Recombinant human Erythropoietin enhanced the cytotoxic effects of tamoxifen toward the spheroid MCF-7 breast cancer cells

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ABSTRACT

Erythropoietin (EPO) is widely used to treat anemia in patients undergoing chemotherapy for cancers. The main objective of this study was to investigate the effect of rHuEPO on the response of spheroid breast cancer, MCF-7, cells to tamoxifen treatment. The MCF-7 spheroids were treated with 10 mg/mL tamoxifen in combination with either 0, 10, 100 or 200 IU/mL rHuEPO for 24, 48 or 72 h. The viability of the MCF-7 cells was determined using the annexin-V, cell cycle, caspases activation and acridine orange/propidium iodide staining. rHuEPO-tamoxifen combination significantly (p > 0.05) increased the number of spheroid MCF-7 cells entering early apoptotic phase after 12 h and late apoptotic phase after 24 h of treatment; primarily the result of the antiproliferative effect tamoxifen. Tamoxifen alone significantly (p < 0.05) increased the caspase-3 and caspase-9 activities in the spheroid MCF-7 cells by 200 to 550% of the control. Combination rHuEPO and tamoxifen produced much lesser effect on the caspase-8 activity. The rHuEPO in the combination treatment had concentration-dependently caused decrease in the caspase activities. rHuEPO-tamoxifen combination markedly increased MCF-7 cells entering the SubG0/G1 phase of the cell cycle by more than 500% of the control, while decreasing those entering the G2 + M and S phases by 50%. After 72 h, the combination treatment produced greater (p < 0.05) change in the SubG0/G1 phase than tamoxifen treatment alone. Morphologically, spheroid MCF-7 cells subjected to combination rHuEPO-tamoxifen treatment showed nuclear condensation and margination, cytoplasmic blebbing, necrosis, and early and late apoptosis. Thus, the study showed that rHuEPO-tamoxifen combination induced apoptosis in the spheroid MCF-7 cells. The apoptotic effect of the rHuEPO-tamoxifen combination treatment on the MCF-7 cells was greater than that produced by tamoxifen alone. The rHuEPO-tamoxifen treatment enhanced the caspase-independent apoptotic effects of tamoxifen on the spheroid MCF-7 cells.

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Abbreviations: rHuEPO, Recombinant human erythropoietin; TAM Tamoxifen ULAT, ultra-low adhesive plate; HD, Hanging drop; poly-HEMA, Poly 2-hydroxyethyl methacyrlate; OLT, Overlay technique; MCF-7, human breast cell line; ESBR, The Eppendorf swing-bucket rotor with tubes rack; ECRB, Eppendorf A-4-62 centrifuge rotor 1 MTP buckets; MCF-7, GMD, geometrical mean diameter; Dc, geometrical mean of diameter; S, surface area; CC, correlation coefficient; CV, coefficient of variance.

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1. Introduction

Although several candidate therapeutic compounds from natural products have been discovered, tamoxifen, well-known for its chemotherapeutic properties, is still the drug of choice for breast cancers. Like most cancers, breast cancers are complicated by anemia, the result of the bone suppressive effects of chemotherapeutics and as one of the manifestations of the disease. Erythropoietin is used in conjunction with chemotherapeutics to alleviate the anemia (Bohlius et al., 2019). However, the use of EPO as adjuvant drug in cancers is hazardous. In cancer therapies, the inclusion of EPO was suggested to promote angiogenesis and tumor growth, invasion, and metastasis (Cao, 2013).

2. Methodology

2.1. 2D monolayer culture

The T-75 flask was used to culture MCF-7 cells. The cells were grown in 10 mL of RPMI1640 media containing 10% heat-inactivated foetal bovine serum (FBS) and 1% of penicillin/streptomycin. MCF-7 cells were maintained in a 5% CO₂ incubator under 37 °C and 95% humidity with medium change every 2 days. After reaching 85% confluence, cold phosphate-buffered saline was used to wash the cells and prepare them to be detached with 0.25% trypsin-EDTA. The cell pellets were then washed twice with 1 mL cold PBS and again centrifuged at 200 g, the supernatant obtained cell pellets. The cell pellets were then suspended in 1 mL cold PBS and centrifuged at 200 g, the supernatant was discarded. The pellets were resuspended, counted, and subjected to assays for cell viability determination or proceeded to spheroid formation.

2.2. Spheroid formation

A modified hanging drop technique (HDT) was used for the development of spheroids (Timmins and Nielsen, 2007; Foty, 2011). A 96-well microplate with conical-shaped bottom (Nunc - MicroWell™ 96 well polystyrene plates, USA, cat. number: P4241J) was used. 0.1 μL of 1·10⁶ MCF-7 cells/mL in RPMI1640 medium were seeded as drops in each well of the microplate. The microplate was gently inverted and incubated in 5% CO₂ incubator at 37 °C and under 95% humidity. To minimize evaporation, four microwell mini-trays were placed inside a plastic container together with a small dish filled with water placed between the plates in the incubator. The plates were regularly examined for spheroids formation. The spheroids were photographed and their sizes determined by calculating the geometrical mean diameter (Kunz-Schughart and Mueller-Klieser, 2000). The homogeneity of spheroids was determined by coefficient of variance (CV) calculation, the CV < 10% is indicating spheroid homogeneity.

2.3. Determination of IC₅₀ of tamoxifen in MCF-7 cells

This experiment first determined the half maximal inhibitory concentration (IC₅₀) of tamoxifen on the MCF-7 cells to be used in the study. Untreated cells served as the negative control. The MTT assay was performed as described elsewhere (Mosmann, 1983). 200 μL of 10⁴ MCF-7 cells/mL in RPMI1640 medium were seeded into each well of the 96-well microplate. The cells were treated with tamoxifen at concentrations of either 0, 0.35, 0.71, 1.41, 2.83, 5.65, 11.30, or 22.60 μg/mL for 24, 48, or 72 h. After treatment, the medium was discarded and cells washed gently with PBS. 20 μL of MTT dye, added to each well, and the plate incubated in the dark for 4 h. After discarding the medium, 100 μL DMSO was added to each well to dissolve the formazan crystals. The plate was incubated for 30 min and read in the ELISA reader (ELx800 Absorbance Microplate Reader, BioTek, US) at 570 nm. The cell viability was calculated using the following formula:

\[
\text{Cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100\%
\]

Where the OD is the optical density. The IC₅₀ of tamoxifen on the MCF-7 cells was calculated by forecast function in Microsoft Excel.

2.4. EPO and tamoxifen treatments

MCF-7 spheroids were treated with either 10 μg/mL (approximated IC₅₀ concentration) tamoxifen alone or with 10 μg/mL tamoxifen in combination with 10, 100 or 200 IU/mL rHuEPO in medium for 12, 24, or 72 h. All cells were in medium supplemented with 2% FBS during treatment. The spheroids were incubated for 5 min with PBS to eliminate traces of drugs and PBS. To obtain the single cell, the spheroids were then detached with TrypLE™ Express Enzyme (Fisher Scientific, Cat. number: 12604013) with pipetting. MCF-7 single cell suspensions were then subjected to the subsequent annexin V-FITC, caspase, cell cycle, and acridine orange (AO)/propidium iodide (PI) assays.

2.5. Annexin V-FITC assay

Single cell harvested from treated MCF-7 spheroids were subjected to annexin V-FITC assay (Sigma Aldrich, USA). Brieﬂy, the cells in 5-mL tubes were washed with cold PBS and centrifuged at 200 × g (Eppendorf AG, Hamburg, Germany) for 10 min to obtain cell pellets. The cell pellets were then washed twice with 1 mL cold PBS and again centrifuged at 200 × g, the supernatant removed, and the cell pellet re-suspended in cold 1X binding buffer. 500 μL of 1 · 10⁶ cell/mL of cell suspension was transferred to a fresh 5 mL tube and 5 μL of annexin V-FITC conjugate and 10 μL of propidium iodide (PI) added. The tubes were gently vortexed and incubated in dark at room temperature for 15 min before subjecting to flow cytometry (BD, USA) using Argon laser (Cyan ADP, DAKO, Glostrup, Denmark). The data were analyzed using the Summit V4.3 software. Untreated cells served as the control.
2.6. Caspase 3, 8 and 9 activity assays

Single-cell suspensions of treated MCF-7 spheroids were subjected to caspase assays as described by manufacturer (Genscript Corporation Inc, Piscataway, NJ, USA). Untreated cells served as the control. The extinction values were determined spectrophotometrically in a microplate reader (ELx800 Absorbance Microplate Reader, US) at 405 nm. Caspase concentrations were expressed as absorbance percentage of the control.

2.7. Cell cycle analysis

Single-cell suspensions of treated MCF-7 spheroids were subjected to cell cycle analysis according to the method describe by Pozarowski and Darzynkiewicz (2004). The cells were washed with PBS containing 0.1% sodium azide and fixed with 500 µL 80% cold ethanol, dropwise with vortexing to prevent aggregation. The cells were again washed with PBS, incubated with 0.1 mg/mL RNase enzyme at 37 °C for 1 h, and then with 10 µg/mL PI at room temperature in the dark for 1 h. The DNA content of cells were determine in the FACS Calibur flow cytometer by laser emitting excitation light at 488 nm (Becton Dickinson, USA) and the graph of gating were generated to determine the percentage of cells in the different cell cycle phases using the CellQuest software (Becton Dickinson, USA). Untreated cells served as the control.

2.8. Acridine orange and propidium iodide double staining

Treated single-cell MCF-7 suspensions of MCF-7 spheroids were subjected to AO/PI double staining assay (Bank, 1988). Briefly, the cells were washed with cold PBS and pelleted by centrifugation at 200 × g (Eppendorf AG, Hamburg, Germany) for 5 min and resuspended to obtain a suspension of 1 × 10^6 cell/mL. Then, 10 µL of the cell suspension were mixed with fresh 10 µL AO/PI stain solution (50 µg/mL) and immediately examined under fluorescence confocal microscopy (FV1000 viewer Olympus, Japan). Untreated cells served as the control. Viable cells were determined from a total of 100 cells and the results expressed as % of untreated control. Nonviable cells showed features of death or undergoing death, which included chromatin condensation and margination, membrane blebbing, apoptotic bodies, early apoptosis, and secondary necrosis.

2.9. Statistical analysis

The experiments were repeated 3 times. Values were expressed as percentage of the controls. Two-way ANOVA test for two factors was used to determine significance difference among study groups for dose and time of exposure. p < 0.05 was considered significance. SPSS software program was used to perform the statistical procedures.

3. Results

3.1. Spheroids

By the modified HDT, the spheroids generated after 120 h of cultivation were round and uniform in shape. The greatest decrease in viability of the MCF-7 cells was after treatment with 22.6 µg/ml tamoxifen. The IC_{50} of tamoxifen on MCF-7 cells after 72 h was approximated as 10 µg/mL and used as the tamoxifen concentration in the treatment of the MCF-7 cells.

3.2. IC_{50} of tamoxifen on MCF-7 cell

The IC_{50} of tamoxifen on the MCF-7 cells decreased in dose- and time-dependent manner (Table 1). The greatest decrease in Tamoxifen markedly increased MCF-7 cells entering the SubG0/G1 phase of the cell cycle by more than 50% of the untreated control, while decreasing those entering the G2 + M and S phases by 50%. Treatment of spheroid MCF-7 cells with the rhHuEPO-tamoxifen combination for 72 h resulted in significant (p < 0.05) greater number of cells entering the SubG0/G1 phase of the cell cycle than treatment with tamoxifen alone. However, the other
Table 1
Half-maximal inhibitory concentrations (IC50) of tamoxifen on MCF-7 cells determined by MTT assay.

| Treatment Period (hour) | Tamoxifen (μg/mL) | IC50 |
|-------------------------|-------------------|------|
| 0.35                    | 0.71              | 1.41 |
| 1.41                    | 2.83              | 5.65 |
| 5.65                    | 11.30             | 22.60|

Cell viability (%)

| Treatment Period (h) | 24 | 48 | 72 |
|----------------------|----|----|----|
| Tamoxifen concentration | 95.48 | 99.77 | 98.13 |
| 0.35                 | 93.67 | 101.04 | 94.85 |
| 1.41                 | 91.18 | 93.87  | 94.85 |
| 2.83                 | 91.40 | 83.93  | 84.87 |
| 5.65                 | 85.75 | 73.08  | 80.11 |
| 11.30                | 73.08 | 37.11  | 24.73 |
| 22.60                | 12.90 | 6.13   | 4.68  |

Fig. 2a. Effect of tamoxifen and rHuEPO treatments on the viability of MCF-7 cells from 3D culture after 12 h, determined by annexin-V assay. Combination rHuEPO-tamoxifen treatment significantly caused increased number of MCF-7 cells in early apoptosis and late apoptosis at high rHuEPO concentration. The effect on MCF-7 cells was dependent on rHuEPO concentration. (B) Tamoxifen and rHuEPO-tamoxifen combination treatments for 12 h. (I) Untreated cells (negative control) and cells treated with (II) 10 μg/mL tamoxifen alone and with combination of 10 μg/mL tamoxifen and (III) 10, (IV) 100 or (V) 200 IU/mL rHuEPO. rHuEPO potentiated the cytotoxic effect of tamoxifen. TAM = 10 μg/mL tamoxifen. *For each cell phase, means significantly different from TAM means at p < 0.05. Values are expressed as % of control. Error bars represent RSE.

Fig. 2b. Effect of tamoxifen and rHuEPO treatments on the viability of MCF-7 cells from 3D culture after 24 h, determined by annexin-V assay. Combination rHuEPO-tamoxifen treatment significantly caused more MCF-7 cells to enter late apoptosis than tamoxifen treatment alone. rHuEPO potentiated the cytotoxic effect of tamoxifen on MCF-7 cells, which was greater at 24 than 12 h of treatment. (D) Tamoxifen and rHuEPO-tamoxifen combination treatments on viability of MCF-7 after 24 h. (I) Untreated cells (negative control) and cell treated with (II) 10 μg/mL tamoxifen and combination treatment with 10 μg/mL tamoxifen and (III) 10, (IV) 100 or (V) 200 IU/mL rHuEPO. The cells were stained with propidium iodide and annexin V were analysed via flow cytometry. *For each cell phase, means significantly different from TAM means at p < 0.05. Values are expressed as % of control. Error bars represent RSE.
phases of the MCF-7 cell cycle were not affected by the presence of rHuEPO in the treatment regimen (Fig. 4).

3.6. Acridine orange and propidium iodide double staining

The AO/PI assay was used to determine effect of treatments on the viability of MCF-7 cells based on morphological changes. Untreated MCF-7 cells showed just a few necrotic cells, presumed to originate from core of the spheroids. Upon treatment with tamoxifen and rHuEPO, the number of viable cells decreased significantly (p < 0.05). The number of viable cells were significantly (p < 0.05) lower after treatment with combination rHuEPO-tamoxifen than with tamoxifen alone (Fig. 5). The effect became more significant (p < 0.05) with increase in rHuEPO concentration and time of exposure. Cells subjected to combination rHuEPO-tamoxifen treatment showed, nuclear condensation and margination, cytoplasmic blebbing, necrosis, and early and late apoptosis.

4. Discussion

Tamoxifen is one of the drugs of choice for the treatment of breast cancers. However, patients often develop resistance to the drug (Ali et al., 2016). In spite of the debatable potential adverse effects, EPO is still being used with tamoxifen in the treatment of breast cancers. The inclusion of EPO in cancer treatment regimens is to alleviate anemia that is often develops in cancer patients. Contrary to previous reports that suggested EPO promote cancer cell survival and growth (Cao, 2013) and inhibits chemotherapy-induced cell death (Pham et al., 2019), we showed that rHuEPO, under appropriate treatment conditions, is not only safe for use in cancer patients but also potentiates the effect of anticancer drugs (Radwan et al., 2016; Beh et al., 2017).

Tamoxifen was shown to induce apoptosis of MCF-7 (ER+) and MDA-MB231 (ER−) breast cancer cells (Salam and Karami-Tehrani, 2003; Liu et al., 2014) and to inhibit proliferation, migration, and invasion of breast tumors (Li et al., 2017). In our study, all
assays also showed that tamoxifen caused death of MCF-7 cells, primarily through apoptosis, and the effect increased with time of exposure. The study also showed that combination rHuEPO-tamoxifen treatment had similarly caused apoptosis of MCF-7 cells, and the effect was dependent on rHuEPO concentration and time of exposure.

Caspase activities in cancer cells is one of the indicators of apoptosis (Elmore, 2007). Caspase activities are among the most often used parameters in the determination of the anti-proliferative effects of anticancer drugs and compounds. Tamoxifen treatment increased the activities of caspase-3 and -9 in the MCF-7 cells by 5- to 6-fold and caspase-8 by 2-fold. These findings suggest that the apoptotic effect of tamoxifen is mainly through the intrinsic or mitochondrial pathway. However, with the inclusion of rHuEPO in the tamoxifen treatment, the activities of these caspases decreased dramatically, particularly at high treatment concentrations of 100 and 200 IU/mL rHuEPO. What is most intriguing in this study is that, in spite of lack of involvement of the caspases, the combination rHuEPO-tamoxifen treatment still caused the MCF-7 cells to undergo apoptosis. This was evident by the development of the typical morphological features of apoptosis in the treated MCF-7 cells. Thus, the evidences point to the combination treatments causing apoptosis via a mechanism other than the caspase pathway. Since the activity of caspase-3 and -9 are promoted by the release of the caspase-activating cytochrome c from mitochondrial membrane depolarization, it is postulated that rHuEPO stabilized the mitochondrial membrane rHuEPO (Nguyen et al., 2018), preventing the release of cytochrome c and up-regulation of caspases and activation of the caspase-dependent apoptotic pathway by tamoxifen. One of our earlier studies showed that 3D MCF-7 spheroids treatment with EPO alone reduced cell viability without caspases activation. In fact, the decrease in caspases activities was EPO-concentration dependent (Shujaa Edin et al., 2021).

Morphological manifestation of apoptosis is not totally dependent on the caspase activities. There are other mediators, including the cathepsins and other proteases that could execute apoptosis. Apoptosis may not only be directed by the mitochondrial organelles, but also by the lysosomes and the endoplasmic reticulum that either act independently or synergistically (Bröker et al., 2005). In one of the caspase-independent pathways, the initiation of apoptosis relies on release of the apoptosis-inducing factors that translocate to the nucleus and promoting chromatin condensation and DNA fragmentation (Koff et al., 2015).

The EPOR are not only located on plasma membranes, but also within the cell, surrounding the nucleus of cancer cells (Beh et al., 2017; Miao et al., 2017). Thus, rHuEPO may cause cellular changes both via its interactions with the cell surface and intracellular EPORs. If rHuEPO interacts with intracellular EPOR, it would evidently bypass the mitochondrial-mediated and caspase-dependent apoptosis; pathways that are reliant on surface receptor-ligand interactions. Among the mechanisms of the anti-breast cancer effect of tamoxifen is the induction of apoptosis via the mitochondrial-caspase pathway (Mandlekar and Kong, 2001). The inclusion of rHuEPO had inhibited the caspase activities while potentiating the caspase-independent apoptotic effect of tamoxifen on the spheroid MCF-7 cells. It appears that rHuEPO potentiated the tamoxifen-induced cancer cell apoptosis via a cytoplasmic pathway rather than the surface receptor-ligand interactions.

One the most significant effects of tamoxifen was to cause increase in number of spheroid MCF-7 cells entering the SubG0/G1 phase of the cell cycle. Cells in the SubG0/G1 phase are those that had undergone DNA fragmentation and apoptosis. Apparently, the inclusion of rHuEPO in the treatment regimen only marginally potentiated the effect of tamoxifen on the cell cycle phase. The effect of the rHuEPO-tamoxifen combination on the spheroid MCF-7 cells was similar to that on the murine mammary gland, LA7, cells. This was shown in our previous study, where the nanostructured lipid carriers double-loaded with rHuEPO and tamoxifen induced apoptosis and G0/G1 arrest of the LA7 cells Beh et al., 2017.

In conclusion, it is conceivable that the rHuEPO-tamoxifen combination is a relevant treatment regimen for breast cancers. This treatment regimen, by the synergistic actions between tamoxifen and rHuEPO, is postulated to cause MCF-7 cell death via a caspase-independent pathway. The findings from this study also dispel the notion that using EPO in the treatment of cancers like breast cancers is detrimental. In fact, the inclusion of rHuEPO can potentiate the anti-breast cancer effects of tamoxifen. To the best our knowledge, this is a first report that showed EPO synergizes with tamoxifen to reduce viability and cause death of MCF-7 cells from 3D cultures.
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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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