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Paclitaxel and apoptosis in breast cancer cells

Paklitaksel i apoptoza w komórkach raka gruczołu piersiowego

INTRODUCTION

In neoplasm cells alterations are observed in the function of genes which are responsible for regulation of cell cycle concerned with the increase of expression of genes participating in neoplasm processes. These changes can be connected with the influence on the activity of genes controlling growth, division and transcription processes in the cells.

Genes concerned with apoptosis belong to a group which can inhibit the process of transformation (Bcl-2, Bcl-XL, BclW, MCL1, BFL-1/A1, BCL-W, BCL-G) or can activate this process (BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B) [5]. Proapoptotic genes of Bcl-2 family are proteins which are a part of intracellular organella, such as mitochondria, reticuloendothelial system, cytosol and cytoskeletal system. In the case of initiation of signal to apoptosis this protein passes through mitochondrial membranes and cause programmed cell death (BAX, BCLX, BAK, BOK, BAD, BIK, BID, BIM, BCL-XS, KRK, MTD, NIP3, NOXA, BCL-B). A gene which inhibits apoptotic process BCL-XL can be joined to APAF-1, and cause inactivation of caspases [2-4,9,16]. In the case of increasing the products of genes activating apoptotic process it causes increase in the permeability of mitochondrial membrane, which can lead do the release of cytochrom c to the cytoplasm.This factor binds with APAF1 and can activate caspases 8 and 9. Caspases 8 and 10 can be activated through TNF. Apoptosis can be induced by passing a signal from some receptors: APO1, IGF1R, TNFR1 inside the cell. This process activates caspases. P-53 protein, which leads to the increase of products connected with apoptosis (BAX, BCLX), can release c cyclin. This process is connected with activation of sphingomielin and release of ceramids. Ceramids inhibit the activity of genes which enable the survival of the cells. In the process of activation of apoptosis transcriptor factor NF-kB, MAPK family, ERK kinases, SAPK/JNK and caspases participate. This phenomenon can be induced through the increase of the activity of membrane receptors connected with FASL ligands. These factors bind with signal proteins FAS and cause degradation of the cells through activation of caspase 8.

Lately, multilevel analysis of gene expression performed by microarray technique has broadened the knowledge about proliferation of neoplasm. Microarray consists of stable plastic or glass grunt of small size on which are localized small points for genes [8].

Our analysis was connected with estimation of changes in the activity of genes participating in apoptotic and neoplasm processes under the influence of paclitaxel applied in the treatment of breast cancer.

Paclitaxel belongs to taxanes, which are a group of drugs binding with units of beta tubulin. Microtubules are structures which have the ability of polimerysation and depolimerysation. Drugs from the group of taxanes cause inhibition of this process. It leads to the inhibition of cell division in G2/M phase. The mechanism of the action of paclitaxel causes inhibition in forming the mitotic spindle. It increases the inhibition of neoplasm through the activation of apoptotic process and the inhibition of angiogenesis. It influences phosphorylation of BCL-2, and causes acceleration of apoptosis in neoplasm cells [2, 4, 6, 14,18].

MATERIAL AND METHODS

In research *in vitro* breast cancer cell line (T47D ECACC 85012201) was used. These cultures were incubated in standard conditions (RPMI 1640, 10% FBS, antibiotics, 37°C, 5% CO_2, 90% humidity of the air). Paclitaxel was administered to such cultures in the following doses: 60 ng/ml (P60 group) and 300 ng/ml (P300 group). These concentrations are equivalent to standard polytherapeutic and monotherapeutic doses. The control group was breast cancer cells *in vitro* incubated without paclitaxel.

TRNA from the cells was isolated and RT-PCR reaction was performed to estimate cDNA. The samples were exposed to specific human gene cancer primers (Human Cancer cDNA Labeling Primers – SIGMA). By exposing a reverse transcription reaction in buffor nucleotides labeled 32P cDNA were estimated. This product was purified in Sephadex column (Sephadex G-25) by centrifugation. Such cDNA were hybridized on the soil in a hybridization chamber during 18 hours. The matrix was washed after this process and stored in a special cassette containing radiosensitive soil. Then the soil of the matrix was scanned on a high resolution scanner detecting 50 microne points. The number of activities from points which indicated the level of expression following the genes was compared with a the model level of activity of E. coli-B1444 gene located on the matrix.

The next step was counting the activity from points of the matrix including the activity of individual genes in a control group and in groups where paclitaxel was administered (15).

Genes participating in apoptotic process were divided into the groups: apoptotic (BAD, BID, BIK, DEDD, BOK, CIDEP, CRADD) and antiapoptotic (BCL1,2,2A1,3,6,7A,9, BAX, BAG-1, DED,) caspases 1-10, 13-14). After estimation of the view of activity it was put in the computer created network.

Genes	Number	Example of Genes
Apoptotic genes	37	BAD, BID, BIK, DEDD, BOK, CIDEP, CRADD
Antiapoptotic genes	12	BCL1,2,2A1,3,6,7A,9, BAX, BAG-1, DED, DAD1,
Caspases genes	13	CASP1-10, 13-14,

RESULTS

Table 1. Expression of apoptotic and caspase genes in breast cancer cell line (T47D) after treatment of paclitaxel

	Control	Dose of paclitaxel 60 ng/ml	Dose of paclitaxel 300 ng/ml	P
Proapoptotic genes N=37	6.05	19.20*	4.15*	< 0.001
Antiapoptotic genes N=12	5.02	15.25*	4.95	< 0.001
Caspase genes N=13	4.85	14.92*	3.55*	< 0.001

The analysis showed that in a group of cells where paclitaxel was administered in a lower do 60 ng/ml it caused a statistically significant increase into the expression of pro and antiapoptotic genes and genes coding caspases in comparison to the control group.

A statistically significant correlation was noted between the control group and the first group for apoptotic genes, and a statistically significant correlation between the control group and the second group for the mentioned genes.

CONCLUSIONS

Results of the study showed that in a group of cells where paclitaxel was administered in a lower dose – 60 ng/ml it caused a statistically significant increase expression of pro and anti- apoptotic genes and genes coding caspases in comparison to the control group

High levels of expression in many genes connected with acceleration of proliferation can be related with apoptotsis of cancer cells which induces sensitivity of this cells to paclitaxel.

A significant decrease of caspases and apoptotic gene levels after administration of paclitaxel in a dose 300 ng/ml indicates the evident cytotoxic effect, which leads to the inhibition of the majority of cellular processes.

Activation of many ways connected with processes preceding G2/M phase can indicate that paclitaxel influences earlier control of cancer cell cycle phases.

DISCUSSION

In our investigation we found an increase of antiapoptotic gene expression derived from Bcl2 family and also a similar increase in the level of proapoptotic genes. It is strange that a similar level in these two opposite group of genes induces only apoptosis processes. Inhibition of cell growth and death of the cells was seen after administration of paclitaxel in a dose 60 ng/ml. There are different explanations of this process. One of them can be connected with some domination of the expression of apoptotic genes. This hypothesis dose not explain entirely why the processes of apoptosis are so intensive in the breast cancer *in vitro*. Another explanation can be connected with a probably greater intensity of metabolic pathways in these cultures (bigger

number of this processes in comparison to antiapoptotic). This suggestion could be supported by described in many reports possibility of phosphorylation of products of Bcl-2 by many proteins, which leads to inhibition of Bcl-2 activity. Such proceses are seen when MAPK is activated. Another kinase which is responsible for the mentioned phosphorylation BCL-2 is PKA (activated by microtubuls fractures) [8] and is also connected with the activation of cRAF kinase which leads to the activation of kinase A and is dependent on the degree of polimerysation of microtubules. In addition, phosphorylated form of BCL-2 can activate caspase 3 which can lead to an irreversible apoptotic process. Apoptotic processes have prevalence because in the cells a very intense process of phosphorylation of BCL-2 products is seen. It leads to inhibition of antiapoptotic products and escalates apoptosis processes in the mechanism of activation of many caspases. Selective activation of apoptosis in T47D cell line should be consider under influence of paclitaxel and sensitivity to this drug. In reports information appears that paclitaxel can activate inhibition of mitotic process [13]. One of the mentioned mechanisms is concerned with serine-threonine kinase cMOS. It was noticed this protein can be stored during mitotic division of the cells and is responsible for phosphorylation of tubulin protein.

Chang et all. [4] noticed an increased level of 78 of 92 genes coding apoptotic and thermal shock proteins in 48% of 24 women with breast cancer treated with paclitaxel. It indicates that this drug influences the activation of products concerned with apoptosis. It can explain why apoptotic processes have so strong manifestation in the treated lines of the cells. In the activation of apoptosis by paclitaxel we can find at least 3 ways: phosphorylation BCL-2, activation of apoptosis during inhibition of cell division and direct activation of apoptotic products. Another explanationof the domination of apoptosis process in these cultures is possible. Research conducted on T47D line reveals that increased expression of BIC product and ER-2 receptors is observed after administration of beta estradiol. In cultures we also observed increased expression of Bic. It is known that BIC can bind with BCL-2 protein and inhibit its activity. Perhaps this way of activation is significant and has strong influence on the inhibition of BCl-2 product. Additionally, another raport [7] described protein DAXX which belongs to apoptotic products. This protein interacts with repressors of transcription such as PAX5, ETS-1 and reduces their expression. DAXX activates many ways concerned with apoptosis [9,10]. They are connected with increased expression of genes coding caspases, transcriptor factors as well histone proteins. In connection with activation of ASK-1 it is a very important way of apoptosis. A high level of this protein induces the activity of caspases 8 and 9, NFk-B and E2 F1. It can prove that in cell cultures irreversible apoptotic processes dominate impossible to inhibition even if the level of BCL-2 is high. These processes are induced by irreversible degradation for cell organella by caspases. It is the reason for advantageous apoptotic process despite the high level of antiapoptotic products even in active forms. Activation of apoptosis is induced by caspase 3. It interacts with ICAD which is the reason for the lower activity of CAD and leads to activation of DNA-se. In this process CIDE participates which, after connection with DFF45, activates DNA-se in the same way. Another pro-apoptotic protein DAP-3 binds with the inner mitochondrial membrane. It activates apoptosis by binding to DISC complex through TRAIL, FAS and FADD [1,3,12]. It probably stops translation process in the matrix of mitochondrium by replacing the active product of translation in the site of initiation.

The increase of the level of apoptotic genes has clinical significance. The statement of percentage of activity pro and antiapoptotic genes in relation to all group of activated genes and also estimation of the degree of their expression and gene classification can estimate the degree of malignancy of the neoplasm, sensibility to treatment. In breast cell cancer MCF-7M increased expression of Bcl-2 an low p-53 and Bax was observed [9,11,17].

The increase of the expression of antiapoptotic genes after administration of paclitaxel in a dose 60 ng/ml requires explanation In spite of the fact that the examined cell showed intense division. These observations suggest that expression of antiapoptotic genes should not be so intense. It is possible that expression of these genes is less that proapoptotic. Another explanation suggests the levels of both group are similar, but because of an unknown cell mechanism prevalence is achieved by proapototic genes.

It is possible that in this process caspase activation has significance, which leads to an irreversible reaction causing degradation of cell structures.

Paclitaxel in a dose 60 ng/ml is activated opposing by groups of pro and antiapoptotic genes. The probable reason for the advantage of apoptotic processes over antiapoptotic processes in these cancer cells may be explained by activation of a significant numbers of ways connected with phosphorylation of Bcl-2 gene products.

It was proved that activation of apoptotic process is connecteded with the proper relation between pro and antiapoptotic genes. It should be examined whether such ways of activation are connected with the influence of paclitaxel, or the type of activity of the following cancer cell lines. It was stated that beginning of apoptosis process is connected with a lasting proper relation the expression of Bax/Bcl-2, which influence the mitochondrial potential and increase of permeability of mitochondrial membrane. The proper relation of these group of genes influence the activation or inactivation of apoptosis. This information indicates that induction of apoptosis is concerned with the proper relation both of these groups.

Chang and al. [4] conducted the analysis of the expression of genes treated with docetaxel in the base of RNA extracted after biopsy conducted in 24 women with breast cancer. Reports showed overexpression of 14 from 92 antiapoptotic genes in 54% patients. It is possible that the expression level is responsible for the difference in the reaction after treatment with paclitaxel. Perhaps this reaction is dependent on the relation between the level of many groups of genes.

Possible reason high activity of antiapoptotic genes, and their simultaneous weak influence on inhibition of apoptotic process can be an effect of higher expression mentioned genes connected with phosphorylation their protein products inducted by paclitaxel.

The reasons for the decrease in the antiapoptotic activity of Bcl-2 can be different. It is possible the an increase of resistance of the cell to this drug caused by the increase of the activity of antiapoptotic genes. Probably it activates enough of these genes and their product sand leads to anti-apoptotic process. In this activity opposite mechanisms can be activated. For instance, BAX can influence Bcl-2 and induce inactivation of this gene. It can activate dimerisation and translocation in mitochondria which leads to programmed cell death.

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SUMMARY

Our analysis was connected with estimation of changes in the expression of genes participating in the apoptotic process under the influence of paclitaxel applied in breast cancer cells *in vitro*. Increase of the expression of antiapototic genes after administration of paclitaxel in a dose 60 ng/ml requires

explanation. These observations suggest that the expression of antiapoptotic genes should not be so intense. It is possible that the expression of antiapoptotic genes is less that proapoptotic. Another explanation suggests the levels of both group are similar, but because of an unknown cell mechanism the prevalence is achieved by proapototic genes. It is possible that in this process caspase activation has significance, which leads to a irreversible reaction causing degradation of cell structures.

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

Keywords: paclitaxel, apoptosis, breast cancer, caspases, microarray method

STRESZCZENIE

Badania nasze miały na celu określenie zmian ekspresji genów uczestniczących w procesie apoptozy pod wpływem paklitakselu dodawanego do komórek raka piersi *in vitro*. Wzrost ekspresji antyapoptotycznych genów po podaniu paklitakselu w dawce 60 ng/ml wymaga wyjaśnienia. Badania sugerują, że ekspresja tych genów nie powinna być tak intensywna. Możliwe jest, że ekspresja antyapoptotycznych genów jest niższa niż genów proapoptotycznych. Inne tłumaczenie to podobne poziomy ekspresji obu grup genów z nieznanym mechanizmem komórkowej regulacji genów proapoptotycznych. W procesie tym dużą rolę może odgrywać aktywacja kaspaz, która prowadzi do nieodwracalnej reakcji powodującej uszkodzenie organelli komórkowych. Paklitaksel w dawce 60 ng/ml aktywuje dwie antagonistycznie działające grupy genów: pro- i anty- apoptotyczne. Przewagę proapoptotycznego procesu nad antyapoptotycznym w komórkach nowotworowych piersi można tłumaczyć aktywacją wielu szlaków związanych z fosforylacją białek kodowanych przez gen Bcl-2.

Słowa kluczowe: paklitaksel, apoptoza, rak piersi, kaspazy, metoda microarray