In vitro study on the effect of doxorubicin on the proliferation markers MCM3 and Ki-67

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Summary

Purpose: Aberrant proliferation is an essential feature of cancer cells, which can be caused by alterations in components of the cell cycle, such as minichromosome maintenance protein-3 (MCM3) and Ki-67. Doxorubicin is a cytotoxic/cytostatic anticancer agent commonly used in chemotherapy. We investigated the effect of this drug on MCM3 and Ki-67 in the KB cell line, which is considered a subline of HeLa cell line.

Methods: KB cells were treated with doxorubicin and its effect on apoptosis, mRNA levels and protein expression of MCM3 and Ki-67 was determined by flow cytometry (annexin V-FITC/PI assay), quantitative real-time RT-PCR (qRT-PCR) and immunocytochemistry, respectively. Cytotoxicity was assessed using the MTT assay. One-way analysis of variance (ANOVA) was used for comparing groups and differences were assessed by a Tukey’s post hoc test.

Results: Protein expression of both biomarkers and MCM3 mRNA were not affected by doxorubicin, but Ki-67 mRNA significantly increased after treatment (p=0.049).

Conclusions: Considering that doxorubicin can influence certain biochemical events that lead to modifications in Ki-67, this factor might be useful in evaluating the impact of anthracycline-based chemotherapeutic agents. Changes in MCM3 following doxorubicin treatment require further investigation.

Key words: doxorubicin, Ki-67, minichromosome maintenance protein

Introduction

Dysregulation of cell growth and proliferation is considered a hallmark of cancer [1], which has been extensively investigated in different tumors, mostly to exploit its prognostic significance [2-5]. Proliferation markers can also be used to evaluate the biologic behavior of neoplasms and their response to various therapeutic agents and even to predict the need for adjuvant chemotherapeutic treatments [6]. Several methods have been applied in different studies to determine cellular proliferation, which mostly rely on their ability to assess specific phases of the cell cycle. Examples of these techniques may be as simple as a mitotic count that shows cells in the M phase to the more sophisticated bivariate flow cytometry subsequent to in vivo pulse labeling [6]. Cell-cycle related proteins such as Ki-67 and replication regulating antigens like the minichromosome maintenance protein (MCM) family have also been employed as proliferation markers [7-9].

The Ki-67 antigen was initially introduced by Gerdes et al. [10] in 1983 and has since received considerable attention. Due to its exclusive expression during the G1 to M phases of the cell cycle, it was suggested to be an acceptable marker when evaluating cell proliferation [4,11,12]. MCM proteins consist of 10 factors involved in DNA synthesis of which MCM2 through MCM7 constitute a family with similar functions and activities [13]. This group has an important role in DNA replication and is expressed in proliferating cells but not in those that have left the cell cycle [2,7]. In addition to its usefulness as a proliferation marker, which has been attested to by several studies
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[2,5,7], MCMs 2-7 are also suggested to exhibit replicative helicase activity in vivo [14]. The distinctive presence of Ki-67 and MCMs in the different phases of the cell cycle [9,11,12,15,16] may affect their application in clinical and research settings. Comparison of these markers in various human tumors has led to diverse results as to which is more reliable in showing proliferating cells [2,3,5,17,18] and most studies have favored the MCM family [2,5,7,19,20]. Doxorubicin (adriamycin) is an anthracycline antibiotic that has been widely used in a variety of malignancies for many years. This drug acts through numerous mechanisms finally leading to cytotoxic and cytostatic effects, which have been suggested to be cell-cycle-dependent [21].

The aim of the present study was to evaluate the mRNA level and protein expression of Ki-67 and MCM3 cell-cycle-associated factors in the KB cell line, before and after doxorubicin therapy. It is noteworthy that the KB cell line, initially isolated from an oral squamous cell carcinoma, has been reported to be contaminated with HeLa cells and is therefore regarded as a subline of these cells [22].

Methods

All tests were performed in triplicate and repeated at least 3 times.

Cells and cell culture

The KB cell line (ATCC CCL-17, USA), was purchased from Pasteur Institute Cell Bank, Tehran, Iran. Cells were cultivated in monolayer culture at 37°C under an atmosphere of 5% CO2 and 95% air in a humidified incubator. Growth medium consisted of RPMI 1640 (BioSera, UK) along with penicillin-streptomycin (100 U/ml).

Cell viability assay

MTT assay was used for estimating the inhibitory effect of doxorubicin on the growth of KB cells [23]. Briefly, KB cells were distributed into 96-well plates at 5000 cells per well for 48 h. Wells were divided into doxorubicin and RPMI control for the assessment of IC50.

Annexin V-FITC and propidium iodide (PI) staining

Analysis of apoptosis was done by incubating the doxorubicin- or RPMI-treated cells with Annexin V-FITC and PI (APOPTEST™-FITC kit, NEXINS RESEARCH, the Netherlands), according to the recommendations of the manufacturer. Data acquired from 104 cells were analysed utilizing a PARTEC flow cytometer equipped with FloMax software [25].

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The mRNA expression of MCM3 and Ki-67 was quantified using qRT-PCR.

KB cells were kept in T25 flasks containing RPMI medium, FBS and penicillin-streptomycin for 24 h prior to treatment with the IC50 concentration of doxorubicin, which continued for a further 48 h. Total RNA was extracted from control and drug-treated cells using Tri-Pure isolation reagent (Roche, Germany). Complementary DNA library was prepared by adding 2 μg total RNA to M-MLV reverse transcriptase (Fermentas) and oligo (dT)18 and incubation for 60 min at 42°C and 10 min at 94°C. Real time PCR was performed for MCM3 with β-actin serving as an internal control. For this purpose, 2 μl complementary DNA along with 0.5 μl of each forward and reverse primers (0.05 millimolar) for MCM3 (forward: 5’–CTGAAGGGCAGAAGATGGTGTG-3’; reverse: 5’–GATGGGAAAGTGGCCGATGAG–3’), Ki-67 (forward 5’–GAGAAAGGATGGAAGTGCACGC–3’; reverse 5’–TCGGAAGACCACCTCTTCTCCT–3’) [23] and β-actin (forward: 5’–TACCTGCGCCCTCAGGAGGAGCAA–3’; reverse: 5’– GTCCGTGGGAATCCAGAATGAA–3’). A reaction mixture (20 μl) containing 10 μl QuantiTect Syber Green Master mix (2x) and DEPC water along with the primers and complementary DNA was prepared. The amplification reaction included 10 min of predenaturation at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, 10 s of annealing at 60°C (57°C for β-actin) and extension for 10 s at 72°C. At the end, a cycle of final extension was carried out for 2 min at 72°C. Relative mRNA expression was analysed using the threshold cycle of both markers relative to that of β-actin.

MCM3 and Ki-67 protein expression assessed by immunocytochemistry

KB cells were maintained in a humidified CO2 incubator for 48 h at 37°C, and then treated with the IC50 concentration of doxorubicin or RPMI 1640 (control) for 72h at the same temperature. Cell fixation was carried out at -20°C for 30 min using methanol: acetone (9:1) after washing them twice with cold PBS. The fixed cells were then incubated with 3% hydrogen peroxide in methanol for 50 min to block endogenous peroxidase activity. Ultra V block (Labvision, USA) was applied for 10 min to reduce non-specific staining. MCM3 primary rabbit polyclonal antibody (1:500 dilution; ab4460; AbCam, USA) and Ki-67 rabbit monoclonal antibody (1:150 dilution; clone SP6; LabVision, USA) were added to the KB cells, incubated overnight at 4°C and visualized employing a streptavidin-biotin immunoperoxidase detection kit with aminoethylcarbozol as the substrate (Labvision, USA) and Mayer’s hematoxylin.
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1. Real time qRT-PCR quantification of MCM3 and Ki-67 before and after administration of doxorubicin

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Cycle threshold (CT) (Mean ± SE)</th>
<th>ΔCT (^a)</th>
<th>2^ΔCt</th>
<th>2^-ΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM3 (RPMI)</td>
<td>16.16±0.93</td>
<td>0.78</td>
<td>0.58</td>
<td>2.08</td>
</tr>
<tr>
<td>MCM3 (Dox)</td>
<td>16.57±1.1</td>
<td>1.64</td>
<td>0.28</td>
<td>0.48</td>
</tr>
<tr>
<td>Ki67 (RPMI)</td>
<td>20.25±1.8</td>
<td>4.87</td>
<td>0.03</td>
<td>0.24</td>
</tr>
<tr>
<td>Ki67 (Dox)</td>
<td>17.36±1.05</td>
<td>2.83</td>
<td>0.14</td>
<td>4.11</td>
</tr>
<tr>
<td>β Actin (RPMI)</td>
<td>15.58±0.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β Actin (Dox)</td>
<td>14.55±0.56</td>
<td></td>
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</tbody>
</table>

\(^a\)ΔCT: (Target gene CT - β-Actin CT), Dox: doxorubicin, SE: standard error

(DakoCytomation, Denmark) as counterstain. Omission of the primary antibody was performed in order to obtain negative controls. The expression of MCM3 and Ki-67 antigens was determined under a light microscope and stain intensity was scored as weak (+), moderate (++), or strong (+++).

Statistics

The data presented in the current investigation (means ± SE) were gathered through performing each test in triplicate and repeating each condition 3 times. One-way analysis of variance (ANOVA) was used for comparing groups and differences were assessed by a Tukey’s post hoc test. P values < 0.05 were considered significant.

Results

Effect of doxorubicin on cell viability

KB cells were treated with doxorubicin under standard laboratory conditions and an IC\(_{50}\) of 150 nM was obtained after 3-day exposure, which was used throughout the current investigation.

Apoptosis induction

KB cells were incubated with RPMI or the IC\(_{50}\) concentration of doxorubicin. The required protocol for Annexin V-FITC and PI staining was followed and the results of total apoptotic cells were recorded as 17.35 ± 5.37 and 5.16 ± 1.88 for doxorubicin and RPMI, respectively. Statistical analysis indicated a significant difference (p<0.0001) in apoptotic cell percentage between doxorubicin-treated (48h) and control cells. As shown in Figure 1, this difference was most prominent in the G2/M phase of the cell cycle.

mRNA expression of MCM3 and Ki-67

The mRNA expression levels of MCM3 and Ki-67 was assessed in doxorubicin-treated KB cells by means of qRT-PCR. Accordingly, a significant increase (p=0.049) in Ki-67 (as reported in a previous study of ours) but no difference (p=0.813) in MCM3 mRNA was observed after administration of doxorubicin in comparison to RPMI (Table 1).

Immunocytochemical expression of MCM3 and Ki-67

Antigen expression of both markers was assessed under a light microscope following immunocytochemical staining with polyclonal or monoclonal antibodies. Both proteins were localized in cell nuclei and demonstrated strong immunostaining (+++) with and without doxorubicin treatment. Reactivity did not differ between the test and control KB cells (Figure 2).

Discussion

In the present study, the proliferation associated factors Ki-67 and MCM3 were evaluated in the KB cell line before and after treatment with doxorubicin.

Ki-67 is considered as one of the most widely used markers for identification of the proliferative activity of neoplastic cells [4,9] and it is thought to participate in cell division through regulation of the nucleoli’s life cycle [4]. According to the results obtained in the current investigation, a significant increase occurred in Ki-67 mRNA following incubation with doxorubicin, but there was no change in the expression of its protein. The exact function and role of Ki-67 in proliferation and cell cycle control remains unclear [5,7,15,19]. A number of studies have suggested that it may not be necessary for proliferation [5,15,24] and it has been implicated in cell cycle independent processes such as ribosome biosynthesis [15,19]. Therefore it does not seem possible to present a definitive explanation for our findings. Sasaki et al. [12] in a study of Ki-67 in HeLa S3 cells, showed an increase in Ki-67 antigen after treatment with doxorubicin. In addition, inhibition of DNA synthesis led to increased Ki-67 protein expression throughout the cell cycle. It was hypothesized that, due to the maintaining role of Ki-67 antigen in the cell cycle, interference in DNA replication might cause a reactive enhancement of the Ki-67 protein. In our study, the increase in Ki-67 mRNA following doxorubicin treatment did not lead to protein augmentation. We used the KB cell line which, despite the fact that is considered a subline of HeLa cells, may still differ from HeLa in some aspects, especially regarding the fact that DNA sensitivity to damage not only varies be-
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Figure 1. Flow cytometric cell cycle analysis of KB cells before and after treatment with doxorubicin (150 nM) for 36 hours depicting percentage of cells in G₁, S and G₂/M. RPMI: Roswell Park Memorial Institute (Culture medium).

Figure 2. Immunocytochemical staining of KB cells with antibodies against MCM3 and Ki-67. a: strong MCM3 immunoreactivity before treatment with doxorubicin. b: strong reactivity to the antibody after incubation with doxorubicin. c: strong immunostaining of Ki-67 in RPMI-treated cells. d: strongly positive Ki-67 nuclei in KB cells following treatment with doxorubicin. c and d have been reproduced from reference 30 with permission. Inset shows the negative control. Original magnification × 400.
between dissimilar cell types, but can even be different between cells in a single sample due to their existence in various proliferative or metabolic states [25]. Also the discordance between Ki-67 mRNA and protein has been reported in at least some cases of previous investigations [4, 26-28], and it has been stated that mRNA levels do not necessarily reflect protein quantities [28]. Moreover it has been suggested that cells lacking Ki-67 antigen may still possess various levels of Ki-67 mRNA [4]. Furthermore, considering that the distribution and expression intensity of Ki-67 protein fluctuates during the cell cycle [9], maybe the KB cells that are in different phases of the cycle, express levels of this antigen that are suboptimal to detection by immunocytochemistry. Posttranslational phosphorylation of Ki-67 protein occurs during the cell cycle, especially at G2/M, which modifies its biochemical features. This may affect the ability of MIB-1 to identify Ki-67 protein; thus, depending on which phase the individual cells are in, Ki-67 expression may be high or low, i.e. expression would be minimal in cells that exit mitosis [4, 29].

It is not simple to precisely determine the effect of a specific intervention on a biologic factor, especially when its complete role and function have not been fully elucidated, as is the case with Ki-67. However possible theories can be proposed that would require extensive investigations to be rejected or accepted; one such hypothesis could be as follows: the expression of Ki-67 is increased at G2/M [11, 12], which is exactly the period during which doxorubicin induced the apoptosis observed in the present study, based on our apoptosis analysis. According to Kausch et al. [30] Ki-67 negative cells are incapable of executing mitosis and are therefore driven to undergo apoptosis. Maybe it could be hypothesized that doxorubicin might have a kind of inhibitory or reducing effect on Ki-67 protein that leads to apoptosis; cancer cells attempt to survive this effect by increasing their mRNA to produce more protein. Ultimately, the production of protein by the cell and induction of apoptosis by doxorubicin reaches an equilibrium, the result of which has been a lack of change in the protein after doxorubicin treatment.

The MCMs include 6 nuclear proteins that are suggested to be valuable markers in the assessment of proliferation, due to their expression during G1 to M phases of the cell cycle and their disappearance in differentiated cells and those that have ceased to proliferate (G0) [7, 19, 31]. In the present investigation, there was no change in MCM3 mRNA and protein after treatment with doxorubicin. We have no clear indication of the biochemical circumstances that have led to these findings; however, a possible theory could be that MCM3 mRNA has not been significantly affected by doxorubicin. This assumption was made because doxorubicin exerted its main effect in G2/M, as shown by our apoptosis assay, while the mRNA and protein levels of MCMs have been shown to increase at the G1/S point following growth stimulation [15, 31]. According to Potter et al. [25] cells situated in the G2/M of the cell cycle are more sensitive to doxorubicin. In addition, based on previous studies, MCM protein levels decrease or their phosphorylation or chromatin binding status becomes altered during G2/M [16, 32]. Further studies are required to elucidate this matter, especially considering that in contrast to the above-mentioned facts, there are also reports that indicate a relatively constant level of MCM proteins throughout the cell cycle, which makes our interpretation even more difficult [2, 33, 34].

We were not able to find similar studies in the KB cell line to compare our results. In a prior study, preoperative radio-hyperthermo-chemotherapy (with doxorubicin as a component of the systemic chemotherapeutic treatment) was administered to patients with different types of sarcomas, which resulted in a non-significant reduction in MCM2 index [35]. In addition, MCM2 gene expression has been shown to decrease in breast cancers that have a favorable response to doxorubicin-based chemotherapy, and to increase in those with a poor prognosis [36, 37]. However, comparison with these reports may not be accurate due to the fact that both of them were conducted in clinical settings and have used MCM2 as a proliferation marker.

In the present study, comparison between MCM3 and Ki-67 showed a higher level of MCM3 mRNA, but no difference in their protein expression. Most previous studies comparing these molecules have been conducted on human tissues and showed a higher protein expression of MCM by immunohistochemical estimation of their labeling indices [2, 3, 20]. This was explained by the fact that MCMs are capable of demonstrating cells that have the potential to proliferate [8], but may not be actively proliferating, while Ki-67 only distinguishes the latter [15, 17]. Also it has been indicated that MCMs are expressed throughout the entire cell cycle, while Ki-67 is undetectable in early G1 phase [2, 7, 19]. The higher mRNA level of MCM3 in comparison to Ki-67 observed
The MCM complex has been suggested to act as a replication helicase [7,19]. In recent years, helicases have been suggested as suitable factors to target during administration of anticancer therapies [38]. Anthracycline agents, including doxorubicin, have been shown to inhibit helicase activity [39]; however, considering that only MCM 4, 6 and 7 display helicase activity \textit{in vitro} [40] we were not able to explore this feature in the present study. Due to the important role of replication helicases in cancer biology, further investigation for development of new anticancer strategies, using this feature of anthracycline drugs is proposed.

Acknowledgements

The present study was supported by Iran National Science Foundation, INSF.

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