Curcumin-Induced Apoptosis Pathways in Positive (BT474) and Negative (MCF-7) Human Epidermal Growth Factor Receptor Breast Cancer Cells

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Abstract

Aim: Curcumin has several advantages and potential health benefits in breast cancer treatment, such as low cytotoxicity and minimal development of resistance, compared to chemotherapy-based therapies. The aim of this study was to elucidate the mechanisms of Curcumin-induced apoptosis in human epidermal growth factor receptor positive (BT474) and negative (MCF-7) breast cancer cell lines.

Methods: Cytotoxic activity of curcumin was measured by the Sulforhodamine B assay. Apoptosis induction was measured by flow cytometry and detection of an apoptosis-associated PARP cleavage product via Western blotting. Western blotting was also used to measure Cytochrome C translocation, which determines mitochondrial involvement, and Caspase-7 activation. The Caspase cascade was measured by activity levels of Caspase-3, Caspase-8, and Caspase-9 using chromogenic substrates.

Results: Curcumin CC50 (concentration that kills 50% of the cells) was 25.92 ± 5.13 µM for MCF-7 and 49.06 ± 5.56 µM for BT474 cells. Flow cytometry results indicated a 13.36 ± 5.96% expression increase and a 6.87 ± 1.42% expression increase in early apoptosis (Annexin V positively stained) in MCF-7 and BT474 cells, respectively. Late stage apoptosis (Annexin V positive stained) in Curcumin-exposed MCF-7 cells was increased by 3.45 ± 0.84%, but no increase was seen in BT474 cells. PARP cleavage product (300 kD) was detected in caspase-9-silenced cells but no Curcumin effect was observed in BT474 cells. Curcumin-exposed MCF-7 cells, but not BT474 cells, showed substantial release of Cytochrome C from the mitochondria into the cytoplasm. Caspase-8 was not detected in MCF-7 cells, but Cytochrome C was detected only in BT474 cells. MCF-7 cells reportedly have a gene deletion for Caspase-3 and therefore do not express it. Caspase-3, an alternate effector caspase, was then tested in MCF-7 cells, but no Curcumin upregulation was detected.

Conclusion: Cytotoxicity of Curcumin is low for both MCF-7 and BT474 cells and concentrations are easily achievable. Apoptosis induction by Curcumin exposure was detected by both flow cytometry and PARP cleavage. Flow cytometry showed that Curcumin-induced early stage apoptosis (Annexin V stained) in both MCF-7 and BT474 cells, but no late-stage apoptosis increase was seen only in MCF-7. Caspase-3 activation and Cytochrome C translocation studies determined an extrinsic pathway (Caspase-8 and Caspase-3 positive, Caspase-9 negative with no Cytochrome C translocation). Caspase-7 was detected in MCF-7, but not in BT474. Caspase-3 activity was upregulated only in BT474 cells. MCF-7 cells reportedly have a gene deletion for Caspase-3 and therefore do not express it. Caspase-3, an alternate effector caspase, was then tested in MCF-7 cells, but no Curcumin upregulation was detected.

Introduction

Breast cancer is a complex and heterogeneous disease caused by both genetic and non-genetic risk factors. Curcumin has potential breast cancer therapeutic activities, reported via cell cycle arrest and apoptosis induction. Apoptosis is a process by which normal cells commit suicide; cancer cells are anti-apoptotic and continue to live indefinitely. Apoptosis is characterized by marked changes in cellular morphology, including loss of plasma membrane asymmetry and attachment, chromatin condensation, membrane blebbing, nuclear breakdown and the appearance of membrane-associated apoptotic bodies, internucleosomal DNA fragmentation, as well as cleavage of Poly(ADP-ribose) polymerase (PARP) and activation of caspases. In order to maximize the efficacy of Curcumin’s natural therapy for cancer treatment, it is important to understand the specific apoptotic mechanisms of action for Curcumin. Studies using a variety of breast tumor cell lines are necessary because there is high cell-to-cell variability in response to Curcumin. The fundamental molecular difference between MCF-7 and BT474 cells is the presence or absence of Human epidermal growth factor receptor 2 (HER2). MCF-7 breast cancer cells express HER2 and BT474 cells express HER2 and HER2. Therefore we determined the minimal mechanisms of Curcumin-induced apoptosis in these breast cancer cell lines.

Material and Methods

Parameters | Assay Methods | Cytotoxicity | Apoptosis/Flow Cytometry | Apoptotic protein analysis
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| | | Sulforhodamine B (SRB) assay | Flow cytometry: Annexin/V/Propidium Iodide staining | PARP Cleavage: Western blotting
| | | | | Cytochrome C translocation: Western blotting

Results Summary

- **Curcumin CC50**: MCF-7: 50µM, BT474: 25µM
- **Flow Cytometry Curcumin Apoptosis Induction**: MCF-7: Increased Early and Late phase Apoptosis
- **Caspase Cascade/Cytochrome C translocation**: MCF-7: Caspase-3 and Caspase-8 upregulated (p<0.001, p<0.000016); no Cytochrome C translocation
- **Caspase Cascade/Cytochrome C translocation**: BT474: Caspase-3 and Caspase-8 upregulated (p<0.001, p<0.00016); no Cytochrome C translocation

Conclusions

- Early stage apoptosis induction is evident with Curcumin exposure reported in both MCF-7 and BT474 cell lines.
- Late-stage apoptosis induction is cell specific; HER2 positive cells show no induction, HER2 negative cells demonstrate induction.
- **PARP cleavage**: a hallmark of this type of cell death. MCF-7 cells are treated with 0, 20 and 30 µM of Curcumin for 24 h.
- **Caspase Cascade/Cytochrome C translocation**: MCF-7: Caspase-3 and Caspase-8 upregulated (p<0.001, p<0.000016); no Cytochrome C translocation
- **Caspase Cascade/Cytochrome C translocation**: BT474: Caspase-3 and Caspase-8 upregulated (p<0.001, p<0.00016); no Cytochrome C translocation

Figure-1: Flow Cytometry analysis of apoptosis. Flow cytometry was used to quantitatively assess number of breast cancer cells (MCF-7 and BT474) that had been treated for 18 h with 0.2% DMSO or 20 µM curcumin in 0.2% DMSO. Cells were then collected by trypsinization, washed 2X with 1% BSA in cold PBS, and suspended in loading buffer (10mMTris). The Annexin/V/PI staining protocol was performed according to the manufacturer’s instructions. In both cell lines Curcumin caused significant increase in early apoptosis. However, late apoptosis was significant only in MCF-7 cells, but not in BT474. The dot blots shown represent one set of experiments. The chart is combined data from 3 independent sets of experiments.

Figure-2: Cleavage of PARP in breast cancer cell lines. Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme that catalyzes the transfer of ADP-ribose polymers onto itself and other nuclear proteins in response to DNA strand breaks. During apoptosis cleavage of PARP into fragments of 85 and 24 kDa has become a useful hallmark of this type of cell death. MCF-7 were seeded at a density of 2X 10^5 cells in 10 cm plate, allowed to grow for 24 h, then treated with 0, 20 and 30 µM of Curcumin for 24 h. Cells were fractionated into cytosolic and mitochondrial fractions using a commercial kit available from Thermo Fisher Scientific. BT474 cells (5 x 10^5) were treated after 24 h growth with 0, 20, 50 and 50 µM of Curcumin and then similarly fractionated.

Figure-3: Effect of Curcumin on activation of Caspase-8. Chromogenic peptide substrate IETD-pNA was used for the quantitation of Caspase-8 activity. Control and Curcumin-treated Caspases were assayed as described in the materials and methods section. A. MCF-7 cells and B. BT474 cells. Columns represent the data from 5 replicates, p<0.05, as determined using a Student’s t test. Results are the fold increase in activity compared to control. Curcumin significantly increases the activation of Caspase-8 in both MCF-7 (Figure 2A: 1.35 ± 0.34 vs 3.85 ± 0.53, p<2 x 10⁻³) and BT474 (Figure 2B: 1.46 ± 0.32 vs 3.61 ± 0.83, p= 1.2 x 10⁻⁷) cells.

Figure-4: Curcumin-induced activation of Caspase-3 in BT474 cells. An in vitro apoptosis activity kit using chromogenic substrate (Ac-DEVD-pNA) was used to measure the Caspase-3 activity. Columns represent the data from 5 replicates, p<0.05, as determined using a Student’s t test. Results are the fold increase in activity compared to control. Curcumin exposure induced a significant increase in Caspase-3 activation in BT474 (1.01 ± 0.13 vs 2.23 ± 0.26 p=1.6 x10⁻⁵).

Figure-5: Curcumin-induced translocation of Cytochrome C. MCF-7 cells were seeded at a density of 2X 10^5 cells in 10 cm plate, allowed to grow for 24 h, then treated with 0, 20 and 30 µM of Curcumin for 24 h. Cells were fractionated into cytosolic and mitochondrial fractions using a commercial kit available from Thermo Fisher Scientific. BT474 cells (5 x 10^5) were treated after 24 h growth with 0, 20, 50 and 50 µM of Curcumin and then similarly fractionated. Equal amounts of protein (100 µg) were loaded onto 8% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-PARP polyclonal antibody (PARP pAb) and anti-actin monoclonal antibody (actin mAb). The results are shown from 3 independent experiments. A: MCF-7 and B: BT474.

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