Constitutive expression of the c-H-ras oncogene inhibits doxorubicin-induced apoptosis and promotes cell survival in a rhabdomyosarcoma cell line

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Summary
Drugs used in anti-cancer chemotherapy are thought to exert their cytotoxic action by induction of apoptosis. Genes have been identified which can mediate or modulate this drug-induced apoptosis, among which are c-myc, p53 and bcl-2. Since expression of oncogenic ras genes is a frequent observation in human cancer, we investigated the effects of the c-H-ras oncogene on anti-cancer drug-induced apoptosis. Apoptosis induced by a 2 h doxorubicin exposure was measured by in situ nick translation and flow cytometry in a rat cell line (R2T24) stably transfected with the c-H-ras oncogene and in a control cell line (R2NEO) transfected only with the antibiotic resistance gene neo. Both cell lines (R2T24 and R2NEO) had nearly identical growth characteristics, including cell doubling time, distribution over the cell cycle phases and plating efficiency in soft agar. Doxorubicin exposure of the R2NEO cells led to massive induction of apoptosis. In contrast, R2T24 cells, expressing the c-H-ras oncogene, showed significantly less apoptosis after doxorubicin incubation. Doxorubicin induced approximately 3- to 5-fold less cytotoxicity in the R2T24 cells than in the R2NEO cells, as determined by clonogenic assay in soft agar. No difference was observed in intracellular doxorubicin accumulation between the two cell lines, indicating that the classical, P-glycoprotein-mediated multidrug resistance phenotype is not involved in the observed differences in drug sensitivity. In conclusion, our data show that constitutive expression of the c-H-ras oncogene suppresses doxorubicin-induced apoptosis and promotes cell survival, suggesting that human tumours with ras oncogene expression might be less susceptible to doxorubicin treatment.

Keywords: apoptosis; c-H-ras oncogene; drug resistance; doxorubicin

Chemotherapy failure due to cellular drug resistance is still a major problem in most cancer patients. A variety of drug resistance mechanisms have been characterised using in vitro cell lines made resistant to the different classes of anti-cancer agents. Qualitative and quantitative alterations in cellular target proteins, drug metabolism, repair mechanisms and drug efflux from the cell, among others, can cause drug resistance in vitro. However, a clear relationship between these cellular biochemical alterations and chemotherapy failure in patients could not be established for most drug resistance mechanisms identified so far. These resistance mechanisms have in common that they concern, directly or indirectly, the interaction of the drug molecule with its intracellular target molecules. A different approach for the elucidation of the mechanisms of cellular drug resistance is to study how cells are killed by cytotoxic drugs and to unravel the events that occur as a consequence of the drug–target interaction that finally leads to cell death.

It is now well appreciated that most anti-cancer drugs can exert their cytotoxic action by triggering a conserved, gene-activated programme for cell death, often referred to as apoptosis (Wyllie et al., 1980; Dive and Hickman, 1991; Eastman and Barry, 1992; Sen and D’Incalci, 1992; Wyllie, 1993). Apoptosis is the normal physiological method of cell death during, for example, embryogenesis and tissue homeostasis, and can also be induced by a large variety of external stimuli, such as viral infections and toxic insults. Therefore, it may well be that the susceptibility of a cancer cell to drug-induced apoptosis is an important determinant in the therapeutic response (Dive and Hickman, 1991). Recent evidence strongly suggests that modulation of the apoptotic cell response can lead to drug resistance. It has been shown that the bcl-2 gene can prevent or markedly reduce cell kill induced by anti-cancer drugs (Reed, 1994). This oncogene is a member of a superfamily of related genes, including bax (Olincy et al., 1993) and bcl-x (Boise et al., 1993), which normally regulate apoptosis in mammalian cells and are thought to induce cytotoxic drug resistance by blocking a final common pathway to apoptotic cell death. Although the molecular mechanism of apoptosis is yet unknown, several other (onco)genes have been shown to mediate or modulate the apoptotic pathway, among which are the tumour-suppressor gene p53 and the proto-oncogene c-myc. In some cell systems, overexpression of these genes induces or facilitates apoptosis (Young-Roush et al., 1991; Evan et al., 1992; Shaw et al., 1992). It is very likely that other (onco)genes involved in cell proliferation will also play a role in the process of apoptosis. Since oncogenic activation of the ras gene is frequently observed in human cancers (Bos, 1989), we investigated, in our effort to characterise drug resistance parameters in human tumours, the effects of the c-H-ras oncogene on chemotherapy-induced apoptosis.

Materials and methods

Cell lines
The rhabdomyosarcoma cell line R2 and the transfectants, R2T24 and R2NEO, have been described previously (Hermens and Bentvelzen, 1992), and were maintained in monolayer culture in Dulbecco’s modified culture medium, supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. The cells were cultured at 37°C in a humidified incubator with 8.5% carbon dioxide. The R2T24 cell line was co-transfected with the plasmid pT24 carrying the c-H-ras oncogene (Reddy et al., 1982) and the plasmid pKH carrying the neo gene (Davies and Gimenez, 1980). The R2NEO cell line was transfected with the neo gene only. Southern blot analysis with the 6.6 kb BamHI fragment of the pT24 plasmid revealed that the R2T24 cell line contains six copies of the c-H-ras oncogene per cell (Hermens and Bentvelzen,
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followed by another incubation for 60 min after the addition of cyclosporin A (3 μM). The incubations were stopped by putting the cells on melting ice. After washing twice with ice-cold PBS (pH 7.4) the cells were stored at 4°C until flow cytometric analysis.

Results

Growth characteristics of the cell lines

Rat rhabdomyosarcoma R2 cells were stably transfected with plasmids containing the ras oncogene and/or the neo gene, resulting in the establishment of the cell lines R2T24 and R2NEO respectively. These cell lines have been described previously (Hermens and Bentvelzen, 1992), and their relevant growth characteristics will be summarised here. The R2T24 cell line contains six copies of the ras oncogene per cell, as estimated by Southern blot analysis. Dot-blot and Northern blot hybridisation showed abundant ras mRNA expression in R2T24 cells. The in vitro doubling time of the R2, R2T24 and R2NEO cell lines is 0.9, 1.0, and 1.0 days respectively. Cell cycle analysis of cells in logarithmic growth showed no differences between the R2, R2T24 and R2NEO cells in the distribution over the cell cycle phases. The mean proportions of cells in G1, S and G2/M were 50%, 31% and 19% for R2 cells, 54%, 30% and 16% for R2T24 cells and 56%, 28% and 16% for R2NEO cells.

Doxorubicin-induced apoptosis

A 2 h incubation of exponentially growing R2 or R2NEO cells with doxorubicin (1 μM) resulted in an apoptotic response, as determined 24 h later by cell morphology, DNA degradation and in situ nick translation assay. The cells had a typical apoptotic feature with condensed chromatin and nuclear fragmentation. DNA degradation in nucleosome-sized fragments could be detected by qualitative gel electrophoresis (Sellins and Cohen, 1987) (data not shown). Prior to drug incubation, the R2 parental and the R2NEO cell lines only showed very low levels of spontaneous apoptosis, as determined by in situ nick translation assay (Figure 1a). However, after doxorubicin incubation apoptotic cells could be distinguished in R2 and R2NEO cultures on the basis of DNA content and biotin-dUTP labelling. In Figure 1b–1d, R2NEO cell cultures are shown at t = 24 h, t = 48 h and t = 72 h after doxorubicin exposure. Extensive biotin-dUTP labelling was observed at t = 24 h (Figure 1b), which was followed by a gradual decrease in labelling at t = 48 h and t = 72 h (Figure 1c, 1d). At t = 24 h, the vast majority (about 90%) of cells were apoptotic, and formed one cluster with predominantly a subnormal DNA content (Figure 1d). In contrast, at t = 24 h, R2T24 cells remained mostly viable after doxorubicin incubation and showed very little DNA degradation upon gel electrophoresis (data not shown). In the in situ nick translation assay a very small fraction (about 7%) of R2T24 cells was labelled above background (Figure 1e). Although, in the ras-transfected cultures the number of apoptotic cells also upon excitation by the laser light was registered on a photomultiplier of the FACSscan flow cytometer. Data analysis was performed using histogram analysis of the LYSYS II software program (Becton Dickinson). The accumulation of doxorubicin was expressed in arbitrary units (a.u.) by calculating the mean fluorescence distribution of each cell sample. Cells (2 × 10^6) in RPMI without phenol red buffered with 10 mM HEPES buffer (pH 7.4) were incubated at 37°C and 8.5% carbon dioxide either for 60 min with doxorubicin (1 μM) or for 60 min with doxorubicin (1 μM), followed by another incubation for 60 min after the addition of cyclosporin A (3 μM). The incubations were stopped by putting the cells on melting ice. After washing twice with ice-cold PBS (pH 7.4) the cells were stored at 4°C until flow cytometric analysis.

Cytotoxicity assay

Doxorubicin-induced cytotoxicity was determined by colony formation in soft agar. Cells were incubated for 2 h with doxorubicin (concentration range 10 nM to 3.3 μM), washed twice and plated in triplicate at a density of 10^3, 10^4 and 10^5 cells per 35 mm Petri dish in 1 ml of fresh medium containing 0.3% soft agar. Colonies were counted after 10 days of incubation at 37°C and 8.5% carbon dioxide.

Intracellular doxorubicin accumulation

Intracellular doxorubicin accumulation was measured as described previously (Nooter et al., 1983). Cellular anthracycline net uptake can be quantitated by flow cytometry by measuring the fluorescence of the anthracycline molecules upon excitation with laser light of 488 nm (Nooter et al., 1983, 1989). The fluorescence which is emitted by the cells
Doxorubicin-induced drug resistance

Doxorubicin-induced drug resistance of R2T24 cells. The extent of DNA strand breaks is estimated by avidin–FITC fluorescence (ordinate) and cellular DNA content by DNA–propidium iodide fluorescence (abscissa), both expressed in arbitrary units (a.u.). The cells were treated with doxorubicin (1 μM) for 2 h and at the indicated time points thereafter labelled with biotin-dUTP by in situ nick translation and counterstained with propidium iodide. Control, untreated R2NEO cells. The position of cells in G1, S, or G2 + M is indicated. AP, apoptotic cells. Data from representative experiments.

Increased in time (up to about 40% at t = 72 h) (Figure 1f), there was a striking difference between the ras-transfected and the neo-transfected cells (compare Figure 1d and f).

Figure 2 shows the time course of drug-induced apoptosis in R2NEO and R2T24 cells at various doxorubicin concentrations. In the R2NEO cells the proportion of apoptotic cells increased with time at all drug concentrations tested, and the highest drug concentrations induced the highest percentages of apoptotic cells. At 1 μM doxorubicin — a concentration that gives more than 4 log cell kill in a clonogenic assay on the R2NEO cells (Figure 3) — 96 h after drug incubation practically all cells were apoptotic (Figure 2d). Compared with the neo-transfected cells, the apoptotic response in the ras-transfected cultures was clearly delayed and less extensive. Ninety-six hours after drug incubation (1 μM) a large population of cells with normal DNA content and only background biotin-dUTP labelling was still present in the R2T24 culture, and these surviving cells started to re-populate the culture flasks. In order to quantitate the differences in cell survival between R2NEO and R2T24 cells after doxorubicin incubation, we performed clonogenic assays.

Doxorubicin-induced cytotoxicity

Doxorubicin-induced cytotoxicity was determined by colony formation in soft agar. Survival was expressed as percentage of colony formation in the control cultures, that is without drug incubation. In the control cultures the plating efficiencies varied between 80% and 90%, and no differences were found in this respect between the parental (R2) cells, the neo-transfected (R2NEO) cells and the c-H-ras-transfected (R2T24) cells. However, in the presence of doxorubicin the R2T24 cells were drug resistant by a factor of about 3–5 as compared with the R2 and R2NEO cells (Figure 3).
Intracellular doxorubicin accumulation

It has been reported (Chin et al., 1992) that the promoter of the human mdr1 P-glycoprotein gene can be activated by the c-H-ras oncogene. Since the mdr1 P-glycoprotein confers resistance to hydrophobic natural product cytotoxic drugs (e.g. anthracyclines) by acting as a drug extrusion pump that actively lowers the intracellular drug accumulation (Chin et al., 1993), these results would imply that in our c-H-ras-transfected R2T24 cells up-regulation of the P-glycoprotein might have occurred. To investigate that possibility, we determined steady-state intracellular doxorubicin accumulation in R2, R2NEO and R2T24 cells by flow cytometry (Nooter et al., 1983, 1989). This technique makes use of the spontaneous fluorescence of the anthracycline molecules upon excitation with laser light at 488 nm. In Figure 4 the results are shown of cells incubated with doxorubicin alone, and of cells that were incubated with doxorubicin plus cyclosporin A. Cyclosporin A is a competitive inhibitor of the mdr1 P-glycoprotein drug pump (Nooter et al., 1989), and causes an increase in intracellular anthracycline accumulation when added to P-glycoprotein-expressing cells. In that way, cyclosporin A can be used in experiments designed to demonstrate a functional mdr1 P-glycoprotein drug pump. The intracellular doxorubicin accumulation in R2, R2NEO and R2T24 cells did not differ statistically after a 60 min incubation period with 1 μM doxorubicin (Figure 4). In all three cell lines (R2, R2NEO and R2T24) the addition of cyclosporin A (final concentration 3 μM) to the incubation medium led to an increase in intracellular doxorubicin accumulation, probably as a result of a reduction in endogenous rat mdr P-glycoprotein molecules (Deuchars et al., 1992), which in rodent cell lines often have a somewhat elevated basal expression level. However, also in the presence of cyclosporin A, no differences were found in intracellular doxorubicin accumulation between the control cell lines (R2 and R2NEO) and the ras-transfected cell line (R2T24). Apparently, in the R2 cells constitutive expression of the c-H-ras oncogene does not enhance mdr P-glycoprotein expression. Thus, the differences in drug-induced apoptosis and cell survival between the control cell lines and the R2T24 cells cannot be ascribed to differences in intracellular drug accumulation.

Discussion

In the present study we showed that cells with constitutive expression of the c-H-ras oncogene were approximately 3-to 5-fold more resistant to doxorubicin when comparing the drug concentrations needed for identical log cell kill in the ras-transfected cells and in control cells. In accordance with these drug resistance data, drug-induced apoptosis, as estimated by the in situ nick translation assay, was delayed and significantly lower in the c-H-ras-transfected cells than in the control cells. This inhibition of drug-induced apoptosis by constitutive c-H-ras oncogene expression was not absolute but relative; since 4 days after a 2 h drug exposure with 1 μM doxorubicin even about 60% of the c-H-ras-transfected cells were killed. In the control neo-transfected culture, about 100% of the cells were apoptotic at that time. In the clonogenic assay, no colonies were scored in the R2NEO cultures at 1 μM doxorubicin, while only a 3 log cell kill was found in the R2T24 cultures at that drug concentration. Apparently, constitutive expression of the c-H-ras oncogene promotes cell survival after cytotoxic drug exposure by inhibiting the apoptotic response. Data on the antiapoptotic effects of the c-H-ras oncogene in line with the observations presented here have also been provided by others (Wyllie et al., 1987; Arends et al., 1993). The first link between ras and apoptosis came from a study by Wyllie et al. (1987), who showed that animal tumours with constitutive expression of the c-H-ras oncogene had a remarkably low incidence of spontaneous apoptotic cell death. Using an in vitro system in which apoptosis is induced by serum deprivation, they showed that constitutive expression of the ras oncogene reduced apoptosis in rat fibroblasts subjected to serum withdrawal (Arends et al., 1993).

Several (proto)oncogenes, including ras (this report; Arends et al., 1993), bcl-2 (Williams et al., 1990; Strasser et al., 1991; Bissonnette et al., 1992; Miyashita and Reed, 1992; Wang et al., 1993), abl (Evans et al., 1993) and raf (Troppmaier et al., 1992), have been shown to inhibit apoptosis in a variety of experimental model systems. Whether these genes interfere with one and the same apoptotic pathway and how they work is not yet known. The best studied example in this respect is bcl-2. When overexpressed bcl-2 blocks apoptosis, including apoptosis induced by: (1) growth factor withdrawal (Williams et al., 1990); (2) overexpression of the wild-type p53 tumour-suppressor gene (Wang et al., 1993); (3) the c-myc proto-oncogene (Bissonnette et al., 1992); (4) chemotherapeutic agents (Miyashita and Reed, 1992); and (5) ionising radiation (Strasser et al., 1991). Different modes of action have been postulated for the inhibitory effects of bcl-2 on induction of apoptosis (reviewed in Reed, 1994). In a recent study, the generation of oxygen free radicals was explored during apoptosis (Hockenbery et al., 1993). bcl-2 did not appear to influence the generation of oxygen free radicals but prevented oxidative damage to cellular constituents, suggesting that bcl-2 functions in an antioxidant pathway to prevent apoptosis. The current hypothesis on anti-cancer drug-induced apoptosis is that the drug-induced DNA damage up-regulates the level of wild-type p53 protein (Fritsche et al.,
1993), which in turn, triggers the apoptotic response (Lowe et al., 1993). From two studies it can be concluded that bel-2 interferes in the apoptotic signal transduction pathway 'downstream' of the events associated with the interactions of the two molecules with the intracellular target molecules (Fisher et al., 1993; Kamesaki et al., 1993). One study particularly worth mentioning here linked bel-2 with one of the ras superfamily (Fernandez-Sarabia and Bischoff, 1994).

In human cell extracts, the bel-2 protein has been found to be associated with the ras-related protein R-ras p23 (Fernandez-Sarabia and Bischoff, 1994). The authors hypothesised that, if R-ras were a component of a signal transduction pathway mediating induction of apoptosis, the up-regulation of ras p23 with bel-2 could thus lead to suppression of apoptosis. However, in the same study, no association was found between bel-2 and other members of the ras superfamily, making a similar scenario for the H-ras oncogene less likely.

The c-ras p21 proteins participate in the control of cell proliferation as signal transducers from cell-surface receptors to the nucleus: the serine/threonine kinase rasf-1 probably acts as an effector of ras function (Dolch et al., 1991; Leevy et al., 1994; Stokoe et al., 1994), and cells triggered into apoptosis by growth factor deprivation can be protected by activated ras (Troppmair et al., 1992). Therefore, an interesting possibility that deserves further study is that the antapoptotic effect of the c-H-ras oncogene is mediated by activated ras.

Mutational activation of ras proto-oncogenes is frequently observed in human tumours (Bijl, 1989), and the inhibitory effect of constitutive ras oncogene expression on drug-induced apoptosis in vitro could have implications for tumour cell response to cytotoxic drug treatment in cancer patients. Our in vitro data showed that in doxorubicin at 1 μM, a concentration within the clinical range of plasma concentrations, the neo-transfected cultures did not survive, whereas a significant proportion of the ras-transfected cells survived indeed and finally repopulated the cultures. If this phenomenon also takes place in tumours expressing endogenous ras oncogenes, these tumours might be less susceptible to anti-cancer drug treatment, and it could be anticipated that such drug-resistant tumour cells contribute to the recurrence of tumours.

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