EPIDERMAL GROWTH FACTOR, PHORBOL ESTERS, AND AURINTRICARBOXYLIC ACID ARE SURVIVAL FACTORS FOR MDA-231 CELLS EXPOSED TO ADRIAMYCIN

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SUMMARY

The ability of epidermal growth factor (EGF), insulinlike growth factor-1 (IGF-1), insulin, 12-O-tetradecanoylphorbol-13-acetate (TPA), and aurintricarboxylic acid (ATA) to protect the human breast cancer cell line MDA-231 from death induced by the anticancer drug adriamycin was investigated. Cell death was induced in the MDA-231 cells either by a short-time exposure to a high dose of adriamycin (2 µg·ml⁻¹·h⁻¹) and further culturing in the absence of the drug, or by continuous exposure to a low dose of adriamycin (0.3 µg/ml). Cell death was evaluated after 48 h of incubation by several techniques (trypan blue dye exclusion, lactic dehydrogenase activity, cellular ATP content, transmission electron microscopy, and DNA fragmentation). EGF, TPA, and ATA, each at an optimal concentration of 20 ng/ml, 5 ng/ml, and 100 µg/ml respectively, substantially enhanced survival of cells exposed either to a high or low dose of adriamycin. Neither IGF-1 nor insulin, each at concentrations of 20 ng/ml, had an effect on cell survival. The three survival factors enhanced protein synthesis in the untreated cells and attenuated the continuous decrease in protein synthesis in the adriamycin-treated cells. Moreover, the three survival factors protected the MDA-231 cells from death in the absence of protein synthesis (cycloheximide 30 µg/ml). These results suggest that EGF, TPA, and ATA promote survival of adriamycin pretreated cells by at least two mechanisms: enhancement of protein synthesis and by a protein synthesis independent process, probably a posttranslational modification effect.

Key words: EGF; TPA; aurintricarboxylic acid; adriamycin; cell-survival.

INTRODUCTION

Previously we have shown that EGF and IGF-1 can protect viability of several cell lines exposed continuously to the protein synthesis inhibitor cycloheximide (15–17). We suggested that the continuous deficiency in some cell vital protein molecules, which resulted from the persisting protein synthesis inhibition induced by cycloheximide, terminates in cell death. Inasmuch as protein synthesis remained inhibited in the presence of either epidermal growth factor (EGF) or insulinlike growth factor-1 (IGF-1), we proposed that the growth factors maintained the amount of those vital proteins by a posttranslational modification effect, from a pool of nonfunctional precursor molecules.

Several anticancer drugs may exert their cytotoxic effect through an inhibition/interference in the normal protein synthesis process, although their primary effect induces damage in the DNA or RNA arrangement. Growth factors may protect those injured cells from death by the above mentioned mechanism, i.e. maintaining the critical level of some vital protein molecules through a posttranslational modification effect. Indeed, we have shown that EGF and IGF-1 enhanced substantially survival of MDA-231 cells exposed to the anticancer drug actinomycin D (18), a powerful inhibitor of RNA synthesis.

In the present study we investigated the ability of the growth factors EGF, IGF-1 and insulin, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), and aurintricarboxylic acid (ATA) to protect the human breast cancer cell line MDA-231 from death induced by adriamycin. Adriamycin belongs to a number of antitumor drugs which interact with DNA topoisomerase II (43). These drugs stop the enzyme in an intermediary reversible complex with DNA, which prevents the final rejoining step of the reaction and results in increased DNA strand cleavage.

Recently, it has been shown that growth factors, apart from their mitogenic effect, can function as survival factors for cells in growth factor-deprived medium or for cells subjected to cytotoxic drugs (28,33,38–42,45). Indeed, for some growth factors it has been shown that their survival effect could be dissociated from the growth effect, which depends on RNA and protein synthesis. In the MDA-231 cells it was shown that IGF-1 enhanced DNA synthesis, whereas EGF and insulin did not (14,36). We have previously shown that EGF and IGF-1 protected MDA-231 cells from death induced either by the protein synthesis inhibitor cycloheximide, or by the powerful RNA synthesis inhibitor actinomycin D, whereas insulin had no effect (17,18).

Tumor promoter phorbol esters, such as TPA, induce various biochemical and biological effects in culture cells, including striking stimulatory or inhibitory effects on cell proliferation and differentiation (6,11). The only currently recognized mediator of the TPA action is the Ca²⁺ and phospholipid-dependent protein kinase C,
which most probably represents the high affinity phorbol ester receptor in target cells (1,7,25,32,34,35). It was shown that TPA can increase cell survival of serum-deprived cells or of cells subjected to cytotoxic drugs (12,13,22,26,41).

The triphenylmethane dye ATA is known as a general inhibitor of nucleases (20). Inasmuch as apoptotic cell death is related to an increase in a certain endonuclease activity, it was proposed that inhibition of these nuclease by ATA may protect cell viability and promote cell survival. Indeed, it has been reported that ATA can rescue both sympathetic neurons from nerve growth factor deprivation-induced death (3) and thymocytes from glucocorticoid- and ionophore-induced apoptosis (36).

In the present study we found that EGF, TPA, and ATA are survival factors for MDA-231 cells exposed to the cytotoxic effect of the anticancer drug adriamycin. The presumable involvement of growth factors in drug resistance is discussed.

**MATERIALS AND METHODS**

**Materials.** EGF and IGF-1 were obtained from Boehringer, Mannheim (Germany). Insulin, adriamycin, TPA, ATA, ATP bioluminescent assay kit, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). [4,5,3H]-Leucine (specific activity 120 to 190 Ci/nmol) was obtained from Amersham International Plc (Buckinghamshire, UK).

**Cell culture.** MDA-231 cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 5% fetal bovine serum, and 100 U each of penicillin and streptomycin. Culture media and supplements were purchased from Biological Industries (Beth Haemek, Israel).

**Cell death estimation.** Cells were plated in 35-mm diameter dishes at a density of 250,000 cells per dish as described previously (17,18). Cells were plated in 2 ml of culture medium, and on Day 3 of culture 1 ml of fresh culture medium was added. Five days after plating (4 days in experiments described in Fig. 2) the medium was removed by aspiration and cells were washed twice by gentle rinsing with culture medium without serum which was aspirated. Cells were incubated in 2 ml of medium containing 1 mg/ml BSA (without serum). After 24 h the medium was aspirated, and cells were washed once by gentle rinsing with culture medium without serum which was aspirated. Cells were further incubated in 2 ml of serum-free medium containing 1 mg/ml BSA as indicated for each experiment.

**Trypan blue exclusion test.** After treatment the medium was recovered and the cells were detached with trypsin as described previously (17,18). Stained and unstained cells were counted on a hemacytometer (10³ cells were counted for each determination).

**Lactic dehydrogenase (LDH) assay.** After treatment of the cells, the amount of LDH was evaluated in the culture medium as described previously (15–18). Results were expressed in international units per liter.

**Assay for decrease in ATP content.** Aliquots from total cell pellets were counted on a hemacytometer to determine the total number of cells, and an aliquot was assayed for ATP content using a commercial bioluminescent assay kit from Sigma. The total ATP content in the cell pellet was divided by the total number of cells in the pellet, to determine the ATP content per cell.

**Measurement of protein synthesis.** To label cell proteins, [3H]-leucine (2 μCi/ml) was added to the culture medium, and the cells were incubated for 1 h at 37°C. The cells were then washed twice with cold phosphate buffered saline (PBS), and the cell monolayers were fixed to the culture plate by exposure to 37°C for 1 h. The cells were then washed twice with cold 10% TCA, and the precipitated material was dissolved in 0.2 N NaOH at 37°C before liquid scintillation counting.

**DNA extraction and electrophoresis.** Cells (6 × 10⁶) were lysed over night at 37°C in 1 ml of solution containing 20 mM Tris-HCl (pH 7.8), 10 mM EDTA, 1% sodium dodecylsulfate, and 0.5 mg/ml of proteinase K (Merk, Germany). The lysed cells were extracted once each in phenol, phenol/chloroform/isoamyl alcohol (25:24.1, vol/vol) and chloroform/isoamyl alcohol (24:1, vol/vol). DNA was precipitated with 100 μl of 3 M sodium acetate and 100% ethanol (2.5 vol) at −20°C. After centrifugation at 13,000 Xg the precipitated DNA was dissolved in 200 μl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) containing 0.1 mg/ml RNase A

**RESULTS**

**Effect of adriamycin on cell cytotoxicity.** MDA-231 cells were exposed for 1 h to increase concentrations of adriamycin, washed thoroughly, and further cultured in the absence of the drug for up to 72 h. As shown in Fig. 1, the percent of dead cells increased continuously with an increase either in the drug concentration, or with the incubation time. Thus 23, 42, and 62% of the cells were killed after 48 h of exposure to 0.5, 1, and 2 μg/ml of adriamycin, respectively. Although the untreated cells (0 μg/ml) were highly crowded, only 13 and 20% of the cells were dead after 48 or 72 h of incubation, respectively.

**Effect of various agents on survival of adriamycin pretreated cells.** Table 1 shows the effect of several agents on survival of cells previously exposed to 2 μg/ml adriamycin for 1 h, then further cultured in the absence or presence of the indicated additives for 48 h. Cell death was evaluated by three different methods: counting the trypan blue-stained cells, measuring the released LDH activity, and determining the depletion in the cellular ATP content. Exposure of the cells to adriamycin resulted in 71% dead cells, a comparable rise in the LDH-released activity, and a depletion in the cellular ATP content. EGF, TPA, and ATA are survival factors which, at the indicated concentrations, reduced cell death to 26, 14, and 12%,