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Multiple cell cycle access to the apoptotic death programme in human neuroblastoma cells

Mauro Piacentini[a], Laszlo Fesus[d] and Gerry Melino[b,c]

Departments of [a]Biology and of [b]Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, Italy, [c]IDI-IRCCS, Rome, Italy and *[d]Department of Biochemistry, University Medical School of Debrecen, Debrecen, Hungary*

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We report the induction of apoptosis in a human neuroblastoma cell line SK-N-BE(2) by cisplatin or retinoic acid, and its relation to cell cycle. Apoptosis was monitored by counting apoptotic bodies and evaluating the activity of 'tissue' transglutaminase (EC 2.3.2.13), one of the genes specifically expressed in apoptotic cells. Data indicate that both agents enhance apoptosis, even though cells arrest at different cell cycle phases. In fact, retinoic acid causes accumulation in G_1, whilst cisplatin induces accumulation of cells in the G_2/M phase. This evidence suggests the presence of multiple start points for the apoptotic death programme within the cell cycle of human neuroblastoma cells.

Apoptosis; Neuroblastoma; Cisplatin; Tissue transglutaminase

1. INTRODUCTION

Apoptosis is the genetically controlled process leading to cell self-elimination in tissues [11,30,31]. It also plays a pivotal role in the control of tumour cell proliferation by counterbalancing the effect of mitosis, hence regulating cell number [11,30,31]. Apoptosis occurs in isolated cells featuring compaction of the cytoplasm and organelles, smoothing of the plasma membrane, and the presence of condensed chromatin in characteristic arrays which correspond at the molecular level to its cleavage at internucleosomal sites, thus causing the characteristic DNA ladder [11,30,31]. This DNA fragmentation is allegedly due to the activation of a yet uncharacterized Ca^{2+}-Mg^{2+}-dependent endonuclease [30,31]. The molecular mechanisms regulating the apoptotic program have not been fully elucidated as yet; however, under physiological conditions, its requirement for RNA and protein synthesis indicates the control by specific genes [11]. We reported the specific expression of 'tissue' transglutaminase (tTG) in several cell types undergoing the apoptotic program including human neuroblastoma cells [11,12,24–26,29]. Transglutaminases (EC 2.3.2.13) are Ca^{2+}-dependent enzymes catalyzing cross-linking reactions among polypeptide chains, leading to the formation of protein polymers insoluble in detergents and chaotropic agents [13]. Indeed, tTG activation results in the assembly of a highly cross-linked protein scaffold which temporarily stabilizes the integrity of the dying cell before its clearance by phagocytosis or shedding into body cavities [11,12].

Neuroblastomas in culture are characterized by the presence of three morphologically and biochemically distinct phenotypes (i.e. neural 'N-type', intermediate 'I-type', and flat substrate-adherent 'S-type') which undergo transdifferentiation [4,20]. Human neuroblastoma SK-N-BE(2) cells differentiate toward a neural phenotype upon retinoic acid (RA) treatment [15,19–21,24,26]. Moreover, we recently demonstrated that during the RA-treatment, a subset (S-type) of SK-N-BE(2) cells undergo apoptosis; the latter specifically express high tTG protein level [20,24,26]. It has recently been suggested that anticancer drugs exert their biological effects by triggering cell death by apoptosis [1,5,7,17,28]. The possibility to evoke a specific cell death pathway in cancer cells has obvious therapeutic importance. The aim of this study was to investigate the effect of cisplatin and RA on apoptosis in human neuroblastoma cells in relation to the cell cycle.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[1,4(*n*)-^{3}H]Putrescine dihydrochloride (26.3 Ci/mmol) was from Amersham, Bucks, UK). Optifluor was from Packard (Zurich, Switzerland). *N,N'*-Dimethylcasein and bovine serum albumin were from Fluka (Switzerland). All-*trans* RA was from Sigma (St. Louis, MO). Cisplatin was from Bristol Labs. (Syracuse, NY). Cell culture media and plastic were from Flow (UK). Other chemicals were of reagent grade and used without further purification.

Correspondence address. G. Melino, Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Roma 'Tor Vergata', Via O. Raimondo, 00173 Rome, Italy. Fax: (39) (6) 723 4793.

Abbreviations: tTG, 'tissue' transglutaminase; RA, retinoic acid; MEM, minimal essential medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

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2.2. *Cell cultures*

The parental SK-N-BE(2) line and its clonally derived lines BE(2)-C [I-type] and BE(2)-M17 [N-type] were grown in a 1:1 mixture of MEM and Hams F-12 media supplemented with 15% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 1.2 g/liter bicarbonate, non-essential amino acids (1% v/v) and 15 mM HEPES in humidified atmosphere with 5% (v/v) CO_2 at 37°C. All the experiments were performed using the cell lines at 70–76th, 33–37th and 26–29th passage, respectively.

Cells were plated at 5×10^4 cells/cm^2 in growth medium containing 5 μM RA (from a 5 mM stock solution dissolved in 70% ethanol) or 5 μM cisplatin (from a 1.7 mM stock solution dissolved in PBS). Medium was replaced daily. Control cultures were treated with 0.07% ethanol. Cell number and viability were evaluated in a Thoma haemocytomer chamber. The cell cycle was evaluated by flow cytometry using propidium iodide staining [21] on a FACScan flow cytometer (Becton-Dickinson, CA, USA).

2.3. *Biochemical determinations*

Mechanically removed cells, washed in (Ca^{2+}/Mg^{2+})-free PBS, were sonicated (4°C for 20 s) and tTG activity was measured by detecting the incorporation of [^{3}H]putrescine into *N,N'*-dimethylcasein as previously reported [25]. The incubation mixture contained 150 mM Tris-HCl buffer pH 8.3, 5 mM $CaCl_2$, 10 mM dithiothreitol, 30 mM NaCl, 2.5 mg *N,N'*-dimethylcasein/ml, 0.2 mM putrescine (containing 1 μCi of [^{3}H]putrescine), and 0.1–0.2 mg of protein in a final volume of 0.3 ml. After 20 min incubation at 37°C, 100 μl of incubation mixture was spotted onto Whatman 3MM filter paper moistened with 20% trichloroacetic acid (TCA). Free [^{3}H]putrescine was eliminated by washing with large volumes of cold 5% TCA containing 0.2 M KCl; dry filters were counted with 8 ml Opti-fluor [18]. Protein concentration was determined as previously described using bovine serum albumin as standard [25].

2.4. *Quantification of apoptotic bodies*

Cross-linked apoptotic bodies were estimated on cells cultured in 25 cm^2 flasks, as described [12,25]. Cells floating in the culture medium for the previous 24 h were collected by centrifugation at $800 \times g$ for 5 min and pooled with the cells mechanically recovered from flasks. Then the cells, washed in PBS, were spun and suspended in lysis buffer (10 mM KCl, 2 mM $MgCl_2$, 0.5% Triton X-100 in 10 mM Tris-HCl, pH 7.5) containing 0.2 mM phenylmethylsulphonyl fluoride to inhibit proteases and 0.4 mM iodoacetamide to inhibit transglutaminase. After centrifugation the pellet was washed in the lysis buffer and suspended in 6 M guanidine hydrochloride to dissolve nuclei [12]. The pellet from the subsequent centrifugation was suspended in 2% SDS solution containing 0.05 mM β-mercaptoethanol, boiled, and the number of detergent-insoluble apoptotic bodies was scored using a phase contrast microscope (Laborlux D, Leitz). Apoptotic bodies were also evaluated by flow cytometry using propidium iodide staining [6,7] on a FACScan flow cytometer (Becton-Dickinson, CA, USA).

3. RESULTS AND DISCUSSION

We demonstrated that human neuroblastoma cells (SK-N-BE(2)) undergo spontaneous apoptosis in culture [24], furthermore, we found that RA treatment for a few days was able to significantly enhance their cell death rate [24]. Apoptosis was associated with a specific increase of tTG expression (mRNA, protein and cross-linking activity) in the apoptotic cells which was restricted to neuroblastoma cells showing the S-morphology [26].

In the present study we have investigated whether other agents, beside RA, are able to enhance tTG activity and apoptotic index. Particularly, it has recently

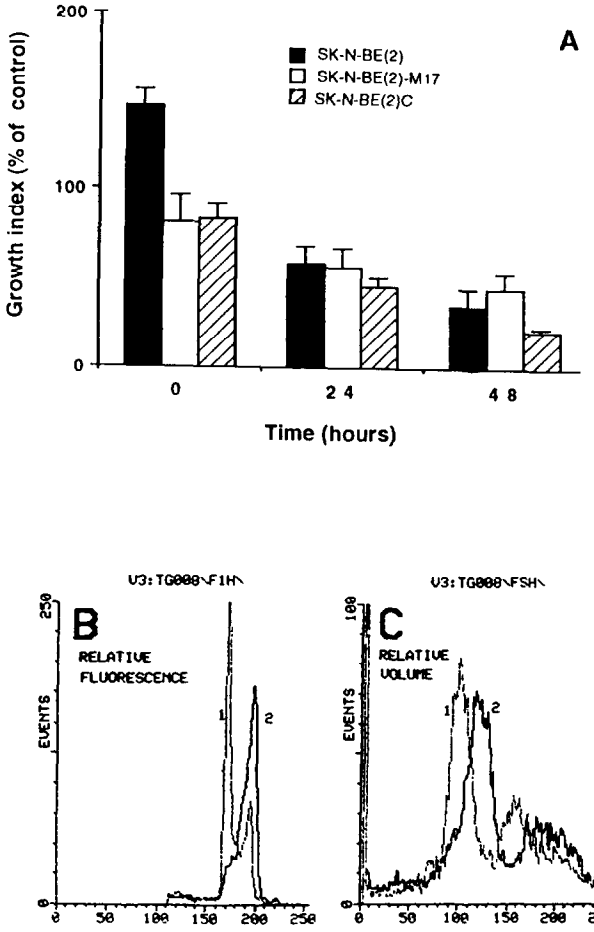

Fig. 1. (A) Growth index of SK-N-BE(2) human neuroblastoma cells and its derivative clones, BE(2)-M17 and BE(2)-C, following 4 h incubation with 5 μM cisplatin. All 3 cell lines show a drastic reduction in growth rate. (B) Cell cycle analysis by flow cytometry of untreated (line 1) and cisplatin treated (5 μM for 4 h; line 2) SK-N-BE(2) cells, showing a sharp G2/M block induced by cisplatin. (C) Cell volume analysis by flow cytometry of untreated (line 1) and cisplatin treated (5 μM for 4 h; line 2) SK-N-BE(2) cells. Note the increase in cell volume in cisplatin treated cells (B; line 2).

been suggested that chemotherapeutic agents such as cisplatin, exert their cytopathic activity by triggering apoptosis in malignant cells [1]. Fig. 1A shows that cisplatin is able to induce a large growth inhibition in SK-N-BE(2) parental cell line as well as in its neuroblastic (BE(2)-M17) and substrate-adherent (BE(2)-C) derived clones. In addition, after 4 h exposure to cisplatin the SK-N-BE(2) cells are blocked in G2-M phase of the cell cycle (Fig. 1B) and show a significant increase in their mean volume (Fig. 1C). Noteworthy, the growth inhibition observed in the SK-N-BE(2) parental cell line as well as in its derived clones (Fig. 1A) is parallelled by a proportional increase in tTG activity (Table I).

Apoptotic cells do not lyse, but shrink and fragment into smaller membrane-limited particles with a characteristic morphology (apoptotic bodies) [11,30,31]; their proteins are extensively cross-linked by tTG which ren-

Table I

Effect of cisplatin on 'tissue' transglutaminase activity and His-dependent cross-linked apoptotic bodies formation in human neuroblastoma cell lines

Treatments	SK-N-BE(2) wild type	BE(2)C intermediate	BE(2)M17 neuroblastic
	tTG activity*		
None	77 ± 11	72 ± 17	23 ± 10
Cisplatin (24 h)	97 ± 12	128 ± 11	70 ± 13
Cisplatin (48 h)	107 ± 15	158 ± 15	75 ± 10
	Cross-linked apoptotic envelopes**		
None	0.1	0.3	0.4
Cisplatin	0.3	2.0	2.5

Cells were cultured as described in the experimental section in the presence of 5 μM cis-platinum for 14 h, after treatment cells were extensively washed in RPMI and then cultured for additional 48 h, then after washing in PBS mechanically removed from flasks.

*In vitro tTG activity was measured as pmol of [^{3}H]putrescine incorporated into protein/h/mg protein. Data are the mean ± S.E.M. of triplicate determinations carried out on three different experiments. **tTG-dependent formation of detergent-insoluble cross-linked apoptotic envelopes was evaluated at the phase microscope as described in section 2. Data are the mean of duplicate determinations with an S.E.M. less than 15%.

ders them resistant to treatment with detergents [12,24,25]. The evidence that tTG expressed in the dying cells derived from cisplatin-treated SK-N-BE(2) cells is actively cross-linking the intracellular protein is also reported in Table I. In fact, the number of the cross-linked apoptotic bodies recovered from the cultures upon cisplatin-treatment is largely increased (3- to over 6-fold) and strictly parallels the increased tTG activity. The induction of tTG activity upon cisplatin-treatment is detectable, even though at lesser specific activity with respect to the BE(2)-C clone, also in the 'N-type' BE(2)-M17 clone (Table I). This result is very interesting since our previous studies demonstrated that RA was unable to induce apoptosis in the neuronal BE(2)-M17 cell clone [24,26], which instead evidenced a relative resistance to RA treatment [21]. By contrast, the results presented in Table I indicate that the neuronal differentiation stage reached by this clone can still be forced by cisplatin into the programmed death pathway, thus implying that the death programme in neuroblastic cells is not irreversibly blocked. In addition, Table II indicates that cisplatin and RA are the only agents tested able to enhance the tTG activity; in fact okadaic acid and TGFβ which have been shown to induce apoptosis in other systems [3,11,14], were unable to increase tTG activity in our model.

Several reports suggest that the 'priming state' for apoptosis could be related to specific phases of the cell cycle, as well as to the differentiation stage [8,25]. In order to investigate this point we analysed the cell cycle phases of human neuroblastoma SK-N-BE(2) cell upon exposure to both RA and cisplatin at different time intervals. Table III indicates that, while RA causes the accumulation of cells in the G_1 phase, cisplatin blocks

Table II

Effect of caffeine, TGFβ, okadaic acid and RA on 'tissue' transglutaminase activity in BE(2)-C cells

	tTG activity* (pmol/h/mg protein)
Control	78 ± 10
Caffeine (2 mM)	
6 h	70 ± 12
14 h	35 ± 3
24 h	54 ± 7
TGFβ (80 pM)	
12 h	30 ± 5
24 h	30 ± 3
48 h	50 ± 3
Okadaic acid (0.5 μM)	
5 h	36 ± 6
16 h	34 ± 4
Retinoic acid (5 μM)	
24 h	87 ± 16
48 h	216 ± 30

*In vitro tTG activity was measured as pmol of [^{3}H]putrescine incorporated into protein/h/mg protein. Data are the mean ± S.E.M. of triplicate determinations carried out on three different experiments.

Table III

Effect cisplatin, caffeine and RA on cell cycle phases of BE(2)-C cells

Treatment	G_0/G_1	S	G_2/M
None	63.6	18.4	18.0
Cisplatin (4 h)	39.4	16.4	44.1
Cisplatin (36 h)	21.2	21.3	57.5
Caffeine (6 h)	65.7	14.3	20.1
Caffeine (12 h)	69.2	14.5	16.0
Caffeine (24 h)	71.2	17.5	11.1
Cisplatin (4 h) + caffeine (24 h)	40.0	16.9	42.5
Cisplatin (36 h) + caffeine (24 h)	21.1	26.4	51.4
Retinoic acid (72 h)	80.1	9.4	10.3
Retinoic acid (96 h)	77.0	8.8	14.2
Retinoic acid (120 h)	79.7	9.8	10.5

Cisplatin (5 μM) was added for 4 h or 36 h and after extensive washing caffeine (2 mM) was added for an additional 6 h or 24 h. RA was added at the final concentration of 5 μM for up to 5 days. Cell cycle analysis was performed on methanol/acethone fixed cells (4:1, at −20°C) incubated with 10 μg/ml iodidum propide and 15 kU/ml RNase for 30 min at 37°C. Fluorescence at 580 nm was collected on a FACScan analyzer and evaluated and 10,000 events using a CellFit programme.

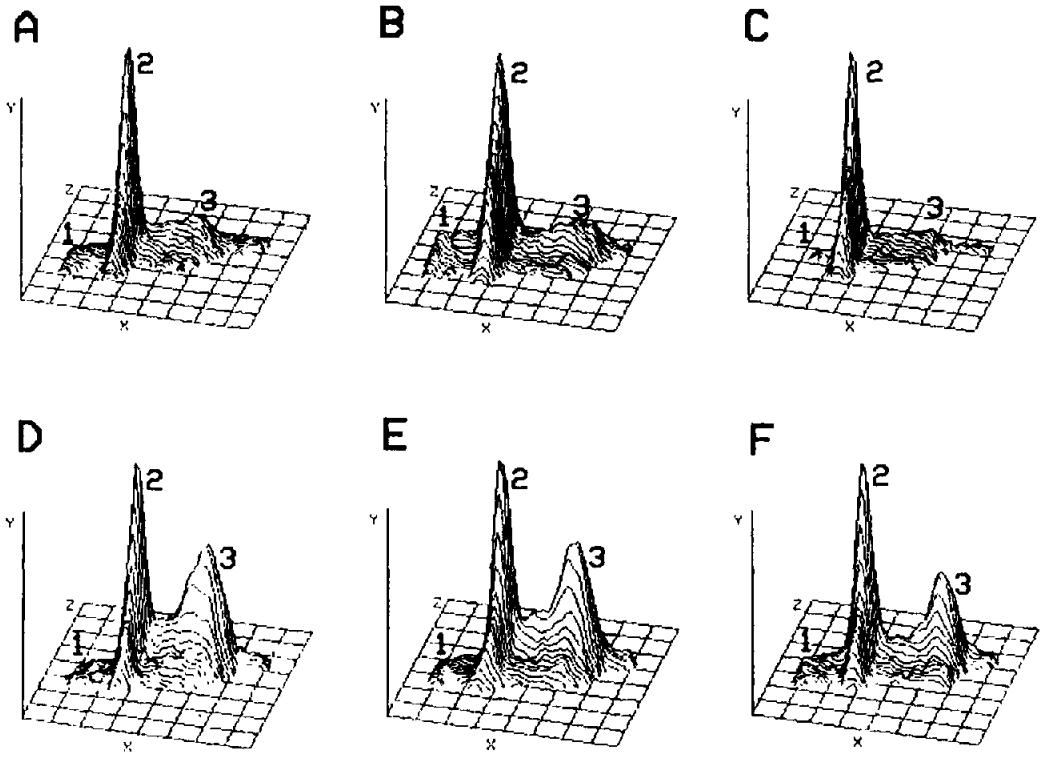

Fig. 2. 3D-evaluation of cell cycle in SK-N-BE(2) cells; untreated (panel A), and treated with 5 mM caffeine for 12 h (panel B) or 24 h (panel C). Cells were also treated with 5 μM cisplatin for 4 h (panel D), followed by 5 mM caffeine for 24 h (panel E) or 48 h (panel F). The axes represent 580 nm fluorescent emission by propidium iodide (x), cell volume (z), and number of events (y). The three main peaks indicate apoptotic cells (peak 1), cells in G0/G1 (peak 2) and cells in G2/M (peak 3). Cisplatin induces a sharp arrest in G2/M (panels A, D), and caffeine removes the G2/M checkpoint both in untreated cells (panels A–C) and in cisplatin-treated cells (panels D–F).

the cells in the G_2/M phase (see also Fig. 2 panels A and D); the percentage of cells accumulated in G_2/M is dependent on the exposure time to cisplatin. Since both RA and cisplatin kill neuroblastoma cells by triggering apoptosis our finding indicate that this programme is accessible from different cell cycle phases, namely G_0/G_1 and G_2/M. The transitions through G_1/S and G_2/M are regulated by the so-called checkpoints control which are surveilled by specific genes [16]. One of the recent most exciting developments in cell cycle regulation is that an unproper completion of its key events (DNA duplication, chromosome segregation and cell cleavage) may result in the induction of cell death programme. P53 has recently been suggested to play a pivotal role in the control of G_1/S transition by switching on the apoptosis in case the cell has damaged DNA [32]. In this regard the occurrence of apoptosis following cisplatin could be envisaged as a defence mechanism to eliminate damaged cells in order to avoid cycling mutated cells undergoing definitive transformation. In order to investigate this hypothesis we attempted to force the G_2/M block of cisplatin-damaged cells by caffeine. Caffeine is a synergistic agent, potentiating slow death by nitrogen mustard in G_2 arrested hamster kidney cells [18]. Caffeine, acting as a partially specific protein kinase inhibitor, allows cells with damaged or unreplicated DNA to enter mitosis, overcoming feedback controls [27]; see the reduction of peak 3 (G_2/M) in panels A–C in Fig. 2 and Table III. This aberrant mitosis may force the cells to death, possibly by inducing apoptosis [18,22]. However, we failed to detect apoptosis both by morphological (Fig. 2 peak 1 in panels D–F) or biochemical (tTG activity and crosslinked apoptotic bodies; data not shown) analysis in cells treated with cisplatin followed by 2 mM caffeine for 6 to 48 h. Thus, the reported potentiation of lethality by caffeine in G_2 damaged cells in our model occurs by mechanisms other than programmed cell death. In keeping with this hypothesis Fig. 2 shows the accumulation of small hypochromic cells with fragmented chromatin upon exposure to cisplatin and subsequently to caffeine for 48 h (peak 1, panels D–F). The cells in peak 1 are gated upon scatter and fluorescence emission, and previous studies have shown that cells presenting these features by FACS analysis indeed have their DNA cleaved at the internucleosomal linker regions [6,17]. In connection with this, it is important to mention that the DNA of cells, especially when blocked in mitosis, could be cleaved in a typical 'ladder' pattern by the DNAI localized in the cytoplasm [23] and this phenomenon is independent from mRNA and protein synthesis [5]. The data reported herein show that the effect of caffeine does not

require tTG induction, thus suggesting that neuroblastoma cells can also die by alternative pathways which only share some features (DNA fragmentation) with the physiological gene-dependent programme of cell death.

The mechanism of action of RA seems to contemplate an arrest of the cells in G_1 followed by an active switch of the neuronal differentiation or apoptotic pathway depending on the maturational stage of the neural crest derived cell [24,26]. In keeping with this is our finding that apoptosis induced by RA is S-phenotype specific [24,26]; while both the neuroblastic and the substrate-adherent cells can be forced to apoptosis by cisplatin, which seems to have broader, less specific effects. The detailed molecular mechanisms of apoptosis are still largely unknown in eukaryotic cells, thus if this difference is due to the expression of specific key regulatory genes represents matter for future investigations. Although the data presented in this report do not enable us to speculate whether RA and cisplatin use the same pathway to reach the common end result, however, the fact that they share an enhanced expression of one of the effector elements of the apoptotic programme (tTG) [11], might suggest that RA and cisplatin have two separate cell cycle startpoints for a common pathway of programmed cell death. In keeping with this it has recently been reported that, in synchronized cells, tTG activity is detectable in all phases of the cell cycle, but with a relative peak during mid S-phase [8].

In conclusion, the present report demonstrates various chemical agents force human neuroblastoma cells to enter the apoptotic death programme from different cell cycle phases. These data are in keeping with those recently reported in Rat-1 fibroblast cells having a deregulated *c-myc* expression and A1.1 T-cell hybridoma which also enter apoptosis from various points through the cell cycle [5,8], thus further underlying the profound connection between apoptosis and cell-cycle regulation [2,9,10].

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