NFκB is a transcription factor discovered by Sen and Baltimore in 1986, in the kappa light chain of immunoglobulins in B cells (1,2). Research over the last few years has revealed that NFκB is an inducible and ubiquitously expressed transcription factor for genes involved in cell survival, cell adhesion, inflammation, differentiation and growth (1, 2).

Cellular responses to a wide variety of diverse stimuli have been identified and have been shown to lead to the activation of NFκB (2). These stimuli reveal that NFκB is a common pathway for cellular adaptation to stress. Several studies have shown that different agents such as, ionizing radiation, reactive oxygen species and antineoplastic drugs, change the expression of many genes by affecting transcription factors including NFκB (3–5).

NFκB is a member of the Rel family of transcription factors present in various cell types. In unstimulated cells it is complexed with the NFκB heterodimer, typically comprising p50 and p65 (Rel A) subunits and the IkBα as the inhibitory subunit. This inactive complex resides in the cytosol. Upon cell stimulation, IkBα is rapidly phosphorylated with the participation of IKK-kinase complex, degraded via the ubiquitin-proteasome pathway and then, the liberated NFκB is able to translocate into the nucleus to activate the target genes by binding to regulatory elements in enhancers and promoters (2, 6). The degradation of inhibitory unit is the prototypical but not the only pathway of NFκB activation (3).

Recently, NFκB has been found to be critically important for control of cell proliferation, apoptosis and tumour development (7). NFκB target genes, implicated in cell death prevention, include those encoding the tumour necrosis factor (TNF); the inhibitor of apoptosis proteins c-IAP1, c-IAP2, X-IAP, the receptor-associated factors TRAF-1 and TRAF-2 (8, 9).

Abstract: NFκB, a member of the Rel family of transcription factors has been found to be critically important for control of cell proliferation and apoptosis. Although rare, there are systems in which NFκB has occurred as pro-apoptotic factor. The potent activators of NFκB are anthracycline anticancer drugs which induce the events of apoptosis. These results could point to the pro-apoptotic role of NFκB. Recent studies have shown that activation of that transcription factor well correlated with cytotoxic activity of anthracyclines. Potential mechanism through which NFκB could play a role in tumorigenesis involves its constitutive activation which was shown in a wide variety of tumour types. The aim of this study was to define the level of activity of NFκB in the native human tumour cells differing in sensitivity to anthracycline analogs to study the mechanism responsible for cytotoxic action of these drugs. Constitutive activation of NFκB was determined with use of ELISA test and confocal microscopy. As was shown, both methods have occurred as quite comparable. Constitutive activation of NFκB was observed in all neoplastic cells tested independently on sensitivity to anthracyclines. However, these results do not exclude the supportive role of NFκB in apoptotic activity of these drugs. It is necessary to study the influence of the compounds tested here on NFκB activation and on the induction and intensity of apoptotic processes.

Keywords: NFκB; anthracyclines; cytotoxicity; constitutive activation

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Cellular responses to a wide variety of diverse stimuli have been identified and have been shown to lead to the activation of NFκB (2). These stimuli reveal that NFκB is a common pathway for cellular adaptation to stress. Several studies have shown that different agents such as, ionizing radiation, reactive oxygen species and antineoplastic drugs, change the expression of many genes by affecting transcription factors including NFκB (3–5).

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Although rare, there are systems in which NFκB has been shown to play a pro-apoptotic role in addition to its more common anti-apoptotic. Examples of its pro-apoptotic activity in cells include B- cells, T- cells, granulocytes, macrophages, neuronal cells (2). The opposing effects of NFκB are thought to be cell type specific and/or dependent on the inducing signal. Different activation pathways of NFκB may cause the expression of proteins that promote or inhibit apoptosis (2).

The potent activators of NFκB are anthracycline anticancer drugs (3, 4, Gruber unpublished). Similarly to other NFκB activators, their effects appear to involve reactive oxygen species but also DNA lesions generation. It has been shown that anthracyclines activate the events of programmed cell death (apoptosis) in cells which may be a mechanism underlying their therapeutic efficacy in certain tumour types. Above studies could point to the pro-apoptotic role of NFκB. Recent studies have shown that activation of this transcription factor induced by anthracycline analogs well correlated with cytotoxic activity of those compounds (4).

Potential mechanism through which NFκB could play a role in tumorigenesis involves its constitutive activation (2). Such kind of activation was shown in a wide variety of tumour types including melanoma, leukemia, pancreatic cancer, prostate carcinoma (2, 10–13) and it was linked to resistance to apoptosis.

It is tempting to believe that in some cases such as anthracycline therapy, constitutive activation of NFκB accounts for the sensitivity of the cells to the drugs.

The aim of this study was to define the level of activity of NFκB in the native human tumour cells i.e., melanoma cells and cervix carcinoma cells differing in sensitivity to anthracycline analogs to study the mechanism responsible for cytotoxic action of these drugs.

The searching of the main cytotoxic activity mechanism of anthracyclines is the pivotal matter because of the drug resistance which is the big obstacle in antitumour therapy.

Constitutive activation of transcription factor was determined with use of confocal microscopy and with ELISA test provided by BD Mercury™ Transfactor Kit.

**EXPERIMENTAL**

**Drugs.** DOX (doxorubicin) purchased from Fluka, Germany; ANN (annamycin) and WP903 new anthracycline analogs received from MD Anderson Cancer Center, Houston TX, USA.

**Cells.** ME18 – human melanoma cells obtained from the Centre of Oncology, Warsaw, Poland; ME18/R- its subline resistant to DOX, obtained experimentally (14); HeLa – human cervix carcinoma cells; KB-V1 – its subline resistant to vinblastine from American Tissue Culture Collection.

Cells were grown in MEM supplemented with 10% fetal calf serum and antibiotics. ME18/R cells were maintained in MEM with DOX, 1 µg/mL and KB-V1 cells in MEM with vinblastine, 0.5 µg/mL.

**MTT test.** The suspensions of the cells were diluted in MEM to 10 cells/mL and 0.05 mL of each suspension was placed into individual wells on a 96-well multiplate. Then, 0.05 mL of each drug solution in MEM was added at double strength dilution for 48h. The final concentrations of DOX, ANN and WP903 ranged from 0.058 to 5.8 µg/mL. The cells treated as controls were kept in drug-free MEM.

MTT test was performed as was given earlier (15). The IC₅₀ values were determined as the concentrations of the drugs required for 50% cell growth inhibition.

**Preparation of nuclear extracts.** The cell pellets were washed with cold PBS and resuspended in lysis buffer A (1 M Hepes, pH = 7.9/ 2 M KCl/ 0.5 M EDTA, pH = 8.0/ 0.1 M EGTA, pH = 7.0) with addition of 0.1 M DTT, 100 mM PMSF, leupeptin 1 mg/mL, aprotinin 1 mg/mL and benzamidine 250 mg. During one hour incubation on ice with intermittent vortexing, the cells were disrupted and nuclei were released visible as the transparent pellet.

Nuclear extracts were obtained by incubation of that pellet on ice for two hours in lysis buffer B (1 M Hepes, pH = 7.9/ 5 M NaCl/ 0.5 M EDTA, pH = 8.0/ 0.1 M EGTA, pH = 7.0) with addition of 0.1 M DTT, 100 mM PMSF, leupeptin 1 mg/mL, aprotinin 1 mg/mL, benzamidine 250 mg and with frequent intermittent vortexing. Supernatants (nuclear extract) were analysed with ELISA test.

**ELISA test.** BD™ Transfactor Kit provides rapid, high-throughput detection of specific transcription factor activity in cell extracts. Using an enzyme-linked immunosorbent assay (ELISA)-based format, the Transfactor kit detects DNA binding by specific transcription factors. Each kit contains a 96-well plate with the consensus binding sequence for NFκB p50 or p65 subunits, coated in 80 wells, and a mutant binding sequence (negative control) coated in 16 control wells.

In our study, nuclear cell extracts were incubated in the wells to allow the NFκB subunits bind to their consensus sequences. Bound transcription factors were then detected by specific primary an-
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Antibodies i.e., p50 – with whole rabbit antiserum; p65 – with rabbit polyclonal IgG, 1 µg/µL. A horseradish peroxidase – conjugated secondary antibody (goat anti – rabbit IgG – HRP, 1 µg/µL) was then used to detect the bound primary antibody. The enzymatic product formed in colorimetric reaction (according to the kit procedure) could be measured with an absorbance microtiter plate reader at 650 nm.

Nuclear extracts of HeLa cells treated with TNFα (the strong NFκB activator) were used as a positive control.

Confocal microscopy. Cells growing on the cover glasses were fixed for 5 min in acetone (20°C), washed with PBS and then, blocked with normal goat serum (Oncogene NSO2L) for 30 min at room temperature. After washing with PBS, the cells were overlaid with polyclonal antibodies (1:66): NFκB (p65RelA) (ab-1) (Oncogene PC137) or NFκB (p50) (ab-1) (Oncogene PC136) and complexed with the fluorochromes Alex 647 (Z-25302 Molecular Probes) or Alex 488 (Z-25302 Molecular Probes), respectively, according to the manufacturer’s procedure. Next, the complexes antibody – fluorochrome were fixed with paraformaldehyde, 4% for 15 min and then washed with PBS. After dying with propidium iodide (PI) and RNase, the glasses were placed in the mounting medium – Prolong™ Antifade kit (Molecular Probes) and analysed with use of confocal microscopy, Olympus FV500 with the lasers: Ar-488nm; He-Ne-543 nm and He-Ne-633 nm.

Microscopy images were analysed with use of program FluoView, ver. 4.3.

Statistical analysis. The statistical evaluation of the results was performed using Student’s test and Cochrane’s Cox test for unrelated samples.

RESULTS

Cytotoxicity assay

Cytotoxic activity of the tested drugs was determined with use of MTT test. As was shown in Table 1, parental ME18 cells exhibited much more sensitivity to all anthracyclines tested than its subline resistant to DOX – ME18/R. HeLa and KB-V1 cells exhibited comparable sensitivity to all drugs tested.

Table 1. IC50 values [µg/mL] determined with use of MTT test

<table>
<thead>
<tr>
<th>DRUG</th>
<th>ME18</th>
<th>ME18/R</th>
<th>HeLa</th>
<th>KB-V1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>7.10±1.85</td>
<td>28.30±1.20</td>
<td>0.52±0.08</td>
<td>0.61±0.09</td>
</tr>
<tr>
<td>ANN</td>
<td>0.58±0.11</td>
<td>28.30±1.45</td>
<td>1.45±0.03</td>
<td>1.38±0.08</td>
</tr>
<tr>
<td>WP903</td>
<td>0.93±0.26</td>
<td>3.40±0.27</td>
<td>0.57±0.04</td>
<td>0.54±0.02</td>
</tr>
</tbody>
</table>

The cells were exposed to the drugs, each used in the range 0.058-5.8 µg/mL for 48 h, then MTT test was done as described in Experimental. Results represent the mean ±SEM (n=8-16).

*IC50 – values statistically different from IC50 defined for the same compound in ME18/R cells (p<0.05 for all results).

Figure 1. Transcription factor profiling in nuclear extracts from 48 h cell cultures. HeLa cells stimulated with TNFα was the positive control. Background control was prepared with all reagents excluding nuclear extract. Results represent the mean of 2–6 experiments.
NFκB activation
ELISA test

In this part of the study, NFκB activation was detected with use of specific primary and secondary antibodies for p50 or p65 as was described in Experimental. The possible changes in activation of NFκB were observed as colorimetric reaction and noted as absorbance (A<sub>650nm</sub>).

As was shown in Figure 1, there are different NFκB constitutive activation dependently on the kind of cells. In this study it was noted the slightly increased level of p50 and p65 in nuclear extracts from HeLa and KB-V1 cells. All levels were comparable except p50 in HeLa cells. Its increase was noted as twice higher than in KB-V1 cells. As far as melanoma cells is concerned, the slight increase of p65 only was observed in ME18 cells. No activation was noted in ME18/R cells.
Confocal microscopy

This method allows to follow the localization of NFκB subunits throughout the individual cell using specific antibodies complexed with fluorochromes. Images visible under microscope were analysed with use of the program FluoView ver. 4.3.

As was shown in Figure 2, all kind of the tested cells characterized with the increased activity of NFκB. The strongest constitutive activation of NFκB was observed in ME18/R cells (the increased level of p65 subunit in the nucleus) (Figure 2b) and in KB-V1 cells (the increased levels of p50 and p65 subunits in the nuclei, simultaneously) (Fig. 2d). The weaker effects of native activation of NFκB were observed in ME18 and HeLa cells (Figures 2a, c).

Figure 2. Translocation of p50 or p65 to the nuclei presented as the intensity of fluorescence signal obtained with use of FluoView ver. 4.3. Cell cultures were prepared as described in Experimental. Light lines – p50; dark lines – p65. Me18 cells (a); ME18/R cells (b); HeLa cells (c); KB-V1 cells (d).
DISCUSSION

One of the potential mechanisms through which NFκB could play a role in tumorigenesis involves its constitutive activation. The activation of this transcription factor occurs as its transport from the cytoplasm to the nucleus upon degradation of the inhibitory subunit. In the nucleus, the activated factor binds to specific κB sites on the DNA and mediates the expression of a number of genes involved in the cellular response to various stresses. When NFκB is found persistently in the nucleus, it is referred to constitutive activation (2, 13).

In the present work, NFκB activation was studied with two new methods, ELISA test and confocal microscopy. The latter one eliminates the necessity of nuclear extract preparation. NFκB is detected in the intact fixed cells. Such procedure does not allow to lose the searched proteins. The same, confocal microscopy seems to be the more reliable method for detection of NFκB subunits present in the nucleus.

In this study, the results obtained with these both methods were qualitatively mostly consistent. In regard to quantitative analysis, the intensities of signals obtained with each of these methods were not comparable. On the basis of ELISA test it is only possible to say that the levels of both subunits of NFκB are a little increased in reference to the background control and this effect points to the activated transcription factor in the tested cells. This discrepancy may be explained with different initial procedure as was mentioned above. Besides, there could be some differences in specificity or sensitivity of antibodies used in both methods. Especially, that much lower level of NFκB proteins with use of ELISA test were observed in all experiments independently on the nuclear extract tested.

Generally, the current studies did not bring the expected results. The expected correlation between the constitutive activation of NFκB and the increased sensitivity to anthracyclines in the cell lines tested was not observed. Conversely, constitutive activation of NFκB seemed to be related to anthracycline resistance which is, in turn, consistent with the results obtained by Arlt et al. (16) concerning human pancreatic carcinoma cells resistant to DOX.

The strong activation of NFκB observed as p65 subunit detection with confocal microscopy in the nuclei of ME18/R cells is in agreement with the literature data concerning human melanoma cells. Many authors point to the fact that melanoma cells have the increased level of p65 subunit (6, 11, 13, 17). Shattuck–Brandt et al. (13) noted that Hs294T melanoma contained 19-fold more immunoreactive NFκB than those observed in normal retinal pigment epithelial cells. This increase in NFκB p65 correlated with increased NFκB DNA binding activity in Hs294T nuclear extracts. Constitutively elevated RelA (p65) was also shown by Mc Nulty et al. (17) in human metastatic melanoma cultures relative to normal melanocytes. The latter effect suggests the positive relationship between NFκB and invasiveness of the neoplastic cells which supports the antipoptotic role of NFκB.

The results obtained here are consistent with the literature data that the persistent activation of NFκB is characteristic for a wide variety of tumours types (2, 10, 18–19). As was shown in here, that observation includes human melanoma cells and cervix carcinoma cells.

On the basis of this study is not possible to draw precise conclusions concerning the role of NFκB in anthracycline cytotoxic action. However, the observations made do not exclude the supportive role of this transcription factor in apoptotic activity of anthracyclines as was exhibited by Ashikawa et al. (4) and Gruber (unpublished). The lack of constitutive activation in the neoplastic cells (human T- cell leukemia Jurkat cells, human non-small cell lung carcinoma cells and human myeloid KBM-5 cells), shown by these authors, did not have any relevance with the anthracycline – stimulated NFκB activation which, in turn, correlated with cytotoxic activity of these drugs.

So, the pivotal step in our further work on that matter will be to study the influence of the chosen anthracyclines on NFκB activation and on the induction and intensity of apoptotic processes in human melanoma and human cervix carcinoma cells.

REFERENCES

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