ng/mL) it caused statistically significant increase of expression of pro and antiapoptotic genes and genes coding caspases in comparison to control group (K).

Statistically significant correlation was noted between control group and the first and the second group for apoptotic genes.

An increase of expression level of antiapoptotic genes derived from Bcl2 family and also similar increase level of proapoptotic genes was found. An inhibition of cell growth and death of the cells after administration of paclitaxel in a dose 60 ng/mL were observed. This could be supported by possibility of phosphorylation of Bcl-2 protein what leads to inhibition of Bcl-2 activity. Such process was seen when MAPK was activated. Another way responsible for the mentioned phosphorylation of Bcl-2 is the activation of protein kinase connected with activation of cRaf kinase what leads to activation of kinase A and is dependent on degree of polymerization of microtubules. Phosphorylated form of Bcl-2 can activate caspase 3 what can lead to irreversible apoptotic process (14). This leads to inhibition of antiapoptotic products and escalates apoptosis processes in the mechanism of activation of many caspases. Selective activation of apoptosis in carcinoma cells after paclitaxel treatment should also be considered. Paclitaxel can activate inhibition of mitotic process. The first mechanism may be an activation of serine-threonine kinase. Another described process connected with activation of P-21 protein leads to inhibition of CDK1 cyclin and its complex with cyclin. This process influences the inhibition of cell division. P-21 causes an increase of MAP which binds with tubulin and leads to bigger polymerization of microtubules (15, 16). This activated product results in acceleration of the apoptosis process. Chang et al. (9) noticed an increased level of 78 out of 92 genes coding apoptotic and thermal shock proteins in 48% of 24 women with breast cancer treated with paclitaxel. This observation may explain why apoptotic processes have so strong manifestation in the cancer cells. In activation of apoptosis by paclitaxel at least 3 ways may be considered: phosphorylation of Bcl-2, activation of apoptosis during inhibition of cell division and direct activation of apoptotic products. Studies conducted on T47D carcinoma cells *in vitro* revealed that an increase of expression of Bic product and ER-2 receptors is observed after administration of beta-estradiol. We also observed an increased expression of *Bic*. This metabolic pathway is similar to the way activated by antiestrogen drugs. It is know that Bic can bind with Bcl-2 protein and inhibits its activity (4, 14, 17-19). This way of activation is significant and has influence on inhibition of BCl-2 product. Additionally, in another report protein Daxx was described which belongs to apoptotic products (20). This protein interacts with repressors of transcription such as PAX5, ETS-1 and reduces their expression (25, 26). Daxx binds with ASK-1 and induces activity of JNK. The transport of Daxx to cytosol increases activity of ASK-1 as a kinase (27). Daxx activates many ways connected with apoptosis, such as increased expression of genes coding caspases and transcriptor factors as

histone proteins (21-25). High level of ASK-1 protein induces activity of caspases: 8 and 9, NFk-B and E2 F1(26, 27). This may suggest that in cell cultures dominate irreversible apoptotic processes which can not be inhibited even if the level of Bcl-2 is high. These processes are inducted by irreversible degradation of cell components by caspases. It is the reason for the advantage of apoptotic process despite high level of antiapoptotic products even in active forms. The activation of apoptosis is induced by caspase 3. It interacts with ICAD what is the reason of lower activity of CAD and leads to activation of DNA-ase. In this process participates CIDE which after connection with DFF45 activates DNA-ase in the same way. Another pro-apoptotic protein DAP-3 binds with inner mitochondrial membrane. It activates apoptosis by binding to DISC complex through TRAIL, FAS and FADD (28, 29).

An increase of level of apoptotic genes has clinical significance. The statement of percentage of activity of pro and antiapoptotic genes in relation to all groups of activated genes can evaluate a degree of malignancy of the neoplasm and its sensibility to treatment. In breast cell cancer MCF-7M it was observed an increased expression of *Bcl-2* an low of *P-53* and *Bax*. The lower level of *P-53* and *Bax* gene*s* expression was observed in MCF-7L cells, and MCF7-7N have high expression level of *P-53* and *Bax* genes, but low of *Bcl-2*. These results aren't in concordance with our results. In carcinoma cell lines we do not observed a coexistence of high level of pro- and anti-apoptotic genes. An increase of expression of antiapoptotic genes after administration of paclitaxel in a dose of 60 ng/mL requires explanation. This observation suggests that the expression of antiapoptotic genes should not be so intense. It is possible that expression of antiapoptotic genes is lower than proapoptotic genes. Another explanation suggests that the expression levels of both groups of genes are similar, but because of unknown cell mechanism the prevalence is achieved by proapoptotic genes. It is possible that in this process caspase activation has its significance, what leads to irreversible reaction causing degradation of cell structures (2, 6).

Paclitaxel in a dose of 60 ng/mL activated opposing groups of pro- and antiapoptotic genes. The probable reason of advantage of apoptotic over antiapoptotic processes in the cancer cells may be explained by the activation of significant number of ways connected with phosphorylation of *Bcl-2* gene products (13, 14).

It was proved that activation of apoptotic process depends on proper relation between pro and antiapoptotic genes. Activation of apoptosis can be connected with influence of paclitaxel, or type of activity of cell lines. It was stated that the beginning of apoptosis process is connected with proper ratio of expression Bax/Bcl-2, which influences on increasing of permeability of mitochondrial membrane (30, 31). Proper relation of these gene expression influences the activation or inactivation of apoptosis. This information indicates that induction of apoptosis is not concerned with the increase of expression, but proper relation between of these groups. It indicates, that high level of expression of antiapoptotic genes does not always influence the programmed cell death. Chang et al. (9) analyzed of expression of genes in 24 women with breast cancer after docetaxel treatment. The report showed overexpression of 14 from 92 antiapoptotic genes in 54% of patients. It is possible that expression level is responsible for difference reaction after treatment with paclitaxel.

The high activity of antiapoptotic genes, and their simultaneous weak influence on inhibition of apoptotic process can be explained by the mechanism of decrease of expression of these genes connected with phosphorylation reaction of gene products caused by paclitaxel.

The reasons for a decrease of antiapoptotic activity of Bcl-2 can be different. It is possible that it increases the cell resistance to this drug. This can be connected with the activity of both of this group of genes. In this expression it can be activated both opposite mechanisms. For instance BAX can influence on Bcl-2 and induct inactivation of this gene. It can activate dimerization and translocation in mitochondria what leads to programmed cell death (31-33).

An increase of expression of oncogenes which are diverse groups having opposing function is concerned with stimulation or inhibition of proliferation processes of cancer cells. It showed that paclitaxel has influence on proliferation processes and causes development of resistance to treatment.

A significant decrease of expression of oncogenes and apoptotic genes after administration of paclitaxel in a dose of 300 ng/mL pointed at cytotoxic effect, which lead to inhibition of a majority of cellular processes.

The activation of many processes proceeding G2/M phase can indicate that paclitaxel influences on control of cycle cell phases in the breast carcinoma cells.

REFERENCES

1. Bacus S.S., Gudkov A.V., Lowe M.: Oncogene 20, 147 (2001).

2. Bal J. Molecular biology in medicine (in Polish). PWN. Warszawa 2001.
3. Baldwin A.S.: Clin. Invest. 197, 241 (2001).
4. Ballif B.A., Blenis J.: Cell Growth Differ. 12, 397 (2001).
5. Bielak-Żmijewska A.: Kosmos 52, 157 (2003).
6. Bodnar L., Wcisło G.: Współczesna Onkologia (in Polish). 9, 435 (2004).
7. Cao Q., Xia Y., Azadniv M., Crispe I.N.: J. Immunol. 173, 1111 (2004).
8. Cavdar Koc E., Ranasinghe A., Burkhart W., Blackburn K.: FEBS Lett. 492, 166 (2001).
9. Chang H.Y., Nishitoh H., Yang X., Ichijo H., Baltimore D.: Science 281, 1860 (1998).
10. Charette S.J., Lambert H., Landry J.: J. Biol. Chem. 276, 36071 (2001).
11. Chen L., Chen J.D.: Mol. Cell. Biol. 23, 7108 (2003).
12. Coller H.A., Grandori C., Tamayo P., Colbert T.: Proc. Natl. Acad. Sci. USA 97, 3260 (2000).
13. Cooper C.S.: Cancer Res. 3, 158 (2001).
14. Desai K.V., Kavanaugh C.J., Calvo A., Green J. E.: Endocrine-Related Cancer 9, 207 (2002).
15. Deveraux Q.L., Reed J.C.: Genes Dev. 13, 239 (1999).
16. Dumontet C., Sikic B.I.: J. Clin. Oncol. 17, 1061 (1999).
17. Emelyanov A.V., Kovac C.R., Sepulveda M.A., Birshtein B.K.: J. Biol. Chem. 277, 11156 (2002).
18. Fuller G.M., Shields D.: Molecular Basis of Medical Cell Biology (Polish translation) Wydawnictwo PZWL, Warszawa 2000.
19. Gligorov J., Lotz J. P.: Oncologist 9, 3 (2004).
20. Green D.R.: Cell 102, 1 (2000).
21. Gross A., Mc Donnel J.M., Korsmeyer S. J.: Genes Dev. 13, 1899 (1999).
22. Hengartner M.O.: Nature 407, 770 (2000).
23. Herr I., Debatin K.M.: Blood 98, 2603 (2001).
24. Hollenbach A.D., Sublett J.E., McPherson C.J.: EMBO J. 18, 3702 (1999).
25. Hu W. H., Johnson H., Shu H.B.: Biol. Chem. 275, 10838 (2000).
26. Hulkko S.M., Wakui H., Zilliacus J.: Biochem. J. 349, 885 (2000).
27. Hur J., Chesnes J.: Proc. Natl. Acad. Sci. USA 101, 2351 (2004).
28. Kaufamnn S.H., Earshaw W.C.: Exp. Cell Res. 256, 42 (2000).
29. Kisiel A., Skąpska A., Markiewicz W.T., Figlerowicz M.: Kosmos 53, 295 (2004).
30. Liuh-Yow C., Don C.: Mol. Cell. Biol. 23, 7108 (2003).
31. Messerschmitt M., Jakobs S., Vogel F., Fritz S.: J. Cell Biol. 160, 55 (2003).
32. Mukamel Z., Kimch A.: J. Biol. Chem. 279, 36732 (2004).
33. Pratt M.A., Bishop T.E., White D., Yasvinski G.: Mol. Cell. Biol. 23, 6887 (2003).