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Involvement of 5-lipoxygenase in programmed cell death of cancer cells

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Abstract

We investigated the involvement of 5-lipoxygenase activity in the early phases of programmed cell death (PCD) induced by H_2O_2 or retinoids in different human tumour cells (erythroleukaemia, neuroblastoma, melanoma). Apoptotic cells showed enhanced 5-lipoxygenase activity which was paralleled by decreased superoxide dismutase activity and increased light emission. Ultraweak luminescence, mainly due to membrane lipid peroxidation by lipoxygenase activation, increased in all cell lines tested within 10–15 min after induction of PCD, in a concentration and time-dependent manner. At the same time, we observed a significant increase in the intracellular steady state level of the 5-lipoxygenase metabolite leukotriene B_4. Furthermore, 5-lipoxygenase metabolite 5-hydroxyeicosatetraenoic acid was able to induce PCD in all cell lines tested. Conversely, the general lipoxygenase inhibitor nordihydroguaiaretic acid and the selective 5-lipoxygenase inhibitor caffeic acid protected the different tumour cells from H_2O_2-induced PCD to a similar extent. These results show the activation of the 5-lipoxygenase pathway in PCD of three different cancer cell lines.

Keywords: cell death; erythroleukaemia; leukotriene B_4; lipoxygenase; melanoma; neuroblastoma

Abbreviations: 5-H(P)ETE, 5-hydro(pero)xyeicosa-6,8,11,14-tetraenoic acid; LTB_4, leukotriene B_4; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; PCD, programmed cell death; RA, all-*trans* retinoic acid; ROS, reactive oxygen species; Tunel, TdT-mediated dUTP-biotin nick end labelling.

Introduction

The mechanisms responsible for the morphological and biochemical hallmarks of programmed cell death (PCD) are not yet fully understood (Raff, 1992; Hale *et al*, 1996). Reactive oxygen species (ROS) and lipid peroxides have been considered crucial elements in PCD (Hockenbery *et al*, 1993; Kane *et al*, 1993); for example, the apoptosis of thymocytes involves oxygen metabolites (Torres-Roca *et al*, 1995; Wolfe *et al*, 1994). A possible mechanism has been suggested to link the redox regulation to the expression of transcription factors such as NF-kB and Fos-Jun (Abate *et al*, 1990; Meyer *et al*, 1993) explaining the connection between oxidative stress and PCD. Despite evidence for the involvement of ROS in PCD, oxygen radicals have not been directly identified in apoptotic cells, furthermore other data seem contradictory. The anti-apoptotic effect of Bcl-2 has been related to its ability to decrease the net cellular generation of ROS and lipid peroxides (Hockenbery *et al*, 1993; Kane *et al*, 1993); however, Bcl-2 is also able to protect cells from apoptotic death induced by several unrelated stimuli, such as ceramide (Martin *et al*, 1995), and Bcl-2ΔC has been shown to have pro-oxidant activity in murine B-cells (Steinman, 1995). On the other hand, anoxia is known to induce endonuclease activity (Stoler *et al*, 1992), typical of PCD, indicating that ROS are not required for PCD, while Bcl-2 still protects from death in these conditions (Jacobson and Raff, 1995; Shimizu *et al*, 1995). Thus, Bcl-2 may act by a mechanism different from the regulation of the oxidative balance, such as the Bcl-2/Bax rheostatic mechanism involving several members of the Bcl-2 family (Boise *et al*, 1993; Chittenden *et al*, 1995; Farrow *et al*, 1995; Klefer *et al*, 1995; Oltvai *et al*, 1993; Takayama *et al*, 1995; Yang *et al*, 1995). Despite some controversy, intracellular oxidants (ROS) seem related to the apoptotic process. Together with mitochondrial electron transport, arachidonic acid metabolites are the major generator of ROS. Consequently, the metabolites of arachidonic acid might contribute to controlling the fate of the cell, via the production of ROS, the damage of important intracellular components and the expression of other genes, including those implicated in PCD. Recently, some evidence has shown the involvement of arachidonic acid metabolites in PCD (Hebert *et al*, 1996; Tang *et al*, 1996). Here, we would like to contribute by evaluating the involvement of 5-lipoxygenase in cell death.

Although the nucleus plays a central role in PCD, cellular membranes are the primary site of action of several PCD inducers and lipid messengers act as regulators of apoptosis (Bissonnette *et al*, 1995; McGahon *et al*, 1995). 5-Lipoxygenase (EC 1.13.11.34) plays a critical role in lipid-mediated signal transduction (Los *et al*, 1995) and its metabolites were recently found to be active in the nucleus of human cells (Jakobsson *et al*, 1995). Mammalian lipoxygenases are also able to directly oxygenate cell

membranes, introducing peroxides in the lipid bilayers (Kühn *et al*, 1990; Schnurr *et al*, 1996). In this study, we used different human cancer cells to demonstrate the involvement of 5-lipoxygenase in PCD.

Results

Modulation of oxygen-related enzyme activities in H_2O_2-induced apoptosis

PCD was induced by H_2O_2-mediated oxidative stress, as described by Hockenbery *et al* (1993) and in our cellular models by De Laurenzi *et al* (1995). In order to use different systems, the cell lines were selected among classic unrelated models to study apoptosis, currently used by several laboratories. The induction of apoptosis, at least in some models, is accompanied by a downregulation of the expression of mRNA of antioxidant enzymes, such as catalase, superoxide dismutase, DT-diaphorase and thioredoxin (Briehl *et al*, 1995). We repeated these measurements in our cell systems extending the observation to 5-lipoxygenase. After the H_2O_2 pulse, all cell lines showed increased 5-lipoxygenase activity and decreased superoxide dismutase activity (Table 1). The activation of 5-lipoxygenase was evident at a very early stage of PCD induction, although at this point there was still no sign of apoptotic body formation (Table 1). Catalase activity sharply increased after exposure to hydrogen peroxide and then decreased towards the control values (Table 1). These data are at variance with the recently reported downregulation of catalase activity in dexamethasone-induced PCD in mouse lymphoid cells (Baker *et al*, 1996).

Neuroblastoma SK-N-BE(2) cells undergo PCD when treated with RA for 3 to 5 days (Melino *et al*, 1994; Piacentini *et al*, 1992, 1993). Table 2 shows that retinoids increased PCD in parallel to a significant enhancement of 5-lipoxygenase activity. In previous experiments, we showed that RA treatment of SK-N-BE(2) cells reduces superoxide dismutase activity and increases catalase activity to 50% and 120% of the control values, respectively (Steinkühler *et al*, 1988). Therefore, modulation of antioxidant enzymes by RA resembles that observed upon H_2O_2 pulse.

Table 2 Changes of 5-lipoxygenase activity and luminescence in RA-induced apoptosis

Sample	Apoptotic bodies (%)	5-lipoxygenase activity	Luminescence (%)
control	4.6 ± 0.4	0.18 ± 0.01 (100%)	100
RA (3 days)	7.3 ± 0.6	0.28 ± 0.02 (155%)	152 ± 15
RA (5 days)	12.6 ± 1.0	0.36 ± 0.04 (200%)	178 ± 20

Effects of 1 μM retinoic acid (RA) on apoptotic body formation, 5-lipoxygenase activity and luminescence of SK-N-BE (2) cells. 5-lipoxygenase activity is expressed as both nmol 5-HPETE formed x min^{-1} x mg protein^{-1} ($\pm$S.D.) and percentage of the control (in brackets). Formation of apoptotic bodies was measured by cytofluorimetry and Hoechst staining. Data are the mean of three independent experiments, each one performed in duplicate, with p values < 0.01 vs. control.

Table 1 Activities of oxygen-related enzymes in H_2O_2-induced apoptosis

Sample	Apoptotic bodies (%)	5-lipoxygenase activity	Superoxide dismutase activity	Catalase activity
K562 control	2.5 ± 0.3	0.36 ± 0.04 (100%)	9.50 ± 1.00 (100%)	40.84 ± 4.10 (100%)
K562+H_2O_2 (15 min)	3.3 ± 0.3[a]	0.61 ± 0.06 (170%)	7.10 ± 0.75[b] (75%)	72.21 ± 7.25 (177%)
K562+H_2O_2 (24 h)	39.7 ± 3.0	0.72 ± 0.07 (200%)	4.75 ± 0.45 (50%)	34.42 ± 3.45[b] (84%)
SK-N-BE (2) control	4.6 ± 0.4	0.18 ± 0.01 (100%)	2.20 ± 0.20 (100%)	19.24 ± 1.95 (100%)
SK-N-BE (2)+H_2O_2 (15 min)	8.0 ± 0.7	0.34 ± 0.03 (189%)	1.15 ± 0.10 (52%)	27.36 ± 2.75 (142%)
SK-N-BE (2)+H_2O_2 (24 h)	13.8 ± 1.2	0.38 ± 0.04 (211%)	0.50 ± 0.05 (23%)	23.58 ± 2.35 (122%)[b]
B-mel control	1.1 ± 0.2	0.18 ± 0.01 (100%)	15.30 ± 1.50 (100%)	6.09 ± 0.61 (100%)
B-mel+H_2O_2 (5 min)	0.9 ± 0.2[a]	0.32 ± 0.03 (178%)	12.10 ± 1.25[b] (79%)	10.79 ± 1.10 (177%)
B-mel+H_2O_2 (24 h)	14.1 ± 1.3	0.38 ± 0.04 (211%)	3.20 ± 0.30 (21%)	7.66 ± 0.75[b] (126%)

Effects of hydrogen peroxide on the activity of 5-lipoxygenase (nmol 5-HPETE formed x min^{-1} x mg protein^{-1}), superoxide dismutase (units x mg protein^{-1}) and catalase (μmol H_2O_2 degraded x min^{-1} x mg protein^{-1}) of K562, SK-N-BE (2) and B-mel cells. Formation of apoptotic bodies was measured by cytofluorimetry (reported in the Table) and Hoechst staining (not shown). 10 mM H_2O_2 was used for K562 and SK-N-BE (2) cell treatment, but 5 mM only for B-mel cells. These conditions also yielded the highest luminescence, as shown in Figure 1. The Table reports the absolute values ($\pm$S.D.) and the percentage with respect to the controls (in brackets). Data are the mean of three independent experiments, each one performed in duplicate, with p values < 0.01 vs. control, if not stated otherwise. [a]Denotes $p > 0.05$ vs. control; [b]denotes $p < 0.05$ vs. control.

Increase of lipid peroxidation in cells treated with H_2O_2

We have measured cellular luminescence in order to evaluate the amount of lipid peroxides formed in the lipid bilayers of cells in response to hydrogen peroxide (Maccarrone *et al*, 1995a,b; Nakano *et al*, 1994). Treatment with H_2O_2 increased the luminescence in all the cell types studied (Figure 1). Light emission after the addition of H_2O_2 was concentration- and time-dependent, but each cell type showed a maximum response at a typical dose and time (Figure 1B and C). Higher concentrations of, or longer incubation with hydrogen peroxide consistently resulted in a decrease of luminescence. The experimental conditions which yielded the largest increases of luminescence in each cell type yielded a significant increase in 5-lipoxygenase activity as well (Table 1). Table 1 also shows that K562, SK-N-BE(2) and B-mel cells exposed to the respective optimal hydrogen peroxide stimulus and then cultured for 24 h had a significant increase in apoptotic body formation, as evaluated by flow cytometry and Hoechst staining. This increase in apoptotic bodies was significantly less pronounced in cells pulsed with other less-than-optimal amounts of hydrogen peroxide and then cultured for 24 h (data not shown).

To better correlate the activation of 5-lipoxygenase with programmed cell death, we separated by gradient centrifugation live cells from dead cells 24 h after treatment with 10 mM H_2O_2. Dying K562 cells (higher density; $70.4 \pm 6.0\%$ apoptotic event by flow cytometry) show an activity of 5-lipoxygenase of 1.18 ± 0.10 nmol 5-HPETE $\times$ min^{-1} $\times$ mg protein^{-1}; while K562 cells before separation (39.7 ± 2.8 % apoptotic event by flow cytometry) show an activity of 5-lipoxygenase of 0.72 ± 0.07 nmol 5-HPETE $\times$ min^{-1} $\times$ mg protein^{-1}. This increase in both apoptotic events (177%) and 5-lipoxygenase activity (164%) in dying cells over the whole cell population, indicates that the activation of 5-lipoxygenase at 24 h does indeed correlate with the death pathway and is not related with other phenomena occurring in the fraction of live cells.

In order to verify whether the activation of 5-lipoxygenase and luminescence also occurred with a different inducer of PCD, we treated neuroblastoma cells with retinoic acid for 3 or 5 days, a model well-characterised in our laboratory (Melino *et al*, 1994; Piacentini *et al*, 1992, 1993). Treatment with retinoids significantly increased light emission from neuroblastoma cells (Table 2), although longer incubation times were needed due to the slower induction of PCD in this model (Melino *et al*, 1994; Piacentini *et al*, 1992, 1993).

Involvement of 5-lipoxygenase in PCD

The activation of 5-lipoxygenase in cells undergoing PCD should result in the overproduction of its intracellular metabolites, such as LTB_4 (Jakobsson *et al*, 1995). K562, SK-N-BE(2) and B-mel cells indeed show a significant increase (in the nM range) in LTB_4 levels within the cell (2.9- to 15-fold, depending on the cell line), when pulsed with hydrogen peroxide (Figure 2A). The increase in LTB_4 was as rapid as the activation of 5-lipoxygenase, though LTB_4 then declined towards the basal level, whereas 5-lipoxygenase activity remained high (compare Figure 2A and Table 1). This finding might suggest a rapid LTB_4 turnover and might be explained by the fact that leukotrienes are not the sole products of 5-lipoxygenase, which might also act in membrane oxygenation (Kühn *et al*, 1990; Schnurr *et al*,

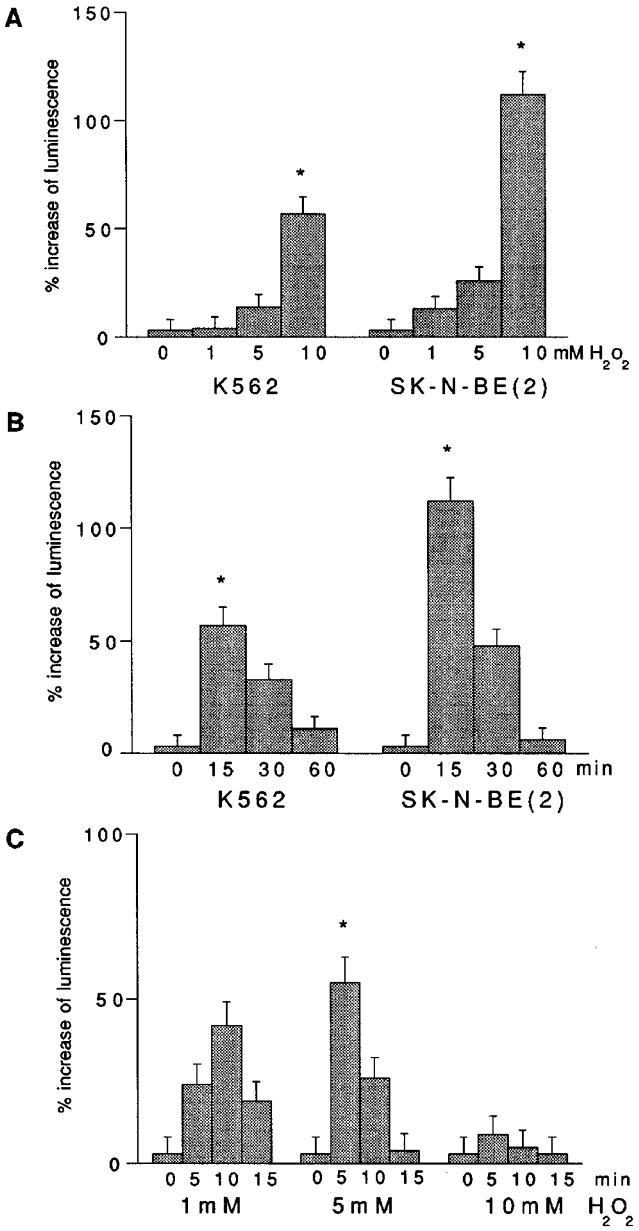

Figure 1 Effect of H_2O_2 on the ultraweak luminescence of K562, SK-N-BE(2) and B-mel cells. (**A**) shows cells incubated for 15 min in the suitable culture media containing the indicated concentrations of hydrogen peroxide. The asterisks indicate the concentrations that were chosen for time-course experiments. (**B**) shows the effect of the incubation time on luminescence of K562 and SK-N-BE(2) cells, incubated with 10 mM H_2O_2. (**C**) shows the effects of hydrogen peroxide concentration and incubation time on luminescence of B-mel cells. The asterisks in (**B**–**C**) indicate the experimental conditions corresponding to the largest increases in 5-lipoxygenase activation.

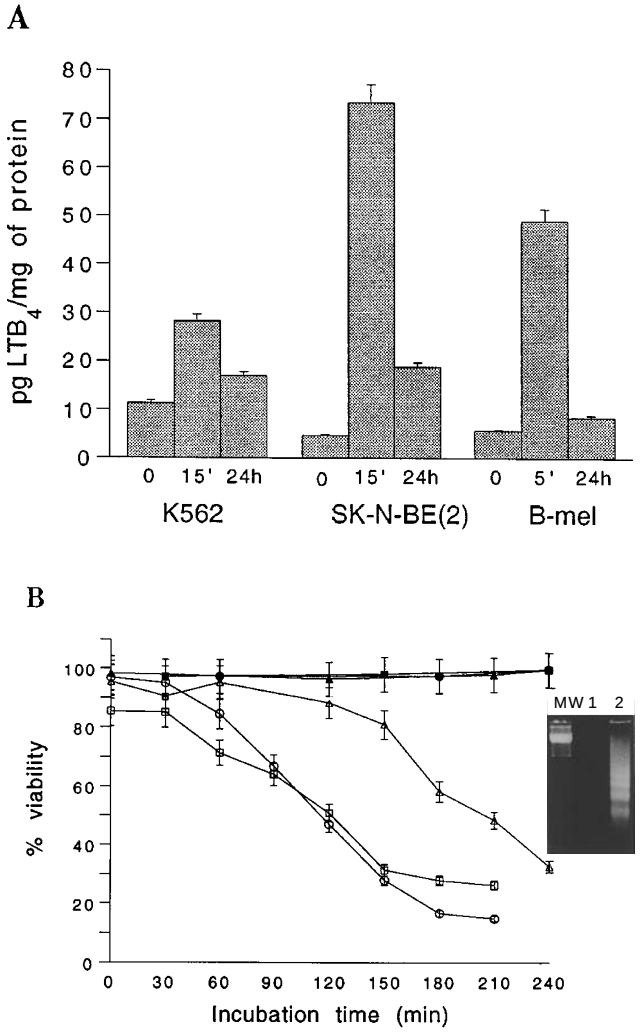

Figure 2 (**A**) Changes in LTB_4 levels in K562, SK-N-BE(2) and B-mel cells. The different cell lines were pulsed with H_2O_2 under conditions inducing maximum activation of 5-lipoxygenase and increase of luminescence (asterisks in Figure 1B and C). (**B**) Cytotoxicity of 5-HETE. The effect of 5-HETE on cell viability was evaluated on K562 (empty triangles), SK-N-BE(2) (empty squares) and B-mel (empty circles) cells, compared to untreated controls (full symbols). 5-HETE was used at a final concentration of 5 μM. Inset. DNA fragmentation of K562 cells, untreated (lane 1) or exposed to 5 μM 5-HETE for 180 min. MW indicates the 123 bp ladder.

1996). Furthermore, leukotriene formation depends on the availability of the substrate, which in turn involves the activity of phospholipases (Yamamoto, 1992).

The possible involvement of 5-lipoxygenase products as effectors of PCD was assessed using a more stable derivative of the enzyme activity, 5-HETE. This metabolite reduced viability (determined by Trypan blue dye exclusion) in all the cell types tested, in a concentration- (not shown) and time-dependent manner (Figure 2B). Cell death induced by 5-HETE was associated with the DNA ladders typical of apoptosis, both in K562 cells (Figure 2B, inset) and in the other cell types (not shown). Conversely, the general lipoxygenase inhibitor NDGA (Ford-Hutchinson *et al*, 1994) and the selective 5-lipoxygenase inhibitor caffeic

Table 3 Effect of lipoxygenase inhibitors on H_2O_2-induced apoptosis

Sample	No inhibitor	Apoptotic bodies (%) NDGA	Caffeic Acid
K562	39.7 ± 3.0 (100%)	23.5 ± 2.50 (59%)	21.83 ± 2.0 (55%)
SK-N-BE (2)	13.8 ± 1.2 (100%)	8.28 ± 0.80 (60%)	7.60 ± 0.77 (55%)
B-mel	14.1 ± 1.3 (100%)	8.60 ± 0.85 (61%)	7.55 ± 0.75 (53%)

The general lipoxygenase inhibitor NDGA and the selective 5-lipoxygenase inhibitor caffeic acid were added, both at 40 μM final concentration, to the culture media of K562, SK-N-BE (2) and B-mel cells. The Table reports the absolute values (± S.D.) and the percentage with respect to the controls (in parentheses). Formation of apoptotic bodies was measured by cytofluorimetry and Hoechst staining. Data are the mean of three independent experiments, each one performed in duplicate, with p values < 0.01 vs. control (i.e. no inhibitor).

acid (Ford-Hutchinson *et al*, 1994; Sud'ina *et al*, 1993; Tang *et al*, 1996) significantly protected the different tumour cells from H_2O_2-induced PCD. Indeed, the addition of either 40 μM NDGA or 40 μM caffeic acid to the culture medium, together with H_2O_2, reduced the number of H_2O_2-induced apoptotic bodies by approximately 55–60% (Table 3). Moreover, in the presence of either lipoxygenase inhibitors, cell luminescence decreased to approximately 70% of the control, i.e. cells pulsed with H_2O_2 as shown in Figure 1 (star in panels B–C), whereas superoxide dismutase and catalase activities were not significantly altered.

Discussion

Treatment with H_2O_2 leads to an early significant increase in ultraweak light emission (Figure 1): the H_2O_2 pulses generating the largest increase in luminescence for each cell type significantly increased PCD after 24 h (Table 1). This increase in luminescence suggests an increase in membrane lipid peroxides (Maccarrone *et al*, 1995a,b; Nakano *et al*, 1994) which occurs before any morphological and biochemical evidence of PCD. Such lipid peroxidation is paralleled by an increase in 5-lipoxygenase activity; an enzyme that, together with other lipoxygenases, has been shown to induce membrane lipid peroxidation (Kühn *et al*, 1990; Schnurr *et al*, 1996). 5-Lipoxygenase activity almost doubled in all cell types only 10–15 min after the H_2O_2 pulse, whereas superoxide dismutase activity rapidly decreased and catalase activity transiently increased (Table 1); these changes in 5-lipoxygenase and superoxide dismutase activities were most significant 24 h after treatment with H_2O_2, at which time PCD was also maximal, suggesting that the change in activity of both enzymes may be relevant for PCD. Similar results were obtained inducing PCD with RA (Table 2), transforming growth factor β1 or cisplatin (not shown), indicating a broader implication of these findings.

First, the involvement of 5-lipoxygenase in apoptosis is supported by the increase in intracellular levels of LTB_4 (Figure 2A). The data in Figure 2 indicate that the intracellular concentrations of LTB_4 rose to 30 nM (K562), 50 nM (B-mel) and 70 nM [SK-N-BE(2)], respectively. This

concentration range was found to evoke a 5-lipoxygenase-dependent signal transduction pathway involving protein kinase activation in human cells (Los *et al*, 1995). More in general, lipoxygenases are key-enzymes for the synthesis of lipid second messengers, which are assuming an increasing importance as PCD-regulators (McGahon *et al*, 1995). The ability of lipoxygenases to modulate membrane properties (Kühn *et al*, 1990; Schnurr *et al*, 1996) might be critical in a process involving the membranes, such as PCD. Along these lines, it has been recently reported that 5-lipoxygenase and its products are active in the nucleus of mammalian cells (Jakobsson *et al*, 1995). Second, direct evidence for the relevance of 5-lipoxygenase activity in our systems is provided by the ability of 5-HETE to induce PCD in the same cell types (Figure 2B). Third, the general lipoxygenase inhibitor NDGA (Ford-Hutchinson *et al*, 1994) and the more selective 5-lipoxygenase inhibitor caffeic acid (Ford-Hutchinson *et al*, 1994; Sud'ina *et al*, 1993; Tang *et al*, 1996) protected from H_2O_2-induced PCD to a similar extent (Table 3), suggesting that activation of 5-lipoxygenase, but not other isoenzymes, occurs during apoptosis. NDGA is also a radical scavenger, but its radical scavenging ability is unable to protect human cells against ROS-induced death (O'Donnell *et al*, 1995; Tang *et al*, 1996). Therefore, the NDGA protection against H_2O_2-induced PCD can be attributed to a true inhibition of lipoxygenase activity. This conclusion is corroborated by the effect of caffeic acid, a specific 5-lipoxygenase inhibitor whose protection against H_2O_2-induced PCD (Table 3) correlates with the extent of inhibition of 5-lipoxygenase activity *in vitro* (Sud'ina *et al*, 1993). These results extend recent findings on the role of the 12-lipoxygenase pathway in regulating rat cell death (Tang *et al*, 1996) and indicate that lipoxygenases are indeed involved in the apoptotic programme. This involvement has been recently implied also by Sandstrom *et al*. (1994), who reported that lipid peroxides induce apoptosis in HIV-infected T cells.

A recent paper describes at morphological level the modulation by LOX-derived eicosanoids during apoptosis of human neutrophils (Hebert *et al*, 1996). Here, LTB_4, contrary to other LOX-derived eicosanoids including 5-HETE, attenuates the spontaneous apoptosis of neutrophils, indicating an involvement in the inflammatory pathway. It is likely that the role of eicosanoids in cell death of inflammatory cells such as neutrophils which are particularly rich in eicosanoids, differs from that in cancer cells. Indeed, LTB_4 has been shown to bind and transactivate the transcription factor peroxisome proliferator-activated receptor (PPARα), thus directly controlling the expression of anti-inflammatory agents and genes involved in the β-oxidation pathway such as acyl Co-A oxidase (Devchand *et al*, 1996). The possibility that such a mechanism is implicated in the absence of inflammatory response in apoptosis, through LTB_4, remains to be investigated.

In conclusion, our results show that early activation of the 5-lipoxygenase pathway is associated with PCD, even though the molecular events which allow the execution of the apoptotic programme remain to be fully elucidated.

Materials and Methods

Materials

Chemicals were purchased from Sigma (St. Louis, Missouri, USA) or Flow Laboratories Ltd. (Ayrshire, Scotland, UK). The Biotrak LTB_4 enzyme immunoassay system was from Amersham (Buckinghamshire, UK).

Cell cultures and treatments

Erythroleukaemia K562, neuroblastoma SK-N-BE(2) and melanoma B-mel cells were grown as described (Melino *et al*, 1994). Briefly, cells were grown in monolayer culture in a 1 : 1 mixture of MEM and Hams F-12 media supplemented with 15% heat-inactivated FBS, sodium bicarbonate (1.2 mg ml^{-1}), HEPES buffer (15 mM), L-glutamine (2 mM) and non-essential amino acids (1% v/v). Cells were grown in 5% (v/v) CO_2 humidified atmosphere at 37°C. Cells were fed every 3-4 days and were split weekly at a ratio of 1 : 3 to 1 : 6 using trypsin (0.025%)-EDTA (0.02%). Cells were routinely fed 24 h before harvest for experiments.

RA- or H_2O_2-induced apoptosis was provoked as reported (De Laurenzi *et al*, 1995; Melino *et al*, 1994). Briefly, cells died within 2–4 h when exposed to 0.5–2 mM H_2O_2 (data not shown); alternatively, cells were treated with either 1 mM RA (5 mM stock solution in 70% ethanol); 0.07% ethanol was added to the control cultures. Lipoxygenase inhibitors (NDGA or caffeic acid) were added at the indicated concentrations just before pulsing with H_2O_2. Treatments with 5-HETE (1 to 5 μM final concentration) were performed by adding hydroperoxide to each cell suspension (2.5×10^6 cells/ml PBS). The micromolar concentration range of 5-HETE was chosen because it has been regularly used to study the effect of lipoxygenase products on mammalian cells (O'Donnell *et al*, 1995; Tang *et al*, 1996).

In some experiments, dying cells were separated from the whole cell population by gradient centrifugation. Briefly, an equal volume of cell suspension (in PBS) was layered on top of lymphoprep (Nycomed Pharma A.S., Oslo, Norway). After centrifugation at 1800 rpm for 20 min in a table centrifuge, dying cells were recovered as a pellet, whereas non-dying cells formed an intermediate band between the two fluids at different densities.

Enzyme activity and luminescence measurements

Cell extracts (Maccarrone *et al*, 1995) were assayed for the activity of 5-lipoxygenase (EC 1.13.11.34), superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6). 5-Lipoxygenase activity was expressed as nmol 5-HPETE (5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid) formed *x* min^{-1} *x* mg $protein^{-1}$ (Van der Donk *et al*, 1991); superoxide dismutase activity was expressed as units *x* mg $protein^{-1}$ (Paoletti and Mocali, 1990) and catalase activity as μmol substrate (H_2O_2) degraded *x* min^{-1} *x* mg $protein^{-1}$ (Aebi, 1984). LTB_4 was determined by immunoassay within the linearity range of the calibration curve (pure LTB_4 0–10 pg/well).

Luminescence (Maccarrone *et al*, 1995), counts *x* s^{-1} *x* mg $protein^{-1}$ (cps *x* mgP^{-1}), was expressed as the percentage of the control values, i.e. 320, 126 and 84 cps *x* mgP^{-1}, for K562, SK-N-BE(2) and B-mel cells, respectively. Ultraweak luminescence was measured by single photon counting in a Lumi-A luminometer (SEAS, Milan, Italy) using 10^6 cells/ml PBS suspended in 1 ml cuvettes. Cellular luminescence is proportional to the peroxidation of membrane lipids (Maccarrone *et al* 1995b; Nakano *et al*, 1994).

Evaluation of cell death

After incubation for 24 h in culture medium, floating and enzymatically-detached cells were collected together by centrifugation at 800 × *g* for 5 min. Apoptosis was estimated in all experiments using standard procedures, such as cytofluorimetric analysis (see also Piacentini *et al*, 1993; Melino *et al*, 1994) and Hoechst staining (Hoechst 33258, 0.1 μg/ml PBS, at 37°C for 1 h, washed, air dried and mounted). Tunel (TdT-mediated dUTP-biotin nick end labeling) (see also Melino *et al*, 1994) and DNA ladder (Martin *et al*, 1995) were also used to assess DNA fragmentation.

Briefly, DNA fragmentation of death cells was evaluated by flow cytometry by staining with propidium iodide (50 μg/ml; pre-treated also with RNase to reduce noise) on a FACScan flow cytometer (Becton-Dikinson, CA, USA). Cells were fixed 1 : 1 in PBS using methanol : acetone (4 : 1 v/v) solution at −20°C and stored at +4°C. Cells were excited at 488 nm using a 15 mW Argon laser, and the fluorescence was monitored at 570 nm. Events were triggered by the FSC signal and gated for FSC-H/FSC-A/SSC to avoid aggregates. Ten thousand events were evaluated using the Lysis II Programme (ibid).

Data reported in this paper are the mean of triplicate experiments, with S.D. values as indicated in the text. Statistical analysis was performed by the Student's *t*-test.

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